MOLECULAR MICROBIOLOGY LABORATORY

SECOND EDITION

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A WRITING-INTENSIVE COURSE

SECOND EDITION

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ELSEVIER

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Introduction

I. WRITING-INTENSIVE COURSE

Students enrolled in this course complete a variety of formal written assignments that develop scientific writing, editing, and reading skills. In completing these assignments, students review one another's writing and revise drafts. Students use sources outside this manual to complete laboratory reports, prepare a research proposal, write an editorial, and solve a thought problem.

A. Goals

This ten-week course teaches undergraduate students molecular biology techniques commonly used in the life sciences and develops the students' scientific writing skills.

B. Means

The course contains four units that introduce procedures most life scientists will encounter during their careers. In the first unit, students prepare plasmid DNA, construct a restriction map of the plasmid, and transform it into *E. coli*. The plasmid contains a luciferase reporter gene, which introduces the concept of reporter genes through first-hand experience. In the second unit, students express, purify, and analyze an affinity-tagged protein. The third and fourth units require intellectual input from students, who isolate bacteria from environments that they choose. Each student selects one unknown bacterium to culture, examine by light microscopy, and identify by DNA sequence analysis. During this experiment, students learn to isolate genomic DNA, perform polymerase chain reaction (PCR), purify PCR products, and analyze DNA sequences. In the fourth unit, students use terminal restriction fragment polymorphism (T-RFLP) analyses to characterize DNA isolated from uncultured microbial communities. The methods in this course are common techniques that introduce the fundamental principles of molecular biology and microbial ecology.

This is also a writing-intensive course. This manual contains a general discussion of scientific writing and critical reading, and it includes detailed instructions for preparation and peer review of laboratory reports. Additional writing exercises based on journal articles accompany each unit. The studies in these articles employ techniques used in the laboratory exercises. By evaluating these papers, students reinforce their understanding of the technology. Students see how diverse authors report their findings and how formats differ from one journal to another. They also discover that all scientific papers share several essential components. Lectures based on the book *How to Write and Publish a Scientific*

Paper by Robert Day and Barbara Gastel discuss each section of a scientific paper in detail. To build their writing skills and enhance their understanding of molecular microbiology, students compose and revise laboratory reports, edit their peers' reports, critique journal articles, and study the writing manual by Day and Gastel.

II. SCHEDULE

Day	Laboratory	Lecture	In-class writing	Hand in	Read
1	—	Overview; lab reports and proposals	Discuss proposal assignment due class 6	—	Manual 1–28, 32–57, 155–160; Day Ch. 1–15
2	**Unit 1:** Purify plasmid; restriction	Restriction mapping	Revise sentences	Flowchart 1	AEM 63: 4920–4928, 1997
3	**Unit 1:** Agarose gel; transform	Transformation; reporter genes	Restriction mapping problems	—	Day Ch. 16–18, 30–32; Appendices 2, 4
3 + 1	**Unit 1:** Examine plates	—	—	—	—
4	**Unit 2**	Affinity-tagged protein purification	Peer review report 1; answer questions	Report 1 draft	Manual 68–83; Day Ch. 40
5	**Unit 2:** Lyse cells and bind Ni resin	—	Discuss questions from class 4	Flowchart 2; lab report 1	Manual 68–83, 232–240
6	**Unit 2:** SDS-PAGE	SDS-PAGE	Review proposals	Proposal	Manual 117–122
7	**Unit 3:** Isolate bacteria; **Unit 4:** Extract DNA	DNA extraction from bacterial communities	Read JBC paper; answer questions	Flowchart 4 (first day only DNA extract)	Manual 86–116; AEM 64: 294–303, 1998
7 + 1	**Unit 3:** Examine plates and streak	—	—	—	—
8	**Unit 3:** Gram stain; microscopy; inoculate broth	PCR; rRNA-based phylogeny	Peer review report 2; describe colonies (step 4)	Report 2 draft	Day Ch. 37
9	**Unit 3:** Prepare genomic DNA	DNA purification	Discuss JBC paper	Flowchart 3; lab report 2	AEM 63: 2647–2653, 1997
10	**Unit 3:** PCR	Primer stock preparation	Write abstract for AEM 63: 2647–2653	—	Day Ch. 9; AEM 76: 8117–8125, 2010
11	**Unit 3:** Purify PCR process	DNA sequencing and editing review	Discuss abstracts and AEM 63: 2647–2653	—	Manual 145–53
12	**Unit 3:** Agarose gel and template preparation	Review	Sample problems	—	Day Ch. 24, 26; manual 257–264, 142

(*Continued*)

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Day	Laboratory	Lecture	In-class writing	Hand in	Read
13	—	—	Test	Test	Manual 145–153
14	**Unit 4:** PCR for T-RFLP	T-RFLP studies ligation; *lacZ-α* complementation	Peer review editorial; edit sequences, Unit 3	Flowchart 4; editorial draft	Unit 4
15	**Unit 4:** Purify amplicon; ligate to plasmid; transform; agarose gel and OD	Bacterial community analysis	—	Editorial	Day Ch. 27
15 + 1	**Unit 4:** Inoculate broth with white colony	—	—	—	—
16	**Unit 4:** Purify amplicon; plasmid DNA isolation	Plasmid DNA purification	Peer review report 4	Report 3 draft	—
17	—	T-RFLP analysis	Discuss editorials; analyze T-RFLP data	Lab report 3	—
18	—	DNA sequence editing	Analyze sequences, Unit 4		—
19	—	—	Peer review report 4	Report 4 draft	—
20	—	Student talks; farewell address	—	Lab report 4; extra credit problem	—

Notes:
Manual = Bruslind, L., Burke, M., and Ream, W., 2001, *Scientific Writing for Microbiology Majors*.
Day = Robert Day and Barbara Gastel, 2011, *How to Write and Publish a Scientific Paper* (Santa Barbara, CA: Greenwood).
AEM = Applied and Environmental Microbiology

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III. ATTENDANCE AND GRADING POLICIES

A. Attendance

Attendance is **mandatory**. Each unexcused absence reduces your final grade 5%. Three absences result in an Incomplete. Arrival more than 15 minutes late counts as half an absence. Requests for an excused absence are considered on a case-by-case basis. Acceptable reasons for an excused absence include illness, a family emergency, or interviews for admission to graduate or professional schools. **Please do not attend class if you have influenza or another contagious illness**. To request an excused absence, contact the instructor **before** class. Students with an excused absence must complete all missed assignments.

B. Grading

Final Grade

A = 94–100% of top score
A– = 90–93%
B + = 87–89%
B = 83–86%
B– = 80–82%
C + = 76–79%
C = 70–75%
C– = 65–69%
D = 50–65%
F = <50%
Lab reports = 20% each × 4 = 80%
Test = 20%

C. Partial Credit

Do not expect partial credit for incorrect answers to questions that involve calculations. The book provides examples of all the test questions that require calculations, along with the solutions. These simple arithmetic problems teach you to prepare reagent solutions correctly.

Attention to detail is important in science and medicine. A careless error when converting units can cause a 1000-fold error in the concentration of a solution. In the laboratory, an error of this magnitude ruins an experiment. In medicine, an error of this magnitude kills a patient. In this class, you learn that careless errors have consequences.

Submit laboratory reports by e-mail before the start of class on the date indicated on the schedule. Do not expect credit for laboratory reports submitted after the deadline.

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IV. LABORATORY RULES

You must prepare a flowchart prior to each experiment. You may not begin an experiment without a flowchart. The flowchart is due at the start of class. Ask questions when you do not understand the instructions or the principles involved.

Observe the following safety rules at all times:

1. **Do not pipette by mouth.** You must have a rubber pipette bulb or another mechanical pipette.
2. Proper attire is required. **Wear a laboratory coat, safety glasses, and closed shoes.** Do not wear shorts or sandals. **Lab coats and protective eyewear are required for the experiments that use phenol**, which causes severe burns when it contacts skin. Wash with water to remove phenol.
3. **Do not eat, drink, or chew gum in the laboratory.**
4. **Disinfect your bench surface before and after you work.**
5. **Insert pipettes into the rubber bulb gently** to avoid breaking the pipette and cutting your hand.
6. **Assume that all bacteria you use may cause disease.**
7. Disinfect contaminated equipment and surfaces.
8. Place used liquid cultures, supernatant solutions, and glassware in autoclave containers. Discard contaminated plates, pipette tips, and plastic tubes in autoclave bags. Discard phenol and chloroform in designated waste containers.
9. **Wash your hands after you finish working.**

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V. DECORUM

This course will prepare students for careers in medicine, industry, or academic science. Decorum is important in these professions. Appropriate behavior is required in this class. When the class period begins, please take your seat, end personal conversations, and turn your attention to the instructor. After the introductory lecture, begin your lab work quietly. During class, limit conversations to work-related matters and discussion of in-class assignments. Idle chatter is distracting and leads to careless errors. Employers do not tolerate idle chatter in the workplace.

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VI. FLOWCHARTS

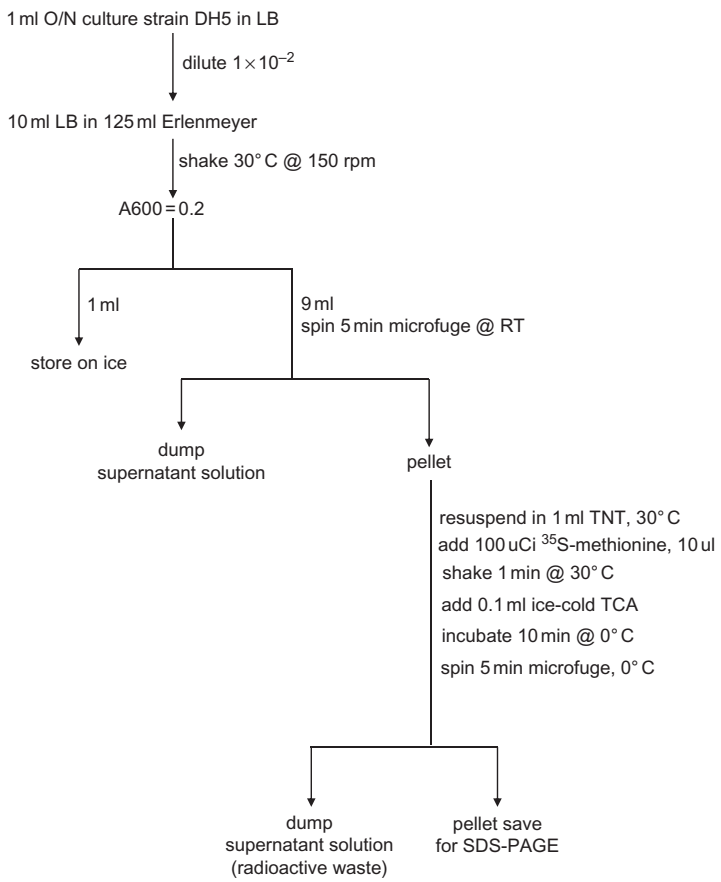
A. How to Prepare a Flowchart

Prepare a flowchart prior to each experiment. You may not participate in the laboratory exercise without a flowchart.

A flowchart outlines each step of the procedure and guides you through the experiment. If you modify a procedure during the course of an experiment, note these changes on the flowchart. Record observations on a separate page as you work.

Flowcharts contain words, symbols, diagrams, and arrows. Begin your flowchart by listing the first step of the procedure. Use an arrow to connect the first step to the second, and so forth. The arrows indicate major procedural steps and direct your attention to the next task. List the steps to proceed from one intermediate to the next beside each arrow. As you complete each step of the procedure, put a check mark on your flowchart beside that step. A sample flowchart follows.

B. Sample Flowchart



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VII. LABORATORY REPORTS

A. Preparing a Laboratory Report

You must prepare a report for each experiment. In class, record data directly in a bound notebook using a **pen**. Date each entry in your notebook. Document your work carefully. Complete, accurate records are crucial in research and medicine; patents worth billions of dollars may depend on notebooks that document the underlying research.

Use a word processor to type the text of your report. Insert graphs, tables, figures, and legends into the report immediately after their first citation in the text. **Word processors have spell check tools.** Spelling errors that your word processor can detect are inexcusable. Some typographical errors, such as *form* in place of *from*, can evade detection by your computer, so proofreading is necessary.

Peer review is essential for high-quality writing. Poor organization or awkward sentences are obvious to most readers. Two peers will review a draft of your report during class, so bring two copies. Due dates for drafts and finished reports are in the schedule. **Write the draft as carefully and completely as possible. Do not submit a first draft or a "rough" draft for review.** Peers cannot evaluate sections that are missing or incomplete.

Submit the final version of your report by e-mail as an attached Word or PDF file. Name the file as follows: seat number (space) last name (space) first name (space) report number. For example, if I sit in seat 8, I would name the file containing my first report "8 Ream Walt report 1.doc."

B. Sections of a Laboratory Report

Laboratory reports must include the following sections (see Appendix A for sample reports):

1. **Name**.
2. **Title**. Make the title concise, specific, and informative. Tell the reader what you did.
3. **Date**. List the dates you performed the experiment.
4. **Purpose**. State the purpose of the experiment in a few sentences. Be specific.
5. **Methods**. *Briefly* describe the procedures used, and reference the appropriate pages in the manual. Do not copy the methods from the manual. Note modifications to the procedure.
6. **Results**. Introduce this section with one or two sentences that describe the experiment. State the rationale for the experiment, and indicate what you hoped to learn. Describe the experiments you did to answer the question. Present the observations you made during the experiment, not what you think should have happened. Do not repeat the methods section.

   Include figures, graphs, and tables in this section. Each figure and table must have a title, a number, and a legend that contains all the information needed to interpret the data. Specify units on the axes of graphs. Label columns and rows in tables. Number each lane on photographs of gels, and indicate the contents of each lane in the

figure legend. Place each figure immediately after the paragraph in which you first cite it.

7. **Discussion**. Offer your interpretation of the data presented in the results section. Begin with a brief introduction that makes the purpose of the experiment clear, then discuss the meaning of your observations. If you can interpret your data in several ways, mention all the possibilities and indicate which alternative you think is correct. Discuss all your results, even if they are unexpected or negative. For example, you should explain the presence of unexpected bands on gels. If the experiment did not work, indicate what went wrong and how you would fix the problem.
8. **Conclusions**. Summarize the meaning of your results in two or three sentences.
9. **References**. List references you used to prepare your report. Use the citation style required for *Journal of Bacteriology*.

   Cite peer-reviewed journal articles and books rather than information from websites. The National Science Foundation prohibits inclusion of universal resource locators (URLs) in grant proposals, because "the sites could be altered or abolished between the time of submission and the time of review." In addition, peers have not evaluated the accuracy of material on websites.

   In *Eats, Shoots and Leaves*, Lynne Truss laments,

   > Despite all the opportunities to "interact", we read material from the internet entirely passively because all the interesting associative thinking has already been done on our behalf. Electronic media are intrinsically ephemeral, are open to perpetual revision, and work quite strenuously against any sort of historical perception. The opposite of edited, the material on the internet is unmediated, except by the technology itself. And having no price, it has questionable value.

10. **Acknowledgments**. Thank (by name) the peers who reviewed your report. If you consulted the Writing Center or other reviewers, acknowledge their help.
11. **Questions**. Answer the study questions at the end of each unit. Show your calculations for mathematical questions. Insert the answers at the end of your report. You may consult the instructor or other sources to answer these questions, but you must write the answer yourself. Use complete, grammatically correct sentences.

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VIII. HOW TO EVALUATE LABORATORY REPORTS

Two classmates review the draft of your report. This gives you an opportunity to improve it before you complete the final version. You review two of your peers' reports for each experiment. The next pages show a sample of the peer review form. Copies of this form are included at the end of this manual. These guidelines will help you write and revise your reports.

Consider organization of the report first. Evaluate each section of the report separately, beginning with the title. Does each section contain the appropriate material? Did the author omit information that belongs in this section, or does it contain material that belongs in another section? For example, did the author include discussion or methods in the results section? Do you understand what the author is trying to say? Do not correct spelling, punctuation, word choice, or sentence structure until you have addressed these global issues.

Each paragraph should focus on one idea and begin with a sentence that describes the content of the paragraph. As you read, suggest paragraph revisions by marking in the text or margins. Do not write the revision yourself; that is the author's responsibility. Indicate statements that are incorrect or unclear. Show the author where you found poor paragraph organization.

Note errors in spelling, punctuation, word choice, and sentence structure. If you are not sure whether a sentence is correct, circle the questionable item and indicate the potential problem. We recommend several books that can help you identify and correct poorly written sentences: *How to Write and Publish a Scientific Paper* by Robert A. Day and Barbara Gastel, *Scientific English* by Robert A. Day and N. D. Sakaduski, *Line by Line* by Claire Kehrwald Cook, and *Elements of Style* by William Strunk and E. B. White. *Eats, Shoots and Leaves* by Lynne Truss explains punctuation.

Use the following checklist to evaluate a draft, including your own. Eight copies of the peer evaluation checklist (two for each lab report) are included in the appendices. Indicate your suggestions on the checklist. Your evaluation matters, so please do it conscientiously. Give the completed checklist and edited draft to the author.

A. Peer Review Checklist

Reviewer _____ Experiment # _____
Author _____ Date _____

1. Look for awkward sentences, poorly organized paragraphs, incorrect grammar, and misspelled words. Even grammatically correct sentences can be awkward and difficult to read. Sentences should be simple and straightforward. Use an active voice instead of a passive voice and eliminate redundant words. Replace vague, qualitative adjectives such as *large* or *small* with numbers. For example, *a tenfold increase* is much more informative than *a large increase*. Explain new terms clearly, and clarify ambiguous statements.

2. Check that the name, title, and date are present. Is the title informative and concise?
3. The purpose statement should be clear, concise, complete, and correct.
4. The methods section should describe the procedures completely, clearly, and concisely. It should cite references properly and indicate modifications made to the procedure.
5. **The results section should begin with an overview of the work.** After reading the results section, a scientist who has not read the methods section should understand the experiment, but it should not include details from the methods section. Strike a balance between too little and too much information by describing the procedures well enough to understand the experiment. Do not include details required to repeat the experiment, which belong in the methods section. For example, do not describe the composition of buffers or media in the results; the methods section should contain this information or reference a publication that does.
6. **Present the results in an order that leads the reader through the experiment.** Is the organization of the data logical? Is the presentation clear? Would changing the organization make the report easier to understand?
7. Figures and tables must have appropriate titles, legends, and citations in the text. Each lane of a gel photograph must have a label, and the figure legend must list the contents of each lane.
8. Does the text accurately describe the figures? The data must support statements made in the text.
9. **The discussion should begin with a brief introduction that makes the purpose of the experiment clear.** This section should flow logically from an introduction through the results to a sound conclusion. The discussion may include background material that puts the need for the experiments in perspective. A good discussion does not merely reiterate information presented in the results section. After a brief recap of the question asked and the work done to answer it, the author should state the meaning of the data and indicate how the findings affect our current understanding of the field. In other words, what was the answer to the question, and what does that answer mean?

   Experiments often produce inconclusive results with more than one interpretation. The discussion should present all possibilities and suggest which one the author believes is correct. If the experiment produced unexpected results, such as "extra" bands on a gel, the discussion must address results that do not fit the author's expectations. The discussion should provide a plausible explanation of such data.
10. The conclusion should summarize the experiment in two or three sentences. The data must support the conclusions.
11. What is best about this writing?
12. What needs improvement?

B. Criteria for Grading Laboratory Reports

	Grade		
Section	**A**	**B**	**C**
Title	Descriptive and brief	Wordy	Vague
Date	Correct: day/month/year	Correct	Missing
Purpose	Clear and concise	Wordy	Vague
	States goal or hypothesis	Unclear	Incorrect
Methods	Clear and succinct	Wordy	Vague or incorrect
	Step by step	Excessive detail	Incomplete
	References correct	Mistakes in references	No references
	Changes noted		Changes ignored
	Prose clear and elegant	Awkward sentences	Poor grammar
	Active voice	Passive voice	Spelling errors
Results	Well organized	Organization confusing	Unorganized
	Describes experiments and results clearly	Complete but difficult to understand	Incomplete
			Incorrect
	Prose clear and elegant	Awkward sentences	Poor grammar
	Figures look neat	Figures look sloppy	Figures unlabeled
	Gel lanes labeled		Gel lanes unlabeled
	Legend lists contents		No figure legends
	Data labeled with correct units		Units incorrect
Discussion	Concise and meaningful interpretation of results	Superficial	Incorrect
	Considers extant theories	Theory not integrated with results	Incomplete
	Reasonable alternative explanations	Importance unclear	
	Cites published papers	Cites Wikipedia	No papers cited
	Prose clear and elegant	Awkward sentences	Poor grammar
	Active voice	Passive voice	Spelling errors

(*Continued*)

(*Continued*)

	Grade		
Section	**A**	**B**	**C**
Conclusion	Correct and concise	Wordy	Vague or incorrect
	Prose clear and elegant	Awkward sentences.	Poor grammar
	Active voice	Passive voice	Spelling errors
References	Read journal articles	Read websites	No outside reading
	Citations in correct format (*J. Bacteriol.* style)	Citations incorrect	No papers cited
Questions	Correct and clearly written	Incomplete; poorly written	Incorrect

C. Checklist for Grading Laboratory Reports

Section/Items Scored	Points	Maximum Score	Comments
Name, Title, Date:			
Informative, brief, correct		10	
Purpose:			
Clear and concise		10	
Correct		10	
Grammar and spelling		10	
Sentence structure		10	
Methods:			
Clear and concise		10	
Correct		10	
Complete; proper references		10	
Grammar and spelling		10	
Sentence structure		10	
Results:			
Overall organization		10	
Paragraph structure		10	
Clear and concise		10	
Grammar and spelling		10	

(*Continued*)

(*Continued*)

Section/Items Scored	Points	Maximum Score	Comments
Sentence structure		10	
Accurate		10	
Thorough		10	
Figures labeled and neat		10	
No methods or discussion		10	
Appropriate style; elegant		10	
Discussion:			
Overall organization		10	
Paragraph structure		10	
Clear and concise		10	
Sentence structure		10	
Grammar and spelling		10	
Logical; supported by data		10	
Thorough		10	
Integrates theory with results		10	
Explains unexpected results		10	
Conclusion:			
Clear and concise		10	
Sentence structure		10	
Grammar and spelling		10	
Logical; supported by data		10	
Questions		70	
Total:		400	

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IX. PROPOSALS TO STUDY ENVIRONMENTAL BACTERIAL POPULATIONS: WRITING ASSIGNMENT (CLASS 6)

A. Overview

In units 3 and 4, the class studies bacterial populations from natural environments. Teams of six students collect bacterial populations from six similar environments. For example, each student in team A may sample a different brand of yogurt, whereas students in team B may sample their own saliva. **You may not take samples from humans other than yourself.** Research on human specimens requires approval by the Institutional Review Board (IRB); you must pass an online course (http://oregonstate.edu/research/ori/hrh/edreq.htm) before you can apply for approval to study human subjects.

In unit 3, you culture one bacterium from each sample and identify the bacterium by sequencing its 16S ribosomal RNA (rRNA) genes. In unit 4, you use a culture-independent approach to study the entire bacterial population in your sample, and you compare this population with those in samples collected by other members of your team.

Each student must write a one- to two-page proposal to study samples from an environment that contains bacteria of interest to their peers, who will evaluate the proposal. Team A reviews and ranks the six proposals written by the members of team B, and vice versa. All team members sample the environment specified in the top-ranked proposal from each team, and the author of the proposal directs the research. Appendix B contains sample proposals.

We live in a world with finite resources. For every research project that we decide to pursue, we abandon others because our resources are limited. Before you can do your research, you must convince your colleagues that it is worthwhile. Your proposal should begin with a general introduction so that your colleagues can understand the experimental system and its importance, even if they do not specialize in that area. Next, your proposal should pose a specific question that the research will answer, and it should indicate why we should care about the answer. Finally, the proposal should demonstrate how the study addresses the question. Most research is justified by direct benefits to people's lives. For example, can we find a species of *Streptomyces* that produces a novel antibiotic? Other research is justified only by curiosity. Can we find evidence of microbial life on Mars? Your research should be important and interesting, and you must be able to convince others that is worthwhile.

B. Site Selection

Select your collection site with the following criteria in mind:

1. **Is the topic important, interesting, and novel?** Doorknobs, handles, keyboards, desktops, counters, toilet seats, cutting boards, money, and similar dry surfaces are not novel or interesting (to me), and their importance in transmission of human disease is questionable. The bacteria living on your cat's rear end may interest you, but why should anyone else care. Choose an environment of general interest. Select an environment that may not occur to others, so that your proposal is novel.

2. **Will comparing multiple samples from similar environments yield interesting information?** Water samples collected upstream and downstream from a sewage treatment facility may identify sources of pollution. Comparing oral bacterial populations from six people may identify species common in most people. Differences between bacterial populations in six brands of yogurt may explain differences in taste.
3. **Is the proposed research feasible?** The search for microbial life on Mars is interesting but not feasible for our class. Dry surfaces (doorknobs, handles, keyboards, desktops, counters, cutting boards, toilet seats, money, and similar objects) are not rich sources of bacteria; chlorine-treated water is also a poor choice. Soil is a rich source of bacteria, but chemicals in some soil samples may interfere with our assays. Yogurt works very well for experiment 4, but the bacterial species used to make yogurt do not grow on the medium we provide for experiment 3. Bacteria from anaerobic environments are a poor choice because you lack the growth chambers required to grow obligate anaerobes.

C. Peer Review of Proposals to Study Environmental Bacterial Populations

Each author solicits comments from at least two classmates. Reviewers comment on the presentation and content. Proposals must not contain typographical or grammatical errors. Sentences should express the author's thoughts clearly, and paragraphs should follow a logical train of thought. The document should have a neat, professional appearance.

Proposals should address several issues:

1. What is the question?
2. Why should the reader care?
3. Will the work answer the question?

Reviewers must ask themselves whether the proposals they read answer these questions to their satisfaction. If they do not, suggest steps that the author can take to correct the deficiencies.

Answers to the question "Why should the reader care?" usually involve work of obvious importance to people's lives, work that addresses a fundamental issue in biology, or work of great general interest.

D. Instructions for Reviewing Proposals

Teams of six students serve as review panels. Each panel receives six proposals (three copies of each) from another team. Each panelist reads three proposals, serving as 1^{o} reviewer for one proposal, 2^{o} reviewer for another, and 3^{o} reviewer for a third proposal.

Written Reviews

Write a brief review of each proposal; address the following points:

1. What does the author propose to do?
2. What question will the research address?
3. How will the study answer the question?
4. Is the proposed research feasible?
5. Is the topic important or interesting?
6. Is the proposal clear and carefully prepared?

Panel Discussion

The instructor appoints a director for each panel. The panel director assigns proposals to panelists, guides the panel discussion, keeps the discussion at an appropriate pace, and presents the results of the panel discussion to the program director (the instructor). The primary (1^o) reviewer presents a summary of the proposal to the panel. The secondary (2^o) reviewer supports or disputes the opinion of the 1^o reviewer. The tertiary (3^o) reviewer writes a summary of the panel discussion. The panel edits and approves the panel summary, which includes a priority rating (high, medium, or low).

The panel ranks all six proposals in order of priority. The panel director provides this list and the reviews to the program director. The program director provides the reviews to the authors and informs the team of the panel's decision.

E. Proposal Review Forms

Reviews are presented using the following forms:

Primary Review

Investigator:
Title:
Synposis:
Comments:
Priority (high, medium, low):

Secondary Review

Investigator:
Title:
Synposis:
Comments:
Priority (high, medium, low):

Panel Summary (by Tertiary Reviewer)

Investigator:
Title:
Synposis:
Comments:
Priority (high medium low):

Panel Summary (by Panel Director)

Rank	Priority	Investigator	Title
1			
2			
3			
4			
5			
6			

Comments:

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X. HOW TO READ A JOURNAL ARTICLE

A. Organization

Title, Authors, Abstract, Introduction, Methods, Results, Discussion, Acknowledgments, and References.

B. Order in Which to Read an Article

Title
Authors
Abstract
Introduction
Results
Discussion
Methods
References

The most important section of the paper is the results. In the abstract, introduction, and discussion, you are reading the authors' interpretations. In the results, you can assess what they found. Your interpretation of the data and its meaning may differ from the authors' ideas. If the title catches your eye and the abstract piques your interest, read the introduction next. This section provides sufficient background for you to understand the rationale behind the experiment. Skip the methods section and continue by reading the results. Make certain that the text accurately describes the data in the figures and tables. Sometimes, the data do not support statements made in the text. You should never assume that the text is correct. As you read the results, decide for yourself what the results mean. In the discussion, the authors try to convince you of the meaning and significance of their data. You must weigh their arguments and decide whether you agree.

C. Information Contained in Each Section

1. **Title.** The title describes the contents of the article using as few words as possible. The title is important for several reasons. It is the first thing that readers see, and it helps them decide whether to read the article. A good title helps readers find articles relevant to their interests because literature databases use key words from the title to list papers.
2. **Authors.** Over time, you will recognize authors doing important work in your field. Literature searches based on their names ensure that you see everything they publish.
3. **Abstract.** The abstract is a condensed version of the article. It explains the rationale for the study, reports the key results, and points out their significance. The abstract lets you decide whether you want to read the entire paper, and it provides an overview that makes the article easier to understand. Literature databases often supply abstracts online.
4. **Introduction.** The introduction supplies background information and puts the paper into perspective. It gives the rationale for the work and may include formal hypotheses.

At the end of this section, the authors briefly state what they did, what they observed, and what they concluded.

5. **Methods.** The methods section describes in detail the procedures used. Read this section to learn about techniques that are unfamiliar to you. Study the methods section carefully if you intent to conduct similar experiments or to better understand possible limitations of the experiments in the paper.
6. **Results.** This section describes the experiments and the observations the authors made. The results section presents the data, often as figures, tables, and graphs. The authors do not discuss the meaning of their findings here.
7. **Discussion.** The authors summarize and interpret their data in the discussion section. They show how their observations relate to each other to form a cohesive story. The authors discuss how their data support or contradict current theories and extend previously published observations. The discussion provides plausible explanations for unexpected results. Often, discussion sections end by stating the significance of the work. In the discussion section, the authors use the past tense to describe data from the results section and the present tense to cite facts established by previously published work.
8. **Acknowledgments.** The authors list agencies that funded the work, and they thank colleagues who provided cultures, materials, equipment, or technical expertise. The authors also thank staff who helped prepare the manuscript and colleagues who critically read it.
9. **References.** The bibliography lists the literature cited in the paper. Depending on the style adopted by the journal, the bibliography may list references alphabetically (by first author) or in the order that they occur in the text. Reference management programs, such as Reference Manager, EndNote, and ProCite, greatly simplify preparation of bibliographies. These programs allow the author to create a database of references and format citations to fit the styles required by different journals.

Notes

Notes

XI. WRITING TIPS

Students in previous classes made the same mistakes frequently. I list them here so that you can avoid them in your writing. See *Scientific Writing for Microbiology Majors*, which is available at http://microbiology.science.oregonstate.edu/files/micro/WIC_WritingManual.pdf, for additional guidance.

A. Begin Each Paragraph with a Topic Sentence

These examples illustrate the importance of a topic sentence at the beginning of a paragraph. A topic sentence is particularly important at the beginning of a section. For example, the results section should begin with the rationale for the experiment. The first sentences should indicate the question the experiment addresses and the approach used to answer the question.

In the first two examples, the Results section begins abruptly; the authors do not indicate what they studied. In the third example, the author states what she did, but she does not indicate the rationale underlying the experiment. In the fourth example, the author indicates the purpose of the experiment, but she uses jargon and provides too much detail.

Example 1

"Results:

A white precipitate appeared after adding the potassium acetate. After centrifugation the chromosomal DNA and cellular debris were separated from the supernatant, which remained clear. No visible DNA pellet was seen at step 11."

The first sentence of this Results section describes a minor observation made during the middle of the experiment. To what did she add the potassium acetate? What was she trying to precipitate? What is step 11? What was the purpose of the experiment? This section should begin with an outline of the experiment, including its purpose and rationale. In the second sentence, a comma should follow the introductory words "After centrifugation," and a noun (*solution*) should follow the adjective *supernatant* (see section D, "Avoid Jargon and Colloquial Wording").

Example 2

"Agarose Gel Electrophoresis

The restriction fragments in lanes 1 and 2 from the agarose gel electrophoresis have a very distinct pattern and thus are the ladders that lanes 3 and 4 will be compared to. Lane 3 is the *Pst*I cut DNA and Lane 4 is the uncut DNA. In lane 3, there are two visible bands, one at the 10,000 bp and one at the 3,500 bp. In lane 4, there is barely one visible band, indicating that the DNA did not get cut."

This Results section begins with details regarding restriction fragments produced from a plasmid that the author has not described. What DNA did he cut? What enzyme did he use? Why did he cut the DNA? What did he hope to learn from the restriction map?

The first sentence has many problems. The author refers to lanes in an agarose gel, but he did not indicate the figure number. The author used the passive voice (*will be compared*),

the sentence ends with a preposition (*to*), and the adverbs *very* and *thus* are not appropriate here.

The second sentence does not make sense. Lane 3 *contains Pst*I-cut DNA, but the lane is not *made* of DNA. The compound adjective *Pst*I-cut requires a hyphen.

The third and fourth sentences begin with *there are* and *there is*. Although these sentences are grammatically correct, they are wordy. As Day and Gastel indicate (p. 214), sentences that begin with *There is* are not as readable as sentences that begin otherwise. The fourth sentence ends with the colloquial expression *did not get cut*. More formal wording (*was not cut*) is appropriate in a laboratory report.

A revised version of these sentences might read,

> "Lane 3 contains *Pst*I-cut pKN800 DNA, and lane 4 contains uncut pKN800 (Fig. 1). We compared the DNA bands in lanes 1 and 2 to those in lanes 3 and 4 (Fig. 1). *Pst*I digestion of pKN800 produced restriction fragments of 10,000 and 3,500 base pairs (bp) (Fig. 1, lane 3). Uncut pKN800 DNA produced a single band (Fig. 1, lane 4)."

The paragraph still needs a topic sentence, and the Results section still needs an introductory paragraph. Example 3 has a better topic sentence than the previous examples.

Example 3

> "Results: Agarose Gel Electrophoresis
>
> Plasmid DNA from the *Escherichia coli* strain NM522 (pKN800) was purified. A portion of the purified DNA was then cut with the *Pst*I enzyme. The cut and uncut plasmids were subjected to gel electrophoresis in order to determine the molecular weight of the DNA fragments. The cut DNA exhibited two bands, one of about 3400 bp and one of about 10000 bp (Figure 1)."

Although the author mentions the plasmid under study, she does not indicate the reason she purified pKN800 plasmid DNA. The second and third sentences are grammatically correct, but they use the passive voice (*was then cut* and *were subjected*).

Example 4

> "Results:
>
> Isolated pKN800 plasmid was digested by *Pst*I and run through a 0.8% agarose gel to determine the orientation of the *lux* operon within the plasmid by RFLP analysis (figure 1.1)."

This sentence indicates the purpose of the experiment and the approach used to answer the question. Although this is a good topic sentence, it has other problems. The phrase *run though* is colloquial jargon, and the concentration of the agarose gel is a detail that belongs in the methods section. The author should indicate that the acronym *RFLP* means "restriction fragment length polymorphism."

WALT'S VERSION:

> Plasmids pKN800-A and pKN800-B contain the luciferase (*lux*) operon from *Vibrio fischeri* on a nine-kilobase (Kb) *Sal*I restriction fragment, which Engebrecht et al. (1983) inserted into the cloning vector pBR322 (4.36 Kb) at its single *Sal*I site in the tetracycline resistance (*tet*) gene. In pKN800-A, the *lux* operon and *tet* gene are transcribed in the same direction, whereas the *lux* operon lies in the opposite orientation in pKN800-B (Engebrecht et al., 1983).

We received an *E. coli* strain harboring an unspecified version of pKN800. To assess whether the orientation of the *lux* operon in pKN800 affects *lux* gene expression, we isolated pKN800 DNA and determined the orientation of the *lux* operon. To confirm that pKN800 encodes functional luciferase proteins, we transformed pKN800 into *E. coli* DH5 and screened ampicillin-resistant transformants for luminescence.

We determined the orientation of the *lux* operon in our isolate of pKN800 by digesting the plasmid DNA with *Pst*I, which cuts once in pBR322 (1.4 Kb from the *Sal*I site) and once in the *lux* operon (0.42 Kb from the 5′ end). pKN800-A yields *Pst*I fragments of 11.54 and 1.82 Kb, whereas *Pst*I digestion of pKN800-B produces 9.98- and 3.38-Kb fragments.

B. Write an Informative Title

These titles convey very little information to the reader. They do not indicate the purpose of the study or its most significant results. They do not indicate that the plasmid pKN800 carries the luciferase operon. Including "pKN800" in the title does not enlighten readers. What is pKN800? Why is pKN800 interesting? Why did we bother to purify, map, and transform pKN800 DNA?

Title 1. "Plasmid Purification and Transformation of *E. coli*"
Title 2. "Transformation of pKN800 into *Escherichia coli*"
Title 3. "Purification, Identification, and Transformation of pKN800 Plasmid"
Title 4. "Plasmid pKN800 Purification, Restriction Mapping and Transformation of *E. coli*"
Title 5. "Purification and Gel Electrophoresis of pKN800 Plasmid and Transformation of *Escherichia coli* Strain DH5α with pKN800 DNA"
Walt's Title. "*Vibrio fisheri* Luciferase Operon Confers Bioluminescence on *Escherichia coli*.

C. Avoid Wordy Phrases and the Passive Voice

Here are examples of the passive voice used excessively. The passive voice is grammatically correct, but it makes writing wordy and dull.

"I'm in agreement with your assessment of the inventions [sic] present commercial value."

This sentence has three problems. *I'm in agreement* is a wordy way to say *I agree*. *I'm* is too informal, and *inventions* requires an apostrophe. An improved sentence would read,

"I agree with your assessment of the invention's present commercial value."

Use was instead of was identified as being, it was observed that, or was determined to be.

Change *identified to be in the B conformation* to *was in the B conformation*.

Use *were* instead of *were found to be*, *were observed to be*, *were determined to be*, *were shown to be*, or *were measured to be*.

Use *is* instead of *is shown to be*, or *is known to be*.

Use *are* instead of h*ave been shown to be*.

Use *may* instead of *is thought to*.

Change the passive voice ("It is estimated by the CDC that") to the active voice ("The CDC estimates that").

Change *would need to be utilized* to *will be used*.

Change *requesting that funds be given for* to *requesting funds for*.

Eliminate *It was found that*, *The results show that*, *In this study*, and *The purpose of this study was to examine*. If you find these phrases in your writing, remove them and begin the sentence with the next word. For example, change *The results show that the* lux *operon was in orientation A* to *The* lux *operon was in orientation A*.

Eliminate words that do not contribute to the meaning of the sentence (e.g,. *absolutely*, *actually*, *fairly*, *quite*, *really*, *very*, and *virtually*).

D. Avoid Jargon and Colloquial Wording

Do not use laboratory jargon or informal wording. For example, scientists often use the word *spin* for *centrifugation* when they converse in the laboratory; do not use *spin* in your written reports. Many students use the slang *run on* or *run through* when they describe gel electrophoresis. The phrase *run through* might describe the outcome of a fencing match, but it has no place in a laboratory report. *Run on the gel* and *ran a gel* are laboratory slang. Also, *electrophorese* is not a word. I suggest that you "separate restriction fragments by agarose gel electrophoresis" rather than "run the cut DNA through an agarose gel."

Colloquial wording is suitable for conversation or informal writing, but scientific writing requires formal wording. For example, "the DNA did not get cut" is appropriate for conversation, whereas "the DNA was not cut" is more formal.

Supernatant is an adjective, not a noun, although scientists often use *supernatant* as a noun. After centrifugation, you remove the *supernatant solution* not the *supernatant*.

E. Avoid Pretentious Wording

Change:

utilize to *use*,
hospital setting to *hospital*,
proved to be to *was*,
was able to find to *found*,
grown up in to *grown in* or *cultured in*,
I would like to propose to *I propose*,
I would like to study to *I will study*,
fairly prevalent to *prevalent*,
is thought to to *may*,
have been implicated in to *cause*.

Day and Gastel (Appendix 2) list additional pretentious phrases to avoid.

F. Do Not Use Nouns as Adjectives

Change *isolated* rabbit *bacteria* to *isolated bacteria from rabbits*.
Change *a* hand *sample of a hospital employee* to *a sample from a hospital employee's hand*.
Day and Gastel (pp. 200–201) provide more examples.

G. Revise Sentences That Do Not Make Sense

Example 1. "Undiluted LB + Amp plates" implies that the author diluted other plates. The author means that she spread undiluted cultures on the plates, but the plates themselves were not "diluted."
Example 2. "LB plates containing no plasmid DNA" implies that other plates contained plasmid DNA.

Cells spread on the plates may contain plasmid DNA, but the plates do not contain DNA.

H. Place Modifiers Properly

Place modifiers next to the word they modify. In the following sentence, a misplaced modifier changes the meaning.

> "The sample was taken from inside a plastic water bottle, so as not to include bacteria from a mouth caught on the rim."

The author means to say that bacteria from the mouth are on the rim. However, in this sentence *caught on the rim* modifies *mouth*. Day and Gastel (pp. 192–193) provide several humorous examples of misplaced modifiers.

I. Shorten Run-on Sentences

Example 1

> "Proof that individuals are not properly cleansing their hands and which potentially dangerous organisms can remain viable on door handles would be valuable information companies seeking to reduce the sick days taken by their employees."

This jumbled sentence contains two distinct ideas. "Companies may reduce sick days taken by employees by encouraging them to cleanse their hands properly. Identifying pathogenic bacteria that remain viable on door handles is important."

Example 2

> "It is important to determine the sanitation of sponges because of their prevalent use in households, and to see what households do use them properly, and the bacteria that are still able to colonize."

This sentence has many problems. A wordy beginning (*It is important to determine*) precedes two incomplete clauses (*to see what household do use them properly* and *the bacteria that*

are still able to colonize). The author should replace the pretentious *do use* with *use*. A simpler version might read, "Sanitation of sponges is important because of their widespread use in households."

J. Convert Fragments into Complete Sentences

> "All of these can be caused by bacteria like *Streptococcus pneumoniae* and *Escherichia coli*: fairly prevalent bacteria in crowded areas."

The last clause of this sentence is a fragment, and the word *fairly* is meaningless here. The word *like* means "similar to" in this context, which is not the meaning the author intended. A revised sentence might read, "These diseases are caused by *Streptococcus pneumoniae* and *Escherichia coli*, which are prevalent in crowded areas."

K. Follow Established Conventions

Cite references included in the bibliography at the appropriate location in the text. Cite references to support all statements of scientific facts.

Italicize only the first three letters of a restriction enzyme name: *Pst*I, *Hin*dIII, *Sal*I, *Eco*RI.

Italicize genus, species, and gene names. Bacterial gene names contain three lowercase letters followed by a capital letter. For example, "We sequenced the *recA* gene from *Escherichia coli*." If a gene contains a mutation, the mutant gene receives an allele number specific for that mutation. The allele number, which is italicized, follows the gene name. For example, "The *recA441* mutation makes *E. coli* temperature sensitive."

Hyphenate compound adjectives. For example, "I gave the nine-year-old boy step-by-step directions to isolate the wild-type gene."

Use the correct symbols for phenotype (Rec), genotype (*recA*), and protein names (RecA). For example, "The wild-type *recA* gene encodes active RecA protein, which confers a Rec^+ phenotype to the cells."

Use capital letters for procedures named after a person (e.g., Gram stain or Southern blot), but do not capitalize procedures not named after a person (e.g., northern blot or western blot). Do not capitalize adjectives such as *gram-positive* bacteria, even though you use the Gram stain to distinguish these types of bacteria.

Data is the plural of *datum*. Therefore, use *these data* instead of *this data*.

Number (or otherwise label) each lane on the photograph of a gel. Write a figure legend that includes the figure number, title, and the contents of each lane.

Do not use *homology* when you mean *identity* or *similarity*. The word *homology* means that two species share a common ancestor. If your data show that the 16S ribosomal RNA genes from two different species are 80% identical, say precisely that: "The genes are 80% identical." Leave arguments about "homology" to evolutionary biologists unless the purpose of your research is to establish evolutionary relationships.

Molecular weight (MW) is a dimensionless number that expresses the mass of a molecule relative to 1/12th of the mass of a ^{12}C atom; MW = (mass of molecule)/[(mass of ^{12}C)/12]. Do not express molecular weight in Daltons (Da) or kiloDaltons (kDa); these

terms are laboratory jargon. *The Handbook of Chemistry and Physics* does not refer to atomic mass units as "Daltons."

L. Use *Which* and *That* Correctly

Which and *that* are not interchangeable. Compare the following sentences.

> "Plasmids *that* contain the ColE1 origin of replication have a high copy number."
> "Plasmids, *which* contain the ColE1 origin of replication, have a high copy number."

In the first sentence, *that* introduces an essential clause. This clause is restrictive because it limits the plasmids under consideration to those with a ColE1 origin of replication. The second sentence contains a nonessential clause introduced by the word *which*. This clause is nonrestrictive and implies that *all* plasmids contain a ColE1 origin of replication, which is incorrect.

Use *which* to introduce a nonessential clause, and set off the clause with commas. For example, "Plasmid pKN800, which contains the luciferase operon, causes *E. coli* cells to glow." Day and Gastel (p. 196) discuss the proper use of *which* and *that*.

Example 1

Here is a single sentence in which both *which* and *that* are misused.

> "*S. mutans* is one variety of bacteria, the other being *S. sobrinus*, which form plaques on teeth, leading to eventual tooth decay that contributes to gingivitis and periodontal disease."

This sentence says that *all* bacteria form plaque on teeth, which is not true. The sentence should begin, "*S. mutans* is one species of bacteria that forms plaques on teeth." The sentence implies that only some forms of tooth decay contribute to gingivitis and periodontal disease. The sentence should read, "Plaque on teeth leads to tooth decay, which contributes to gingivitis and periodontal disease."

The clause about *S. sobrinus* disrupts the flow of the sentence. I like these versions better than the original.

> "*S. mutans* and *S. sobrinus* are bacterial species that form plaque on teeth. Plaque leads to tooth decay, which contributes to gingivitis and periodontal disease."
> "*S. mutans* and *S. sobrinus* are bacterial species that form plaque on teeth, which leads to tooth decay and contributes to gingivitis and periodontal disease."

Example 2

Improper use of *that* in the following sentence changes the meaning.

> ". . . plasmid pKN800 that contains a luciferase gene . . ."

This sentence implies that only some pKN800 plasmids have the luciferase gene, which is not true; all pKN800 plasmids have the luciferase gene. Use *which* instead of *that*, and set the clause off with commas.

"... plasmid pKN800, which contains a luciferase gene, ..."

M. Do Not End a Sentence with a Preposition

Prepositions (*with*, *for*, *to*, *of*, etc.) normally should not come at the end of a sentence; see Day and Gastel (p. 193).

"... germs we share our environment with."
"... what strain of bacteria I have isolated for."
"... may be compared to."

N. Use the Correct Homophone

Homophones have the same pronunciation but differ in meaning. *To*, *two*, and *too* are homophones. Scientists frequently misuse *complimentary* when they refer to complementary base pairs in DNA. *Complementary* means "completing" or "forming a complement." *Complimentary* means "praising," "flattering," or "given for free."

O. Do Not Use the Same Word Twice in One Sentence

Change *fecal contamination contaminates* to *feces contaminates*.

Notes

Notes

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EXPERIMENT

1

Plasmid Purification and Restriction Mapping

I. INTRODUCTION

In this unit, you isolate a plasmid from *Escherichia coli*, determine its structure by restriction mapping, and transform it into another *E. coli* strain.

II. BACKGROUND: PLASMID DNA PREPARATION

In this course, you learn to work with plasmids, because you may use them often in your career. We have many methods to purify plasmid DNA from *Escherichia coli* and other bacteria. These procedures lyse cells and separate plasmid DNA from other cellular components by a variety of means. Detergents, organic solvents, alkali, or heat can lyse bacteria. Three factors dictate which method to use: the size of the plasmid, the bacterial strain, and the technique used to purify plasmid DNA from the lysate.

Large plasmids (>50 kb, or kilobases) are easily damaged and must be released from cells by gentle lysis that minimizes osmotic shock. This reduces the shear forces that arise from disruption of the pressurized bacterial cells. We may use more severe methods to lyse bacteria containing smaller plasmids, such as the plasmid you purify in this unit. These procedures usually use EDTA to chelate divalent cations and make the outer membrane permeable. Because nucleases require divalent cations (Mg^{2+}) for activity, EDTA in the lysate protects DNA from degradation. Many procedures use lysozyme to hydrolyze the peptidoglycan layer (Fig. 1.1). Addition of a detergent, sodium dodecyl sulfate (SDS), breaks the weakened cells by dissolving the lipid bilayers. Adding alkali (NaOH) denatures both circular plasmid DNA and linear chromosomal DNA fragments. When the pH of the lysate returns to neutral, linear chromosomal DNA fragments remain denatured,

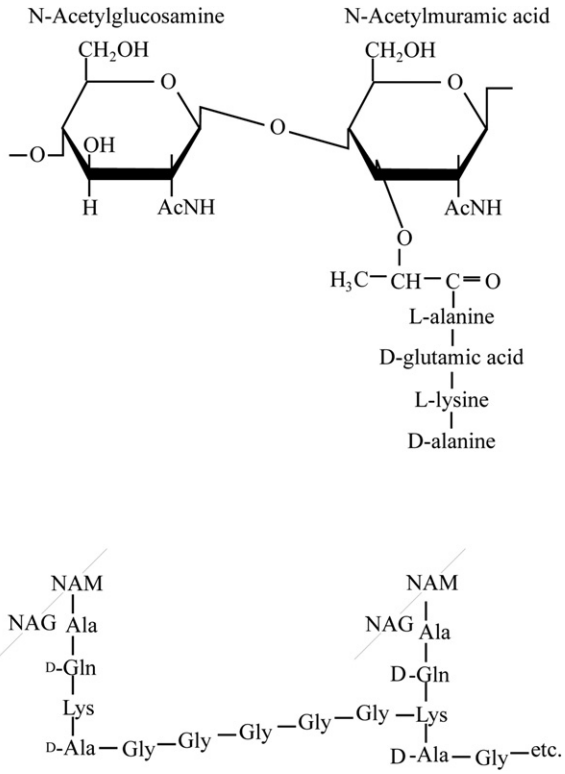


FIGURE 1.1 **Peptidoglycan structure.** *Source: Drawing by Trina Shanks.*

whereas the intertwined strands of covalently closed circular plasmid DNA "snap back" into duplex DNA molecules. Most of the denatured chromosomal DNA precipitates along with the cellular debris, leaving the plasmid DNA in solution.

In this unit, you isolate a plasmid, either pKN800-A or pKN800-B, from *E. coli*. These plasmids carry a β-lactamase gene (*bla*) and the luciferase (*lux)* operon from *Vibrio fischeri* (Engebrecht et al., 1983). The *bla* gene confers ampicillin resistance to cells containing the plasmid. The *lux* operon consists of five structural genes and two regulatory genes, all of which are required for luminescence. Figure 1.2 shows how these plasmids were constructed; the A and B designations indicate different orientations of the *lux* operon relative to the vector plasmid (pBR322). You will create a restriction map of the plasmid you isolate to determine whether it carries *lux* in the A or B orientation.

To isolate one of these plasmids, you use EDTA, lysozyme, alkali, and SDS to lyse the cells. After lysis occurs, you add potassium acetate to neutralize the lysate and precipitate denatured chromosomal DNA and cellular debris, which you remove by centrifugation. The supernatant solution contains RNA from the cells in addition to plasmid DNA. Because these RNA molecules obscure small restriction fragments (<1 kb) on agarose gels,

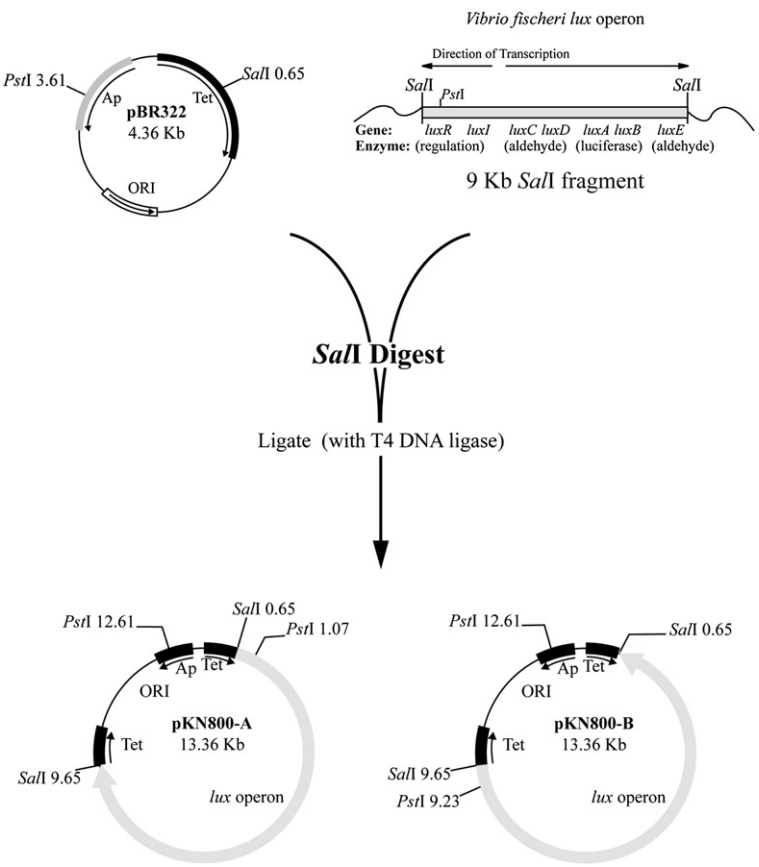


FIGURE 1.2 **Construction and structure of pKN800-A and -B.** *Source: Engebrecht et al., 1983.*

you add ribonuclease A (RNase A) to eliminate the RNA. Extraction with a mixture of phenol and chloroform removes RNase and cellular proteins from the DNA preparation. In the presence of salt (potassium acetate), ethanol causes DNA to precipitate from solution, allowing you to concentrate and desalt the DNA. The plasmid DNA will be pure enough to use for restriction mapping, cloning, DNA sequence analysis, or transformation into other strains of *E. coli*.

Restriction endonucleases cut both DNA strands at specific sequences, leaving ends that are either staggered or blunt, depending on the enzyme used. Commonly used type II restriction endonucleases cut within palindromic target sequences 4–8 bp (base pairs) long. One strand of a palindromic DNA sequence is identical to its complement read 5′ to 3′. *Sal*I recognizes the palindrome 5′-GTCGAC-3′. Engebrecht et al. used *Sal*I to construct the pKN800 plasmids. *Sal*I, isolated from *Streptomyces albus*, cuts between the G and T bases on each strand ($G^{\nabla}TCGAC$) leaving four unpaired bases (TCGA) at the 5′ end of each restriction fragment.

$$5'-GTCGAC-3'$$
$$3'-CAGCTG-5'$$

are cut by *Sal*I to give:

$$5'-G \qquad\qquad TCGAC-3'$$
$$3'-CAGCT \qquad\qquad G-5'$$

The single-stranded ends of a *Sal*I restriction fragment can form base pairs with the identical ends of any other DNA fragment produced by *Sal*I cleavage. Once the ends of two restriction fragments form base pairs (in vitro), DNA ligase [plus adenosine triphosphate (ATP)] can create covalent phosphodiester bonds that join the fragments. A second ligation event (between the other ends of the two linked fragments) forms a circular DNA molecule, which remains intact when transformed into *E. coli*. The RecBCD nuclease destroys linear DNAs introduced into *E. coli*, so circle formation is critical for successful cloning into plasmid vectors.

The entire *Vibrio fischeri lux* operon lies within a 9000-bp *Sal*I fragment, which Engebrecht et al. inserted into the single *Sal*I site of the plasmid cloning vector pBR322 to form the pKN800 plasmids (Fig. 1.2). Insertion of foreign DNA into the *Sal*I site of pBR322 disrupts the gene encoding tetracycline resistance. Thus, cells harboring recombinant plasmids are resistant to ampicillin but sensitive to tetracycline. Cells transformed with recircularized pBR322 exhibit resistance to both antibiotics. Recircularized vector lacking an insert is the most prevalent product of the simple ligation experiment depicted in Figure 1.2. Screening for insertional inactivation of the *tet* gene simplifies the search for cells that contain recombinant plasmids. In our case, we can also detect cells that contain recombinant plasmids by looking for cells that glow in the dark, which is the phenotype conferred by the *lux* operon.

The *lux* operon can insert into pBR322 in two possible orientations (Fig. 1.2). To distinguish orientation A from B, you cut the plasmid DNA with *Pst*I. The *lux* operon and pBR322 each contains a single *Pst*I site positioned asymmetrically relative to the *Sal*I sites. Although *Pst*I cuts each plasmid into two fragments, the sizes of the *Pst*I fragments produced differ depending on the orientation of *lux* relative to pBR322.

The restriction endonuclease *Pst*I (isolated from *Providencia stuartii*) recognizes the sequence

$$5'-\text{CTGCAG}-3'$$
$$3'-\text{GACGTC}-5'$$

which is cut by *Pst*I to give:

$$5'-\text{CTGCA} \qquad \text{G}-3'$$
$$3'-\text{G} \qquad \text{ACGTC}-5'$$

*Pst*I cuts between the A and G bases on each strand (CTGCA$^{\text{v}}$G), leaving four unpaired bases (TGCA) at the 3′ end of each restriction fragment. Note that *Pst*I cleavage generates fragments with unpaired bases at their 3′ ends, whereas *Sal*I fragments have single-stranded 5′ ends.

Agarose gel electrophoresis separates DNA molecules based on their size and structure. DNA, which has a negative charge, migrates from the cathode (negative, black lead) to the anode (positive, red lead) when an electrical field is applied across the gel. Agarose concentration and the ionic strength of the electrophoresis buffer affect the mobility of DNA through an agarose gel. The agarose gel acts as a molecular sieve that retards the migration of long DNA molecules more than short ones. Linear DNA molecules usually migrate through agarose gels faster than circular DNAs, which occur in two forms: covalently closed circular (ccc) and open circular (oc). Covalently closed circular DNAs form supercoils; picture a twisted telephone cord. Small supercoiled DNAs migrate more rapidly than linear DNAs of the same length, but large supercoiled DNAs migrate slower than the corresponding linear molecules. Most of the plasmid DNA in your preparation should be supercoiled. A break (nick) in one strand of a circular DNA molecule relieves the torsional strain that causes supercoiling and produces "relaxed" or "open" circular DNA, which migrates through agarose much more slowly than other topological forms (linear or supercoiled DNA) of the same size. Which would you rather try to pull through a chain-link fence, a piece of pipe (linear DNA), a hula hoop (relaxed circular DNA), or a hula hoop twisted into a straight (albeit fat) cylinder (supercoiled DNA)?

We use ethidium bromide, a fluorescent dye, to stain DNA in an agarose gel. The planar structure of ethidium bromide allows it to intercalate (stack) between the nucleotide bases of DNA (Fig. 1.3). Ethidium-stained DNA exposed to ultraviolet light emits visible (orange) light, allowing us to detect ~1 ng of DNA.

In this unit, you estimate the size of restriction fragments based on their electrophoretic mobility relative to linear DNA fragments of known size (Fig. 1.4). Measure the migration distance of each band of the size standard (Fig. 1.5). Create a semilogarithmic standard curve by plotting size (in bp) on the log (Y) axis versus migration distance (in cm) on the linear (X) axis (Fig. 1.6). Figure 1.7 contains semilogarithmic graph paper. Next, measure the migration distance of each restriction fragment produced by *Pst*I digestion of pKN800 (Fig. 1.5), and use the standard curve to estimate their sizes (Fig. 1.6).

Commercial size standards contain known amounts of DNA in each band (Fig. 1.4). Estimate the amount of DNA in each band of your sample by comparing its intensity to that of the standards. Fluorescence is directly proportional to the quantity (ng) of DNA present, regardless of the size of the DNA molecules in the band.

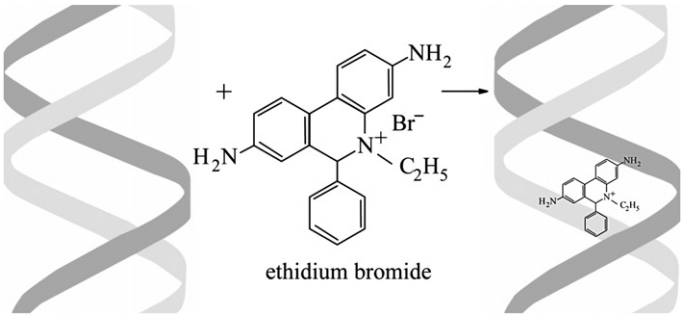


FIGURE 1.3 **Chemical structure of ethidium bromide.** *Source: Drawing by Trina Shanks.*

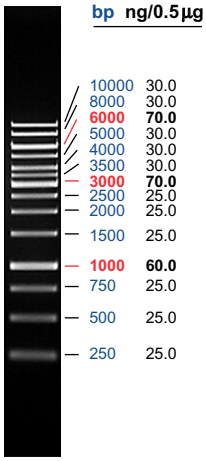


FIGURE 1.4 **Fermentas GeneRuler 1-kb DNA ladder.** The left-hand column indicates the size of each DNA molecule in base pairs. The right-hand column indicates ng of DNA in each band for lanes loaded with 500 ng of ladder. *Source: Image from Fermentas online catalog.*

Transformation is a simple, inexpensive, and effective way to introduce recombinant plasmids into *E. coli*. In this unit, you transform pKN800 DNA into a different strain of *E. coli*, select ampicillin-resistant transformants, and score colonies for luminescence. Some gram-positive (*Streptococcus* and *Bacillus)* and gram-negative (*Neisseria* and *Haemophilus*) bacteria normally become **competent** to take up extracellular DNA during specific stages of their growth cycle. Other bacteria, such as *Escherichia coli*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa*, become competent under artificial conditions. Most transformation methods use ice-cold solutions of $CaCl_2$ or $RbCl_2$ followed by a brief heat shock to transform plasmid DNA into *E. coli*. **Electroporation**, which involves damaging the cell envelope with pulses of high voltage, introduces DNA into *E. coli* and bacterial species that are difficult to transform with $CaCl_2$ treatment.

Micropipettor Technique

You use micropipettors (20 µl, 200 µl, and 1 ml capacity) with sterile tips (yellow for 20- and 200-µl pipettors; blue for 1-ml pipettor) to measure and transfer small volumes of

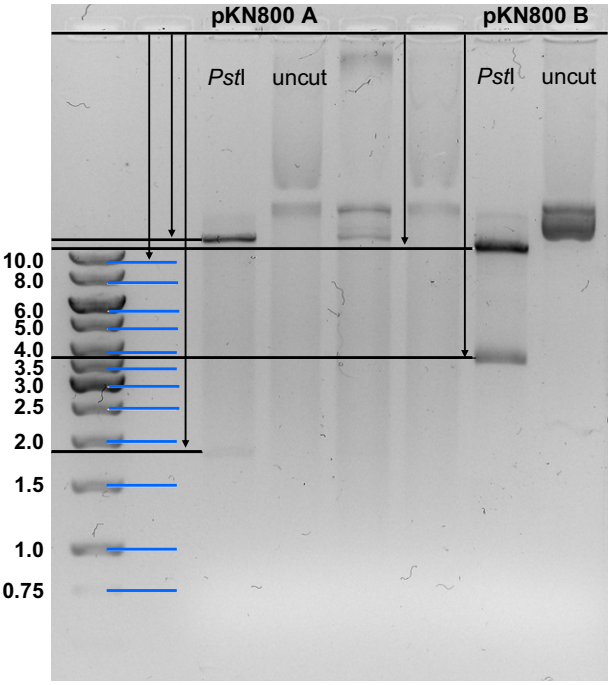


FIGURE 1.5 Agarose gel electrophoresis of uncut and *Pst*I-cut pKN800-A and -B plasmid DNA. The left-hand lane contains 500 ng of Fermentas GeneRuler 1-kb DNA ladder (Fig. 1.4); numbers indicate the size of DNA molecules in kb. Blue lines mark positions of bands in the size standard. Horizontal black lines indicate positions of restriction fragments produced by *Pst*I digestion of pKN800-A or pKN800-B. Vertical arrows show migration distances from wells. Lane 1: 1-kb DNA ladder; lane 3: pKN800-A cut with *Pst*I; lane 4: uncut pKN800-A; lane 7: pKN800-B cut with *Pst*I; lane 8: uncut pKN800-B. The gel contained 1% agarose.

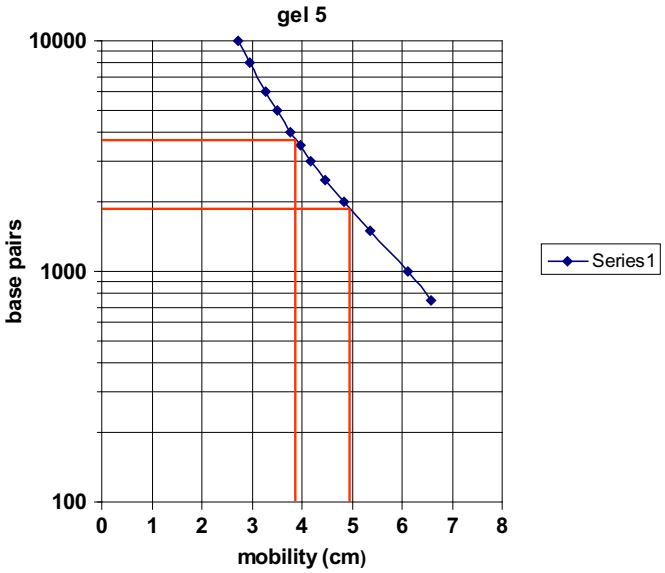


FIGURE 1.6 Electrophoretic mobility of DNA size standards. Semilogarithmic plot of DNA fragment size (bp) versus mobility (cm) during agarose gel electrophoresis (Fig. 1.5). Blue triangles represent size standards from the Fermentas GeneRuler 1-kb DNA ladder (Fig. 1.4). Vertical red lines indicate distances migrated by the diagnostic (smaller) *Pst*I restriction fragments from pKN800-B (left line) and pKN800-A (right line). Horizontal red lines indicate estimated sizes of 3.38 kb *Pst*I fragment from pKN800-B (top line) and 1.82 kb *Pst*I fragment from pKN80-A (bottom line).

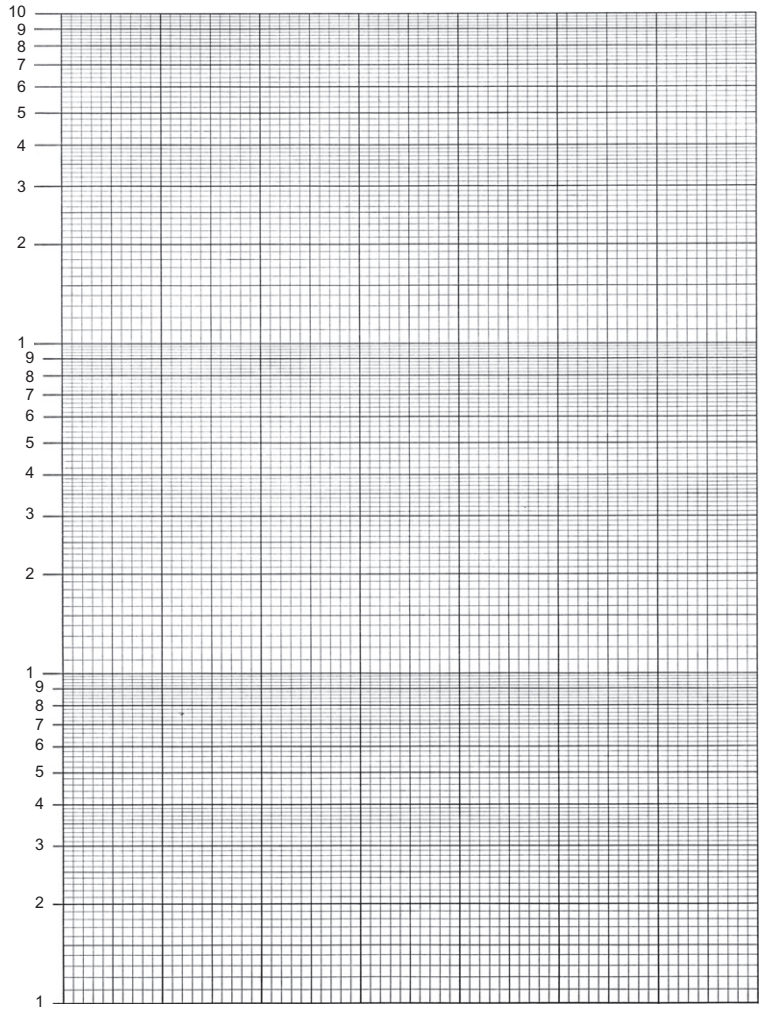


FIGURE 1.7 **Semilogarithmic graph paper.**

liquid. Accurate measurements require proper technique. Sterile tips come in racks held inside closed plastic boxes. To place a tip securely on the pipette barrel, press the barrel firmly inside a tip (while it is still in the rack) and withdraw the tip from the box. **Do not** use your hand to put the tip on the pipettor. Use a clean tip each time you pipette from a reagent stock.

The pipettor has a two-stage plunger; use the stage with low resistance to measure and the high-resistance stage to expel all the liquid from the pipette tip. Press the plunger to the bottom of the low-resistance stage **before** you insert the tip in the liquid. Submerge the

mouth of the tip in the solution you wish to pipette, but do not go deeper than necessary. This prevents drops from clinging to the outside of the tip, which can occur when you measure viscous solutions, such as restriction endonucleases. Storage buffers for most enzymes contain glycerol, which is especially viscous at −20°C, the temperature typically used to store enzymes.

Draw the liquid into the tip by releasing the plunger gradually; this should take 2 seconds. If you allow the spring to snap the plunger back too quickly, your measurements may be inaccurate. With your free hand, pick up the tube into which you will transfer the solution; this allows you to see where the liquid goes. Move the tip to the bottom of the tube and expel the solution as a single drop. Ensure that subsequent additions go into the same drop. After you transfer all the components into a single drop at the bottom of the tube, set the pipettor to approximately the volume of the drop. Insert the tip into the drop, and mix the reagents by pipetting up and down several times. Do not create bubbles, which may cause enzymes in the mixture to oxidize.

Notes

Notes

III. PROCEDURE

A. Purify Plasmid pKN800 DNA (Class 2)

Work in pairs; wear gloves to protect plasmid DNA from skin-borne nucleases and to protect your skin from phenol. Lab coats and eye protection are **required** when you (or others) use phenol. Open containers of phenol only in the fume hoods. Do all steps aseptically.

1. **TAs do step 1.** Grow 5-ml cultures of *E. coli* strains NM522 (pKN800-A) and NM522 (pKN800-B) in Luria broth +50 μg/ml ampicillin; shake at 37°C overnight. Prepare a 5-ml culture for each pair of students. Centrifuge cells at 4000 rpm for 10 minutes. Discard the supernatant solution into a container that we autoclave, and resuspend the cell pellet in 150 μl of lysis buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris·HCl, pH 8.0). Transfer the cells to a sterile 1.5-ml microcentrifuge tube and store on ice until class.
2. **Students start here.** Add 60 μl of a freshly prepared lysozyme solution (80 mg lysozyme/ml in 10 mM Tris•HCl, pH 8) to the cell suspension and mix vigorously. Incubate at room temperature for 10 minutes. Mark the tube with your name and sample number.
3. Add 200 μl of 1% SDS, 1 M NaOH solution to the cell suspension. Invert the tube **gently** several times and place on ice for 5 minutes. The cells lyse at this point, increasing the viscosity of the solution. **Treat the sample gently, because you do not want to shear the chromosomal DNA and release it from the membrane.** If you shear chromosomal DNA into fragments, it will copurify with the plasmid DNA. You want to isolate only plasmid DNA. The detergent (SDS) lyses the cells, and the NaOH increases the pH so that the DNA denatures.
4. Add 150 μl of 5 M potassium acetate to the lysate. Invert the tube **gently** several times and place on ice for 10 minutes. You should see a white flocculent precipitate. The potassium acetate returns the pH to neutral and helps precipitate chromosomal DNA.
5. Centrifuge the tube at maximum speed (14,000 rpm) for 10 minutes. Place another sample on the opposite side of the rotor to balance it. The pellet contains chromosomal DNA and debris from lysed cells. Use a micropipette to transfer the supernatant solution to a clean, sterile 1.5-ml microcentrifuge tube. Mark the tube with your name and sample number. The supernatant solution contains plasmid DNA; you should have approximately 500 μl of this solution. Discard the pellet in a container that we will autoclave.
6. Add 10 μl RNase A (1 mg/ml in 25 mM Tris·HCl, pH 7.4) to the supernatant solution and invert the tube several times; incubate at room temperature for 10 minutes. This step degrades RNA.
7. Wear gloves, goggles, and lab coats. Add 500 μl of phenol–chloroform–isoamyl alcohol (25:24:1) to the supernatant solution. Vortex vigorously and centrifuge at full speed for 3 minutes to separate the aqueous (top) and organic (bottom) phases.
8. Transfer the **top** (aqueous) phase, which contains plasmid DNA, into a clean, sterile 1.5-ml centrifuge tube. Mark the tube with your name and sample number. Avoid the

interphase region, which is a white layer of protein that forms between the aqueous and organic phases. Cap the tubes containing the organic (bottom) phase; discard the phenol in the organic waste container.

9. Add 10 μl of 3.5 M sodium acetate to the aqueous phase containing the plasmid DNA. Invert the tube several times. Sodium acetate (and potassium acetate added in step 4) provides the high salt concentration required for ethanol precipitation of DNA.
10. Add 500 μl of ice-cold 95% ethanol to the DNA−salt solution. Invert the tube several times; incubate on ice for 10 minutes.
11. Centrifuge at full speed for 10 minutes. Place the hinge of the tube lid to the outside of the rotor; this will help you locate the DNA pellet, which will adhere to the outer wall at the bottom of the tube. Pour off the alcohol gently; do not dislodge the DNA pellet from the tube. Centrifuge the tube for 10 seconds. Carefully remove residual ethanol with a sterile pipette tip. Label the tube with your name and sample number, and write "pure pKN800" on the tube. Incubate the tube, with the cap open, in a vacuum desiccator or Speed Vac centrifuge until the ethanol has evaporated.
12. Dissolve the pellet in 50 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8). Place in an ice bucket. Store this sample in the freezer until next class.

B. Restriction of Plasmid pKN800 (Class 2)

1. Label two sterile 1.5-ml microcentrifuge tubes with your name and sample number. Write "pst" on one tube, and "uncut" on the other.
2. Add 3 μl of 10X reaction buffer (supplied by the manufacturer) to both tubes (one labeled "pst" and the other labeled "uncut"). Use fresh sterile tips.
3. Add 10 μl of sterile distilled water to the tube labeled "pst" and 12 μl to the tube labeled "uncut." Use fresh sterile tips for each addition.
4. Transfer 15 μl of pKN800 DNA into each tube. Use fresh sterile tips. Store these tubes and the rest of your plasmid DNA on ice.
5. Use a new sterile pipette tip to add 2 μl (20 units) of *Pst*I enzyme to the tube labeled "pst." Set the pipettor to 15 μl and pipette the reagents up and down (inside the tube) several times. Mix thoroughly, but do not make bubbles. Most companies sell restriction enzymes at a concentration of 10−20 units/μl. A unit of restriction endonuclease is the amount required to cut 1 μg of a particular DNA in 1 hour under specified reaction conditions.
6. Centrifuge the tube for 10 seconds to bring the liquid to the bottom.
7. Incubate both samples in a 37°C water bath for 45−60 minutes. Use a floating rack to hold the tubes.
8. Incubate the samples at 70°C for 10 minutes to inactivate the *Pst*I enzyme; store the samples at 4°C until next class. **The heat treatment is optional** for this experiment. However, if you want to clone the restriction fragments, you must inactivate the restriction enzyme before you ligate the DNA fragments to a vector. Heat inactivates *Pst*I and many other restriction endonucleases. Heating to 70°C does not inactivate some enzymes, such as *Bgl*II; extract with phenol to remove heat-stable enzymes.

C. Agarose Gel Electrophoresis of *Pst*I-Digested pKN800 (Class 3)

1. **Cast an agarose gel.** Use a 0.8% agarose gel to separate the restriction fragments in your DNA samples. This percentage of agarose separates linear DNA molecules that range from 0.6 to 10 kb.
   - **a.** Seal the edges of a clean, dry gel-casting tray supplied with the electrophoresis apparatus. Our apparatus comes with a sealing clamp. Install the well former (comb) on the casting tray; set the comb depth so that a microscope slide just fits between the tray and the tips of the comb's teeth. Make certain the comb is parallel to the edge of the casting tray.
   - **b.** Add 0.8 grams of agarose to a flask containing 100 ml of 1X TAE buffer (0.04 M Tris-acetate with 0.001 M EDTA). Mark the level of the liquid on the flask. Boil the liquid and swirl until the agarose dissolves completely. **Use heat-resistant gloves to hold the flask, and swirl the flask gently.** Undissolved agarose appears as small, clear particles floating in the solution. Add distilled water to replace the liquid that evaporated. Use a microwave oven, hot plate, or steamer to melt the agarose.
   - **c.** Cool the agarose solution to 55°C; the flask should be cool enough to touch. Ethidium bromide is a planar molecule that intercalates into DNA by stacking between the bases. This causes DNA polymerase to make frameshift mutations during DNA replication. Because ethidium bromide is mutagenic, you should **wear gloves and a lab coat.** Add 4 μl of ethidium bromide (10 mg/ml stock solution) per 100 ml agarose solution; swirl to mix. Pour the cooled agarose solution into the casting tray. Pour a gel 3–5 mm thick.
   - **d.** After the agarose has solidified, remove the casting tray from the clamp and place the tray in the electrophoresis apparatus. Add just enough TAE electrophoresis buffer to fill the buffer reservoirs and cover the gel to a depth of 1 mm. Lift the comb straight up to remove it from the gel. The buffer fills the wells and prevents them from tearing as you remove the comb. The wells must be full of electrophoresis buffer before you load your samples into them.
2. **Load the DNA samples onto the agarose gel.**
   - **a.** Centrifuge the samples 15 seconds.
   - **b.** Label two new tubes "pst" and "uncut."
   - **c.** Transfer 15 μl of each of your samples into the appropriate tube. Store the remaining plasmid DNA on ice.
   - **d.** Add 2 μl of loading solution to each of the 15-μl samples. Pipette up and down a few times. The loading solution contains bromophenol blue for tracking the progress of the electrophoresis and glycerol to increase the density of the sample so that it will sink to the bottom of the well in the gel. **Do not add loading solution to the other tubes.**
   - **e.** Load the samples into different wells of the submerged gel. Place the pipette tip just above the well and gently expel the sample, which will sink to the bottom of the well and displace the electrophoresis buffer. If you insert the tip too deeply into the well, you may pierce the bottom, allowing your sample to leak out of the well.
   - **f.** Load 500 ng of Fermentas GeneRuler 1-kb DNA ladder in one lane. This size standard provides DNA molecules of known size that range from 10,000 to 250 bp

(Fig. 1.4). You use these standards to estimate the size of the restriction fragments in your samples.

g. Attach the electrical leads so that the positive (red) lead is at the bottom of the gel and the negative (black) lead is at the top, nearest your samples. DNA, which has a negative charge, migrates toward the positive lead (anode). Apply 10 volts/cm (75 volts) to the gel. Turn the rheostat on the power supply gradually from zero until the meter indicates the desired voltage. If you apply the full load all at once, you may blow the fuse. **[Begin the transformation (step D) while you wait for the electrophoresis to finish.]**

h. Turn off the current when the bromophenol blue dye is 1 cm from the bottom of the gel.

i. View the ethidium bromide–stained gel on an ultraviolet light transilluminator. **You must wear UV-blocking eyewear; a face shield is best.** Do not look at the light source without proper eye protection, and protect your skin from UV light.

j. Photograph the gel. Each student will receive a digital image.

D. Transform *E. coli* Strain DH5α with pKN800 DNA (Class 3)

- **Competent cell preparation.** You will use purchased frozen competent cells, but you may prepare competent cells yourself as follows. Grow *E. coli* DH5α (or any other *E. coli* strain) to mid-log phase ($OD_{600} = 0.4$) in 10 ml LB broth then chill on ice. Centrifuge cells (3700 rpm, 10 minutes, 4°C) in a clinical centrifuge. Discard the supernatant solution into a container that we will autoclave and place the cells on ice. Resuspend the cell pellet in 10 ml of ice-cold 100 mM $CaCl_2$ and incubate on ice for 30 minutes. Centrifuge the cells (3700 rpm, 10 minutes), discard the supernatant solution, and place the cells on ice. Resuspend the cell pellet in 5 ml of ice-cold 100 mM $CaCl_2$ and incubate the cells on ice for 30 minutes. Centrifuge the cells, pour off the supernatant solution, and place the cells on ice. Resuspend the cell pellet in 1 ml of cold 100 mM $CaCl_2$ and incubate on ice for 15 minutes. The cells are now competent for transformation.

1. **Students start here.** Work in pairs; use aseptic technique throughout the procedure. Label one sterile 1.5-ml microcentrifuge tube "cut," a second tube "uncut," and a third tube "no DNA." Place the tubes on ice. Thaw frozen competent cells on ice immediately before use.
2. Stir ice-cold competent cells with a sterile pipette tip; transfer 50 μl to each chilled tube.
3. Add 5 μl of uncut or *Pst*I-cut plasmid DNA to the appropriate tube. Use a fresh sterile pipette tip for each transfer. Mix the DNA with the competent cells. Do not let the cells become warm. Incubate on ice for 40 minutes. Do the same incubation with the cells in the tube labeled "no DNA."
4. Place the tubes in a 37°C water bath for **exactly** 20 seconds. Do not shake the tubes.
5. Place the tubes on ice for 2 minutes **immediately** after the heat shock.
6. Add 0.95 ml of room-temperature LB broth to each microcentrifuge tube and shake at 225 rpm for 60 minutes at 37°C.

TABLE 1.1 Dilutions for Plating on LB-Ampicillin and LB Agar

	Culture Transformed with		
Plates	**Uncut DNA**	***Pst*I-cut DNA**	**No DNA**
LB + Amp	0.1 ml of undiluted	0.1 ml of undiluted	0.1 ml of undiluted
LB + Amp	0.1 ml of 10^{-1} dilution	—	—
LB	—	—	0.1 ml of 10^{-5} dilution
LB	—	—	0.1 ml of 10^{-6} dilution

7. Spread cells from each culture on LB agar containing 50 μg/ml ampicillin. To ensure that you obtain single colonies, dilute some of the cultures before you spread them on the LB-ampicillin plates; see Table 1.1 for the appropriate dilutions. Use room-temperature LB broth to dilute the cells. Spread two plates for each dilution. The culture transformed with **uncut DNA** should contain the largest number of ampicillin-resistant transformants, because supercoiled plasmid DNA transforms competent *E. coli* cells efficiently. Spread 0.1 ml of undiluted cells from this transformation on LB-ampicillin plates. Also, dilute this culture tenfold and spread 0.1 ml/plate on LB-ampicillin agar.
8. The culture labeled "**no DNA**" is a negative control; this culture should not produce colonies on LB-ampicillin plates. Spread 0.1 ml of undiluted cells on LB-ampicillin plates. This control serves several purposes. It ensures that the competent cells were not resistant to ampicillin prior to transformation with pKN800 DNA, and it shows that the plates contained enough antibiotic to kill the untransformed cells. For these controls to be meaningful, you must prove that the culture contained viable (ampicillin-sensitive) cells. To do this, you plate this culture on LB plates without ampicillin. Prepare 10^{-5}- and 10^{-6}-fold dilutions of the cells in the tube labeled "no DNA," and plate 0.1 ml of each dilution on LB agar **without ampicillin**. The number of colonies on these LB plates allow you to calculate the number of viable cells in the culture.
9. The culture transformed with ***Pst*I-cut DNA** is a control to test whether *Pst*I cut all the plasmid DNA. Linear DNA does not transform *E. coli* because exonuclease V (ExoV, also called the RecBCD nuclease), destroys linear DNA in the bacterial cell. ExoV destroys *Pst*I-cut DNA when it enters *E. coli*, and no ampicillin-resistant colonies grow. However, if *Pst*I did not cut all of the plasmid molecules, uncut plasmid DNA molecules can transform competent *E. coli* and produce ampicillin-resistant transformants. To assess the extent of the *Pst*I digestion, spread LB-ampicillin plates with 0.1 ml of undiluted cells transformed with *Pst*I-cut DNA.
10. Incubate all plates at 30°C overnight. Examine the plates the next afternoon. *E. coli* grows best at 37°C, but the luciferase enzyme works better at 30°C.

E. Observe Luciferase Reporter Gene Expression (Day after Class 3)

1. Take your plates from the 30°C incubator to a dark room.
2. Turn off the light and let your eyes acclimate to the dark for about 2 minutes.
3. Count the number of ampicillin-resistant transformants that glow in the dark.
4. Turn on the lights and count the colonies on each plate. Record the data.

Notes

Notes

IV. LABORATORY REPORT [CLASS 4 (DRAFT) AND CLASS 5]

Include the following in your lab report:

1. In the Results section of your report, describe the pattern of restriction fragments you observed.
2. Include a photograph of your gel as a figure in your report. Give the figure a title and a legend. Label each lane on the photograph, and indicate the contents of each lane in the legend.
3. Measure the distance that each size standard migrated during the electrophoresis. On semilogarithmic graph paper (Fig. 1.7), plot the size (in base pairs) of each standard (log scale) versus the distance migrated (linear scale). If your gel did not work, use one from another group or from the example in Figure 1.5. Figure 1.4 shows the size standards used for this gel.
4. In a table, record the average number of ampicillin-resistant and luminescent transformants obtained in each transformation.
5. Calculate the efficiency of transformation, expressed as the number of transformants per μg of plasmid DNA. Divide the total number of transformants (contained in the entire 1-ml culture) by the amount of DNA used to transform. Take into account the dilutions you made. To estimate the concentration of your plasmid DNA, compare the intensity of the uncut pKN800 band with that of the bands in the size standard. Figure 1.4 indicates the amount of DNA (in ng) contained in each band of the standard.

Notes

Notes

V. QUESTIONS (CLASS 5)

1. Did the uncut plasmid DNA form more than one band during agarose gel electrophoresis? What does each band represent?
2. Which orientation of the *lux* operon did your plasmid have, A or B (see Fig. 1.2)? How can you tell?
3. Did your *Pst*I digestion go to completion? How can you tell?
4. Did restricted plasmid DNA produce ampicillin-resistant transformants? Explain.
5. Would you expect any transformations to yield ampicillin-resistant nonluminescent transformants? Justify your answer.
6. Why did you analyze uncut plasmid on the gel?
7. Why did you plate bacteria that did not receive plasmid DNA?
8. If no colonies grew on any of the LB-ampicillin plates, how could you distinguish between extremely poor transformation efficiency and incorrect medium? What control would indicate whether the plates were at fault?

Notes

Notes

VI. IN-CLASS WRITING EXERCISE (CLASS 2)

Rewrite these sentences, making them more succinct. Eliminate as many words as possible without sacrificing meaning.

1. From the results of this experiment, it was indicated that blue dextran (blue band) had a greater molecular weight than DNP-glycine (yellow band), as it was eluted first.

2. The procedures on pages 27–34 of the lab book were generally followed with the following three deviations as listed below:

3. Colonies that were yellow in color and white in color were seen.

4. A possible explanation for this result could be due to the fact that molecules larger then the largest pore size of the gel cannot diffuse into the gel pores.

Notes

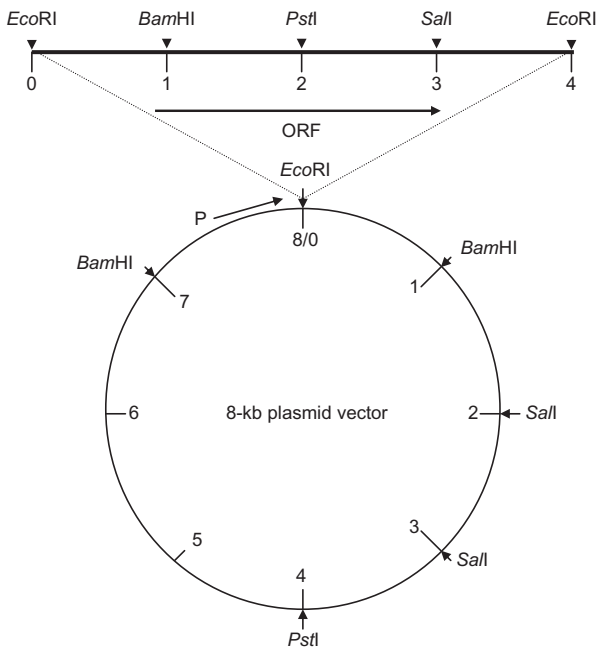
Notes

VII. RESTRICTION MAPPING EXERCISES (CLASS 3)

1. This linear 10-kb DNA fragment contains both *Bam*HI and *Hin*dIII restriction sites. Draw the sites on the map. Locate the sites relative to each other and the ends of the fragment.

Digestion with	Fragment Sizes (kb)
*Bam*HI	5.0, 3.0, 2.0
*Hin*dIII	5.5, 4.5
*Bam*HI and *Hin*dIII	5.0, 3.0, 1.5, 0.5

2. You have an 8-kb plasmid vector with a single *Eco*RI site (circle; see the following figure). You digested the plasmid with *Eco*RI and ligated the plasmid to a 4-kb *Eco*RI restriction fragment (bold line), forming a 12-kb recombinant plasmid (not shown). The plasmid has a promoter (P + arrow) that drives transcription clockwise through the *Eco*RI site. The 4-kb *Eco*RI fragment contains an open reading frame (bold arrow labeled ORF) that you want to express from this promoter. The *Eco*RI fragment could have inserted into the vector plasmid in either orientation relative to the promoter. What restriction enzymes do you need to determine the orientation of the 4-kb fragment relative to the promoter? Do the **minimum number of digestions required** to determine the orientation. What size restriction fragments do you expect for the desired orientation?



Notes

VIII. IN-CLASS WRITING EXERCISE (CLASS 4)

Critique of Kragelund, L., Hosbond, C., and Nybroe, O. 1997. Distribution of metabolic activity and phosphate starvation response of *lux*-tagged *Pseudomonas fluorescens* reporter bacteria in the barley rhizosphere. *Appl. Environ. Microbiol.*, 63, 4920–4928.

1. What is the rationale for this work, and where did you find it?

2. Did the abstract contain statements that you did not understand? Did the introduction clarify these statements?

3. Were any of the results unclear?

4. What is the connection between this paper and experiment 1?

5. Did you notice an unusual finding in the second paragraph of the results section?

Notes

Notes

EXPERIMENT

2

Affinity Purification of Histidine-Tagged FnbA Protein

I. INTRODUCTION

In this unit, you express the *Staphylococcus aureus* fibronectin-binding protein gene (*fnbA*) in *Escherichia coli* (Signas et al., 1989). Next, you purify FnbA protein by affinity chromatography and examine it by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Signas et al. used purified FnbA protein to immunize rabbits and obtain polyclonal antisera, which they used to detect FnbA. We are interested in FnbA because it is required for infection of host tissue.

II. BACKGROUND

S. aureus causes bovine mastitis. The pathogen attaches to fibronectin, a secreted host protein holding together the epithelial cells that line the mucosal surface of the udder. *S. aureus* fibronectin-binding protein contains a 115-amino-acid domain that allows the bacteria to bind host cells. We use a plasmid with a 345-bp portion of the *fnbA* gene that encodes the fibronectin-binding domain fused, in frame, to six histidine codons, which constitute the affinity tag. This gene fusion lies downstream from a strong *E. coli* promoter called *tac*, which is a hybrid of the *trp* and *lac* promoters. A lactose analog, isopropyl thio galactoside (IPTG), induces the *tac* promoter. The plasmid that carries the His-tagged *fnbA* gene encodes resistance to ampicillin. *E. coli* cells harboring this plasmid produce the histidine-tagged fibronectin-binding protein when cultured in broth containing IPTG.

Affinity tags, such as the six histidine residues, simplify purification of tagged proteins. Affinity tags may or may not affect the normal activity of the tagged protein; however, this is not a concern when the goal is to purify a protein for use as an antigen. Histidine residues that are arranged properly within a protein bind nickel (Ni), permit purification of histidine-tagged proteins by chromatography over a nickel-containing resin. In this unit, you disrupt *E. coli* cells with a proprietary detergent and incubate the extracts with a nickel resin. In theory, only the histidine-tagged protein binds, and the other proteins in the extract wash off the resin. Addition of imidazole, which competes with the histidine-tagged protein for binding sites on the Ni resin, releases the tagged protein from the resin. You assess the purity and yield of the eluted protein by SDS-PAGE. To learn more about this technology, see www.qiagen.com/. Under "Products & Services," select "QIAGEN Product Guide," then under "Protein Purification," select "The Ni-NTA System."

You use **SDS-polyacrylamide gel electrophoresis** to analyze your protein samples. SDS-PAGE separates **most** proteins by size, although some proteins migrate faster or slower than predicted. You heat your protein samples in a solution that contains SDS and 2-mercaptoethanol. SDS is an ionic detergent that denatures proteins and imparts a uniform negative charge, and 2-mercaptoethanol reduces disulfide bonds in proteins. Treatment with this solution disrupts the structure of proteins, so that their mobility during SDS-PAGE usually reflects their molecular weight. An SDS-polyacrylamide gel consists of two layers (Fig. 2.1). The stacking gel (top layer) contains 4% acrylamide,

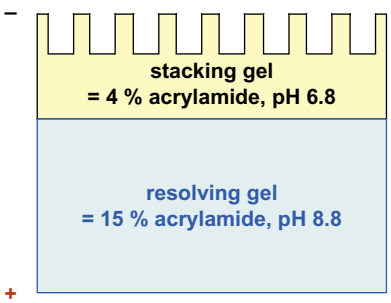


FIGURE 2.1 **SDS-polyacrylamide gel.** + and – symbols indicate positive (red) and negative (black) electrodes.

MRGSHHHHHHGSGGQNSGNQSFEE
DTEEDKPKYGQGGNIVDIDFDSVP
QIHGQNKGNQSFEEDTEKDKPKYE
HGGNIIDIDFDSVPHIHGFNKHIT
EIIEEDTNKDKPSTQFGGHNSVDF
EEDTLPKVSSGLQPSLISstop

M = start; HHHHHH = 6 His tag
RGS...GSG; SGL...LIS = vector
GQN... KVS = FnbA

FIGURE 2.2 **Amino acid sequence of His-tagged FnbA.** Letters indicate the amino acid sequence in the IUPAC single-letter code. The green *M* indicates the amino-terminal methionine. Blue letters represent vector-encoded amino acids, fuchsia letters indicate the histidine tag, and black letters indicate amino acids encoded by the *fnbA* gene.

pH 6.8, whereas the resolving gel (bottom layer) contains 15% acrylamide, pH 8.8, in the gels you use. The optimum concentration of the resolving gel depends on the sizes of the proteins that you want to resolve.

The histidine-tagged FnbA protein contains 138 amino acids and has a predicted molecular weight (MW) of 15,341 (Fig. 2.2). **Molecular weight** is a dimensionless number defined as the mass of a molecule divided by 1/12th of the mass of a ^{12}C atom [MW = relative molecular mass (M_r) = (12) × (mass of molecule)/(mass of ^{12}C)]. Do not express MW in Daltons (Da) or kiloDaltons (kDa). The *fnbA* gene sequence is in GenBank accession J04151 and in Signas et al. (1989). The "D repeat region," which is the portion of FnbA fused to the tag, begins at nucleotide 2350 and ends at nucleotide 2694.

Notes

Notes

III. PROCEDURE

A. Lyse Bacteria (Class 5)

1. Each pair of students receives 1.5 ml of IPTG-induced culture expressing histidine-tagged FnbA. Each group also receives 1.5 ml of an uninduced culture as a negative control.
2. Centrifuge each culture for 2 minutes at maximum speed (14,000 rpm) in a microcentrifuge. Discard the supernatant solution into a flask that we autoclave.
3. Label a fresh microcentrifuge tube for each culture. Write "total" and either "I" (for induced) or "U" (for uninduced) on the tubes.
4. Suspend each cell pellet in 297 μl of Tris-buffered B-PER detergent plus 3 μl of phenyl methyl sulfonyl fluoride (PMSF) solution, which is a proteinase inhibitor. **Caution: PMSF is a poison; do not allow it to contact your skin. Use gloves.** Vortex vigorously; suspend the cells completely.
5. Transfer 10 μl of each suspension to the labeled tubes from step 3 and hold on ice.
6. Centrifuge the remaining 290 μl for 5 minutes at maximum speed to separate soluble and insoluble proteins. Always place microcentrifuge tubes into a fixed-angle rotor in a specific orientation so that you know where the pellet is located, even if it is not visible. I always orient tubes so that the lid's hinge is on the outside of the rotor.
7. For each culture label a fresh tube "sol" and either "I" or "U."
8. Transfer the supernatant solutions (soluble fractions) to the tubes labeled "sol."
9. Suspend the pellet (insoluble material), which may be invisible, in 284 μl of Tris-buffered B-PER. Vortex vigorously for 1 minute.
10. Add 6 μl of lysozyme solution to the resuspended pellet. **Do not add lysozyme to the supernatant solution.**
11. Vortex vigorously for 1 minute.
12. Add 1 ml of diluted (1:10) B-PER reagent to the suspension and vortex for 1 minute.
13. Centrifuge for 10 minutes at maximum speed in a microcentrifuge.
14. Discard the supernatant solutions.
15. Repeat steps 12, 13, and 14.
16. Dissolve pellets, which may be invisible, in 290 μl of water, and label the tubes "insol-I" or "insol-U."

B. Adsorb Histadine-Tagged FnbA Protein to Nickel-Agarose (Class 5)

1. Combine the soluble and insoluble fractions from the induced culture. Do the same for the fractions from the uninduced culture.
2. Mix the nickel-chelated agarose (50% suspension) thoroughly before use. Add 100 μl of nickel-chelated agarose to each of the combined fractions.
3. Close the tubes and agitate by rocking for 10 minutes at room temperature.
4. Centrifuge for 10 seconds.
5. Transfer supernatant solutions to tubes labeled "non-adsorb-I" or "non-adsorb-U."

C. Wash Resin and Elute Histidine-Tagged FnbA Protein (Class 5)

1. Add 100 μl of wash buffer to each pellet and vortex; suspend the resin completely.
2. Incubate 5 minutes at room temperature.
3. Centrifuge for 10 seconds.
4. Discard supernatant solutions.
5. Repeat steps 1–4.
6. Add 50 μl of elution buffer to each pellet, vortex vigorously, and incubate 5 minutes at room temperature.
7. Centrifuge for 10 seconds.
8. Transfer supernatant solutions into tubes labeled "eluate 1-I" and "eluate 1-U." Store at −20°C.
9. Repeat steps 6–8. Place the second supernatant solutions in tubes labeled "eluate 2-I" and "eluate 2-U." Store at −20°C.
10. Label all tubes with your seat number. Store your samples at −20°C until next class.

D. SDS-Polyacrylamide Gel Electrophoresis (Class 6)

1. You use cast gels and a Mini-Protean II gel apparatus from BioRad. Unwrap the gel and remove the comb from the top of the gel.
2. Remove the tape from the bottom of the gel.
3. Insert the gel into the clamp; a TA will demonstrate how to do this. Two groups of students share one gel; each apparatus holds two gels.
4. Align the clamp and gel such that their tops are flush.
5. Tighten the four screws on the clamp. **Do not overtighten the screws.** Excessive pressure may distort the gel or crack the plates.
6. Install the gel and clamp into the electrophoresis apparatus. Insert the notches at the top of the clamps into the apparatus, then snap the lower ends of the clamps together.
7. Place both gels in the apparatus to form the upper chamber. Add electrophoresis buffer to the top buffer chamber. Check for leaks. If necessary, tighten the screws on the clamp to stop leaks. Fill the bottom buffer chamber.
8. Label three tubes for each culture: "nonadsorb," "eluate 1," and "eluate 2."
9. Transfer 10 μl from each of these samples to the appropriate tube.
10. Add 5 μl of SDS sample buffer to each of the 10-μl samples: "total," "nonadsorb," "eluate 1," and "eluate 2." You should have eight samples (four from each culture). BioRad gels have 15 lanes. Reserve one lane for size standards. If your team shares a gel with another group, each group has only seven lanes and must omit one sample. Omit the "nonadsorb" sample from the uninduced culture. Eluates 1 and 2 from the induced culture contain similar amounts of His-tagged FnbA protein (Fig. 2.3); omit one of these samples, if necessary.
11. Close the tubes and incubate at 95°C for 5 minutes, then cool to room temperature.
12. Centrifuge the samples for 10 seconds.

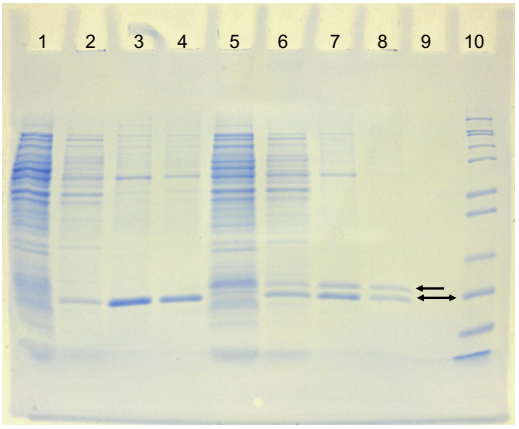


FIGURE 2.3 SDS-PAGE of His-tagged FnbA protein. Total cells (lane 1), flow through (lane 2), eluate 1 (lane 3), and eluate 2 (lane 4) from uninduced cells; total cells (lane 5), flow through (lane 6), eluate 1 (lane 7), and eluate 2 (lane 8) from induced cells; size standards (lane 10) (see Fig. 2.4). Single-headed arrow indicates His-tagged FnbA; double-headed arrow indicates lysozyme.

13. Use a micropipette with long, narrow tips (designed for loading thin gels) to transfer each sample to a different well of the SDS-polyacrylamide gel. Record the well into which you load each sample.
14. Load 15 μl of protein standards in one lane (in the center of the gel).
15. Place the gels and electrode assembly in the electrophoresis tank.
16. Place the top on the tank to connect the electrodes to the power cables.
17. Connect the leads to the power supply and adjust the voltage to a constant 200 V. The current should start at about 100 mA.
18. Turn off the power supply when the blue dye nears the bottom of the gel.

E. Stain Gel (Class 6)

1. Remove the gel sandwich from the apparatus.
2. Separate the glass plates of the gel sandwich. Cut the tape that holds the plates together and use a spatula to pry the plates apart. As the glass plates separate, let the gel stick to the bottom plate.
3. Remove the top plate, and submerge the gel and bottom plate in a tray that contains 100 ml of distilled water. Gently rock the tray until the gel floats off the bottom plate. Mechanical stress may break the gel.
4. Rock the tray gently for 5 minutes.
5. Use a pipette or an aspirator to remove the water from the tray. Do not touch the gel with your hands or allow powder from gloves to contact the gel.
6. Rinse the gel in distilled water two more times for 5 minutes each. Adequate washes are important for subsequent staining; do not reduce wash times.
7. After the last rinse, add 20 ml of Coomassie G-250 stain.
8. Agitate the tray gently overnight at room temperature. The background should remain colorless.
9. Photograph the gel for your report.

Notes

IV. LABORATORY REPORT

A. Draft (Class 8)

Include the following in your laboratory report:

1. Include the photograph of the SDS-polyacrylamide gel as a figure in your report.
2. Estimate the purity of your preparation of His-tagged FnbA protein. Did you detect contaminating proteins? How many contaminants did you see? What fraction of the protein in each eluate is FnbA?
3. Estimate the size of your purified protein. Measure the distance that each protein in the size standard migrated. On semilogarithmic graph paper (see Fig. 1.7 in Experiment 1), plot a standard curve of molecular weight (log scale) versus migration distance (linear scale). Figure 2.4 shows the size of each protein in the molecular weight standard. Measure the migration distance of His-tagged FnbA in the eluates derived from the induced culture. Use the standard curve to estimate the molecular weight of His-tagged FnbA.
4. Does the fraction you eluted from the Ni-agarose resin contain a contaminating protein that you did not observe in the total cellular protein? If so, estimate its molecular weight and speculate about its source. Could this protein be lysozyme? Look at the legend for Figure 2.4.

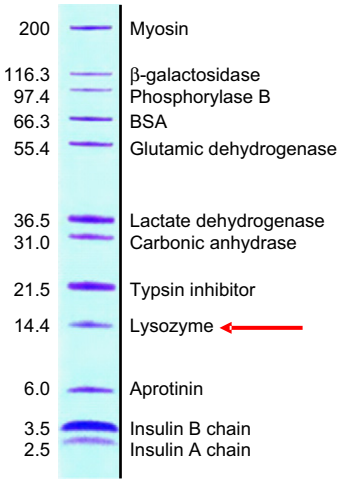


FIGURE 2.4 **Invitrogen Mark12 protein size standards.** Numbers indicate molecular weights (in thousands) of protein size standards: myosin (200,000), β-galactosidase (116,300), phosphorylase B (97,400), bovine serum albumin (66,300), glutamic dehydrogenase (55,400), lactate dehydrogenase (36,500), carbonic anhydrase (31,000), trypsin inhibitor (21,500), lysozyme (14,400), aprotinin (6,000), insulin B chain (3,500), and insulin A chain (2,500). *Source: Image adapted from Invitrogen online catalog.*

B. Questions (Class 9)

1. What is the purpose of SDS in SDS-polyacrylamide gel electrophoresis?
2. Does the apparent molecular weight of your affinity-purified His-tagged FnbA protein agree with the molecular weight predicted from the coding sequence of the gene fusion? Explain.
3. An affinity tag may or may not affect the function of the tagged protein. How could you determine whether His-tagged FnbA retained its function?
4. What are some advantages of the 6xHis tag in protein purification, compared to alternative methods? What are some disadvantages of affinity tags?

Notes

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V. CRITIQUE OF PAPER BY BUSH ET AL.

Critique of Bush, G. L., Tassin, A. M., Friden, H., and Meyer, D. I. 1991. Secretion in yeast: purification and *in vitro* translocation of chemical amounts of prepro-α-factor. *J. Biol. Chem.*, 266, 3811–3814.

1. What is the rationale for this work?

2. What is the connection between this paper and experiment 2?

3. The second paragraph of the introduction contains a number of unnecessary phrases and words. Simplify this paragraph without altering its meaning.

4. In the Results section, the authors state, "Prepro-alpha-factor (His)6 was translocated and glycosylated with approximately the same efficiency as the wild-type prepro-alpha-factor (Fig. 1B)." Do these data support this conclusion? Explain your answer.

5. Would you change the experiment depicted in Figure 1? How would you change it, and why?

6. In the last paragraph of the discussion, the authors note that the glycosylation reaction (Fig. 3) did not go to completion. They proposed that the microsomes lacked sufficient substrate to glycosylate all the preprotein added to the assays. What positive control would test whether the microsomal extracts remained active?

Notes

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EXPERIMENT

3

Polymerase Chain Reaction and DNA Sequence Analysis of Bacterial Ribosomal RNA Genes

I. INTRODUCTION

You begin experiments 3 and 4 simultaneously by collecting a bacterial population from a natural environment. In unit 3, you culture one bacterium from the population and purify genomic DNA from this organism. Use this DNA as a template for polymerase chain reaction (PCR) amplification of the 16S ribosomal RNA (rRNA) genes contained in the bacterial genome. Next, you examine your PCR products by agarose gel electrophoresis. Finally, you prepare the DNA for sequence analysis, which enables you to identify the bacterium that you isolated.

In unit 4, you use a culture-independent approach to study the entire bacterial population in your sample. You purify genomic DNA from this uncultured community and use this DNA as a template for PCR amplification of the 16S rRNA genes contained in this bacterial metagenome. The introduction to unit 4 outlines this procedure.

II. BACKGROUND

The sequences of 16S rRNA genes provide a means to identify bacterial groups. The 16S rRNA is necessary for protein translation. This gene has several features that make it ideal for identification of bacterial groups and study of bacterial evolution. Several sites within the gene are nearly identical over a broad range of bacterial groups, and these sites flank variable regions shared by smaller groups (Fig. 3.1). The highly conserved regions

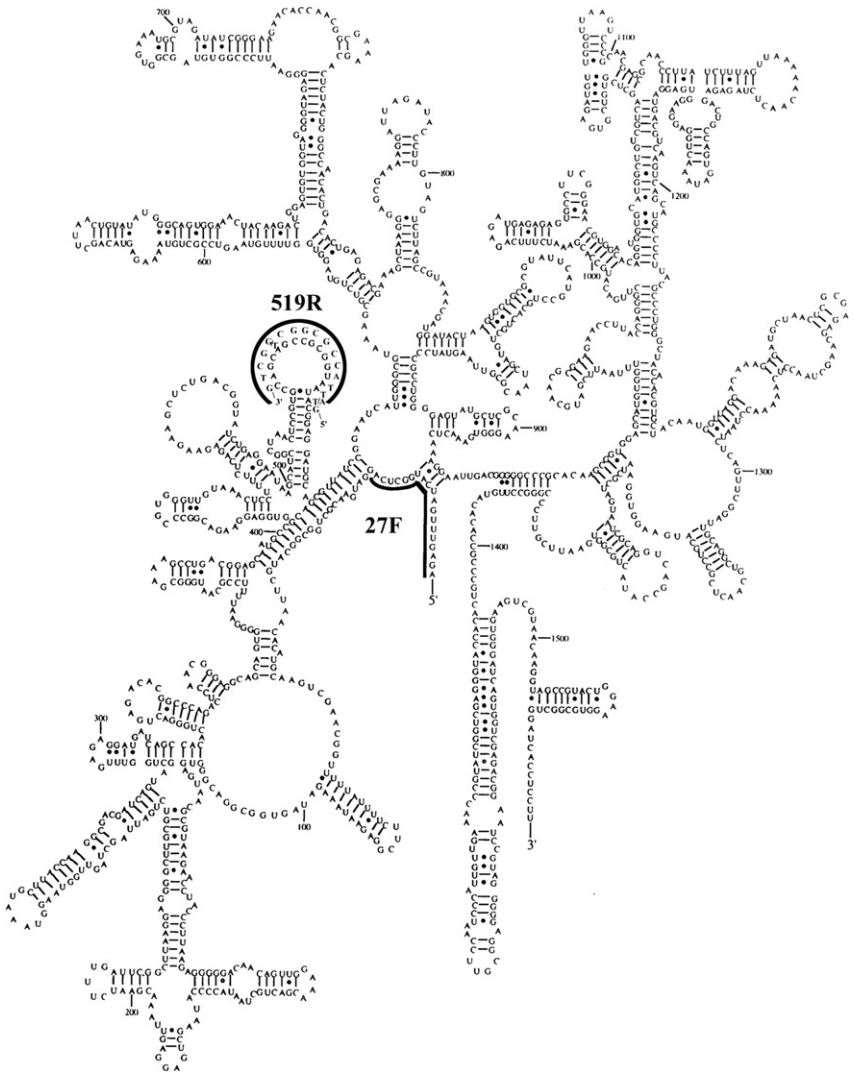

FIGURE 3.1 **Proposed secondary structure for a 16S rRNA.** Bars labeled 27F and 519R indicate the PCR primers used in Experiment 3. *Source: Adapted with permission from M. S. Rappe, M. T. Suzuki, K. L. Vergin, and S. J. Giovannoni. 1998. Phylogenetic diversity of ultraplankton plastid small-subunit rRNA genes recovered in environmental nucleic acid samples from the Pacific and Atlantic coasts of the United States. Appl. Environ. Microbiol. 64, 294–330.*

serve as priming sites for PCR amplification of more variable sequences that lie between two conserved sites. Primers based on these highly conserved sites can prime PCR amplification of 16S rRNA genes from most bacteria. The sequence of the variable regions of this PCR product identifies groups.

DNA amplification by PCR results in rapid in vitro synthesis of many copies of a specific DNA sequence from a large population of different DNA molecules (Fig. 3.2) (Saiki et al., 1988). The reaction contains template DNA (in this case an entire bacterial genome), heat-stable *Taq* DNA polymerase (from *Thermus aquaticus*), deoxynucleoside triphosphates (dNTPs), magnesium, buffer, and two primer oligonucleotides. One primer complements a region upstream of the sequence we want to amplify; the other primer complements a region on the opposite strand downstream of this sequence (Fig. 3.2). Heating the PCR reaction mixture to 94°C denatures the template DNA, then cooling the reaction allows the primer oligonucleotides to anneal to their target sequences. Next, we heat the reaction to 72°C, which is the optimum temperature for *Taq* DNA polymerase activity. We repeat this cycle 35 times for our study.

During the first cycle, the primers anneal to the target sequence, and DNA polymerase extends each primer beyond the other primer-binding site (Fig. 3.2). In the second cycle, these new strands become templates, generating strands running from primer to primer. Therefore, the final amplification product contains DNA molecules of a specific length. In theory, 35 cycles of amplification can produce 8,589,934,592 (2^{33}) copies from a single template molecule; the first two cycles do not produce products of a specific length.

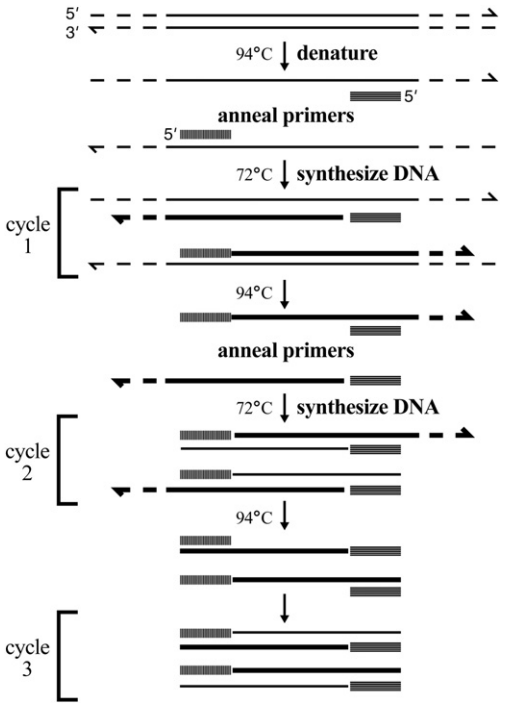

FIGURE 3.2 **Polymerase chain reaction.** The diagram shows the first three cycles of a standard PCR reaction.

Although some investigators have detected a single copy of template DNA, PCR reactions never yield the theoretical maximum amount of DNA.

Because a single molecule of contaminating DNA can amplify many times, you must take precautions to eliminate extraneous DNA. Use clean gloves, a clean work surface, pipette tips with aerosol barriers, and pipettors reserved only to prepare PCR reactions. Prepare PCR cocktails in a work area used only for that purpose; extract template DNA and process PCR products elsewhere. Shanks et al. (2005) and Leonard et al. (2007) recommend strategies to avoid extraneous DNA in PCR reactions. Always perform at least one negative control reaction with no added DNA for **each** reaction that contains template. The PCR kit we use contains template and primers for a positive control reaction, which allows you to verify that the DNA polymerase is active and the dNTPs are not degraded.

DNA sequencing reactions require purified template DNAs free of PCR reagents and primers. You will use cartridges that contain a silica matrix (Fig. 3.3) to separate your PCR product from the rest of the reaction cocktail. DNA binds silica (glass) in the presence of high salt, whereas the other components of a PCR reaction do not. This will allow

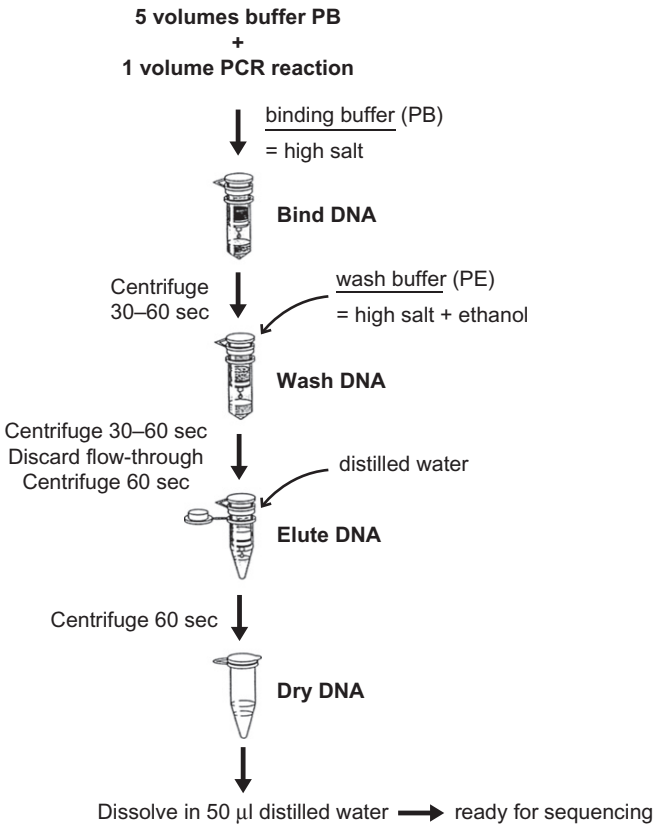

FIGURE 3.3 Purification of PCR products with a Qiaquick cartridge. *Source: Figure adapted from Qiagen online catalog.*

you to bind your PCR product to the silica matrix in the cartridge and wash away other components of the PCR reaction with a high-salt buffer. You will then elute your purified DNA with distilled water.

The "dideoxy" method of DNA sequencing is similar to PCR, but several important differences distinguish these procedures. First, the template for DNA synthesis is a specific DNA molecule, not a complex mixture. Second, a sequencing reaction uses only one primer oligonucleotide. Third, sequencing reactions contain both standard deoxynucleoside triphosphates (dNTPs) and dideoxynucleoside triphosphates (ddNTPs; Fig. 3.4), which lack the 3′-hydroxyl group required for continued DNA synthesis (Fig. 3.5). Incorporation of a ddNTP into a DNA chain prevents further DNA synthesis because the dideoxynucleotide lacks a 3′-OH group to form a phosphodiester bond with the 5′-phosphate of the next dNTP (Fig. 3.5). Finally, either the primer or ddNTPs ("terminators") contain fluorescent labels (Fig. 3.6).

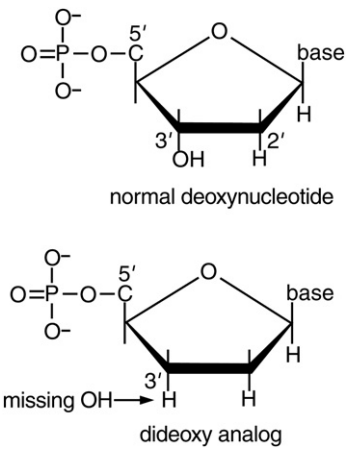

FIGURE 3.4 Structures of normal and dideoxy nucleotides.

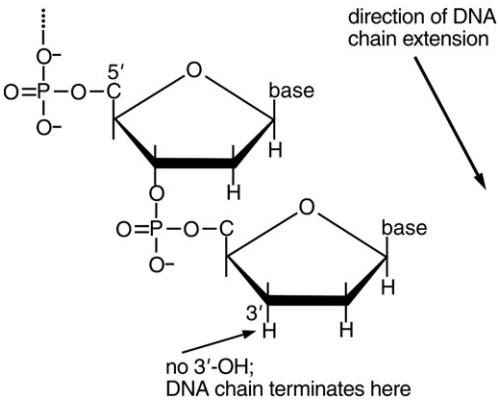

FIGURE 3.5 DNA chain termination by dideoxynucleotide incorporation.

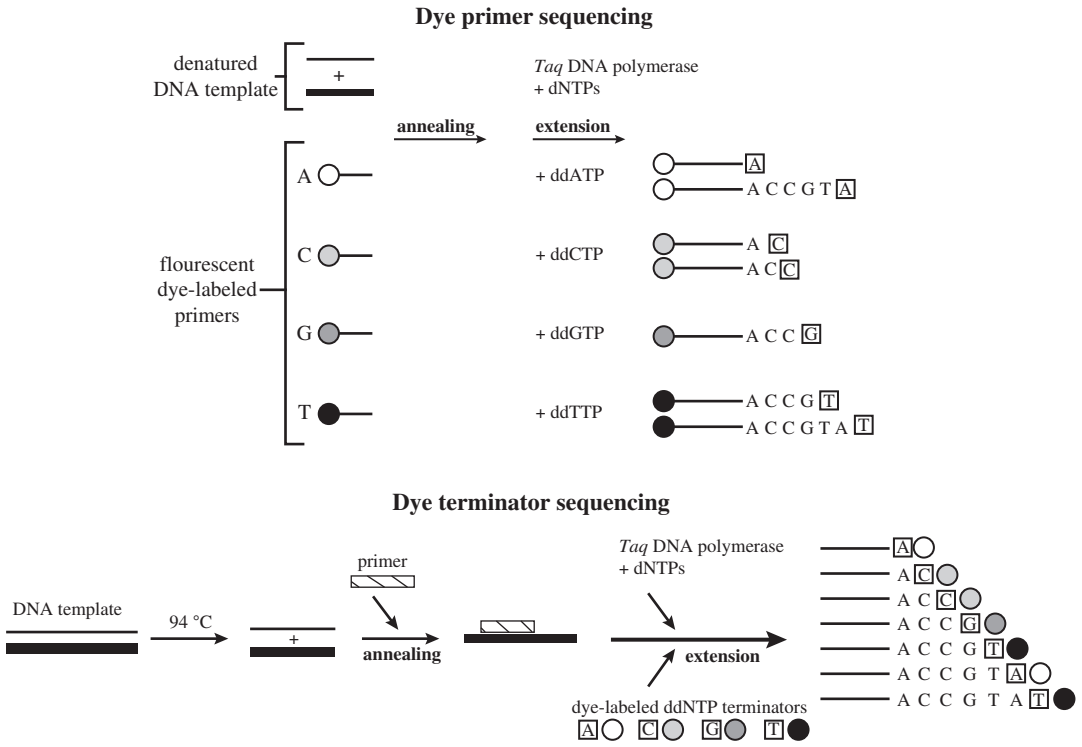

FIGURE 3.6 Dye-primer and dye-terminator DNA sequencing.

In the "dye-terminator" method that we use, each ddNTP contains a fluor that emits a different wavelength (color) of light (Fig. 3.6). The ratio of dNTPs to ddNTPs allows adequate DNA synthesis, but very few reactions continue to the end of the template. This produces a population of DNA molecules that vary in length and extend from the primer at the 5′ end to a labeled ddNTP at the 3′ end. Ideally, the shortest molecule contains the primer with a single ddNTP added to the 3′ end. The synthesis products should also contain molecules that terminated at the second, third, fourth, and fifth incorporated base, and so on, to the end of the template (Fig. 3.6). The sequencing laboratory separates the newly synthesized DNAs according to their length by capillary electrophoresis, which resolves single-stranded DNA molecules that differ in length by a single nucleotide. As each DNA band passes a light source–detector, the sequencer records the color and intensity of the bands and plots a four-color electropherogram (Fig. 3.7).

The success of a sequencing reaction depends on the reagents, primer, reaction conditions, and the amount, length, sequence, and quality of the template. An excellent sequencing reaction yields 900 nucleotides of readable sequence; many sequencing facilities provide about 600 bases of sequence from a typical reaction.

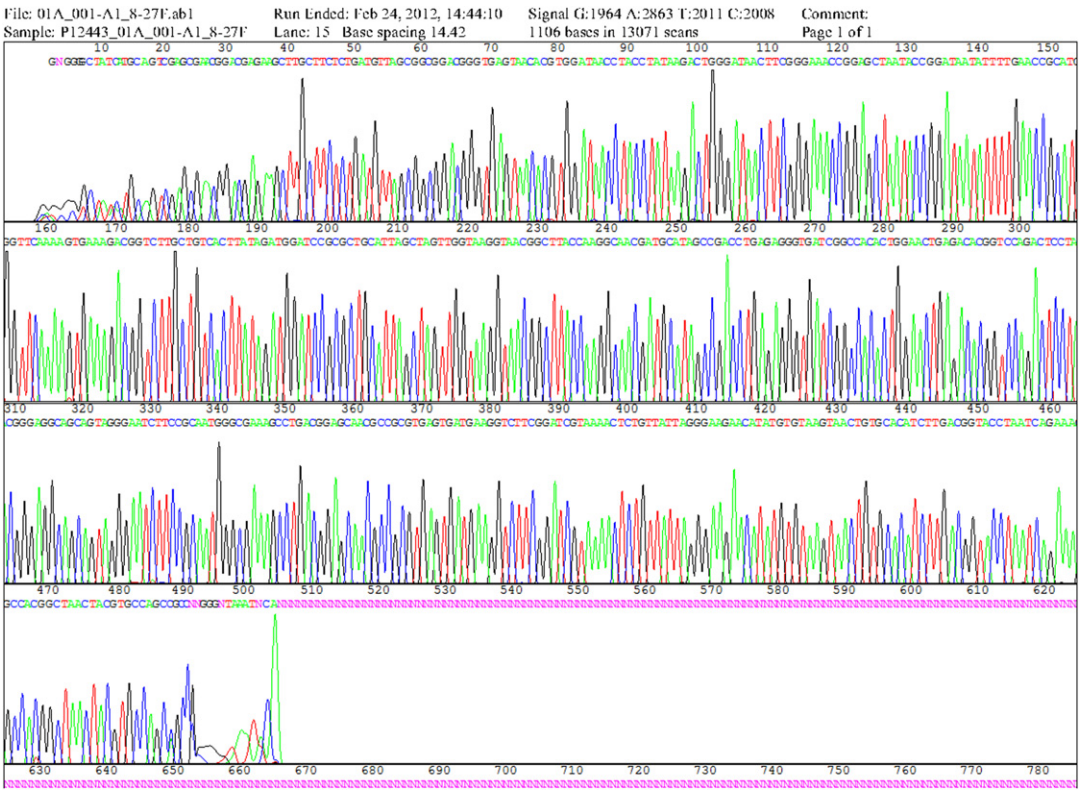

FIGURE 3.7 Partial sequence of 16S rRNA gene from *Staphylococcus aureus* cultured from human saliva.

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III. PROCEDURE

Collect a sample from the environment specified in the top-ranked proposal from your team (class 6); the author of the proposal provides specific instructions. From the population of bacteria that you isolate, select one species to grow in pure culture. You purify genomic DNA from these cells and use the DNA as a template for PCR.

Some of the bacterial species in your sample may **not** grow on LB agar. Other species may grow poorly on this medium; avoid isolates that do not grow vigorously. Consider how the inability to culture all of the bacteria from a particular environment affects interpretation of your data. The culture-based approach you use in unit 3 is not the approach commonly used to study bacterial populations. Most microbiologists studying bacterial populations in natural environments do not attempt to grow bacteria in culture. Instead, they extract DNA directly from the mixture of bacteria present in the sample; you will use this approach in unit 4.

A. Sample Collection for Experiments 3 and 4 (Class 7)

1. Label an LB agar plate and a Bead Solution tube (from the Mo Bio Ultra Clean Soil DNA Isolation Kit) with your seat number.
2. Use a sterile BD BBL Culture Swab EZ to collect the sample. If you sample a dry surface, first submerge the swab head in 0.5 ml of sterile distilled water. If you want to study a soil sample, add 0.25 grams of soil directly to the Bead Solution tube.

   Remove the swab from its container, collect the sample, and streak it across one edge of the LB agar. Next, insert the swab head into the Bead Solution tube and swirl it vigorously for 15 seconds. Return to the lab and complete streaking the plate with sterile toothpicks. Make three additional streaks, each with a fresh toothpick; this creates three "dilutions" of your original sample. Work from one edge of the original streak to make the second streak, make the third streak from the edge of the second, and so forth.
3. Incubate the LB agar plate at an appropriate temperature (25°C, 30°C, or 37°C). Bacteria from soil or water probably grow best at room temperature or 25°C; incubate samples from your body (or an animal) at 37°C. Incubate the plate overnight and **examine it the next day**.
4. **One day after class 7.** Write a brief description of the bacterial (and fungal) colonies on the plate. Note the color, shape, size, and abundance of each colony type. Choose two different isolated bacterial colonies, and use sterile toothpicks to streak each on fresh LB agar. The teaching assistants provide sterile toothpicks and a container for used toothpicks in each growth room. Work in the growth room; another class may be in the laboratory. Use at least three toothpicks for each colony so that you can spread the cells enough to obtain single colonies. Incubate the streaked plates overnight at the proper temperature. **Two days after class 7,** the teaching assistants move streaked plates that show bacterial growth to the cold room.

   Handle these cultures (and all subcultures) as though they are human pathogens. They may be pathogenic, particularly if you sampled an environment conducive to

growth of human pathogens. Avoid all contact with the bacteria, and autoclave all cultures, supernatant solutions, and contaminated tubes, pipettes, and tips.

5. **Class 8.** Use an isolated colony from each streak to inoculate LB broth.
   a. Label two sterile test tubes with the sample source and your seat number.
   b. Pipette 3 ml of LB broth into each tube.
   c. Use aseptic technique to inoculate each tube with an isolated colony from the streaked plates. You may use sterile inoculating sticks, toothpicks, or a loop. (I prefer sticks.) Note the locations of the two colonies you picked, and leave a portion of each colony on the plate so that you can transfer the cells to a microscope slide (see section B).
   d. Incubate your cultures overnight at the appropriate temperature with vigorous aeration (shaking). The **next day,** the TAs move the cultures that show bacterial growth to the cold room. During class 9**,** you will freeze 1 ml of one culture and prepare genomic DNA from 1.5 ml of culture.

B. Gram Stain and Light Microscopy (Class 8)

1. Prepare smears on clean microscope slides using a few cells from the isolated colonies that you inoculated into broth.
2. Air dry and heat fix the smears.
3. Place the slides in a staining rack and immerse them in crystal violet for 1 minute, then rinse the slide with distilled water and drain.
4. Immerse the slides in iodine for 1 minute, then rinse and blot dry.
5. Decolorize with 95% ethanol for 15 seconds, then rinse with distilled water and drain.
6. Counterstain with safranin for 20 to 30 seconds, then rinse, blot dry, and examine under a 100× oil-immersion lens. Photograph the bacterial cells: Place a digital camera near the ocular lens to record the image. We used this method to capture the image on the cover of this book. If you do not have a digital camera, your cell phone camera will suffice. Describe (in writing) the morphology of the bacteria; draw diagrams if necessary. Are they rods, cocci, or another shape? Do the cells form filaments or clusters? How large are the cells? Did the bacteria take the Gram stain? Leave this writing assignment on your bench at the end of class.

   Focusing a light microscope involves several steps. Place the slide on the stage and move it so that a promising area is over the light source. Move the low-power (10×) objective lens close to the slide. Raise the lens with the coarse adjustment knob until the specimen comes into focus. Put a drop of oil directly over the light path through the slide. Shift the oil-immersion lens (100×) into place; the lens should barely touch the oil on the slide. Use the fine adjustment knob to focus on the specimen. Use the stage control knobs to find optimal viewing areas. Clean the oil immersion lens with lens paper when you finish.
7. If you isolated bacteria from soil, stain a sample with malachite green to look for spores. First, smear bacteria on a clean microscope slide, then air dry and heat fix the smears.

8. Place the slides over a beaker of boiling water and cover the specimen with malachite green. Steam for 5 minutes. Add additional stain if it boils off.
9. Wait until the slide cools, then rinse with distilled water for 30 seconds.
10. Counterstain with safranin for 20 seconds, then rinse with distilled water, blot dry, and observe under an oil-immersion lens. Spores appear intense green whereas cells stain red.

C. Freeze Cultures (Class 9)

You complete experiment 3 with one isolate. Choose your favorite culture. Pick a culture that grew to high density, and avoid cultures that form clumps of cells. Transfer 1 ml of culture to a sterile 1-dram, screw-cap vial that contains 0.2 ml of dimethylsulfoxide (DMSO). Shake the vial to mix the culture with the DMSO. Label the vial with your name, seat number, and sample identification. Lab tape adheres to a glass vial in the freezer if you wrap it around the entire vial so that the ends overlap. Place the sample in the freezer box, which we store at −80°C. Note the location of your specimen in the box. The cultures remain viable indefinitely. If necessary, you can start a fresh culture by inoculating broth with a small chunk of frozen culture scraped from the frozen stock with a sterile stick. Do not allow the freezer stock to thaw. Place the remaining culture in a labeled 1.5 ml microcentrifuge tube and purify genomic DNA from these cells (see section D).

D. Purify Genomic DNA (Class 9)

1. Transfer 1.5 ml of your culture to a 1.5 ml microcentrifuge tube; label the tube with your seat number. You prepare DNA from only one of your isolates.
2. Centrifuge at maximum speed for 1 minute at room temperature. Discard the supernatant solution in a container that we will autoclave.
3. Suspend the cells in 450 μl of 25 mM Tris + 10 mM EDTA, pH 8; **disperse the pellet completely**.
4. Add 20 μl of lysozyme (30 mg/ml); dissolve lysozyme in 25 mM Tris, pH 8 immediately before use. Incubate 20 minutes at 37°C.
5. Add 10 μl of proteinase K (50 mg/ml; dissolve in distilled water immediately before use); incubate 20 minutes at 50°C.
6. Add 20 μl of 25% SDS; incubate 10 minutes at 68°C.
7. Add 57 μl of 5 M NaCl; vortex at full power for 1 minute.
8. Incubate 5 minutes at 68°C; vortex at full power for 1 minute.
9. Add 0.5 ml phenol−chloroform−isoamyl alcohol (25:24:1) equilibrated with 1 M Tris, pH 8. Mix well. Avoid all contact with these organic solvents, and do not inhale vapors; work in a fume hood. Lab coats, gloves, eye protection, closed shoes (no sandals), and long pants (no shorts) are required. If phenol contacts skin, wash thoroughly with water and seek medical attention.
10. Centrifuge at maximum speed in a microcentrifuge for 5 minutes at room temperature.

11. Transfer the aqueous (top) phase into a clean 1.5 ml microcentrifuge tube that contains 0.5 ml of chloroform–isoamyl alcohol (24:1); label the tube with your seat number. As you remove the top phase, take care to leave proteins and other cell debris at the interphase. DNA attached to the membrane will tug at material in the interphase as you withdraw the aqueous phase. Discard organic solvents in an organic waste container.
12. Mix well. Centrifuge at maximum speed in a microcentrifuge for 2 minutes at room temperature.
13. Transfer the aqueous (top) phase into a clean 1.5 ml microcentrifuge tube; label the tube with your seat number. As you remove the top phase, leave proteins and other cell debris at the interphase. Discard organic solvents (bottom phase) and interphase in an organic waste container.
14. Add 1 ml of ethanol (ice cold), mix thoroughly, and hold at 0°C or 4°C for 15 minutes.
15. Centrifuge 5 minutes at maximum speed in a microcentrifuge; orient the tube in the centrifuge rotor so that you know where the pellet will form. Discard the supernatant solution.
16. Wash the DNA–RNA pellet with 0.5 ml of 70% ethanol. Let the tube stand for 1 minute; do not disturb the pellet.
17. Centrifuge at maximum speed for 2 minutes at room temperature. Discard supernatant solution carefully; the pellet will not stick tightly to the tube and may come loose.
18. Centrifuge the tube for 10 seconds to bring residual ethanol to the bottom of the tube. Use a clean, sterile yellow pipette tip to remove all ethanol, which inhibits PCR. Avoid the pellet. Orient the tube in the centrifuge rotor so that you know where the pellet will form. Place tubes with lids open in Speed Vac (a heated low-speed centrifuge connected to a vacuum system) for 5 minutes or air dry the pellet.
19. Dissolve the DNA–RNA pellet in 50 μl of DNA buffer. Ensure that the label on your tube is legible. Store frozen at −20°C in the box provided by the teaching assistants.

E. PCR Amplification of 16S rRNA Genes (Class 10)

1. The teaching assistants supply each student with a tube that contains 190 μl of PCR reaction mixture, which includes all components except template DNA. When you set up your PCR reaction, wear clean gloves and use pipette tips that have aerosol barrier filters. P20 and P200 pipettors require different barrier tips. These precautions help you avoid contaminating your PCR reactions with extraneous DNA.
2. Place 95 μl of reaction mixture in each of two 0.2-ml PCR reaction tubes. Handle these tubes gently; they are fragile because they have thin walls to permit rapid heat transfer. Label these tubes at the top of the conical portion. Do not label the lid; markings on the lid will burn off during PCR.
3. Add 5 μl of template DNA to one PCR reaction mixture and mix well. Add 5 μl of sterile distilled water to the other PCR reaction and mix well; this is your no-template

control, which allows you to detect extraneous DNA in the PCR reagents. Store these tubes on ice until the entire class is ready to load the PCR machine.

Each PCR reaction should contain

10× buffer	10 μl
2 mM (each) dNTP mix	10 μl
25 mM $MgCl_2$	6 μl
50% acetamide[1]	10 μl
27F primer, 10 μM	2 μl
519R primer, 10 μM	2 μl
Taq DNA polymerase	0.5 μl (2.5 units)
template DNA	100 ng (about 1–10 μl)
water	amount required to bring total volume to 100 μl

Primer sequences:

8-27F: 5′ AGA GTT TGA TCM TGG CTC AG 3′ (M = A or C) (MW = 6,161)

536-519R: 5′ GWA TTA CCG CGG CKG CTG 3′ (W = A or T and K = G or T)(MW = 5,525)

The *E. coli* 16S rRNA gene is 1542 bp long. Most other organisms have 16S rRNA genes of similar length (±50 bp). Primer numbers refer to coordinates in the *E. coli* 16S rRNA gene (*rrsH*). This PCR primer set produces a 529-bp product from an *E. coli* template; other species produce amplicons of similar lengths. The region between coordinates 8 and 536 contains sufficient variability to distinguish most bacterial species.

4. Most PCR thermal cyclers, including the ones we use, have a heated lid, which prevents condensation of water on the lid of the PCR reaction tube. This obviates the need to overlay the sample with mineral oil, which is used to prevent sample evaporation in machines without a heated lid.
5. Cycle at 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute. Repeat for 35 cycles. Place your tubes in the heat block at the positions designated by the teaching assistants. The TAs will remove the reactions on completion and store them frozen until the next lab period.

F. Purify PCR Product (Class 11)

1. Transfer 10 μl of each completed PCR reaction into a clean microcentrifuge tube labeled with your seat number. Store the samples at 4°C in the box provided by the teaching assistants. Save samples from your PCR reaction and the no-template control. You will

[1]Acetamide helps maintain single-stranded DNA and is useful for templates with high GC contents, which is typical for 16S rRNA genes.

examine these samples by agarose gel electrophoresis to estimate the yield of amplified DNA and to see whether the no-template control indicates the presence of contaminating DNA in the reagents.

2. DNA sequence analysis of a PCR product requires removal of the other components of the PCR reaction. We use cartridges (from Qiagen) that contain a silica membrane, which binds DNA in the presence of a high concentration of a chaotropic salt (a proprietary reagent, called *buffer PB*, supplied with the kit).
3. Mix the completed PCR reaction with 5 volumes (0.5 ml) of buffer PB. Pipette the mixture into the cartridge; label the cartridge with your seat number. Centrifuge at full speed for 1 minute; discard the filtrate.
4. Add 750 μl of buffer PE (a proprietary wash buffer containing 70% ethanol). Centrifuge 1 minute; discard the filtrate.
5. Centrifuge again for 1 minute to remove all traces of ethanol.
6. Transfer the cartridge to a fresh microcentrifuge tube labeled with your seat number. Add 50 μl of elution buffer (10 mM Tris, pH 7.5). Pipette the elution buffer onto the center of the membrane. Let the cartridge stand for 5 minutes at room temperature. Centrifuge 1 minute.
7. Place samples in the Speed Vac for 30 minutes to remove all traces of ethanol. Bring the volume to 50 μl with distilled water.

G. Electrophoretic Analysis of PCR Product (Class 12)

1. Remove 5 μl of purified PCR product to estimate yield by agarose gel electrophoresis. Examine 10 μl (each) of the unpurified PCR reaction and the no-template control reaction. Include a known quantity (470 ng in 4 μl) of the low DNA mass ladder (Invitrogen) in separate lanes so that you can estimate the amount and size of your PCR product. Mix the DNA samples with 1 μl of loading solution (50% glycerol + 0.05% bromophenol blue). Include the two 10 μl samples (unpurified PCR product and no-template control PCR reaction) you removed prior to purification. Load your samples in the lanes assigned to you; on the gel-loading sheet, record the samples you loaded in each lane.
2. Cast and load a 2% agarose gel (use 3:1 agarose from ISC Bio Express) for electrophoresis essentially as described in Experiment 1, section III.C. The PCR products you want to analyze in this unit are smaller than the restriction fragments you studied previously. For optimum resolution of shorter DNA fragments, use a higher percentage of agarose (2% instead of 0.8%) and a different type of agarose ("3:1" instead of standard).

H. DNA Sequence Analysis (Class 14)

A commercial laboratory performs the DNA sequence analysis; currently, we use Functional Biosciences in Madison, Wisconsin (http://functionalbio.com). To sequence a 500-bp PCR product once, they need 500 ng of purified template DNA in 10 μl of water or 10 mM Tris. Normally, you would sequence both strands of the DNA molecule to ensure

that the sequence is correct. To sequence both strands of the PCR product, you would supply the sequencing facility with 10 μl (per template) of each primer (8-27F and 536-519R) at 10 μM. To save money, we sequence only one strand of each template using the 8-27F primer.

You examine the DNA sequences manually to look for ambiguities. If you had sequences from both strands, you use the sequence of one strand to resolve ambiguities in the other. After you have confirmed and corrected the sequence, you perform a computer search of the GenBank database to identify the bacterium you isolated. The BLAST (Basic Local Alignment Search Tool) program can search public databases for DNA or protein sequences from any source.

Remove the reverse complement of the 536-519R sequence before you perform the BLAST search. Bases in red are the reverse complement of the 536-519R primer, which will appear at the 3′ end of the sequence because we used the 8-27F primer for sequencing.

```
Reverse Complement: 5′-CAG C(A/C)G CCG CGG TAA T(A/T)C-3′
536-519R: 3′-GTC G(T/G)C GGC GCC ATT A(T/A)G-5′
```

Do **not** include this portion in the sequence you select for the BLAST search. If your sequence has questionable bases (shown in lowercase letters) or ambiguous bases (shown as *Ns*) near the beginning (5′ end), omit that region from your BLAST search. Delete **all** sequences from the beginning through the last questionable base. Sequences may have untemplated *As* at the 3′ end. This is the basis for topoisomerase-mediated ligation of a PCR product to "TOPO-TA" plasmid DNA; you will use this method in experiment 4. Delete untemplated *As* from the 3′ end along with the reverse complement of the 519R primer. Sequences in this size range (~530 bases) rarely have questionable or ambiguous bases near the 3′ end unless the overall sequence quality is poor. If necessary, remove **all** questionable sequence near the 3′ end. See the following examples for guidance. Underlined type indicates questionable bases (at the 5′ end of unedited sequence >12) or the reverse complement of the 536-519R primer (at the 3′ ends of both unedited sequences). In both examples, the 3′ end of the 536-519R primer is absent.

These sequences are in FASTA format. The first line begins with a "greater than" symbol (>) and contains information that identifies the sample. In this case, the student's seat number (47 or 12) and the sequencing primer (8-27F) identify the samples. Subsequent lines contain the sequence, which is printed 5′ to 3′. You may enter multiple sequences into the BLAST search window simultaneously, provided they are in FASTA format with a blank line between each sequence. The BLAST program aligns each sequence with the closest matches in the database, and it presents a drop-down list that allows you to view each sequence alignment individually.

Before editing,

```
>47_827F TRIM QUALITY: 20
CCAGCTTGCTGGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTAACTCTGGGATAAGCCTGGGAAAC
TGGGTCTAATACCGGATAGGAGCGCCGACCGCATGGTGGGTGTTGGAAAGATTTATCGGTTTTGGATGGACTCGCGGCCTATC
AGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGA
TGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAA
GCACCGGCTAACTACGTGCCAGcagCCGCGGTAAT
```

After editing,

```
>47_827F TRIM QUALITY: 20
CCAGCTTGCTGGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCTTAACTCTGGGATAAGCCTGGGAAAC
TGGGTCTAATACCGGATAGGAGCGCCGACCGCATGGTGGGTGTTGGAAAGATTTATCGGTTTTGGATGGACTCGCGGCCTATC
AGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGA
TGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAA
GCACCGGCTAACTACGTGC
```

Before editing,

```
>12_827F TRIM QUALITY: 20
AGagaaGCTTGCTTCTTGGGTGGCGAGTGGCGAACGGGTGAGTAATATATCGGAACGTACCGAGTAATGGGGGATAACTAATC
GAAAGATTAGCTAATACCGCATATTCTCTGAGGAGGAAAGCAGGGGACCTTCGGGCCTTGCGTTATTCGAGCGGCCGATATCT
GATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCAGTAGCGGGTCTGAGAGGATGATCCGCCACACTGGGACTG
AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGT
CTGAAGAAGGCCTTCGGGTTGTAAAGGACTTTTGTCAGGGAAGAAAAGGCTGTTGCTAATATCGACAG
CTGATGACGGTACCTGAAGAATAAGCACCGGCTAACTACGTGCCAG
```

After editing,

```
>12_827F TRIM QUALITY: 20
GCTTGCTTCTTGGGTGGCGAGTGGCGAACGGGTGAGTAATATATCGGAACGTACCGAGTAATGGGGGATAACTAATCGAAAGA
TTAGCTAATACCGCATATTCTCTGAGGAGGAAAGCAGGGGACCTTCGGGCCTTGCGTTATTCGAGCGGCCGATATCTGATTAG
CTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCAGTAGCGGGTCTGAGAGGATGATCCGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTCTGAAG
AAGGCCTTCGGGTTGTAAAGGACTTTTGTCAGGGAAGAAAAGGCTGTTGCTAATATCGACAGCTGATGACGGTACCTGAAGAA
TAAGCACCGGCTAACTACGTGC
```

To perform a BLAST search,

1. Access the National Center for Biotechnology Information (NCBI) website at www.ncbi.nlm.nih.gov/SNP/ and select BLAST, or access BLAST directly at http://blast.ncbi.nlm.nih.gov/Blast.cgi. You can also use the NCBI website to search PubMed and many other databases.
2. Select "nucleotide BLAST."
3. Paste your edited sequence into the search window labeled "Enter accession number(s), gi(s), or FASTA sequence(s)" (Fig. 3.8). To do this,
   a. Highlight your sequence in the Word file.
   b. Press "Ctrl-C" to copy your sequence.
   c. Put the cursor in the search window at the BLAST site and press "Ctrl-V" to paste your sequence into the search window.
4. Reset the database to "Nucleotide collection (nr/nt)."
5. Select the box to **exclude** "uncultured/environmental sample sequences."
6. Leave the "Optimize for" button set to "Highly similar sequences (megablast)."
7. Select BLAST.
8. Select "formatting options" at the top of the next window that appears (Fig. 3.9).
9. Find "Limit Results" on the next window that appears.
10. Set the "Descriptions" drop-down menu to 10.
11. Set the "Graphical overview" drop-down menu to 0.
12. Set the "Alignments" drop-down menu to 10.
13. Select "View Report." Wait for the list of sequences producing significant alignments to appear (Fig. 3.10); scroll down to view the aligned sequences (Fig. 3.11).

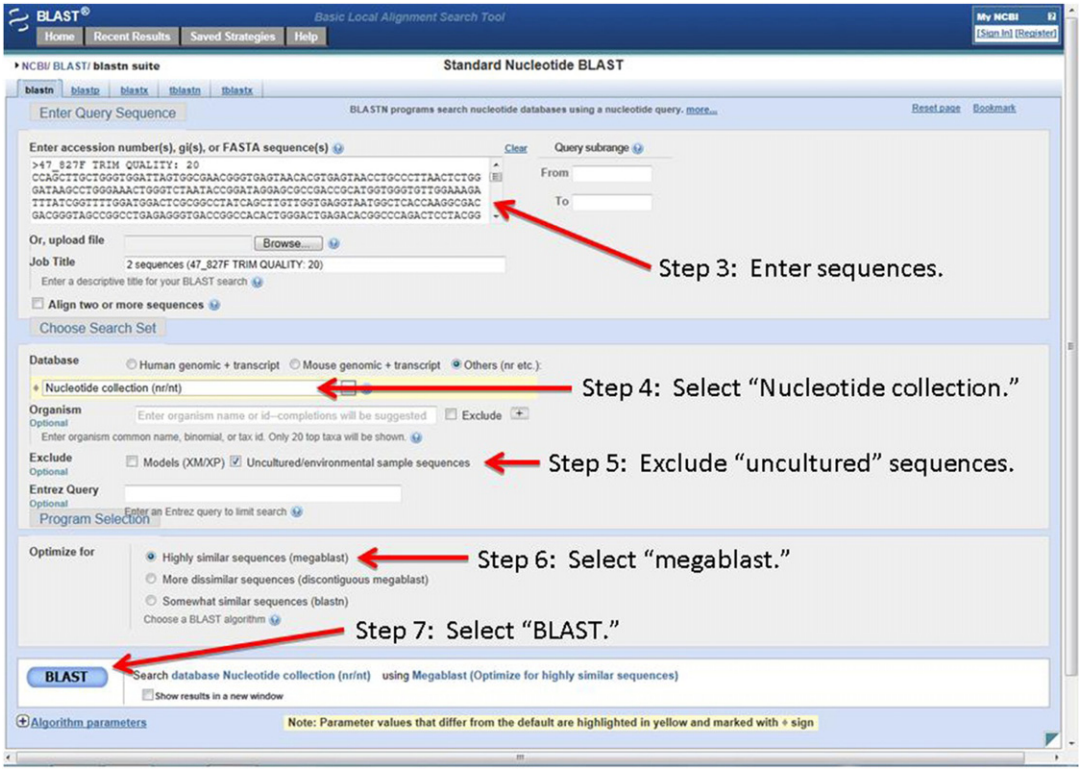

FIGURE 3.8 **BLAST search window.**

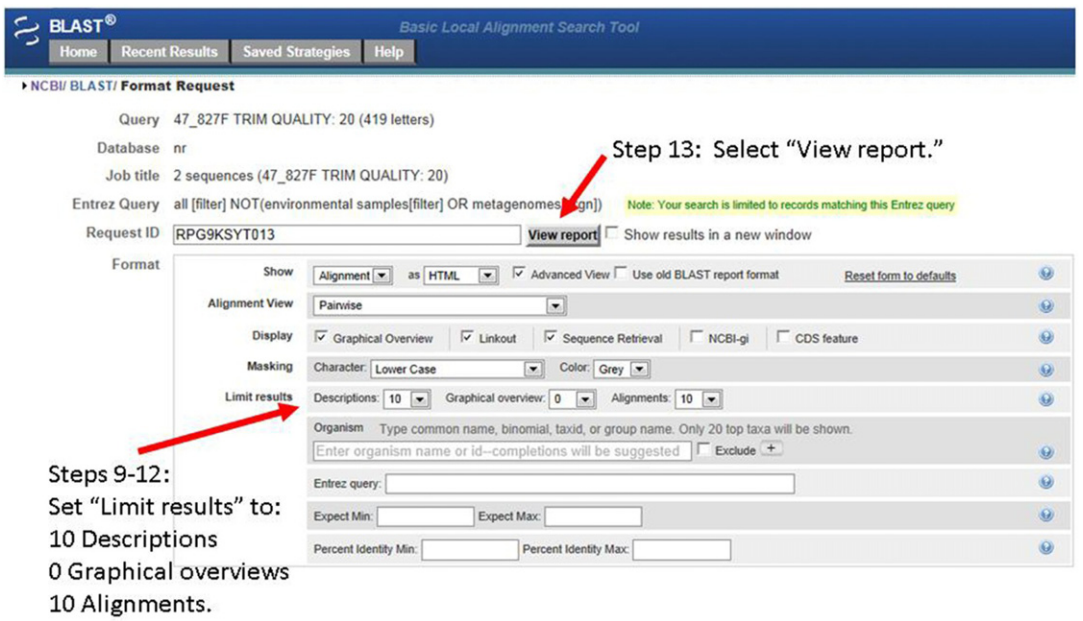

FIGURE 3.9 **BLAST formatting options window.**

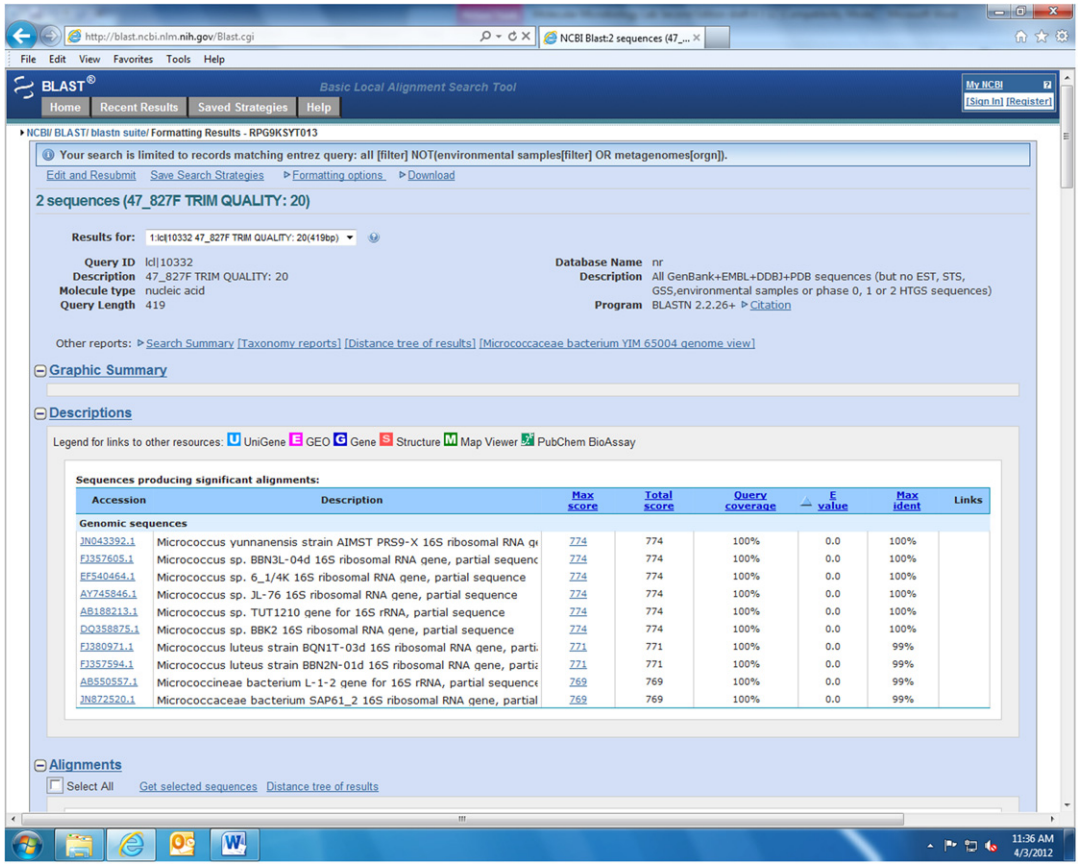

FIGURE 3.10 **BLAST results window.**

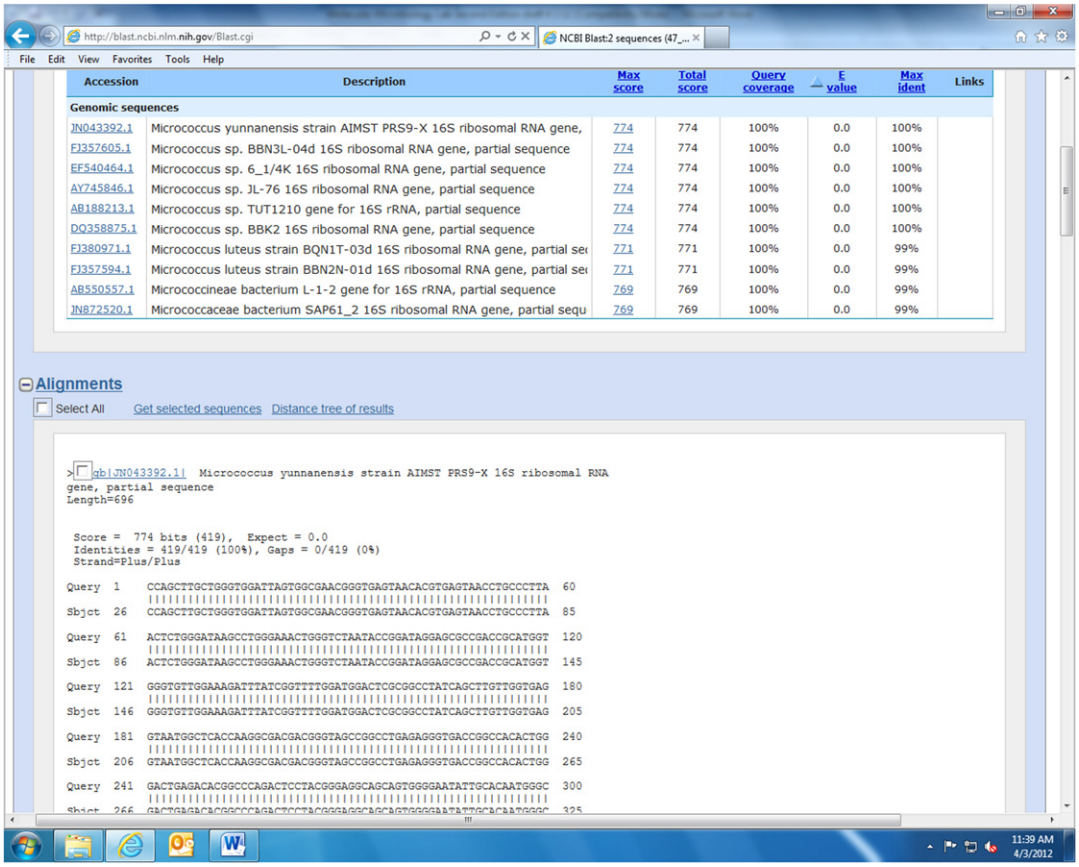

FIGURE 3.11 **BLAST alignments.**

Notes

Notes

IV. LABORATORY REPORT [CLASS 16 (DRAFT) AND CLASS 17]

Include the following in your laboratory report:

1. Describe the source of your bacterium and the colony types on the initial streak plate. How many different colony types did you see based on color and morphology? How rapidly did they grow under the conditions you used?
2. Discuss the results of your microscopy. Include a photograph of the bacterium you used to isolate DNA.
3. Was your PCR amplification successful? How many different PCR products did you detect by gel electrophoresis? How many did you expect? Did you see any products in the "no-template" negative control? If your PCR reaction did not produce a detectable product, discuss possible explanations.
4. Use semilogarithmic graph paper (see Experiment 1) or Excel to plot base pairs (log scale; Y axis) versus distance migrated (linear scale; X axis) for the "low DNA mass ladder" (Fig. 3.12) that you included on your gel. Include this graph in the results section; see Fig. 1.6 for an example. Measure the distances migrated from the bottom of the wells to the front edges of the bands; see Fig. 1.5 for an example. Estimate the size of your PCR product by comparing its migration distance to the standard curve. Indicate on your curve where the PCR product migrated. Include a photograph of your gel. Label each lane, and indicate the contents in the figure legend. If your gel did not work, use one from another group or from Fig. 3.13.
5. Estimate the quantity of product in your PCR reaction before and after purification. Compare the intensity of the PCR product band with those of the "low DNA mass ladder" markers on your gel. Fig. 3.12 shows the amount of DNA in each band of the "low DNA mass ladder." The intensity (brightness) of each band is directly proportional to the amount of DNA in the band.

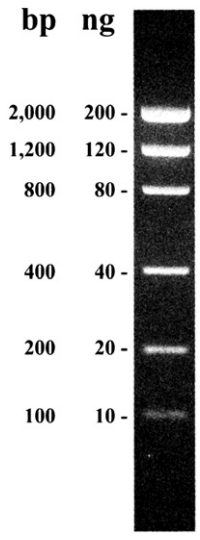

FIGURE 3.12 **Invitrogen low DNA mass ladder size standards.** *Source: Image from Invitrogen online catalog.*

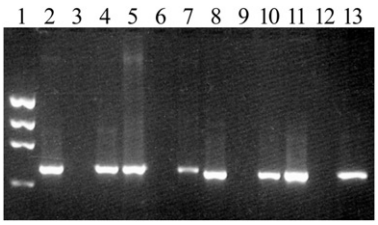

FIGURE 3.13 Agarose gel electrophoresis of PCR products. Lane 1 contains 4 μl (470 ng) of low DNA mass ladder (Fig. 3.12). Lanes 2, 5, 8, and 11 contain unpurified PCR product from experiment 3. Lanes 4, 7, 10, and 13 contain purified PCR product recovered from the samples in lanes 5, 8, 11, and 2, respectively. Note the excellent recovery in lane 4 and the poor recovery in lane 7. Lanes 3, 6, 9, and 12 contain samples from no-template control PCR reactions.

6. Estimate the yield (percent recovery) of purified PCR product. In step 5, you estimated the amount of PCR product present before and after purification. Divide the amount of purified product by the amount in the PCR reaction prior to purification. These numbers are directly comparable because you loaded 10% of each sample (10 μl of the original 100 μl PCR reaction, and 5 μl of the 50 μl final volume of purified product). Show your calculation.
7. Based on the results of the previous two steps, calculate the final concentration (in ng/μl) of your purified PCR product. Show your calculation.
8. Report the DNA sequence of the 16S rRNA gene from the bacterium you isolated. Did the sequence contain ambiguities (Ns)? How did you resolve them? What would you do next to resolve remaining ambiguities? Identify this organism based on the sequence.

Notes

Notes

V. QUESTIONS (CLASS 17)

1. What were the purposes of lysozyme, proteinase K, SDS, and EDTA in the DNA preparation?
2. Why did you do a PCR reaction without added DNA? What do you conclude from your results?
3. An absorbance reading at 260 nm (OD_{260}) of 1 unit indicates a concentration of 33 μg/ml for a short single-stranded oligonucleotide; the figure is 50 μg/ml for double-stranded DNA. You receive a 20-nucleotide oligonucleotide (MW = 6600) from the DNA synthesis facility as a dry powder, which you dissolve in 1 ml of water. You pipette 5 μl of this into 295 μl of water and measure the absorbance at 260 nm; the reading is 2.0. What is the concentration of the primer stock? How much must you dilute it to make a 10 μM stock for use in PCR? Show your calculations.
4. How many ng of each PCR primer constitutes 12 pmol? How many μg/ml of each primer does a 10-μM solution contain? Show your calculations. The molecular weight of the 8-27F primer is 6161; the MW of the 536-519R primer is 5525.
5. Use the following formula to estimate the melting temperature (T_m) of each primer that you used in your PCR:

$$T_m = 16.6 \log [\mathrm{Na}] + 0.41(\% \mathrm{G} + C) + 81.5 - 500/\mathrm{bp}$$

   where [Na] = molar salt concentration, % G + C = percentage expressed as a whole number (e.g., 50), not a fraction (e.g., 0.5), and bp indicates oligonucleotide length in base pairs. Assume the salt concentration in your PCR was 50 mM and calculate the T_m for each primer. Show your calculations.
6. PCR reactions can fail for several reasons. One or more of the reagents (*Taq* DNA polymerase, dNTPs, reaction buffer, or primers) may be faulty or present at the wrong concentration. Alternatively, the template DNA preparation may contain too little intact DNA, or the DNA may contain foreign substances (extracted from the specimen) that inhibit PCR. Finally, the sequences of the primer oligonucleotides may not complement the template sequences well enough to form stable duplexes under the reaction conditions used. How would you distinguish among these possibilities?

Notes

Notes

VI. IN-CLASS WRITING EXERCISE (CLASS 10)

Read the article by J. Borneman and E. W. Triplett. 1997, Molecular microbial diversity in soils from Eastern Amazonia: Evidence for unusual microorganisms and microbial population shifts associated with deforestation. *Appl. Environ. Microbiol.*, 63, 2647–2653. You can find this paper in the library. You could copy the abstract from the paper, but that would defeat the purpose of this exercise. Other students have tried this; your instructor **will** notice if you copy the original abstract.

Write an abstract for this paper.

What is the most significant difference between the procedure used by these authors to prepare template DNA and the method you used in Experiment 3? Explain why this difference is important.

Notes

Notes

EXPERIMENT

4

Terminal Restriction Fragment Length Polymorphism Analysis of 16S Ribosomal RNA Genes from Uncultured Bacterial Communities

I. INTRODUCTION

In this unit, you collect a bacterial population from a natural environment and purify genomic DNA from this uncultured community. You use this DNA as a template for PCR amplification of 16S ribosomal RNA genes contained in this bacterial metagenome. One of the PCR primers contains a fluorescent label. You purify the amplicon, measure its concentration, and examine it by agarose gel electrophoresis. You treat the amplicon with restriction endonucleases and submit the digested DNA to the Central Services Lab for capillary electrophoresis. This allows you to observe restriction-fragment-length polymorphisms (RFLPs) among the fluorescently labeled terminal fragments (T-RFLPs). In addition, you ligate a portion of the intact amplicon to a plasmid vector, and you transform this DNA into *E. coli* to create a molecular clone library of the 16S rRNA genes present in the PCR product. This library will contain a mixture of 16S rRNA genes representing the different bacterial species present in your sample. You prepare plasmid DNA from one clone and submit it for sequencing.

II. BACKGROUND

Culture-independent identification of bacterial species is useful, because many bacteria are currently unculturable. For example, more than 99% of the bacterial species present in a typical soil sample do **not** grow on laboratory media (Borneman and Triplett, 1997), and about half of the species that inhabit the human colon are **not** culturable on known media. Because terminal RFLP analysis is rapid and inexpensive, microbiologists use it to study uncultured bacterial communities. T-RFLP analysis allows microbiologists to monitor changes in bacterial populations over time or in response to treatments or other environmental factors.

T-RFLP analysis is similar to the method you used in unit 3: Both rely on PCR amplification of 16S rRNA genes. In contrast to unit 3, the PCR product synthesized in this unit likely contains a mixture of different amplicons derived from 16S rRNA genes from each of the bacterial species present in the sample. The T-RFLP method uses one fluorescently labeled primer and one unlabeled primer so that one end of the amplicon has a fluorescent tag (Fig. 4.1). You digest the amplicons with a restriction endonuclease (Fig. 4.2) and use capillary electrophoresis to detect restriction fragments containing the labeled primer.

We use a primer pair that produces longer amplicons than those in unit 3. This prevents amplicons synthesized in unit 3, which may contaminate our equipment, from serving as templates in this experiment. Longer amplicons increase the probability that each DNA molecule in the PCR product contains a target site for the restriction endonuclease. Usually, we use a restriction enzyme that cuts a four-base-pair sequence, because these enzymes cut DNA frequently.

T-RFLP analysis measures the length and relative quantity of each labeled (terminal) restriction fragment (Fig. 4.3); you will not detect unlabeled restriction fragments. Prior to restriction endonuclease digestion, you purify the amplicons to remove the reaction buffer, unincorporated primers, and dNTPs. Agarose gel electrophoresis confirms that the amplicon has the anticipated length (~880–940 bp), and we measure the concentration of the PCR product using a "nanodrop" spectrophotometer, which can measure the optical density (OD) of a 1-μl sample. This permits us to digest the proper amount of PCR product (100 ng) with *Rsa*I, which cleaves (GT/AC), or *Msp*I, which cuts (C/CGG). A service

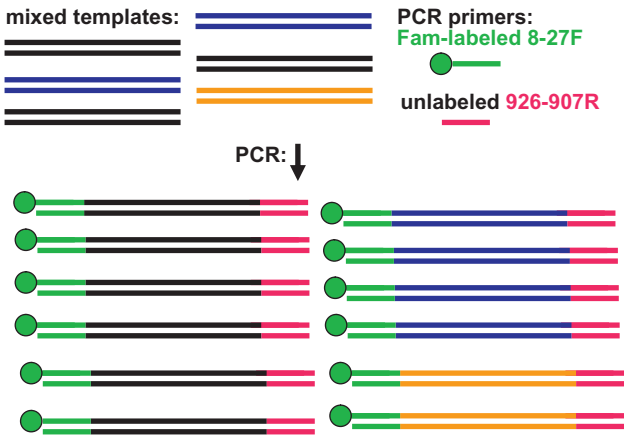

FIGURE 4.1 **Labeled 16S rDNA amplicons for T-RFLP analysis.** Colored bars represent DNA strands. Green and red bars represent fluorescently labeled (Fam-8-27F) and unlabeled (926-907R) PCR primers; numbers indicate coordinates in the *E. coli* 16S rRNA sequence. Green circles represent the fluorescent label (Fam). Black, blue, and gold bars represent 16S rDNA from different bacterial species. Note that the quantities of the templates differ, and the corresponding PCR products reflect these differences.

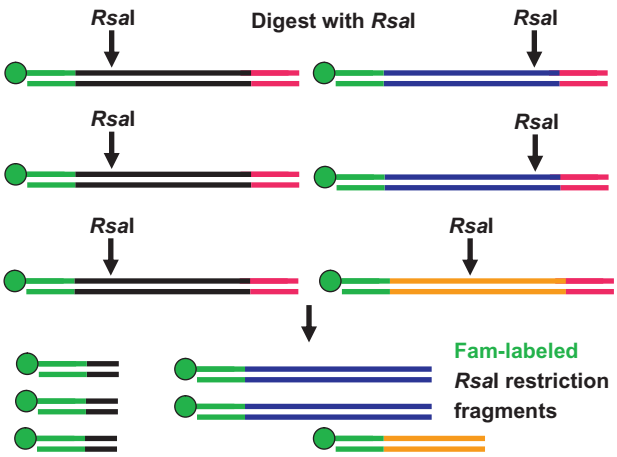

FIGURE 4.2 Restriction fragments of Fam-labeled 16S rDNA amplicon for T-RFLP analysis. Colored bars represent DNA strands. Green and red bars represent fluorescently labeled (Fam-8-27F) and unlabeled (926-907R) PCR primers. Green circles represent the fluorescent label (Fam). Black, blue, and gold bars represent 16S rDNA amplicons derived from different bacterial species. Note that the quantities of the PCR products differ. Arrows indicate *Rsa*I sites in the DNA.

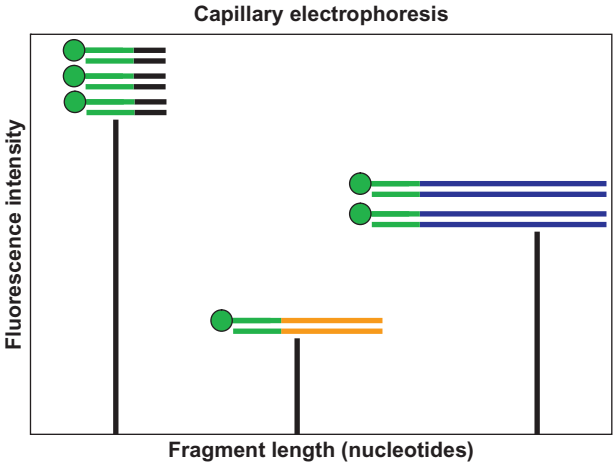

FIGURE 4.3 T-RFLP profile of a bacterial population comprising three species. Vertical bars indicate the fluorescence intensity and length of each labeled *Rsa*I restriction fragment. Horizontal bars represent DNA strands (Fig. 4.1). Green bars represent fluorescently labeled (Fam-8-27F) PCR primer. Green circles represent the fluorescent label (Fam). Black, blue, and gold bars represent labeled (terminal) *Rsa*I restriction fragments of 16S rDNA amplicons derived from different bacterial species (Fig. 4.2). Note that the quantities of the labeled fragments differ.

laboratory analyzes digested DNAs by capillary electrophoresis, which can separate DNA molecules that differ in length by a single nucleotide. Currently we use the Core Laboratory in the Center for Genome Research and Biocomputing at Oregon State University (http://corelabs.cgrb.oregonstate.edu/genotype); this laboratory provides genotyping services to customers worldwide.

The Ribosomal Database Project (RDP) contains over 350,000 16S rDNA sequences, although not all entries are unique (http://rdp.cme.msu.edu/). For a given primer set and restriction endonuclease, the RDP staff predicts the T-RFLP fragment produced by each 16S rRNA gene in the database. More than one species can produce a particular fragment, so T-RFLP fragment length cannot identify a species. Even though T-RFLP analysis cannot identify the species present in a bacterial community, it indicates the complexity of the population. Differences (and similarities) between two bacterial populations may become obvious by comparing their T-RFLP patterns (Fig. 4.4).

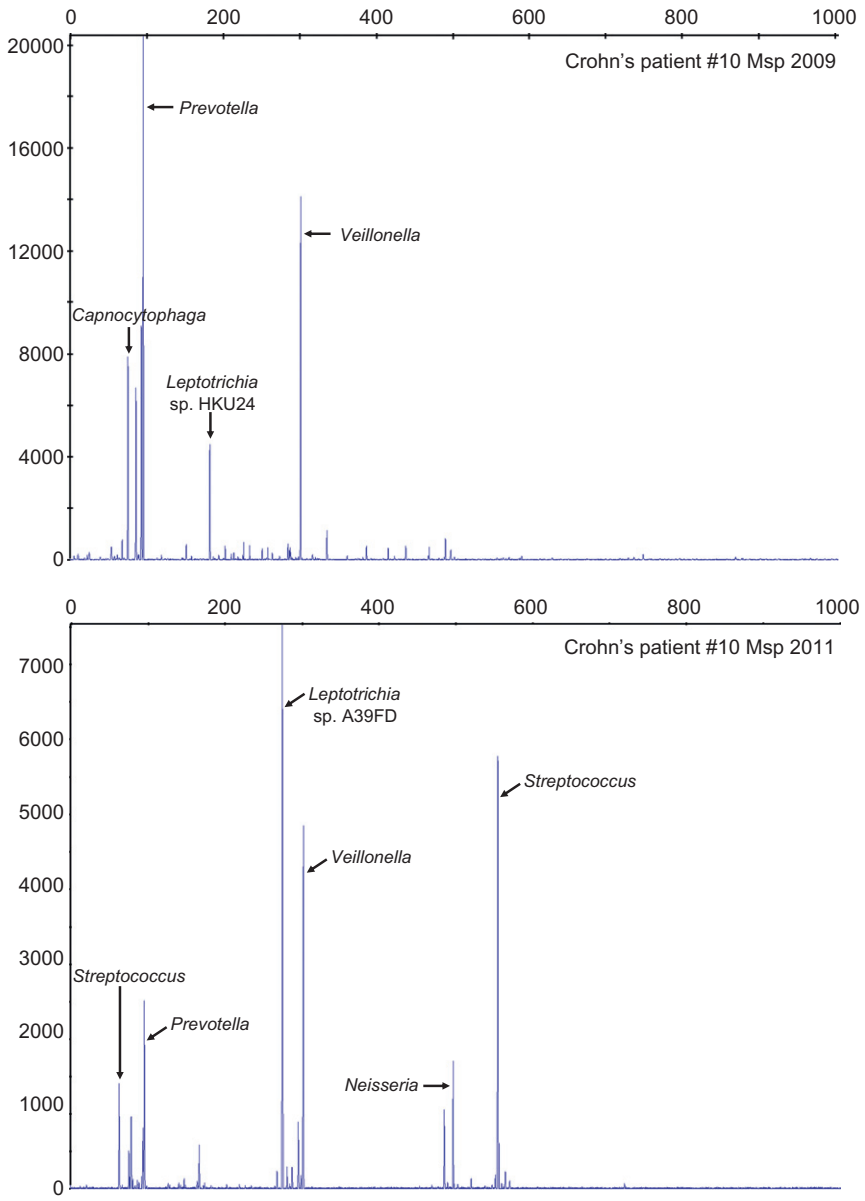

FIGURE 4.4 T-RFLP profiles of gastric bacterial populations from a Crohn's patient in 2009 (top panel) and 2011 (bottom panel). We extracted DNA from gastric biopsies and used PCR to amplify bacterial 16S rRNA genes for T-RFLP and DNA sequence analyses. Numbers on the ordinate indicate lengths of Fam-labeled *Msp*I restriction fragments; numbers on the abscissa indicate fluorescence units. To identify genera corresponding to major T-RFLP peaks, we sequenced 116 cloned 16S rRNA genes from each amplicon. The 16S rRNA genes from two *Leptotrichia* species produced labeled *Msp*I fragments of different lengths: Species HKU24 produced a 187-bp fragment (top panel), whereas species A39FD produced a 278-bp fragment (bottom panel). Note the striking absence of *Streptococcus* species in the sample from 2009 (top panel) and the sharp decrease in the abundance of *Prevotella* species in the sample from 2011 (bottom panel).

Positive identification of species associated with particular T-RFLP peaks requires molecular cloning and sequence analysis. Direct sequencing of the PCR product (as you did in unit 3) is not possible in this case, because you amplified the DNA from a mixed template. You must make a molecular clone library of individual DNA molecules from the PCR product, which allows you to sequence individual 16S rRNA genes.

You use a plasmid vector developed for cloning PCR amplicons. Invitrogen sells plasmid DNA cleaved at a specific site by topoisomerase, which remains covalently bound to the linear plasmid DNA (Fig. 4.5). The double-stranded plasmid DNA has a single T nucleotide extension at each end (Fig 4.5). PCR products synthesized using *Taq* DNA polymerase contain A nucleotides added to each 3′ end. Thus, the plasmid and amplicon DNA molecules have complementary ends, and topoisomerase joins (ligates) these two DNAs into a single circular molecule (Fig. 4.6). You transform competent *E. coli* with this circular plasmid DNA.

Most plasmid vectors provide markers that allow users to select transformed cells and to distinguish transformants containing plasmids with inserts from cells containing intact vector with no foreign DNA inserted. The plasmid (pCR4-TOPO) contains genes for ampicillin and kanamycin resistance, providing a selection for transformed cells (Fig. 4.7). The amplicon inserts into a cloning site within a reporter gene (a portion of *lacZ*). The LacZ enzyme (β-galactosidase) is active as a tetramer (Fig. 4.8). The competent *E. coli* TOP10 strain carries the *lacZ*ΔM15 deletion, which removes codons 11–41 from the *lacZ* gene (Fig. 4.9). This mutation prevents tetramer formation but does not affect the active site of the LacZΔM15 protein, so untransformed cells have a Lac^- phenotype. Plasmid pCR4-TOPO, without foreign DNA inserted into the cloning site, encodes the tetramer-forming domain of LacZ, but this domain lacks the active site. Expression of these two domains of LacZ in the same cell allows the LacZΔM15 protein to form tetramers and regain its enzymatic activity. *E. coli* TOP10 cells transformed with the empty pCR4-TOPO vector exhibit a Lac^+ phenotype because the two mutant LacZ proteins complement each other (Fig. 4.10). Foreign DNA inserted into the cloning site of pCR4-TOPO abolishes production of the tetramer-forming LacZ peptide, so ampicillin- and kanamycin-resistant transformants **with** DNA inserted into the vector have a Lac^- phenotype.

A colorimetric assay allows us to distinguish Lac^+ and Lac^- transformants visually. The agar plates contain a colorless substrate for LacZ, called *X-gal* (5-bromo-4-chloro-3-indolyl-β-D-galactoside; Fig. 4.11). In Lac^+colonies, which contain the empty vector, LacZ cleaves the bond between the indole ring and the sugar; this releases the indole rings, which react with each other to form indigo (a blue dye; Fig. 4.12). The desired Lac^- transformants, which contain amplicon DNA inserted into the vector, remain white (Fig 4.13).

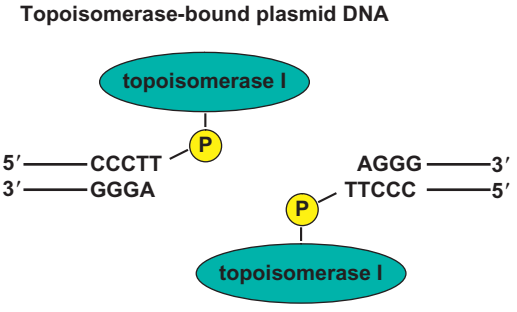

FIGURE 4.5 **Topoisomerase-bound plasmid DNA used for cloning PCR amplicons.** Blue ovals depict topoisomerase I subunits covalently attached to plasmid DNA by phosphodiester bonds with 3′ phosphate groups (yellow circles) of the cut (linear) plasmid DNA. Topoisomerase I recognizes the sequence 5′ CCCTTAGGG 3′. Black bars represent vector plasmid DNA.

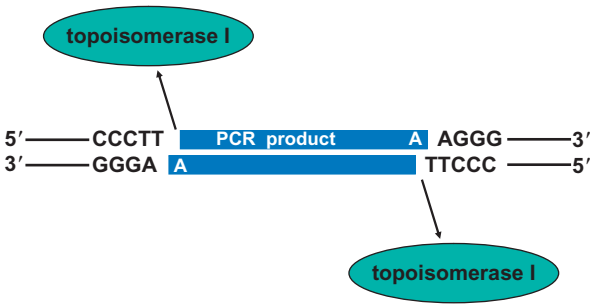

FIGURE 4.6 Topoisomerase-mediated cloning of PCR products amplified with *Taq* DNA polymerase. Blue bars depict a PCR product with untemplated A nucleotides on each 3′ end. Blue ovals represent topoisomerase I released from the plasmid DNA as topoisomerase joins the PCR product to the plasmid. The sequence `5' CCCTTAGGG 3'` indicates the recognition sequence at which topoisomerase inserts the PCR product into the plasmid. Black bars represent vector plasmid DNA.

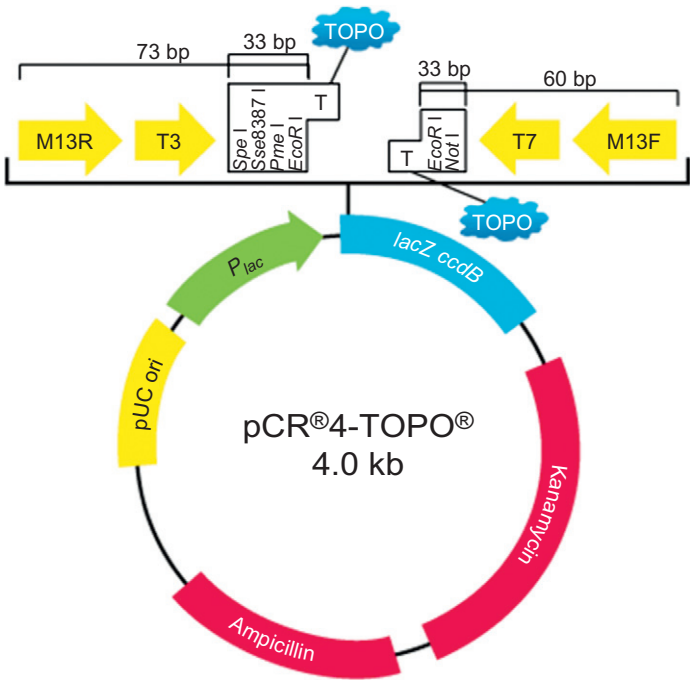

FIGURE 4.7 Schematic diagram of TOPO-TA cloning vector pCR4. Red boxes indicate genes encoding resistance to ampicillin and kanamycin. The yellow box indicates the ColE1 origin of replication. The blue box indicates the sequence encoding the amino-terminal (alpha) portion of the β-galactosidase (*lacZ*) gene, and the green box indicates the *lac* promoter/operator. Yellow arrows indicate commercially available sequencing primers. Large Ts indicate topoisomerase I cleavage sites, and blue clouds represent covalently attached topoisomerase proteins. *Source: Image is from Invitrogen online product literature.*

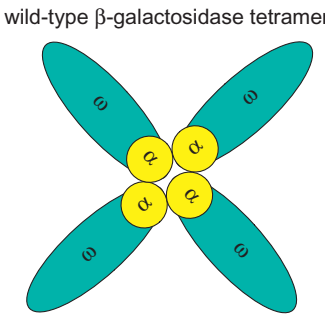

FIGURE 4.8 LacZ (β-galactosidase) protein is active as a tetramer. The α domain (amino acids 1-92; yellow circles) mediates the interaction between LacZ subunits. The ω domain (blue ovals) contains the active site of LacZ.

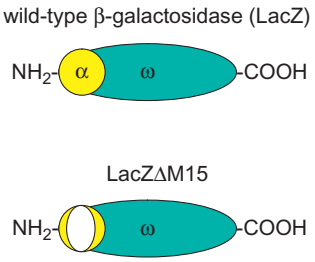

FIGURE 4.9 The *lacZ*ΔM15 mutation prevents formation of LacZ tetramers. The white oval superimposed on the yellow circle indicates that amino acids 11-41 are absent from the LacZΔM15 protein, which is unable to form active tetramers. The yellow circle labeled α represents the α (tetramerization) domain of wild-type LacZ protein; the blue ovals represent the ω (active site) domains of LacZΔM15 and wild-type LacZ.

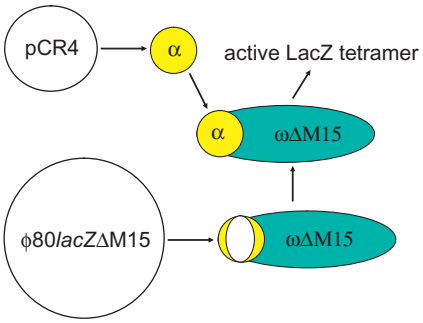

FIGURE 4.10 *E. coli* cells that express the LacZ α and LacZΔM15 proteins have a Lac$^+$ phenotype. Vector plasmid pCR4 (without foreign DNA inserted) encodes the LacZ α protein (yellow circle). In *E. coli* TOP10, the chromosome (large black circle) contains a ϕ80 prophage that encodes the LacZΔM15 protein. The white oval superimposed on the yellow circle indicates that amino acids 11-41 are absent from the α domain of LacZΔM15. The ω domain (blue oval) contains the active site of β-galactosidase.

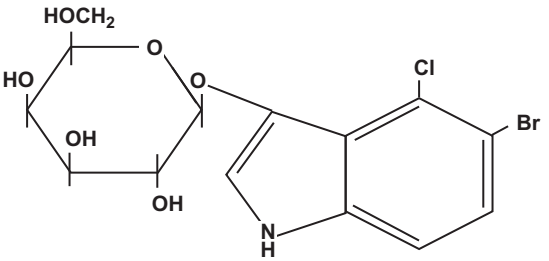

FIGURE 4.11 Chemical structure of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside).

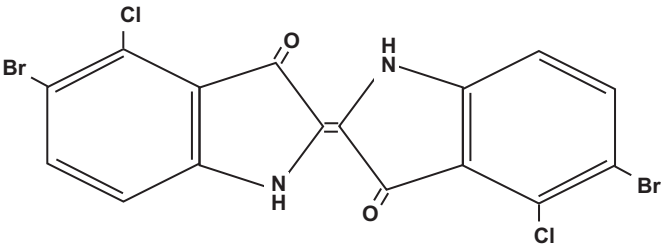

FIGURE 4.12 Chemical structure of indigo. The figure shows a blue compound derived from X-gal after cleavage by β-galactosidase; indigo lacks the bromine and chlorine atoms shown here.

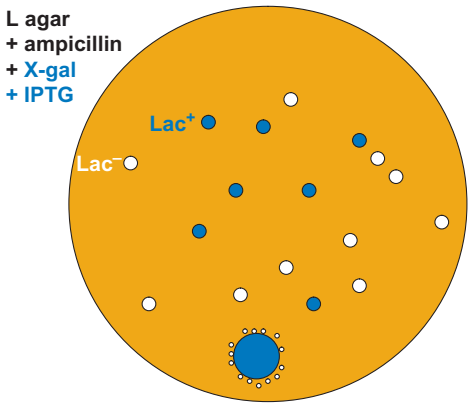

FIGURE 4.13 Agar plate with Lac$^+$ and Lac$^-$ colonies. Lac$^+$ (blue) colonies represent *E. coli* TOP10 cells transformed with intact pCR4, which expresses LacZ α protein. Lac$^-$ (white) colonies represent *E. coli* TOP10 cells transformed with pCR4 plasmid containing a PCR product inserted into the cloning site, which lies in the *lacZ* gene. Insertion of the PCR product into pCR4 prevents expression of LacZ α protein. During topoisomerase-mediated cloning, occasionally pCR4 may suffer a deletion in the *lacZ* gene (without insertion of a PCR product); *E. coli* TOP10 cells transformed with deleted versions of pCR4 form Lac$^-$ (white) colonies also. Small white satellite colonies surrounding the large blue colony arise from untransformed (Lac$^-$ ampicillin-sensitive) *E. coli* TOP10 cells growing slowly near a colony of ampicillin-resistant cells. The transformed cells secrete β-lactamase, which destroys enough ampicillin in the agar near the colony to permit untransformed cells to grow. We include kanamycin in the agar to eliminate growth of untransformed cells. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) produces an indigo pigment on cleavage by β-galactosidase (LacZ). IPTG (isopropyl-β-D-thiogalactoside) induces expression of the *lacZ* genes.

Each ampicillin- and kanamycin-resistant Lac$^-$ transformant represents a different DNA molecule from the PCR product. You streak transformants for single colonies and grow cultures in broth for plasmid DNA isolation. Sequence analysis of cloned amplicons identify both the bacterial species present and the corresponding T-RFLP peak. You use the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to align the sequence of your cloned 16S rRNA gene with its closest match in the databases. You can locate *Msp*I and *Rsa*I restriction sites in the 16S rDNA sequence and calculate the length of each labeled restriction fragment. These analyses allow you to attribute the corresponding T-RFLP peaks to a particular species.

Sequencing multiple cloned 16S rRNA genes may allow you to identify most of the species present in your sample and most of the peaks in the T-RFLP profile. To save time and money, select a single Lac$^-$ transformant for sequencing. Sequencing a molecular clone of a single DNA molecule from a PCR product creates a potential problem: Molecular cloning propagates PCR-generated mutations. We do not detect PCR-induced mutations when we sequence the PCR product directly, as we did in unit 3. The sequencing laboratory we use (Functional Biosciences; http://functionalbio.com/web/index.php) offers a cost-effective service that allows us to sequence 96 (or more) cloned 16S rRNA genes from a clone library. We send the company agar plates containing a library of *E. coli* TOP10 cells transformed with pCR4 plasmid containing a PCR product inserted into the cloning site. A robot picks Lac$^-$ (white) colonies, grows broth cultures, and purifies plasmid DNA for sequencing. This high-throughput method uses 96-well plates to process samples.

Notes

Notes

III. PROCEDURE

Collect a sample from an environment that you believe contains bacteria of interest to you and your colleagues. Select your collection site carefully; in one writing assignment, you must justify your choice. You streak a portion of your sample on LB agar (for unit 3), then you extract DNA directly from bacteria present in the sample (for unit 4). Extraction procedures depend on the properties of the sample; you use an extraction method designed for soil samples. This method works for samples from some other sources (for example, oral swabs or yogurt cultures), but it may not (or may) work well for other samples. **You may not take samples from humans other than yourself**. All research on human specimens requires approval by the Institutional Review Board; you must pass an online course before you can apply to the IRB for approval to study human subjects.

Methods used to take a census of microbial populations suffer from representational biases: Some species are overrepresented whereas others are missed or underrepresented. Culture-based approaches suffer from our inability to grow many bacterial species under laboratory conditions. However, culturing is a very sensitive way to detect species that we know how to grow, because we can detect single cells. PCR-based approaches rarely achieve this level of sensitivity. In addition, PCR-based approaches suffer from well-known sources of bias. For example, DNA extraction methods that work well for one species may work poorly for another; the same is true of PCR primer sets and reaction conditions. In experiments 3 and 4, you use culture-based and PCR-based methods to study a single bacterial community, providing you with first-hand experience of the advantages and drawbacks of each approach.

A. Sample Collection for Experiments 3 and 4 (Class 7)

1. Label an LB agar plate and a Bead Solution tube (from the Mo Bio Ultra Clean Soil DNA Isolation Kit) with your seat number.
2. Use a sterile BD BBL Culture Swab EZ to collect the sample. If you sample a dry surface, first submerge the swab head in 0.5 ml of sterile distilled water. Alternatively, if you want to study a soil sample, add 0.25 grams of soil directly to the Bead Solution tube. Remove the swab from its container, collect the sample, and streak it across one edge of the LB agar. Next, insert the swab head into the Bead Solution tube and swirl it vigorously for 15 seconds. Return to the lab and complete streaking the plate with sterile toothpicks. Make three additional streaks, each with a fresh toothpick; this creates three "dilutions" of your original sample. Work from one edge of the original streak to make the second streak, make the third streak from the edge of the second, and so forth.
3. Incubate the LB agar plate at an appropriate temperature (25°C, 30°C, or 37°C). Bacteria from soil or water probably grow best at room temperature or 25°C; incubate samples from your body (or an animal) at 37°C. Incubate the plate overnight and **examine it the next day**.
4. **One day after class 7**. Write a brief description of the bacterial (and fungal) colonies on the plate. Note the color, shape, size, and abundance of each colony type. Choose

two different isolated bacterial colonies, and use sterile toothpicks to streak each on fresh LB agar. The teaching assistants provide sterile toothpicks and a container for used toothpicks in each growth room. Work in the growth room; another class may be in the laboratory. Use at least three toothpicks for each colony, so that you can spread the cells enough to produce single colonies. Incubate the streaked plates overnight at the proper temperature.

Two days after class 7, the teaching assistants move your streaked plates to the cold room. Handle these cultures (and all subcultures) as though they are human pathogens. They may be pathogenic, particularly if you sampled an environment conducive to growth of human pathogens. Avoid all contact with the bacteria, and autoclave all cultures, supernatant solutions, and contaminated tubes, pipettes, and tips.

5. **Class 8**. Follow the instructions for experiment 3, step 5, to complete unit 3.

B. Extract DNA from an Uncultured Bacterial Community (Class 7)

Wear clean gloves at all times and use aerosol-resistant (ART) pipette tips.

1. Use a sterile BD BBL Culture Swab EZ to collect the sample. If you sample a dry surface, first submerge the swab head in 0.5 ml of sterile distilled water. Insert the swab head into the Bead Solution tube and swirl it vigorously for 15 seconds. Alternatively, if you want to study a soil sample, add 0.25 grams of soil directly to the Bead Solution tube. Remove the swab from the Bead Solution tube and secure the lid.
2. Vortex the bead solution tube for 15 seconds.
3. Add 60 μl of solution S1 and vortex 15 seconds. (S1 contains SDS, an ionic detergent that disrupts lipids in the cell membrane and promotes cell lysis.)
4. Add 200 μl of solution IRS (Inhibitor Removal Solution), which precipitates humic acid and other PCR inhibitors.
5. Secure bead tubes horizontally to the vortex platform. Vortex the Bead Solution tube at maximum speed for 10 minutes. This "bead beating" step causes mechanical lysis of bacterial cells, and it shears DNA.
6. Centrifuge the tubes at 10,000 × g (**not** 10,000 rpm) for 30 seconds.
7. Transfer 500 μl of supernatant solution to a clean 2 ml centrifuge tube (provided with the kit).
8. Add 250 μl of solution S2 and vortex for 5 seconds. Incubate at 4°C for 5 minutes. (Solution S2 precipitates proteins.)
9. Centrifuge the tubes for 1 minute at 10,000 × g.
10. Transfer 700 μl of supernatant solution into a clean 2 ml centrifuge tube (provided with the kit) containing 1.3 ml of solution S3. Vortex the tube for 5 seconds. (Solution S3 is a high salt buffer, which allows DNA to bind silica.)
11. Load 700 μl of DNA−solution S3 mixture (from step 10) into a spin filter cartridge and centrifuge at 10,000 × g for 1 minute. Discard the flow through and repeat this process until all of the solution has passed through the filter; this requires three loads per sample.

12. Add 300 μl of solution S4 and centrifuge for 30 seconds at 10,000 × g. Discard the flow through. (Solution S4, which contains ethanol, washes impurities from the DNA bound to the silica filter.) Repeat the wash with another 300 μl of solution S4. Discard the flow through.
13. Centrifuge for 1 minute at 10,000 × g to remove the ethanol completely. Use a yellow tip to remove drops of ethanol from the rim (inside the cartridge) above the filter.
14. Place the filter cartridge in a clean centrifuge tube; add 50 μl of solution S5 (10 mM Tris, pH 8.0) **to the center of the white filter membrane**. Let stand for 1 minute. Centrifuge for 30 seconds at 10,000 × g. (Elution buffer S5 lacks salt, which releases DNA from the silica filter.)
15. Discard the filter cartridge, secure the lid, write your seat number on the tube, and freeze the DNA at −20°C until class 13. The teaching assistants provide a freezer box to store samples.

C. PCR Amplification of 16S rRNA Genes for Terminal Restriction Fragment Length Polymorphism Analysis (Class 14)

Wear clean gloves at all times and use aerosol-resistant (ART) pipette tips.

1. The teaching assistants supply each student with a tube that contains 190 μl of PCR reaction mixture, which includes all components except template DNA. When you set up your PCR reaction, wear clean gloves and use pipette tips that have aerosol barrier filters (ART tips). Note that P20 and P200 pipettors require different barrier tips. These precautions help you avoid contaminating your PCR reactions with extraneous DNA.
2. Place 95 μl of reaction mixture in each of two 0.2-ml PCR reaction tubes. Handle these tubes gently; they have thin walls to permit rapid heat transfer, and this makes them fragile. Label these tubes (with your seat number) only at the top of the conical portion; do not label the lid. Markings on the lid will burn off during the PCR.
3. Add 5 μl of template DNA (from class 7) to one PCR reaction mixture and mix well. Add 5 μl of sterile distilled water to the other PCR reaction and mix well; this is a no-template control, which allows you to detect extraneous DNA in the PCR reagents. Store these tubes on ice until the entire class is ready to load the PCR machine. Table 4.1 shows what each PCR reaction should contain.
4. Place your tubes in the thermal cycler; the initial block temperature is 94°C. Once the program begins, the instrument will hold the block at 94°C for 1 minute, 51°C for 1 minute, and 72°C for 1 minute; this program repeats for 35 cycles. After the final cycle, the instrument holds the block at 72°C for 10 minutes then at 4°C indefinitely. The teaching assistants remove the reactions and store them frozen until the next class.

The thermal cycler that we use has a heated lid, which prevents condensation of water on the lid of the PCR reaction tube. This obviates the need to overlay the sample with mineral oil, which prevents evaporation of the sample in machines without a heated lid.

TABLE 4.1 Contents of PCR Reaction

Stock	Volume/100 µl	Final Concentration
10 × buffer*	10 µl	1×
25 mM $MgCl_2$	14 µl	3.5 mM
dNTP mix, 10 mM each	2 µl	0.2 mM each
8-27 F "Fam"-labeled primer, 10 µM	1.5 µl	0.15 µM
8-27 F unlabeled primer, 10 µM	0.5 µl	0.05 µM
926-907 R primer, 10 µM	2 µl	0.2 µM
BSA, 0.4% w/v	16 µl	0.064%
Taq DNA polymerase, 5U/µl	0.5 µl	2.5 units
Distilled water	50.5 µl	
Template DNA	5 µl	Unknown
Total volume	100 µl	

**10 × buffer = (100 mM Tris, pH 8.3 + 500 mM KCl + 0.1% w/v gelatin)*
8-27 F "Fam" primer = 5′ AGA GTT TGA TCM TGG CTC AG 3′; (MW of DNA = 6,161)
This primer has a 5′ "Fam" label.

926 − 907 R primer = 5′ CCG TCA ATT CCT TTR AGT TT 3′; (*M* = A or C and R = A or G) (MW = 6, 042)

D. Purify PCR Product (Class 15)

Wear clean gloves at all times and use aerosol-resistant (ART) pipette tips.

1. Mix the template-containing PCR reaction with 5 volumes (0.5 ml) of buffer PBI, which is a proprietary solution from Qiagen that facilitates DNA binding to the silica filter in the QIAquick spin column. The yellow color of buffer PBI indicates a pH <7.5, which is necessary for DNA binding to the silica membrane. Save the no-template control PCR reaction for agarose gel electrophoresis (later this class); do not purify the control reaction.
2. Place a spin column in a 2-ml centrifuge tube and pipette the DNA/PBI mixture into the cartridge. Write your seat number on the tube.
3. Centrifuge at full speed for 1 minute; discard the flow through.
4. Add 750 µl of buffer PE to the column; buffer PE is a proprietary wash buffer containing 70% ethanol.
5. Centrifuge at full speed for 1 minute; discard the flow through.
6. Centrifuge again at full speed for 1 minute to remove all traces of ethanol. Use a yellow tip to remove drops of ethanol from the rim (inside the cartridge) above the filter.
7. Transfer the cartridge to a clean 2-ml centrifuge tube, then add 50 µl of buffer EB (elution buffer = 10 mM Tris, pH 8.5). Make certain that you **pipette the elution buffer into the center of the white filter membrane**. Let the cartridge stand for 1 minute. Write your seat number on the tube.
8. Centrifuge at full speed for 1 minute.

E. Ligate PCR Product to Plasmid: Topoisomerase-Mediated Cloning Reaction (Class 15)

1. You will receive a 0.5-ml centrifuge tube that contains 1 μl of topoisomerase-bound vector plasmid and 1 μl of salt solution (1.2 M NaCl + 60 mM $MgCl_2$). Mix 4 μl of purified PCR product with the contents of the tube.
2. Incubate the mixture at room temperature for 5 minutes. Write your seat number on the tube.

F. Transform Ligated DNA (Class 15)

1. Thaw, on ice, one vial of One Shot TOP10 chemically competent *E. coli* cells. Add the entire 6 μl mixture of ligated DNA to the vial of thawed cells and mix gently. **Do not mix by pipetting**. Use aseptic technique.
2. Incubate the vial on ice for 30 minutes.
3. Place the vial in a 42°C water bath for 30 seconds; do **not** agitate the tube.
4. Place the tube on ice for 2 minutes.
5. Add 250 μl of SOC broth warmed to 37°C.
6. Incubate the tube at 37°C for 1 hour with shaking.
7. You will receive two LB agar plates that contain ampicillin (50 μg/ml), IPTG (40 μM), and X-gal (0.004%). Spread 50 μl of transformed cells on one plate. Centrifuge the tube for 1 minute at full speed to pellet the remaining cells. Discard 200 μl of the supernatant solution into a container that we will autoclave. Suspend the cell pellet in the remaining 50 μl, and spread the concentrated cells on the second agar plate. Incubate at 37°C overnight.
8. The next day, retrieve the agar plate containing your transformants from the 37°C room. Record the number of Lac^- (white) and Lac^+ (blue) colonies in the clone library. For most purposes, you should streak transformants for single colonies before you analyze them. To save time, you inoculate broth with an isolated single colony taken directly from the original plate. This practice is routine for sequencing projects. Sequencing facilities use robots to pick single (unstreaked) colonies from plates. The robots also inoculate broth and prepare plasmid DNA from the resulting cultures, which permits high-throughput sequence analysis.

   The teaching assistants provide tubes of broth, inoculating loops or sterile sticks, and a container for used sticks in the 37°C room. Work in the growth room; another class may be in the laboratory. Pick an isolated single white colony, and inoculate a tube containing 2 ml of LB broth + ampicillin (50 μg/ml). Write your seat number on the tube. Incubate the inoculated tubes in a shaker at 37°C overnight. The next day the TAs will move the tubes to the cold room until class 16.

G. Electrophoretic and Spectrophotometric Analyses of PCR Product (Class 15)

1. Remove 5 μl of the purified PCR product to examine amplicon length and estimate yield by agarose gel electrophoresis. Examine 10 μl of the unpurified no-template negative control reaction. Include a known quantity (470 ng in 4 μl) of Invitrogen low

DNA mass ladder in a separate lane so that you can estimate the quantity and size of your PCR product. Mix each DNA sample with 1 μl of loading solution (50% glycerol + 0.05% bromophenol blue).

2. The TAs cast a 1% agarose gel containing 1 μg/ml ethidium bromide. Load your samples into separate wells and apply 80 volts of current until the dye front migrates approximately halfway down the gel. The TAs place the gel on a UV transilluminator and photograph the gel.
3. The TAs will measure the concentration (OD_{260} = optical density at 260 nm) of each PCR product using a "nanodrop" spectrophotometer. They record the concentration (in ng/μl). Small groups of students may accompany the TAs to observe the operation of this instrument.
4. Place the rest of the purified PCR product in the freezer box provided by the teaching assistants.

H. Restrict Purified PCR Product (Class 16)

1. You will receive a 0.5-ml tube containing 1 μl (10 units) of *Rsa*I restriction endonuclease + 2 μl of 10 × buffer + 1 μl of BSA (1 mg/ml) and a second tube containing 1 μl (10 units) of *Msp*I restriction endonuclease + 2 μl of 10 × buffer + 1 μl of BSA. Write your seat number and "M" (for *Msp*I) or "R" (for *Rsa*I) on the tubes. You also receive a tube with sterile distilled water. Based on the concentration of your PCR amplicon (see section F, class 14), calculate the volume that contains 100 ng of your PCR product. Add this amount of your purified PCR product to each of these tubes of restriction enzyme, and add water (if necessary) to bring the final volume to 20 μl. If your PCR product contains <6.25 ng/μl, add 16 μl to each restriction reaction. Incubate the tubes at 37°C for 2 hours.
2. You will receive six empty 0.5-ml tubes. Label each tube with (a) your seat number, (b) "M" or "R" to indicate the enzyme used to digest the DNA, and (c) "1," "3," or "5" to indicate the volume of restriction digest added to the tube. Mix 1 μl of each digested DNA sample with 19 μl of water in the appropriate tubes. Similarly, mix 3 μl of each sample with 17 μl of water, and mix 5 μl of each sample with 15 μl of water. You have 11 μl of each restricted DNA remaining in the original tube, which you labeled with your seat number and "M" or "R." Add 9 μl of water to each of these tube and add "11" to the label. All four tubes should contain 20 μl of liquid; place these tubes in the rack provided by the TA. Organize the tubes in order, beginning with the least concentrated sample on the left. Your instructor will load these samples into a 96-well plate and submit them to a genotyping service laboratory for cleanup and T-RFLP analysis.

I. Plasmid DNA Purification (Class 16)

1. Pour the "2-ml" overnight broth culture into a 2-ml centrifuge tube. Centrifuge at maximum speed for 1 minute. Discard the supernatant solution into an autoclave waste container.
2. Add 250 μl of buffer P1 (containing RNase A) and vortex to suspend the cell pellet.

3. Add 250 μl buffer P2 (an alkaline SDS solution) and invert 6 times to mix. **Do not vortex**. The solution will become viscous, indicating that the cells have lysed.
4. Add 350 μl of buffer N3 and invert 6 times to mix. This solution contains a high salt concentration and returns the pH to neutral. The solution becomes cloudy as proteins and cell debris precipitate.
5. Centrifuge for 10 minutes at maximum speed.
6. Pipette the supernatant solution into a QIaprep spin column. Centrifuge for 1 minute at full speed and discard the flow through.
7. Add 0.5 ml of wash buffer PB and centrifuge for 1 minute at full speed. Discard the flow through.
8. Add 0.75 ml of wash buffer PE (which contains ethanol). Centrifuge for 1 minute at full speed. Discard the flow through.
9. Centrifuge at full speed for 1 minute to remove residual ethanol. Use a yellow tip to remove drops of ethanol from the rim (inside the cartridge) above the filter.
10. Transfer the cartridge to a clean 2-ml centrifuge tube, then add 50 μl of buffer EB (elution buffer = 10 mM Tris, pH 8.5). Make certain that you **pipette the elution buffer into the center of the white filter membrane**. Let stand for 1 minute. Write your seat number on the tube. Centrifuge for 1 minute at full speed.
11. The TAs will measure the concentration (OD_{260} = optical density at 260 nm) of each purified plasmid DNA sample using a "nanodrop" spectrophotometer. They record the concentration (in ng/μl). Small groups of students may accompany the TAs to observe the operation of this instrument. We submit these plasmid DNA preparations for sequencing. Place your plasmid DNA in the freezer box provided by the teaching assistant.

Notes

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IV. LABORATORY REPORT

Include the following in your laboratory report:

1. Describe the source of the DNA sample you used for T-RFLP analysis. Discuss its importance.
2. Was the PCR amplification successful? Did you detect a PCR product by agarose gel electrophoresis? Estimate the length of the amplicon (in base pairs) based on its electrophoretic mobility relative to the low DNA mass ladder (Fig. 4.14). Is the amplicon the length you expected? Include a photograph of the agarose gel (see Fig. 4.15 for an example) and the semilogarithmic plot you used to estimate the length of the amplicon. Use Excel or semilogarithmic graph paper (shown in experiment 1) to draw your plot. If your PCR reaction did not produce a detectable product, discuss

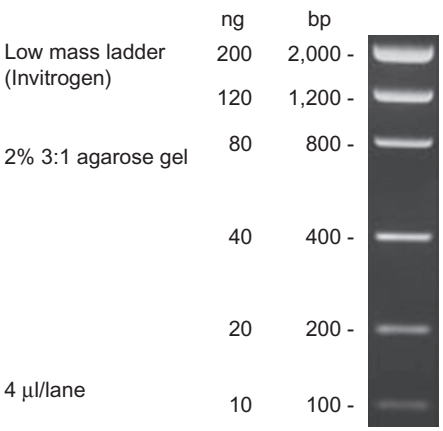

FIGURE 4.14 **Invitrogen low DNA mass ladder size standards.** *Source: Image from Invitrogen online catalog.*

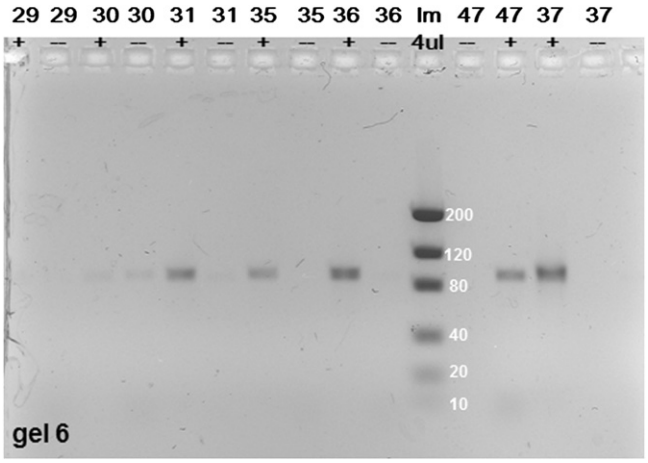

FIGURE 4.15 **Agarose gel electrophoresis of PCR products.** Lane "lm" contains 4 µl (470 ng) of Invitrogen low DNA mass ladder (Fig. 4.14). Numbers above each lane are sample numbers; + indicates lanes containing 5 µl of purified PCR product; – indicates lanes containing 10-µl samples from no-template control reactions. White numbers beside the size standards indicate ng of DNA in each band.

possible explanations and suggest approaches to overcome these problems; for your report, use another gel from the class or from Figure 4.15.

3. Estimate the concentration of the purified PCR product (in ng/μl) based on the intensity of the band relative to the size standards (Fig. 4.14). How does this estimate compare to the concentration measured with the nanodrop spectrophotometer?
4. Did you see a PCR product in your no-template negative control reaction? If so, discuss possible sources of extraneous DNA and steps that may eliminate it.
5. Report the number of Lac^- (white) and Lac^+ (blue) colonies in the clone library you made from your T-RFLP amplicon. What was the concentration of the plasmid DNA you prepared from one member of this library?
6. We used two sequencing primers, M13 reverse (M13R) and T7 promoter (T7), to sequence the 16S rRNA amplicon inserted into pCR4 (Fig. 4.7). You will receive sequences from each strand of your plasmid. Report the sequence of the 16S rRNA gene contained in the plasmid you isolated. Align the complementary sequences where they overlap, and use this information to resolve ambiguities. Indicate which portions of each sequence correspond to the plasmid vector, PCR primers, and the amplified 16S rRNA gene. Remove vector and primer sequences before you perform a BLAST search to identify the species from which the 16S rRNA gene came. Appendix G, "Sequence Editing Tutorial," provides detailed examples of these procedures.
7. Locate the *Rsa*I (`GT/AC`) and *Msp*I (`C/CGG`) restriction sites in the 16S rRNA gene sequence, and determine the length of the corresponding T-RFLP fragments for each enzyme; remember that the *Fam* label was on the 27F primer. See Appendix G for guidance. Do these predicted T-RFLP fragments correspond to peaks observed on the T-RFLP plots?
8. Include both (*Rsa*I and *Msp*I) T-RFLP plots in your report (see Fig. 4.4 for examples); include a table with the numerical data (use Table 4.2 as an example). Use the Applied Biosystems Peak Scanner program to generate T-RFLP plots and data tables; see Appendix H, "Peak Scanner Tutorial," for instructions. Use Excel to calculate how much each peak contributes to the total signal; determine the sum of the "Peak Area" column and divide the signal for each peak by this total. How many major peaks (>2% of the total signal) did each restriction enzyme produce? Did each enzyme produce the same number of major peaks? How many bacterial species did you observe using T-RFLP analysis? Compare this to the number of species you detected using a culture-based approach in experiment 3.
9. Compare the bacterial population in your sample with those in samples collected by other members of your team. Use the PRIMER (Plymouth Routines in Multivariate Ecological Research) program to calculate and plot the similarity of each pair of samples collected by your team. Construct an Excel table that contains the T-RFLP data from all the samples studied by your team. Follow the format in Appendix J, "PRIMER Tutorial," which explains the theoretical basis and operation of the PRIMER program. See the laboratory report entitled 'Identification of probiotics, *L. delbrueckii* and *S. thermophilus*, in Yoplait® Original Vanilla using a culture-independent approach' in Appendix A for an example. These students compared the bacterial populations in six different yogurt samples. They also compared these populations with those analyzed by another team, which studied human saliva. I encourage you to compare your team's samples with those studied by another team.

TABLE 4.2 *Msp*I T-RFLP Profile of Gastric Bacterial Populations from a Crohn's Patient

Peak	Sample	Size	Area	Fraction	Genus
B, 3	IBD 45	63	20981	0.038	
B, 4	IBD 45	67	701	0.001	
B, 5	IBD 45	75	6691	0.012	
B, 6	IBD 45	78	14907	**0.027**	*Streptococcus*
B, 7	IBD 45	80	1487	0.003	
B, 8	IBD 45	86	1855	0.003	
B, 9	IBD 45	88	1195	0.002	
B, 10	IBD 45	91	1938	0.004	
B, 11	IBD 45	93	14065	**0.026**	*Prevotella*
B, 12	IBD 45	95	34169	**0.063**	*Prevotella*
B, 13	IBD 45	126	1316	0.002	
B, 14	IBD 45	128	673	0.001	
B, 15	IBD 45	140	937	0.002	
B, 16	IBD 45	142	773	0.001	
B, 17	IBD 45	147	1764	0.003	
B, 18	IBD 45	148	544	0.001	
B, 19	IBD 45	164	2115	0.004	
B, 20	IBD 45	166	8358	0.015	
B, 21	IBD 45	172	523	0.001	
B, 22	IBD 45	173	1192	0.002	
B, 23	IBD 45	202	767	0.001	
B, 24	IBD 45	219	956	0.002	
B, 25	IBD 45	268	3631	0.007	
B, 26	IBD 45	275	113989	**0.209**	*Leptotrichia*
B, 27	IBD 45	281	4486	0.008	
B, 28	IBD 45	284	1273	0.002	
B, 29	IBD 45	287	5355	0.010	
B, 30	IBD 45	295	15563	0.028	
B, 31	IBD 45	298	3128	0.006	

(*Continued*)

Table 4.2 (*Continued*)

Peak	Sample	Size	Area	Fraction	Genus
B, 32	IBD 45	301	85990	**0.157**	*Veillonella*
B, 33	IBD 45	469	939	0.002	
B, 34	IBD 45	485	20581	0.038	
B, 35	IBD 45	490	1419	0.003	
B, 36	IBD 45	496	32460	**0.059**	*Neisseria*
B, 37	IBD 45	498	587	0.001	
B, 38	IBD 45	502	1486	0.003	
B, 39	IBD 45	520	2974	0.005	
B, 40	IBD 45	547	976	0.002	
B, 41	IBD 45	551	3836	0.007	
B, 42	IBD 45	554	112433	**0.206**	*Streptococcus*
B, 43	IBD 45	556	7153	**0.013**	*Streptococcus*
B, 44	IBD 45	559	1444	0.003	
B, 45	IBD 45	564	5116	0.009	
B, 46	IBD 45	570	2210	0.004	
B, 47	IBD 45	719	1316	0.002	
			546252		

Note: These data correspond to the T-RFLP profile in the bottom panel of Fig. 4.4. To identify genera corresponding to major T-RFLP peaks, we sequenced 116 cloned 16S rRNA genes from the amplicon. The "Size" column indicates the T-RFLP fragment length in nucleotides. The "Area" column indicates the number of fluorescence units in each peak. Cells in the "Fraction" column show each peak area divided by the total signal (546,252 fluorescence units).

Notes

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V. QUESTIONS (CLASS 20)

1. Did you detect more bacterial species using a culture-based approach (experiment 3) or a culture-independent approach (experiment 4)? Explain.
2. Can the approach used in experiment 4 detect dead bacteria? How does the answer to this question affect your interpretation of T-RFLP data?
3. Does a T-RFLP profile accurately reflect the bacterial population present in a sample? Explain.
4. Did you select a Lac^- (white) or a Lac^+ (blue) colony from your clone library for sequence analysis? Explain.
5. Without using DNA sequence analysis, were you able to identify specific bacterial species based on the T-RFLP peaks they produced? Explain.

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VI. WRITING EXERCISE: EDITORIAL ON GENETICALLY MODIFIED CROPS [CLASS 14 (DRAFT) AND CLASS 15]

Read the editorials listed in the Introduction to this book (section XI, Required and Suggested Readings, E. Required Editorials). These articles raise scientific and ethical issues, and they exemplify the critical role that scientific decisions play in everyday life. They also show that science does not operate in a vacuum devoid of politics or human considerations.

Write a brief (1- to 2-page) editorial on this issue. Support your opinion with facts from these articles and other sources. Begin with a brief summary of the problem. State your position on the controversy. Support your position by constructing arguments based on facts. Conclude the paper with a statement that summarizes your position.

Notes

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VII. WRITING ASSIGNMENT: LITERATURE SEARCH PROBLEM [CLASS 14 (BEGIN) AND CLASS 20]

The correct answer to this problem is worth 100 points. It is due at the beginning of class on the last day. Send your answer to your instructor by e-mail.

You are **free to read anything**, but **do not discuss this question or your answer with others.**

Your instructor wants **your** ideas.

Pretend you have just taken a job at New England Biolabs, and Dr. Richard J. Roberts, director of Research, has instructed you to clone (in *E. coli*) the gene for a new type II restriction endonuclease that he discovered in a strain of *Haemophilus aegyptius*.

Dr. Roberts has provided you with:

1. Genomic DNA from this strain
2. The purified restriction endonuclease
3. The target sequence for this endonuclease

You have access to reagents (ligase, DNA polymerases, cloning vectors, etc.) sold by New England Biolabs. However, you *do **not** know the sequence of the gene* that encodes this endonuclease, and you *do **not** have the genome sequence* for this species.

In a seminar, you heard Dr. Roberts say that, for more than 2800 known type II restriction endonucleases, the gene encoding an endonuclease lies adjacent to the corresponding modification methylase gene.

Dr. Roberts wants you to have the endonuclease gene cloned by the morning of your third day at work, or **you are fired**. This *does **not** leave you time to screen a library* of clones for the one Dr. Roberts wants. How do you impress Dr. Roberts and keep your job?

Notes

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VIII. IN-CLASS WRITING EXERCISE (CLASS 15)

The following questions refer to articles on genetically altered foods:

Do you think people from underdeveloped nations would have a different opinion on genetically modified foods than Europeans or Americans?

Do you think people are less likely to accept genetically engineered foods than genetically engineered medicines? Explain.

Which values, ethical or scientific, are most important to you in this controversy? Rank them in terms of importance in your decision-making process.

Notes

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APPENDIX

A

Sample Laboratory Reports

The "Professor's Comments" on each laboratory report are shown in red in square brackets and refer to the preceding underlined words.

REPORT 1

Name: Connie Lee
Title **[[Professor's Comment:** Good.**]]**
Vibrio fischeri Luciferase Operon on Plasmid pKN800 Contributes **[[Professor's Comment:** Confers**]]**Bioluminescence to *Escherichia coli*
Date: January 12, 2012–January 18, 2012

Purpose

To study expression of the *Vibrio fischeri* luciferase (*lux*) operon in *Escherichia coli* (*E. coli*) by purifying a plasmid DNA from *E. coli*, determining the structure of the plasmid using restriction mapping, and introducing the recombinant plasmid into another *E. coli* strain using transformation. **[[Professor's Comment:** Nice, but too long. Break into two or three sentences.**]]**

Methods

We received a strain of *E. coli* containing unspecified pKN800 plasmid DNA. We purified the plasmid DNA and used *Pst*I restriction enzyme to cut the plasmid DNA. We loaded *Pst*I-cut and uncut pKN800 plasmid DNA into an agarose gel for electrophoresis. We transformed *E.coli* strain DH5α with the pKN800 plasmid DNA.

We followed the procedures on p. 43–54 of the laboratory manual with the following exceptions (2): Step A-11 on p. 45: The tube was centrifuged again for five seconds after centrifuging the tube for 10 seconds. Step A-11 on p. 45: The cap of the tube was open to evaporate the ethanol. Step C-1 on p. 48: We used an 1.0% agarose gel to separate the restriction

fragments in the DNA samples. Step D-6 on p. 52: We incubated the samples at 225 rpm for 45 minutes at 37 °C. Steps E-1 to E-4 on p. 54: We examined the plates for two days.

Results

We received an *E. coli* strain containing an unspecified pKN800 plasmid DNA. To determine whether the orientation of the *lux* operon in pKN800 affects luminescent ability in *E. coli*, we purified pKN800 plasmid DNA and determined the orientation of the *lux* operon. To confirm the function of pKN800 plasmid DNA in luciferase production, we transformed pKN800 plasmid DNA into *E. coli* DH5α and recorded the number of ampicillin-resistant and luminescent transformants. **[[Professor's Comment: Excellent.]]**

To determine the orientation of the purified pKN800, we digested the plasmid with *Pst*I, which cuts the plasmid cloning vector pBR322 once and the *lux* operon once. We separated the restriction fragments of the *Pst*I-cut and uncut pKN800 plasmid DNA by agarose gel electrophoresis. Lane one had distinctive bands, which were the 1000-bp molecular weight standard (Fig. A1.1). Lane two had two fragments, containing restriction fragments of the *Pst*I-cut pKN800 plasmid DNA; the top band was more intense than the bottom band. Lane three had only one band, containing the supercoiled uncut pKN800 plasmid DNA; the intensity of the band was low (Fig. A1.1).

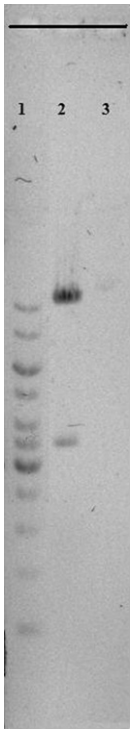

FIGURE A1.1 **Agarose gel electrophoresis of *Pst*I-cut and uncut pKN800 plasmid DNA:** lane 1, 1000-base pair (bp) molecular weight standard; lane 2, pKN800 plasmid DNA cut with *Pst*I; lane 3, pKN800 plasmid DNA not cut with *Pst*I.

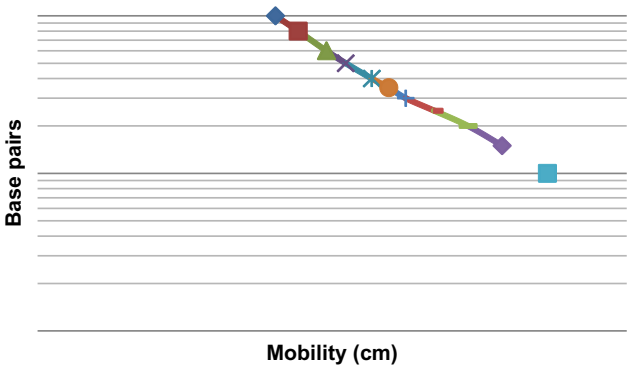

FIGURE A1.2 **Standard curve of 1000-bp DNA molecular weight standard.**

To estimate the size of the restriction fragments cut by *Pst*I, we constructed a standard curve of the 1000-bp DNA molecular weight standard and used it to estimate the size of the restriction fragments (Fig. A1.2). We compared the migration distances of the bands of lane two with the 1000-bp DNA molecular weight standards (Fig. A1.1). The migration distances of the restriction fragments of *Pst*I-cut pKN800 plasmid were 2.05 cm and 3.1 cm. *Pst*I digestion of pKN800 plasmid DNA produced two restriction fragments of 10,500 and 3,500 bp. We did not estimate the size of the uncut pKN800 plasmid DNA as we only used this sample to check whether *Pst*I digested the plasmid completely.

To confirm the function of pKN800 in the production of luciferase protein, we transformed pKN800 plasmid DNA into competent *E. coli* DH5α and observed luciferase reporter gene expression in ampicillin-resistant transformants (Table A1.1).

We calculated the efficiency of transformation to determine whether the transformation was efficient. To calculate the efficiency of transformation, we used the following formula:

$$\frac{\text{The number of ampicillin} - \text{resistant and luminescent transformants}}{\text{The amount of DNA used to transform}}$$

The number of ampicillin-resistant and luminescent transformants from 10^{-1} diluted culture transformed with the uncut pKN800 plasmid DNA was insignificant, so the efficiency of transformation only used the data from the undiluted culture transformed with the uncut pKN800 plasmid DNA. We estimated the amount of the uncut pKN800 plasmid DNA by comparing it with the intensity of the bands of the 1000-bp molecular weight standard (Fig. A1.3).

The amount of pKN800 plasmid DNA was 0.025 μg. The efficiency of transformation was

$$\frac{172 \text{ units}}{0.025\ \mu g} = 6880 \text{ transformants per microgram of plasmid DNA}$$

Discussion

Plasmid pKN800 contains the *lux* operon, which contains α and β subunits that are responsible for luminescence (1). **[[Professor's Comment:** Nice! You may be the only student to cite more than the lab manual.**]]** Insertion of the *lux* operon into the plasmid

TABLE A1.1 The Average Number of Ampicillin-Resistant and Luminescent *E. coli* DH5α Transformants in Each Transformation

Plate	Type of Medium	Number of Ampicillin-Resistant and Luminescent Transformants (units[b])	Number of Colonies
Undiluted culture with uncut pKN800 plasmid DNA		172[c]	178
10^{-1} diluted culture with uncut pKN800 plasmid DNA	LB + Amp agar[a]	Too few to count[d]	Too few to count[d]
Undiluted culture with *Pst*I-cut pKN800 plasmid DNA (positive control)		Too few to count[e]	Too few to count[f]
Undiluted culture with no pKN800 plasmid DNA (negative control)		0	0
10^{-5} diluted culture with no pKN800 plasmid DNA	LB agar	0	Too numerous to count[g]
10^{-6} diluted culture with no pKN800 plasmid DNA		0	221

[a]*This medium contained LB and ampicillin.*
[b]*One unit is one colony.*
[c]*The average was 171.5 units.*
[d]*The average numbers of transformants and colonies were both 10.5 units.*
[e]*The average number of transformants was 1.5 units.*
[f]*The average number of transformants was 4.5 units.*
[g]*The average number of transformants was 274 units.*

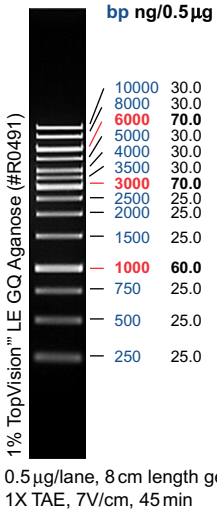

FIGURE A1.3 **The 1000-bp molecular weight standard containing the size and intensity of each band.** The bands with red numbers have twice as much DNA as the bands with blue numbers. The right column is the amount of DNA in each band.

cloning vector pBR322 confers bioluminescence and disrupts tetracycline resistance. A pKN800 plasmid DNA can have one of the two orientations. pKN800-A contains the *lux* operon in the same direction as the *tet* gene, and pKN800-B contains the *lux* operon and the *tet* gene in the opposite direction (2). To investigate whether the orientation of the *lux* operon in pKN800 plasmid affects light production, we purified the plasmid, determined the orientation of the plasmid, and transformed another *E. coli* strain to screen for ampicillin-resistant and luminescent transformants.

*Pst*I digestion of pKN800 plasmid DNA produces two fragments, and the size of each restriction fragment depends on the orientation of the *lux* operon on pKN800 plasmid DNA. To determine the orientation of the *lux* operon on the pKN800 plasmid, we compared the sizes of the restriction fragments with Fig. 1.2 on p. 35 of the laboratory manual (2). According to Fig. 1.2, *Pst*I-cut pKN800-A plasmid DNA produces restriction fragments of 11,540 bp and 1,400 bp; *Pst*I-cut pKN800-B plasmid DNA produces restriction fragments of 9,980 bp and 3,380 bp. Since the result matches closer to the theoretical sizes of the restriction fragments of *Pst*I-cut pKN800-B plasmid, we concluded that the orientation of the *lux* operon in the unspecified pKN800 plasmid is pKN800-B (antisense). *Pst*I digested all pKN800 plasmids completely because there were no unexpected bands on the agarose gel.

Transformation of *E. colii* **[[Professor's Comment:** Spelling.**]]** DH5α with pKN800 plasmid DNA helps us to confirm that pKN800 plasmid DNA encodes functional luciferase proteins. Transformation of pKN800 plasmid DNA into *E. coli* DH5α was successful as we observed growth of ampicillin-resistant and luminescent transformants on LB + Amp agar plates. The culture transformed with uncut pKN800 plasmid DNA contained genes coding for both ampicillin resistance and luminescent ability. The high efficiency **[[Professor's Comment:** In fact, the transformation efficiency you achieved is very low compared to that obtained with the very best cells (~10E9/μg).**]]** of the transformation indicates that the transformation of *E. colii* **[[Professor's Comment:** Spelling.**]]** DH5α with pKN800 DNA was efficient.

The positive control **[[Professor's Comment:** This is a type of negative control, not a positive one.**]]** indicates that *Pst*I successfully cut the pKN800 plasmid DNA as we observed no significant growth. (The few colonies in the positive control indicate that some uncut pKN800 plasmid DNA may be still present. However, the number of transformants is **[[Professor's Comment:** Wrong tense.**]]** too few to count and therefore insignificant.) The culture transformed with *Pst*I-cut pKN800 plasmid DNA cannot grow on LB + Amp agar plates because exonuclease V in *E. coli* destroys linear *Pst*I-cut pKN800 plasmid DNA when the linear DNA enters the bacteria. The culture did not contain cells transformed with the *Ap* gene, which is responsible for ampicillin resistance.

There were **[[Professor's Comment:** This is correct, but see Day's book, page 214, or example 2 in section A of Writing Tips.**]]** viable (ampicillin-sensitive) cells in the cultures as we observed growth on LB agar plates containing cultures that had no cells transformed with the pKN800 plasmid DNA. The negative control ensured that the competent cells are not resistant to ampicillin prior to transformation with pKN800 plasmid DNA as the culture without the pKN800 plasmid DNA did not grow on the LB + Amp plates. The cells in the culture did not contain the *Ap* gene, which is responsible for ampicillin resistance.

The orientation of the *lux* operon on pKN800 is antisense, and we transformed *E. coli* DH5α successfully and efficiently. However, we are not able to determine whether the orientation of the *lux* operon affects the luminescent ability in *E. coli* as all students received the pKN800-B plasmid DNA. We can only determine that the antisense orientation of the *lux* operon on pKN800 plasmid does not affect luminescent ability in *E. coli*.

Conclusion

The orientation of the pKN800 plasmid DNA is antisense, and the pKN800-B plasmid DNA introduces ampicillin resistance and luminescent ability to *E. coli* DH5α efficiently.

References

1. Engebrecht J, Nealson K, Silverman M. 1983. Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*. *Cell* **32**:773–81 **[[Professor's Comment:** Nice!**]]**.
2. Ream W, Geller B, Trempy J, Field K. 2003. *Molecular Microbiology Laboratory Manual*. San Diego, CA: Academic Press; pp. 32–54.

Questions

1. The uncut pKN800 plasmid DNA only formed one band during agarose gel electrophoresis. The band represents the circular supercoiled pKN800 plasmid DNA.

2. My pKN800 plasmid DNA is pKN800-B. I calculated the size of the restriction fragments of a *Pst*I-cut pKN800 plasmid DNA based on the information provided on Fig. 1.2 on p. 35 of the laboratory manual (2). The two restriction fragments on pKN800-B in Fig. 1.2 were 9,980 bp and 3,380 bp, which match with the sizes of the restriction fragments of my *Pst*I-cut pKN800 plasmid DNA.

3. My *Pst*I digestion of pKN800 plasmid DNA went to completion. We observed no colonies[h] on undiluted *Pst*I-cut DNA on LB + Amp agar plates.

4. We got ampicillin-resistant transformants with restricted pKN800 plasmid DNA. We observed colonies on uncut pKN800 plasmid DNA on the LB + Amp plates. If we had no ampicillin-resistant transformants with restricted pKN800 plasmid DNA, we would have no growth on the LB + Amp plates.

5. I would *not* expect any of the transformations to yield ampicillin-resistant nonluminescent transformants. If the given pKN800 plasmid DNA contains recircularized cloning vector pBR322 **[[Professor's Comment:** I do not understand how this could happen.**]]**combined with the *lux* operon, the transformants would still be resistant to ampicillin (it still has the *Ap* gene) but could not glow in the dark. -9

6. I analyzed the uncut pKN800 plasmid DNA on the agarose gel because I wanted to confirm whether the *Pst*I-cut pKN800 plasmid was cut successfully. The uncut pKN800 plasmid DNA served as a comparison. Also, I wanted to estimate the amount of DNA used to transform *E. coli* by comparing the intensity of the band with the 1000-bp DNA molecular weight standard.

[h]A small number of colonies grew on the plates, but the average number was 4.5 colonies, which was too few to count and insignificant.

7. I plated bacteria that did not receive any plasmid DNA to set a negative control. The negative control on LB + Amp agar plate ensures that the competent cells were not resistant to ampicillin before the transformation with pKN800 DNA, and it also shows that the plates contained enough antibiotic to kill the untransformed cells. We also plated bacteria with no plasmid DNA on LB agar to ensure that the culture contained ampicillin-sensitive cells.
8. I would plate the uncut pKN800 plasmid transformants on LB agars. If we observe no growth on LB agar, then the problem is due to incorrect medium. If the medium is incorrect, the uncut plasmid transformants should still not be able to grow and glow in the dark as they cannot survive on the plates. If we observe colonies on LB agars, then the problem is due to poor transformation efficiency. If the transformation is inefficient, then some of the transformants can still grow on the plate and glow in the dark. Streak a **known ampicillin-resistant strain** on the plates; it should grow. For example, you could streak the strain you used to prepare pKN800 plasmid DNA because those cells were grown in ampicillin. -3

REPORT 2

Name: Connie Lee
Title
Identification of probiotics, *L. delbrueckii* and *S. thermophilus*, **[[Professor's Comment:** Use the complete genus name the first time you mention it.**]]** in Yoplait® Original Vanilla using a culture-independent approach
Date: January 31, 2012–March 6, 2012

Purpose

Probiotics in yogurt have many beneficial effects on human health, such as improving human's **[[Professor's Comment:** the**]]** immune system and lowering the risk of cancer and gastrointestinal disorders (1). Identification of probiotics in yogurt may help researchers to indicate which strain is the most efficient one in making yogurt while providing health benefits. Since many bacteria are unculturable, we used a culture-independent approach to identify and study the probiotic community in yogurt, and we compared the result we got **[[Professor's Comment:** Colloquial..**]]**from the culture-dependent approach. Using the culture-independent approach, we identified the probiotic community in yogurt by purifying genomic DNA directly from a sample collected for terminal restriction fragment length polymorphism (T-RFLP) analysis and DNA sequence analysis.

Methods

We collected samples from Yoplait® Original Vanilla yogurt and extracted genomic DNA directly from the uncultured bacterial sample. We amplified 16S rRNA genes by polymerase chain reaction (PCR). We separated the PCR amplicons by agarose gel electrophoresis. We digested the purified PCR products with restriction enzymes and submitted

the digested DNA to the Central Services Lab for capillary electrophoresis. The data from capillary electrophoresis allowed us to perform terminal restriction fragment length polymorphism (T-RFLP) analysis on the PCR amplicons. We also transformed the PCR amplicons into *Escherichia coli* to create a molecular clone library of the 16S rRNA genes present in the PCR product. We sequenced the clones to identify the probiotics detected in T-RFLP analysis.

We followed the procedures on pp. 12–19 of the laboratory manual with the following exceptions (2): Step D-7 on p. 16: We added 50 μL of deionized water into the microcentrifuge tube. Step F-8: We did not re-streak the transformed *E. coli* on a fresh LB agar plate. Step H-1: We received 1 μL of restriction endonucleases +2 μL pf 10x buffer with BSA. Step I-1: For our group, we also sequenced additional clones from the agar plate.

Results

Identification of probiotic community in yogurt may help researchers to indicate which strain is the most efficient one in making yogurt while providing health benefits. However, since many bacteria are currently unculturable in the laboratory, we used a culture-independent approach to identify the probiotic community in yogurt. **[[Professor's Comment:** You already said this.**]]** To collect DNA from the probiotic community in yogurt, we purified genomic DNA directly from the uncultured sample collected from yogurt. We observed the diversity of bacteria present in the yogurt by analyzing restriction-fragment-length polymorphisms (RFLPs) among the fluorescently labeled terminal fragments of the extracted **[[Professor's Comment:** amplified.**]]** DNA. To identify the bacteria detected in T-RFLP analysis, we created a molecular clone library and sequenced the clones.

To determine the most abundant probiotic in yogurt, we collected samples from Yoplait® Original Vanilla yogurt. Yoplait® Original Vanilla was a wet sample source. **[[Professor's Comment:** Unnecessary.**]]** We opened the seal of the yogurt bottle immediately before we collected the samples.

To determine the purity, size, and concentration of the purified PCR product, we separated the PCR products by agarose gel electrophoresis. Lane one had three bands, which were the low DNA mass ladder. Lane two had one band with smearing. Lane three was the no-template negative control, and the sample produced no band (Fig. A2.1).

To estimate the size of the purified PCR product, we constructed a standard curve of the low DNA mass ladder molecular weight standards (Figs. A2.2 and A2.3). To estimate the size of the PCR product, **[[Professor's Comment:** You just said this.**]]** we measured the migration distance of the band. We compared the migration distance of the band in lane two with the bands of the low DNA mass ladder. The band of lane two had a migration distance of 11.7 cm (Fig. A2.1). Extrapolating from the standard curve, we estimated that the band in lane two was 942 base pairs (Fig. A2.3).

To estimate the concentration of the purified PCR product, we estimated the intensity of the band in lane two relative to the molecular weight standards. The PCR amplicons had a concentration of 5 ng/μL. **[[Professor's Comment:** How did you calculate this number. The band on your gel is slightly stronger than the 80-ng standard, and your band is much weaker than the 120-ng standard. So, you have ~90 ng/5 ul loaded in the

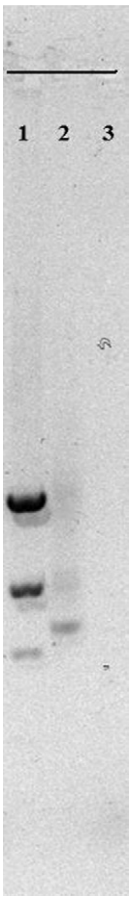

FIGURE A2.1 **Agarose gel electrophoresis of PCR products.** Lane one contains DNA mass ladder (Invitrogen). Lane two contains purified PCR products. Lane three contains a sample from the no-template PCR reaction.

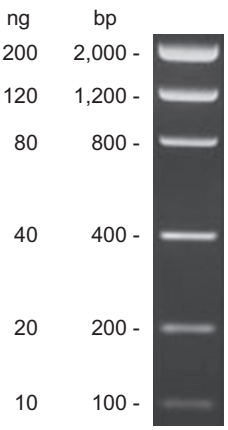

FIGURE A2.2 **Low DNA mass ladder (Invitrogen) molecular weight standards on a 2% 3:1 agarose gel.** Each lane contains 4 μL.

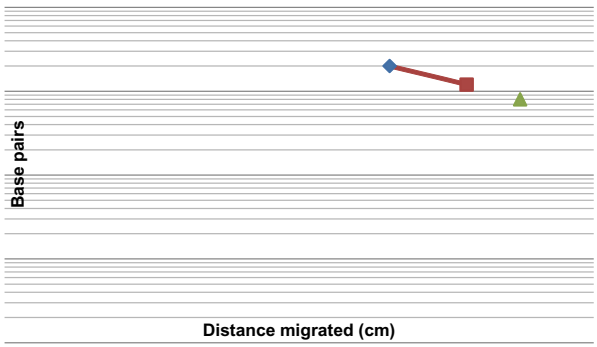

FIGURE A2.3 **Standard curve of the low DNA mass ladder molecular weight standards.**

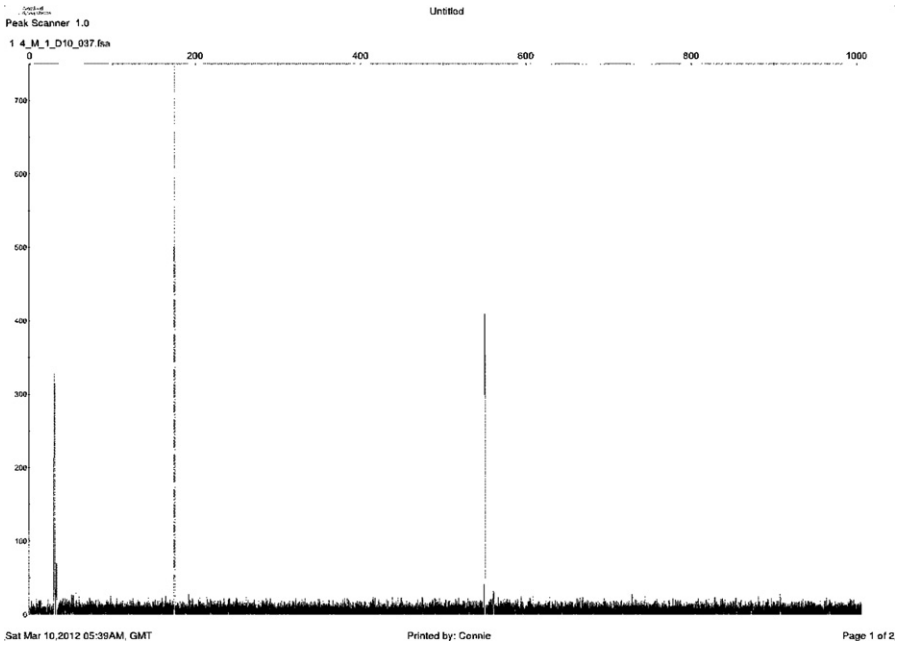

FIGURE A2.4 **T-RFLP profile of *Msp*I-cut PCR amplicons of the bacterial population from Yoplait® Original Vanilla yogurt.** We cut the purified PCR amplicons with *Msp*I restriction enzyme and the submitted sample **[[Professor's Comment:** Unnecessary.**]]** was in a dilution factor of **[[Professor's Comment:** Awkward.**]]** 20. The x-axis is the fragment length (nucleotides), and the y-axis is the fluorescence intensity (height of the peak).

lane = 18 ng/ul.**]]** The purified PCR product's concentration measured by the nanodrop spectrophotometer was 38 ng/μL.

To observe the complexity of the bacterial population in yogurt, we submitted our purified PCR products for T-RFLP analysis. The *Msp*I restriction endonuclease produced four **[[Professor's Comment:** I see only 3 peaks.**]]** major peaks, indicating that there were at least four different bacterial species in the sample (Fig. A2.4). The *Rsa*I endonuclease produced three major peaks, indicating there were at least three different bacterial species

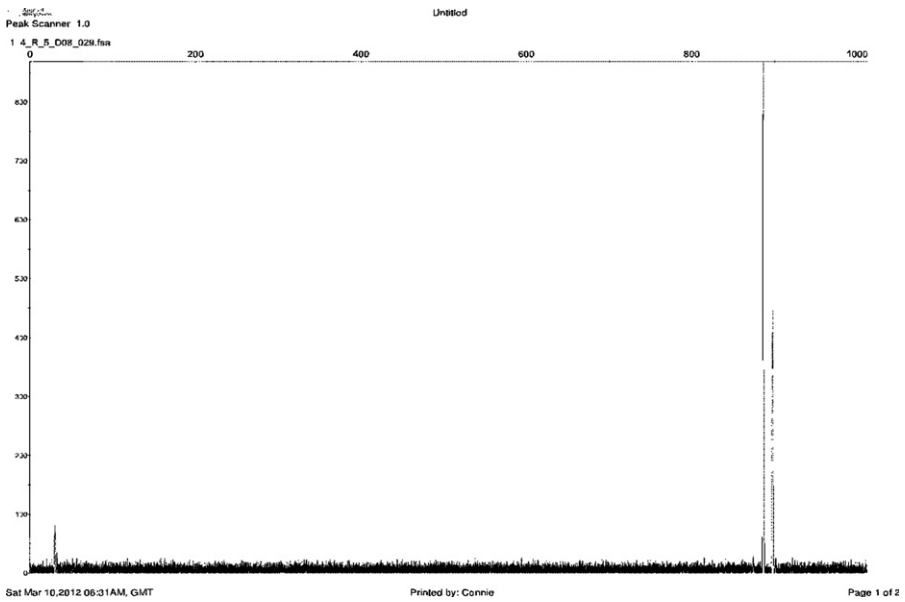

FIGURE A2.5 **T-RFLP profile of *Rsa*I-cut PCR amplicons of the bacterial population from Yoplait® Original Vanilla yogurt.** We cut the purified PCR amplicons with *Rsa*I restriction enzyme and the submitted sample **[[Professor's Comment:** Unnecessary.**]]** was in a dilution factor of **[[Professor's Comment:** Awkward.**]]** four. The x-axis is the fragment length (nucleotides), and the y-axis is the fluorescence intensity (height of the peak).

TABLE A2.1 Length and Intensity of the *Msp*I-Digested DNA Fragments of the Bacterial Population in Yoplait® Original Vanilla Yogurt

Size of the Fragment (base pairs)	Intensity of the Fragment (height of the peak)
30	329
32	68
174	750
550	410

The submitted sample was in a dilution factor of **[[Professor's Comment:** Awkward.**]]** 20.

in the sample (Fig. A2.5). The *Msp*I and *Rsa*I restriction endonucleases did not produce the same number of major peaks. **[[Professor's Comment:** I disagree.**]]** Analyzing the T-RFLP files, we obtained the length and relative quantity of each labeled terminal restriction fragment digested by either *Msp*I or *Rsa*I restriction endonucleases (Tables A2.1 and A2.2).

To identify the species associated with particular T-RFLP peaks, we constructed a molecular clone library and submitted the clones for DNA sequence analysis. To identify

TABLE A2.2 Length and Intensity of the *Rsa*I-Digested DNA Fragments of the Bacterial Population in Yoplait® Original Vanilla Yogurt

Size of the Fragment (base pairs)	Intensity of the Fragment (height of the peak)
30	78
886	869
897	445

The submitted sample was in a dilution factor of **[[Professor's Comment:** Awkward.**]]** four.

TABLE A2.3 Number of Lac^- and Lac^+ Colonies in the Clone Library Made from T-RFLP Amplicons

Concentration of the Transformants	Number of Lac^- (white) Colonies	Number of Lac^+ (blue) Colonies
Original (not concentrated) transformed *E. coli* cells	Too numerous to count	10
Concentrated transformed *E. coli* cells	Too numerous to count	20

The concentration of the transformed cells spread on the agar plate was not measurable. Lac^- colonies were white because they did not produce active β-galactosidase to cleave ×-gal due to the inserted DNA. Lac^+ colonies were blue because they produced active β-galactosidase to cleave ×-gal.

the transformants with vectors that have DNA inserted, we observed the effect of the inserted DNA on *Lac*Z gene expression in ampicillin-resistant transformants (Table A2.3).

We sequenced the 16S rRNA gene to identify the bacteria corresponding to particular T-RFLP peaks. We indicated the portions of each sequence corresponding to the plasmid vector, PCR primers, and amplified 16S rRNA gene (Figs. A2.6 and A2.7). Appendix A shows the additional sequences of the molecular clone library. The bacteria were *Streptococcus thermophilus* and *Lactobacillus delbrueckii*. In a total of 22 DNA sequences, 16 sequences were *S. thermophilus*. Five out of the 22 sequences **[[Professor's Comment:** 16 + 5 = 21 What was #22?**]]** were *L. delbrueckii*. All sequences had 99–100% identity match in the BLAST search.

To determine the length of the corresponding T-RFLP fragments for each enzyme, we located the *Rsa*I (GTAC) and *Msp*I (CCGG) restriction sites in the 16S rRNA gene sequence (Figs. A2.6 and A2.7 and Appendix B). The *Msp*I-cut DNA fragment of *S. thermophilus* contained 555 nucleotides. The *Rsa*I-cut DNA fragments of *S. thermophilus* could contain either 32–34 nucleotides or 891 nucleotides. The *Msp*I-cut DNA fragment of *L. delbrueckii* had 179 nucleotides. The *Rsa*I-cut DNA fragments of *L. delbrueckii* had 905 nucleotides. Using the length of the fragments cut by each restriction enzymes, we identified the bacteria corresponding to particular T-RFLP peaks (Figs. A2.8 and A2.9). On the T-RFLP profile of *Rsa*I-cut PCR amplicons, both *S. thermophilus* and *L. delbrueckii* could correspond to the peak with the size of 897 base pairs (Fig. A2.9).

We used the Plymouth Routines in Multivariate Ecological Research (PRIMER) program to compare the bacterial population in the samples collected from yogurt and human saliva

>04A_4_M13R-27 TRIM QUALITY: 20
GTCCTGCAGGTttaACGAATTCGCCCTTCCgtaATTCCTTTGAGTTTCAACCTTGCGGTCGTAC
TCCCCAGGCGGAGTGCTTAATGCGTTTGCTGCGGCACTGAGGACCGGAAAGTCCCCAACACCTA
GCGCTCATCGTTTACGGCATGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGA
GCCTCAGCGTCAGTTACAGACCAGAGAGCCGCTTTCGCCACCGGTGTTCCTCCATATATCTACG
CATTTCACCGCTACACATGGAATTCCACTCTCCCCTTCTGCACTCAAGTTTGACAGTTTCCAAA
GCGAACTATGGTTGAGCCACAGCCTTTAACTTCAGACTTATCAAACCGCCTGCGCTCGCTTTAC
GCCCAATAAATCCGGACAACGCTCGGGACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGC
CGTCCCTTTCTGGTAAGCTACCGTCACAGTGTGAACTTTCCACTCTCACACCCGTTCTTGACTT
ACAACAGAGCTTTACGATCCGAAAACCTTCTTCACTCACGCGGCGTTGCTCGGTCAGGGTTGCC
CCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGT
GTGGCCGATCACCCTCTCAGGTCGGCTATGTATCGTCGCCTAGGTGAGCCATTACCTCACCTAC
TAGCTAATACAACGCAGGTCCATCTTGTAGTGGAGCAATTGCCCCTTTCAAATAAATGACATGT
GTCATCCATTGTTATGCGGTATTAGCTATCGTTTCCAATAGTTATCCCCCGCTACAAGGCAggT
TACCTACGCGTTACTCACCCGTTCCGCAACTCATCCAagaaCagCaaCGTCCTCTCTTCACCCTT
CTA

FIGURE A2.6 ***S. thermophilus*'s DNA sequence from the M13R primer.** Underlined nucleotides are part of the vector sequence. Blue nucleotides are 926-907R primer used in PCR amplification. Green nucleotides are the *Rsa*I restriction site. Pink nucleotides are the *Msp*I restriction site. Crossed-off nucleotides are ambiguities in the sequence, and we removed them before the BLAST search. Bold and italicized bases are the sequence we used for the BLAST search.

>04A_4_T7Prom TRIM QUALITY: 20
AGAGTTTGATCATGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACGCTG
AAGAGAGGAGCTTGCTCTTCTTGGATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCC
TTGTAGCGGGGGATAACTATTGGAAACGATAGCTAATACCGCATAACAATGGATGACACATGTC
ATTTATTTGAAAGGGGCAATTGCTCCACTACAAGATGGACCTGCGTTGTATTAGCTAGTAGGTG
AGGTAATGGCTCACCTAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGA
CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACC
CTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGTCAA
GAACGGGTGTGAGAGTGGAAAGTTCACACTGTGACGGTAGCTTACCAGAAAGGGACGGCTAACT
ACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGC
GAGCGCAGGCGGTTTGATAAGTCTGAAGTTAAAGGCTGTGGCTCAACCATAGTTCGCTTTGGAA
ACTGTCAAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAT
ATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGACGCTGaggCTCGAAAG
CGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTaaacgATGCACGCTAGGTGT
TCGGCACTTTCGGGTCGTCAgtgccgcagcAAACGCATTAAGCACT

FIGURE A2.7 ***S. thermophilus*'s DNA sequence from the T7 promoter primer.** In this sequence, we did not observe vector sequence. Yellow nucleotides are the 8-27F primer used in PCR amplification. Red nucleotides are the rest of the sequence of 8-27F primer that are not present in the sequence. There is no *Rsa*I restriction site. Pink nucleotides are the *Msp*I restriction site. Crossed-off nucleotides are ambiguities in the sequence, and we removed them before the BLAST search. Bold and italicized bases are the sequence we used for the BLAST search.

(Table A2.4).. **[[Professor's Comment:** One period will do.**]]** We transformed the data by finding the square root of the data. **[[Professor's Comment:** Redundant**]]** Samples with similar populations lie closer together than samples with dissimilar populations (Fig. A2.10). The bacterial populations from yogurt samples lied **[[Professor's Comment: lay.]]** close to

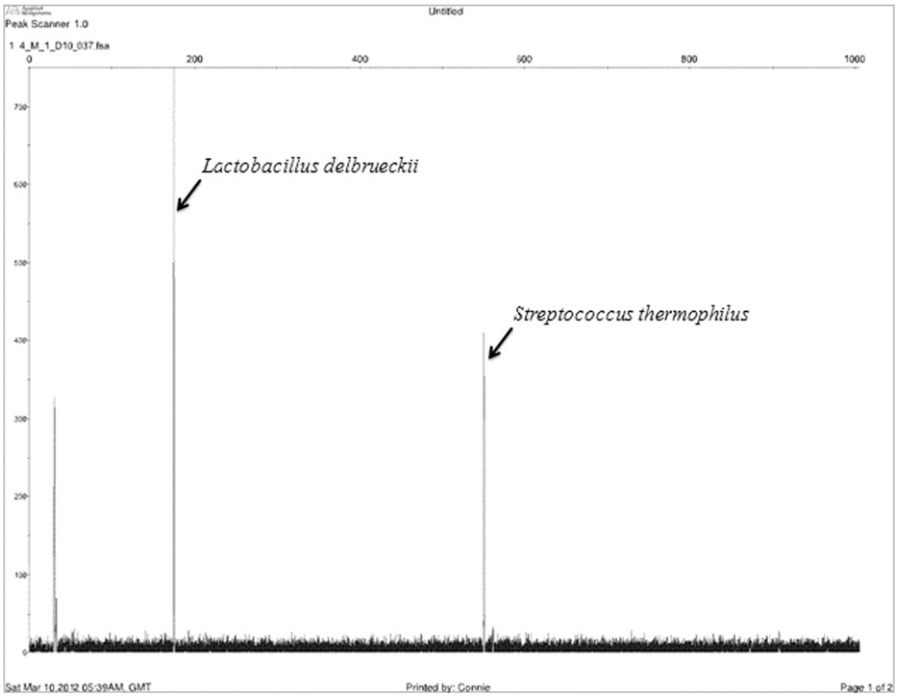

FIGURE A2.8 **T-RFLP profile of *Msp*I-cut PCR amplicons of identified bacterial population from Yoplait® Original Vanilla yogurt.** We cut the purified PCR amplicons with *Msp*I restriction enzyme and the submitted sample was in a dilution factor of 20. **[[Professor's Comment:** Unnecessary.**]]** The x-axis is the fragment length (nucleotides), and the y-axis is the fluorescence intensity (height of the peak).

each other. However, the bacterial populations sampled from human saliva lied **[[Professor's Comment:** lay or were.**]]** far away from each other.

Discussion

Probiotics can help lower cholesterol level, reduce risk of cancer and gastrointestinal diseases, and improve the immune system (1). Probiotics are active ingredients that make yogurt a healthy food. Identification of probiotic populations in yogurt may help researchers to indicate which strain is the most efficient one in making yogurt while providing health benefits. Since many bacteria are unculturable in laboratory, we used a culture-independent approach to study the probiotic population in yogurt. To identify and study the probiotic population and determine the most abundant probiotic in yogurt, we collected samples from Yoplait® Original Vanilla, extracted genomic DNA directly from the sample for PCR amplification, purified PCR amplicons for agarose gel electrophoresis, digested PCR amplicons with restriction enzymes for T-RFLP analysis, and transformed competent *E. coli* cells with PCR amplicons to construct a clone library to identify T-RFLP peaks. **[[Professor's Comment:** This is the third time you said this.**]]**

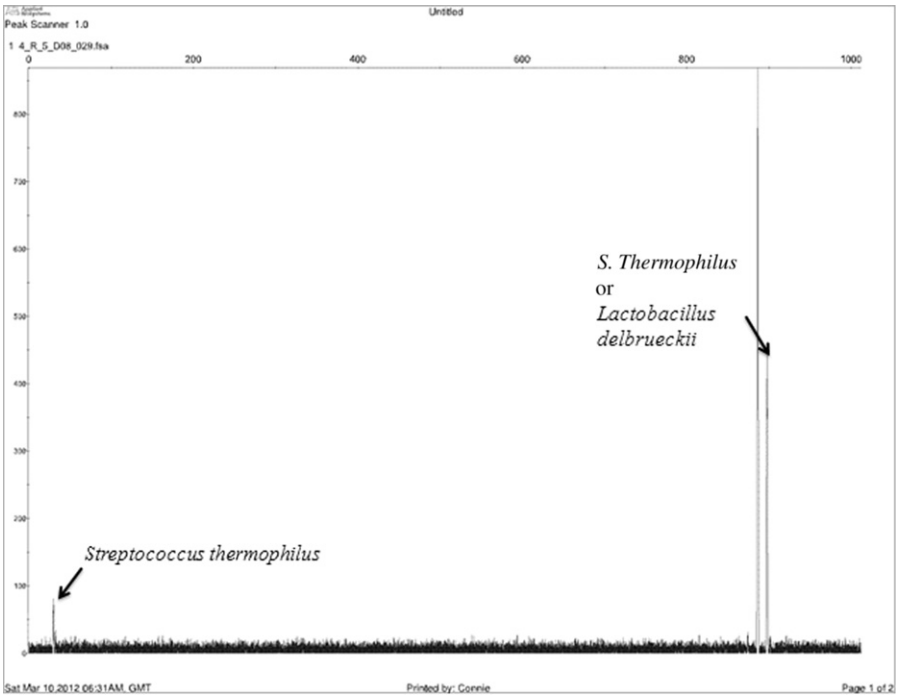

FIGURE A2.9 **T-RFLP profile of *Rsa*I-cut PCR amplicons of identified bacterial population from Yoplait® Original Vanilla yogurt.** We cut the purified PCR amplicons with *Rsa*I restriction enzyme and the submitted sample was in a dilution factor of four. **[[Professor's Comment:** Unnecessary.**]]** The x-axis is the fragment length (nucleotides), and the y-axis is the fluorescence intensity (height of the peak). **[[Professor's Comment:** You should attribute each peak to a particular species. Do not capitalize thermophilus.**]]**

The PCR amplification of the genomic DNA of the uncultured sample from Yoplait® Original Vanilla was successful. The agarose gel electrophoresis detected PCR product, indicating that the PCR reagents successfully reacted and amplified the DNA. The size of the band was 942 base pairs, confirming that the PCR amplicons had the anticipated length of 880 to 940 base pairs. The no-template negative control produced no band, indicating that the PCR reagents were pure.

The concentration of the PCR amplicons was inconsistent between two measurements. The concentration of PCR product estimated by agarose gel electrophoresis was lower than the concentration of PCR product measured by the spectrophotometer. Agarose gel electrophoresis can only give an estimation of the concentration of the PCR product. Instead of physically measuring the amount of product present in each band, we only estimated the intensities of the bands relative to the molecular weight standards. A nanodrop spectrophotometer **[[Professor's Comment:** This measured the signal in the smear as well as the band.**]]** gives a more accurate measurement **[[Professor's Comment:** I disagree.**]]** of the concentration of the PCR product.

To study the complexity of the bacterial population in yogurt, we submitted digested PCR amplicons for analysis by capillary electrophoresis and obtained T-RFLP data. The

TABLE A2.4 Bacterial Genera in Yogurt and Human Saliva

Species Abundance Cut by Mspl								
Genus	**Seat 1**	**Seat 2**	**Seat 3**	**Seat 4**	**Seat 5**	**Seat 6**	**Seat 001**	**Seat 005**
Lactobacillus	75	12.5	8.69	5	16.7	45.8	0	0
Streptococcus	25	87.5	91.3	16	79.2	54.2	34.43	46.5
Anoxybacillus	0	0	0	0	4.17	0	0	0
Rothia	0	0	0	0	0	0	4.34	0
Veillonella	0	0	0	0	0	0	17.39	0
Lachnospiraceae	0	0	0	0	0	0	4.35	0
Campylobacter	0	0	0	0	0	0	8.69	0
Haemophilus	0	0	0	0	0	0	2.17	25.6
Capnocytophaga	0	0	0	0	0	0	2.17	2.32
Fusobacterium	0	0	0	0	0	0	2.17	2.32
Prevotella	0	0	0	0	0	0	8.69	0
Porphytomonas	0	0	0	0	0	0	4.35	0
Gemella	0	0	0	0	0	0	2.17	0
Catonella	0	0	0	0	0	0	2.17	0
Kingella	0	0	0	0	0	0	2.17	0
Lautropia	0	0	0	0	0	0	0	4.65
Neisseria	0	0	0	0	0	0	0	16.3
Actinomyces	0	0	0	0	0	0	0	2.32
Source	Yogurt	Yogurt	Yogurt	Yogurt	Yogurt	Yogurt	Human Saliva	Human Saliva

Numbers indicate the percent of the bacterial population comprised of each genus. The "source" row indicates the type of sources of each sample. The yogurt used by seats 1 and 2 was Tillamook® Vanilla. The yogurt used by seat 3 was Kroger® Plain. The yogurt used by seats 4, 5, and 6 was Yoplait® Original Vanilla. Seats 001 and 005 were sampled from human saliva.

T-RFLP data indicated that there are at least three or four bacteria present in the yogurt. However, more than one species can produce a particular fragment, so each T-RFLP peak may represent more than one species. Although T-RFLP can only give a minimal estimation of the number of species present in the sample, T-RFLP analysis is still useful in observing the complexity of the bacterial community in the yogurt.

The culture-independent approach in this experiment detected more species than the culture-dependent approach in experiment 3, which only detected one species. The

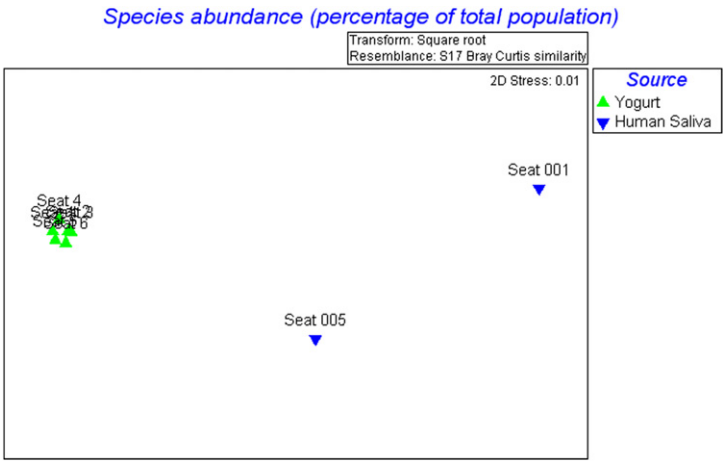

FIGURE A2.10 **Comparison of species abundance of bacterial genera in yogurts and human saliva.** The yogurt used by seats 1 and 2 was Tillamook® Vanilla. The yogurt used by seat 3 was Kroger® Plain. The yogurt used by seats 4, 5, and 6 was Yoplait® Original Vanilla. Seats 001 and 005 were sampled from human saliva.

difference in the number of species detected by the two approaches confirms that many bacteria are unculturable **[[Professor's Comment:** The species you found grow in the laboratory, but they do not grow on LB agar.**]]** in the laboratory. The culture-independent approach ensures that the analysis includes the genomic DNA of all bacteria present in the sample. The culture-independent approach is the more appropriate method for studying a bacterial community in an environment.

Transformation of *E. coli* with PCR amplicons helps us construct a molecular clone library of individual DNA molecules from the PCR product. Transformation of plasmid vector with DNA inserted into *E. coli* was successful as we observed growth of ampicillin-resistant transformants that had the Lac^- phenotypes. The transformants with DNA inserted into the plasmid vector had the Lac^- phenotypes because they lost the gene coding for β-galactosidase, so the Lac^- transformants could not cleave X-gal and remained white.

DNA sequences of the Lac^- transformants help us identify species associated with particular T-RFLP peaks. We cannot sequence the PCR product directly because we amplified the DNA from a mixed template. The BLAST search of the DNA sequences indicates that *S. thermophilus* and *L. delbrueckii* were present in the bacterial population sampled from the yogurt. We successfully indicated the species associated with particular T-RFLP peaks. However, the predicted length of the fragments did not perfectly correspond to the T-RFLP peaks. The predicted length of the fragments and T-RFLP peaks had a difference of three to six nucleotides. The differences may be due to the limitation of T-RFLP analysis. T-RFLP estimates the size of each labeled restriction fragment, and these estimations may differ from the true length by several nucleotides (2).

T-RFLP and DNA sequences help indicate the relative quantity of each species in the sample. However, we cannot determine the most abundant probiotic in the yogurt due to inconsistency in the data. The T-RFLP plot indicated that there were more *L. delbrueckii* in the yogurt, but the molecular clone library indicated that there were more *S. thermophilus* in the yogurt. The inconsistency may be due to incorrectly labeled PCR amplicon,

unsuccessfully digested amplicons, mutation in PCR or DNA sequencing primers, **[[Professor's Comment:** No. Unlikely.**]]** or the limitation of T-RFLP analysis. Also, since we only randomly chose colonies for DNA sequences, the inconsistency may be due to the probability of choosing a species more than the other species. Although we successfully observed the complexity of the bacterial population in the yogurt, we cannot determine the most abundant probiotic in the yogurt.

The presence of *L. delbrueckii* and *S. thermophilus* in the yogurt is logical as *L. delbrueckii* and *S. thermophilus* are the most commonly used probiotics in yogurt production (1). Compared to experiment 3, which only found one species in the sample, the culture-independent approach successfully included unculturable bacteria in the analysis. Also, since we did not detect *Bacillis pumilus* or *Bacillus safensis* in the culture-independent approach, it further confirms that the presence of *Bacillis pumilus* or *Bacillus safensis* in the sample in experiment 3 was due to contamination during the experiment.

The NMDS plot helped us compare the bacterial populations in two different environments. The bacterial populations sampled from yogurt were close to each other, indicating that the bacterial populations in yogurts are similar. However, the bacterial populations sampled from human saliva were far from each other, indicating that the bacterial populations in human saliva did not resemble each other. We attributed the differences of the bacterial populations in human saliva to different food consumed by the students or the health condition of the students. Similar environments had similar bacterial populations, and different environments had dissimilar bacterial populations.

Conclusions

We collected and sampled bacteria from Yoplait® Original Vanilla. The PCR amplification was successful as the agarose gel electrophoresis detected PCR products. T-RFLP profile and DNA sequences indicated that *L. delbrueckii* and *S. thermophilus* were present in the yogurt. Since there were inconsistencies between T-RFLP data and DNA sequences, we could not determine the most abundant probiotic in the yogurt. The presence of *L. delbrueckii* and *S. thermophilus* in yogurt was logical because these bacteria are the most commonly used probiotics in yogurt production. The culture-independent approach successfully includes unculturable bacteria in the analysis.

References

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2. Ream W, Geller B, Trempy J, Field K. 2003. *Experiment 4—Terminal Restriction Fragment Length Polymorphism Analysis of 16s Ribosomal RNA Genes from Uncultured Bacterial Communities*. San Diego, CA: Academic Press.

Questions

1. I detected more bacterial species using a culture-independent approach. In the culture-independent approach, we detected at least three to four species present in the sample collected from the yogurt. However, in the culture-dependent approach, we only detected one species. The culture-independent approach allows us to extract

bacterial genomic DNA directly from uncultured sample. Therefore, we were able to do analysis on the mixed DNA sample that contains genomic DNA from both culturable and unculturable bacterial species.

2. Yes, the approach used in experiment 4 can detect dead bacteria. Since we extracted DNA directly from the sample rather than from a culture, we will get DNA from both live and dead bacteria as long as the bacteria are not lysed. Also, PCR amplification amplifies both dead and live bacteria. Since we used PCR amplicons for our T-RFLP analysis, the T-RFLP profile also cannot distinguish dead and live bacteria. It means that T-RFLP peaks on the plot can indicate bacteria that are either dead or alive.
3. No. A T-RFLP profile only gives minimal reflection of the bacterial population present in a sample. More than one species can produce a particular fragment, meaning that a single T-RFLP fragment detected can actually be fragments from more than one species. Without DNA sequences, a T-RFLP profile only indicates the complexity of the bacterial population but cannot accurately reflect the bacterial population in a sample.
4. I selected a Lac^- (white) colony from my clone library for sequence analysis. Lac^- (white) colonies were transformants that contained amplicon DNA inserted into the plasmid vector. We used the plasmid (pCR4-TOPO) to transform competent *E. coli* cells. The plasmid contains genes for ampicillin resistance, providing a selection for transformed cells. However, some transformed cells may contain empty vectors, and some may contain plasmid vectors with DNA inserted. Transformed cells that contained empty vectors still had intact *lacZ* α domain, so they can produce active β-galactosidase inside the transformed *E. coli* cells. Thus, transformed cells that contained empty vectors were blue because they can cleave X-gal. On the other hand, transformed cells with DNA inserted into the vector did not have an intact *lacZ* α domain, so they cannot product active β-galactosidase inside the transformed *E. coli* cells. Thus, transformed cells with DNA inserted into the vector remained white because they cannot cleave X-gal.
5. No. More than one species can produce a particular fragment, so T-RFLP fragment lengths alone cannot identify a species. We need to construct a molecular clone library and sequence the clones in order to identify the species that correspond to particular T-RFLP peaks.

Appendix A.A

The additional DNA sequences of the plasmid DNA from T7 promoter primer

Note: In all sequences, we did not observe vector sequence. Yellow nucleotides are 8-27F primer used in PCR amplification. Green nucleotides are *Rsa*I restriction site. Pink nucleotides are *Msp*I restriction site. Crossed-off nucleotides are ambiguities in the sequence, and we removed ambiguities before the BLAST search. Bold and italicized bases are the sequence we used for the BLAST search.

Sequence 1 — *L. delbrueckii*

TCCTGGCTCAG*GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCTGAATTCAAAGA~~tccCTTcgGG~~*
~~GTGATTTGTTGGACGCTAGCGGCGGATGGGTGAGTAACacgTGGGCAATCTGCCCTAAAGACTGGGATACCACTTGG~~
~~AAACAGGTGCTAATA~~CCGC~~ATAACAACATGAATCGCATGATTCaAGTTTGAAAGGCGGCGCAagCTGTCACTTTaGG~~
~~ATGAGCCCGCGGCGCATTagcTAGTTGGTGGGGTAAAGGCCTACCAAGGCAATGATGCGtagccgagtTGAGAGACT~~
~~GATCGGccaCATTGGGACTGagaCACGGCCCAAACTCCTACGGGAGGCagcagTAGGGAATCTTCCACAATGGAcgc~~
~~AAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTcggATCGTAAAGCTCTGTTGTTGGTGAagaAGGATAG~~
~~AGGCAGTAACTGGTCTTTATTTGACGGTAATCAACcataaaGTCACGGCTAACTACGTGCCagcagCCGCGGTAATA~~
~~CGTaggTGGCAAGCGTTGT~~CCGG~~ATTTATTGGGCGTAAAGCGAGCGCAggcggAATGATAAGTCTGATGTGAAAGCC~~*
~~CACGGCTCAACCGTGGAACTGCATcggaAACTGTCATTCTTGagtGcaGaataggAGAGTGGAACTCCATGTGTAGc~~
*~~ggtGGAATGCGTAcaTATATGGAAGaacACCAGtggCgaacgcgGCTCTCTGGTCTGCaACTGACGC~~

Sequence 2 — *S. thermophilus*

TCCTGGCTCAG*GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACGCTGAAGAGAGGAGCTTGCTCTTCTT*
GGATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTTGTAGCGGGGGATAACTATTGGAAACGATAGCTA
ATACCGCATAACAATGGATGACACATGTCATTTATTTGAAAGGGGCAATTGCTCCACTACAAGATGGACCTGCGTTG
TATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGAGC
AACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGTCAAGAACGGGTGTGAGAGTGGAAAGT
TCACACTGTGACGGTAGCTTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAG
CGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTGATAAGTCTGAAGTTAAAGGCTGTGGCTCAACCA
TAGTTCGCTTTGGAAACTGTCAAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAG
ATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGCTG~~aggCTCGAAAGCGtgngGaGCG~~
*~~AACAGGATTAGATACCCTGGTAGTCCACGCCGTaaaCGATGAGTGCTAGGTGTTGGATCCTTT~~CCGG~~GATTCAGTGC~~

Sequence 3 — *S. thermophilus*

~~Gtca~~TTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCGGCACTGAATC
CCGGAAAGGATCCAACACCTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCAC
GCTTTCGAGCCTCAGCGTCAGTTACAGACCAGAGAGCCGCTTTCGCCACCGGTGTTCCTCCATATATCTACGCATTT
CACCGCTACACATGGAATTCCACTCTCCCCTTCTGCACTCAAGTTTGACAGTTTCCAAAGCGAACTATGGTTGAGCC
ACAGCCTTTAACTTCAGACTTATCAAACCGCCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTCGGGACCT
ACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTCCCTTTCTGGTAAGCTACCGTCACAGTGTGAACTTTCCACT
CTCACACCCGTTCTTGACTTACAACAGAGCTTTACGATCCGAAAACCTTCTTCACTCACGCGGCGTTGCTCGGTCAG
GGTTGCCCCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCC
GATCACCCTCTCAGGTCGGCTATGTATCGTCGCCTAGGTGAGCCATTACCTCACCTACTAGCTAATACAACGCAggT
CCATCTTGTAGTGGAGCAATTGCCCCTTTCAAATAAATGACATGTGTCATCCATTGTTATGCGGTATTAGCTATCGT
TTCCAATAGTTATCCCCCGCTACAAGGCAGGTTACCTACGCGTTACTCACCCGTTCGCAACTCATCCA~~agaagagca~~
*~~agctCCTCTGCttcagcgTtcTACTTGCATGTATTaggcaCgccgccaGCGTTCGTCCTGagcCaTGA~~

Sequence 4 — *S. thermophilus*

GatatGGCTCAG*GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACGCTGAAGAGAGGAGCTTGCTCTTCT*
TGGATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTTGTAGCGGGGGATAACTATTGGAAACGATAGCT
AATACCGCATAACAATGGATGACACATGTCATTTATTTGAAAGGGGCAATTGCTCCACTACAAGATGGACCTGCGTT
GTATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGAG
CAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGTCAAGAACGGGTGTGAGAGTGGAAAG
TTCACACTGTGACGGTAGCTTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGA
GCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTGATAAGTCTGAAGTTAAAGGCTGTGGCTCAACC
ATAGTTCGCTTTGGAAACTGTCAAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTA
GATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGACGCTG~~aggCTCGAAAGCgtggggaGC~~
~~GAACAGGATTAGATACCCTggTAGTCCACGCcGTAAACGATGAGTGCTaggtGTTGGATCCTTT~~CCGG~~GATTcAGTG~~
~~CC~~

Sequence 5 — *S. thermophilus*

ATCATGGCTCAG*GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACGCTGAAGAGAGGAGCTTGCTCTTCT*
TGGATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTTGTAGCGGGGGATAACTATTGGAAACGATAGCT
AATACCGCATAACAATGGATGACACATGTCATTTATTTGAAAGGGGCAATTGCTCCACTACAAGATGGACCTGCGTT

GTATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGAG
CAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGTCAAGAACGGGTGTGAGAGTGGAAAG
TTCACACTGTGACGGTAGCTTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGA
GCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTGATAAGTCTGAAGTTAAAGGCTGTGGCTCAACC
ATAGTTCGCTTTGGAAACTGTCAAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTA
GATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGACGCTGaggCTCGAAAGCGTggngagCG
AACAGGATTAGATACCCTGGTAGTCCACGCCgtaaacgATGAGTGCTAGGTGTTGGATCCTTTCCGGGATTCAgtgC

Sequence 6 — *L. delbrueckii*

GATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCTGAATTCAAAGATCCCTTCG
GGGTGATTTGTTGGACGCTAGCGGCGGATGGGTGAGTAACACGTGGGCAATCTGCCCTAAAGACTGGGATACCACTT
GGAAACAGGTGCTAATACCGGATAACAACATGAATCGCATGATTCAAGTTTGAAAGGCGGCGTAAGCTGTCACTTTA
GGATGAGCCCGCGGCGCATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCAATGATGCGTAGCCGAGTTGAGAGA
CTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGAC
GCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGAT
AGAGGCAGTAACTGGTCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAA
TACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAATGATAAGTCTGATGTGAAAG
CCCACGGCTCAACCGTGGAACTGCATCGGAAACTGTCATTCTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTA
GCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAaggCGGCTCTCTGGTCTGCAACTGACGCtgaggCTC
GAAAGCATGGGTAGCGAACAGGATTAGaTACCCTGGTAGTCCATGCCGTaaaCGATGAGCGCT

Sequence 7 — *S. thermophilus*

TCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACGCTGAAGAGAGGAGCTTGCTCTTCTT
GGATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTTGTAGCGGGGGATAACTATTGGAAACGATAGCTA
ATACCGCATAACAATGGATGACACATGTCATTTATTTGAAAGGGGCAATTGCTCCACTACAAGATGGACCTGCGTTG
TATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGAGC
AACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGTCAAGAACGGGTGTGAGAGTGGAAAGT
TCACACTGTGACGGTAGCTTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAG
CGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTGATAAGTCTGAAGTTAAAGGCTGTGGCTCAACCA
TAGTTCGCTTTGGAAACTGTCAAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAG
ATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGACGCTGAggCTCGAAAGCGTggnGaGCG
AACAGGATTAGATACCCTGGTAGTCCACGCCgtanacgATGAGTGCTaggTGTTGGATCCT

Sequence 8 — *S. thermophilus*

TCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACGCTGAAGAGAGGAGCTTGCTCTTCTT
GGATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTTGTAGCGGGGGATAACTATTGGAAACGATAGCTA
ATACCGCATAACAATGGATGACACATGTCATTTATTTGAAAGGGGCAATTGCTCCACTACAAGATGGACCTGCGTTG
TATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGAGC
AACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGTCAAGAACGGGTGTGAGAGTGGAAAGT
TCACACTGTGACGGTAGCTTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAG
CGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTGATAAGTCTGAAGTTAAAGGCTGTGGCTCAACCA
TAGTTCGCTTTGGAAACTGTCAAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAG
ATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGACGCTGaggCTCGAAAGCGTGGGGAGCG
AACAGGATTAGATACCCTGGTAGTCCACGCCgtaaacgATGagtGCTAGGTGTTGGATCCTTTCCGGGATCAGTGCC
GAAGCTAACGC

Sequence 9 — *L. delbrueckii*

GATCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCTGAATTCAAAGATCCCTTCG
GGGTGATTTGTTGGACGCTAGCGGCGGATGGGTGAGTAACACGTGGGCAATCTGCCCTAAAGACTGGGATACCACTT
GGAAACAGGTGCTAATACCGGATAACAACATGAATCGCATGATTCAAGTTTGAAAGGCGGCGCAAGCTGTCACTTTA
GGATGAGCCCGCGGCGCATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCAATGATGCGTAGCCGAGTTGAGAGA
CTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGAC
GCAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGAT
AGAGGCAGTAACTGGTCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAA

TACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAATGATAAGTCTGATGTGAAAG
CCCACGGCTCAACCGTGGAACTGCATCGGAAACTGTCATTCTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTA
GCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAggCGGCTCTCTGGTCTGCAACTGACGCTgaggcTC
GAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCgtaaacgATGagCGCTAGGTGTCGGGGACT
TTCCGGTTCTCagtGCCGC

Sequence 10 — *L. delbrueckii*

TTCGTCATTCCTTTGAGTttaACCTTGCGGTCGTACTCCCCAGGCGGAGCGCTTAATGCGTTTGCTGCGGCACTGAG
GACCGGAAAGTCCCCAACACCTAGCGCTCATCGTTTACGGCATGGACTACCAGGGTATCTAATCCTGTTCGCTACCC
ATGCTTTCGAGCCTCAGCGTCAGTTGCAGACCAGAGAGCCGCCTTCGCCACTGGTGTTCTTCCATATATCTACGCAT
TCCACCGCTACACATGGAATTCCACTCTCCTCTTCTGCGCTCAAGAATGACAGTTTCCGATGCAGTTCCACGGTTGA
GCCGTGGGCTTTCACATCAGACTTATCATTCCGCCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTTGCCA
CCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGACTTTCTGGTTGATTACCGTCAAATAAAGACCAGTTA
CTGCCTCTATCCTTCTTCACCAACAACAGAGCTTTACGATCCGAAAACCTTCTTCACTCACGCGGCGTTGCTCCATC
AGACTTGCGTCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTTTGGGCCGTGTCTCAGTCCCAATGTGG
CCGATCAGTCTCTCAACTCGGCTACGCATCATTGCCTTGGTAGGCCTTTACCCCACCAACTAGCTAATGCGCCGCGG
GCTCATCCTAAAGTGACAGCTTACGCCGCCTTTCAAACTTGAATCATGCGATTCATGTTGTTATCCGGTATTAGCAC
CTGTTTCCAAGTGGTATCCCAGTCTTTAGGGCAGATTGCCCACGTGTTACTCACCCATCCGCCGCTAGCGTCCAaca
aatcaccccgaagGGATCTTTgaatTcAGCTCGCTCGACTTGCATGTA

Sequence 11 — *S. thermophilus*

GGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACGCTGAAGAGAGGAGCTTGCTCTTCTTGGAT
GAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTTGTAGCGGGGGATAACTATTGGAAACGATAGCTAATAC
CGCATAACAATGGATGACACATGTCATTTATTTGAAAGGGGCAATTGCTCCACTACAAGATGGACCTGCGTTGTATT
AGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGAC
TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGAGCAACG
CCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGTCAAGAACGGGTGTGAGAGTGGAAAGTTCAC
ACTGTGACGGTAGCTTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTT
GTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTGATAAGTCTGAAGTTAAAGGCTGTGGCTCAACCATAGT
TCGCTTTGGAAACTGTCAAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATAT
ATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGACGCTGaggCTCGAAAGCGTgnggAGCGAACA
GGATTAGATACCCTGGTAGTCCACGCCGtaaacgATGAGTGCtaggtgTTGGATCCTTTCCggattCAGTGCCGcA
GCTaACGCATTAAGcACTCCG

Sequence 12 — *S. thermophilus*

ATCATGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACGCTGAAGAGAGGAGCTTGCTCTTCT
TGGATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTTGTAGCGGGGGATAACTATTGGAAACGATAGCT
AATACCGCATAACAATGGATGACACATGTCATTTATTTGAAAGGGGCAATTGCTCCACTACAAGATGGACCTGCGTT
GTATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGAG
CAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCACTGTTGTAAGTCAAGAACGGGTGTGAGAGTGGAAAG
TTCACACTGTGACGGTAGCTTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGA
GCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTGATAAGTCTGAAGTTAAAGGCTGTGGCTCAACC
ATAGTTCGCTTTGGAAACTGTCAAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTA
GATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGACGCTGaggCTCGAAAGCGTgngGaGC
GAACAGGATTAGaTACCCTGGTAGTCCACGCCGTanaCGATGAGTGCTaggTGTTGGATCCTTTCCGGGATTCAGTG
CCGCAGCTAACGCATTAAGcACT

Sequence 13 — *S. thermophilus*

TCCTGGCtcgGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACGCTGAAGAGAGGAGCTTGCTCTTCTTG
GATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTTGTAGCGGGGGATAACTATTGGAAACGATAGCTAA
TACCGCATAGCAATGGATGACACATGTCATTTATTTGAAAGGGGCAATTGCTCCACTACAAGATGGACCTGCGTTGT
ATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGAGCA
ACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGTCAAGAACGGGTGTGAGAGTGGAAAGTT
CACACTGTGACGGTAGCTTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGC
GTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTGATAAGTCTGAAGTTAAAGGCTGTGGCTCAACCAT

AGTTCGCTTTGGAAACTGTCAAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGA
TATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGACGCTGaggCTCGAAAGCGTgnggaGCGA
ACAGGATTAGATACCCTGGTAGTCCACGCCGTanacGATGAGTGCTAGgTGTTGGATCCTTTCCGGGATTCAG

Sequence 14 — *S. thermophilus*

GATCATGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACGCTGAAGAGAGGAGCTTGCTCTTC
TTGGATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTTGTAGCGGGGGATAACTATTGGAAACGATAGC
TAATACCGCATAACAATGGATGACACATGTCATTTATTTGAAAGGGGCAATTGCTCCACTACAAGATGGACCTGCGT
TGTATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACAC
TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGA
GCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGTCAAGAACGGGTGTGAGAGTGGAAA
GTTCACACTGTGACGGTAGCTTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCG
AGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTGATAAGTCTGAAGTTAAAGGCTGTGGCTCAAC
CATAGTTCGCTTTGGAAACTGTCAAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGT
AGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGACGCTGaggCTCGAAAGCGTGGGAGC
GAACAGGATTAGATACCCTGGTAGTCCACGCCgTanaCGATGAGTGCTAGGTGTTGGATC

Sequence 15 — *S. thermophilus*

TTCGTCATTCCTTTGAGTttaACCTTGCGGTCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTTCGGCACTGAA
TCCCGGAAAGGATCCAACACCTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCC
ACGCTTTCGAGCCTCAGCGTCAGTTACAGACCAGAGAGCCGCTTTCGCCACCGGTGTTCCTCCATATATCTACGCAT
TTCACCGCTACACATGGAATTCCACTCTCCCCTTCTGCACTCAAGTTTGACAGTTTCCAAAGCGAACTATGGTTGAG
CCACAGCCTTTAACTTCAGACTTATCAAACCGCCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTCGGGAC
CTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTCCCTTTCTGGTAAGCTACCGTCACAGTGTGAACTTTCCA
CTCTCACACCCGTTCTTGACTTACAACAGAGCTTTACGATCCGAAAACCTTCTTCACTCACGCGGCGTTGCTCGGTC
AGGGTTGCCCCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGG
CCGATCACCCTCTCAGGTCGGCTATGTATCGTCGCCTAGGTGAGCCATTACCTCACCTACTAGCTAATACAACGCAG
GTCCATCTTGTAGTGGAGCAATTGCCCCTTTCAAATAAATGACATGTGTCATCCATTGTTATGCGGTATTAGCTATC
GTTTCCAATAGTTATCCCCCGCTACAAGGCAGGTTACCTACGCGTTACTCACCCGTTCGCAACTCATCCAagaagag
cAAGCTCCTCTCTTCAGCGTTCTACTTGCATGTA

Sequence 16 — *S. thermophilus*

TCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACGCTGAAGAGAGGAGCTTGCTCTTCTT
GGATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTTGTAGCGGGGGATAACTATTGGAAACGATAGCTA
ATACCGCATAACAATGGATGACACATGTCATTTATTTGAAAGGGGCAATTGCTCCACTACAAGATGGACCTGCGTTG
TATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGAGC
AACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGTCAAGAACGGGTGTGAGAGTGGAAAGT
TCACACTGTGACGGTAGCTTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAG
CGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTGATAAGTCTGAAGTTAAAGGCTGTGGCTCAACCA
TAGTTCGCTTTGGAAACTGTCAAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAG
ATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGACGCTGaggCTCGAAAGCGTgngGaGCG
AACAGGATTAGATACCCTGGTAGCCCACGCCGTanaCGATGAGTGCTAGGTGTTGGATCCTTTCCGGGATTCAGTGC
CGCAGCTAACGCATTAAGcACTCcgcCTGG

Sequence 17 — *S. thermophilus*

GGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACGCTGAAGAGAGGAGCTTGCTCTTCTTGGAT
GAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCCTGTGGCGGGGGATAACTATTGGAAACGATAGCTAATAC
CGCATAACAATGGATGACACATGTCATTTATTTGAAAGGGGCAATTGCTCCACTACAAGATGGACCTGCGTTGTATT
AGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGAC
TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGAGCAACG
CCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGTCAAGAACGGGTGTGAGAGTGGAAAGTTCAC
ACTGTGACGGTAGCTTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTT
GTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTGATAAGTCTGAAGTTAAAGGCTGTGGCTCAACCATAGT
TCGCTTTGGAAACTGTCAAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATAT
ATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGACGCTgaggcTCGAAAGCGTgngGAGCGAACA
GGATTAGATACCCTGGTAGTCCACGCCgtaaacGATGagcGCTAGGTGTTGGGgACtTT

Sequence 18 — *S. thermophilus*

GTCATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGCGCTTAATGCGTTTGCTGCGGCACTGAGGA
CCGGAAAGTCCCCAACACCTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCAC
GCTTTCGAGCCTCAGCGTCAGTTACAGACCAGAGAGCCGCTTTCGCCACCGGTGTTCCTCCATATATCTACGCATTT
CACCGCTACACATGGAATTCCACTCTCCCCTTCTGCACTCAAGTTTGACAGTTTCCAAAGCGAACTATGGTTGAGCC
ACAGCCTTTAACTTCAGACTTATCAAACCGCCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTCGGGACCT
ACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGACTTTCTGGTTGATTACCGTCAAATAAAGACCAGTTACTG
CCTCTATCCTTCTTGACTTACAACAGAGCTTTACGATCCGAAAACCTTCTTCACTCACGCGGCGTTGCTCGGTCAGG
GTTGCCCCCATTGCCGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCG
ATCACCCTCTCAGGTCGGCTATGTATCGTCGCCTAGGTGAGCCATTACCTCACCTACTAGCTAATACAACGCAGGTC
CATCTTGTAGTGGAGCAATTGCCCCTTTCAAATAAATGACATGTGTCATCCATTGTTATGCGGTATTAGCTATCGTT
TCCAATAGTTATCCCCCGCTACAAGGCAGGTTACCTACGCGTTACTCACCCGTTCGCAACTCATCCAagaagagcAA
GCTCCTCTCTTCAGCgTtcT

Sequence 19 — *L. delbrueckii*

TGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCTGAATTCAAAGATTCCTTCGGGATG
ATTTGTTGGACGCTAGCGGCGGATGGGTGAGTAACACGTGGGCAATCTGCCCTAAAGACTGGGATACCACTTGGAAA
CAGGTGCTAATACCGGATAACAACATGAATCGCATGATTCAAGTTTGAAAGGCGGCGTAAGCTGTCACTTTAGGATG
AGCCCGCGGCGCATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCAATGATGCGTAGCCGAGTTGAGAGACTGAT
CGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAG
TCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTGGTGAAGAAGGATAGAGG
CAGTAACTGGTCTTTATTTGACGGTAATCAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT
AGGTGGCAAGCGTTGTCCGGAATTTATTGGGCGTAAAGCGAGCGCAGGCGGAATGATAAGTCTGATGTGAAAGCCCAC
GGCTCAACCGTGGAACTGCATCGGAAACTGTCATTCTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGT
GGAATGCGTAGATATATGGAAGAACACCAGTGGCGaaggcGGCTCTCTGGTCTGCAACTGACGCTGaggCTCGAAAG
CATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGtaaacGATGagcGCTAGGTGTtggggactTTCCC
CTCCTCagtGCCGC

Sequence 20 — *S. thermophilus*

ATCATGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACGCTGAAGAGAGGAGCTTGCTCTTCT
TGGATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTTGTAGCGGGGGATAACTATTGGAAACGATAGCT
AATACCGCATAACAATGGATGACACATGTCATTTATTTGAAAGGGGCAATTGCTCCACTACAAGATGGACCTGCGTT
GTATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGAG
CAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGTCAAGAACGGGTGTGAGAGTGGAAAG
TTCACACTGTGACGGTAGCTTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGA
GCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTGATAAGTCTGAAGTTAAAGGCTGTGGCTCAACC
ATAGTTCGCTTTGGAAACTGTCAAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTA
GATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGACGCTGaggCTCGAAAGCGTgngGAGC
GAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTaggTGTTGGATCCTTtcggggatcagTGC
CGCAgcTaACGCATTAAGCACTC

Sequence 21 — *S. thermophilus*

TCCTGGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACGCTGAAGAGAGGAGCTTGCTCTTCTT
GGATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTTGTAGCGGGGGATAACTATTGGAAACGATAGCTA
ATACCGCATAACAATGGATGACACATGTCATTTATTTGAAAGGGGCAATTGCTCCACTACAAGATGGACCTGCGTTG
TATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGAGC
AACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTGTAAGTCAAGAACGGGTGTGAGAGTGGAAAGT
TCACACTGTGACGGTAGCTTACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAG
CGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTGATAAGTCTGAAGTTAAAGGCTGTGGCTCAACCA
TAGTTCGCTTTGGAAACTGTCAAACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAG
ATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGTCTGTAACTGACGCTGaggCTCGAAAGCGTGGGGAGCG
AACAggATTAGATACCCTGGTAGTCCACGCCgtaaacgATGagtGCTAGgTgttggATCCTTTCcgggattCAGTGC
CGCAGCTAACGCAT

Appendix A.B

Lengths of the corresponding T-RFLP fragments for each enzyme (*MspI* and *RsaI* restriction enzymes) in the additional sequences (*S. thermophilus* and *L. delbrueckii*) of the molecular clone library

Sequence	Identification	Length of *Msp*I-cut Fragment (base pairs)	Length of *Rsa*I-cut Fragment (base pairs)
1	*L. delbrueckii*	179	714
2	*S. thermophilus*	555	Not applicable (NA)
3	*S. thermophilus*	78	32
4	*S. thermophilus*	555	NA
5	*S. thermophilus*	555	NA
6	*L. delbrueckii*	179	NA
7	*S. thermophilus*	555	NA
8	*S. thermophilus*	555	NA
9	*L. delbrueckii*	179	NA
10	*L. delbrueckii*	80	34
11	*S. thermophilus*	555	NA
12	*S. thermophilus*	555	NA
13	*S. thermophilus*	545	NA
14	*S. thermophilus*	555	NA
15	*S. thermophilus*	80	34
16	*S. thermophilus*	555	NA
17	*S. thermophilus*	555	NA
18	*S. thermophilus*	78	32
19	*L. delbrueckii*	179	NA
20	*S. thermophilus*	555	NA
21	*S. thermophilus*	555	NA

APPENDIX

B

Sample Proposals

AN OUTSTANDING PROPOSAL

Isolation of Antibiotic-Producing *Streptomyces* from Soil

Streptomyces is a bacterial genus represented by over 500 species, a number of which are antibiotic producers. Some studies have reported that 50 percent of streptomycetes isolated are antibiotic producers. This genus is responsible for the manufacture of over 500 antibiotic substances (2), including chloramphenicol, erythromycin, neomycin, nystatin, streptomycin, and tetracycline (1, 3, 4), which play a major role in the treatment, control, and cure of human and animal diseases and which are used extensively for research (selectivity) in microbiological laboratories.

We have obtained a specimen isolated from pine soil that we believe is *Streptomyces* and have cross streaked it with *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus* with excellent inhibition. This technique involves streaking one-third trypticase soy agar plate with the test organism and placing cross streaks of known bacterial species in close proximity to it. A clearing on the agar between the test and known bacteria indicates antibiotic production by the test organism.

The colony morphology, cell morphology, and earthy smell are all consistent with this organism. Also, a pathologist at Oregon State University examined the specimen under the microscope and stated that he believed it was *Streptomyces*.

Precise identification by DNA sequence analysis would tell us which strain we have isolated and consequently what antibiotics the strain generally produces. Chemical analysis is probably necessary to definitely identify these antibiotics. We request that funds be allotted for only the DNA sequence analysis at this time to help us narrow the range of possible antibiotics this organism is generating.

References

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2. Mathews CK, van Holde KE. *Biochemistry*. 2nd ed. Menlo Park, CA: The Benjamin/Cummings Publishing Co; 1996. pp. 1026–1027.

3. Prescott LM, Harley JP, Klein DA. *Microbiology*. 4th ed. Dubuque, IA: Wm.C. Brown Publishers; 1996. pp. 498–500.
4. Stryer L. *Biochemistry*. 4th ed. New York, NY: W.H. Freeman and Company; 1995. pp. 902–903.

Notes

Notes

A POOR PROPOSAL

Polution: Man made? Natural? A combination of both?

Who's t say that polution in todays world is a result of mankind. The possibility of naturally occuring polution I believe is out there. As can be seen with the human population, species population explossion is possible for any species. With the great increase of a population, be it humans, plants, or microorganisms, such as bacteria, the ecosystem in which they reside in is put to the limits of support. So, is todays polution problem strickly due to mankind? Or could it be a result of nature and mankind working together? If they are working together to create the polution, who's to say that they can not work together to clean it up.

The two environmental samples that were taken may in a way help to answer these questions, by way of a polymerase chain reaction (PCR) of the 16S rRNA of both samples. If any environmental influence, e it natural or man made, have caused chemical alterations, of any sort, it will be detecable at the variable region or V2 site. If the chemical alterations are found to have some degree of homology in bothe V2 sites then some factor that is the same for both samples has influance a mutation in the RNA sequance. The samples were taken from similar ecological nitches, aquatic, which contained very different ecosystems. The first sample was taken from a rock pit pool. The pool is located in solid rock and is stagnent unless it is raining, then naturally occring springs and rain water disterb it, as do animals that use it. With the spring water silt, clay is added along with organic matter from surrounding grass, trees, and brush. The pool was selected on the bases that humans were in no way involved in the ecosystem that it support or that the pool is apart of. The second sample was taken from a man made pond in an industrial park area. The only water source for the pond is rain water and rain run off from surrounding areas. The pond is located on top of a mud, sand, and silt bottom and has various plant forms growing within along with fish. The ecosystem that the pond supports is in constent interaction with industrial by-products and humans as is the ecosystem that the pond is a apart of.

Perliminary tests, such as TNA plate cultivation and gram stain analysis has identified some unique characteristics of the microbes that were collected. Both plates cultured presented with very few and isolated colonies when incubated at 37 degrees celsius. Both sets of colonies showed small pin point colonies to about 2 mm in diameter. The colonies that developed from the rock pit pool were visibly a cloudy white in color with some clear spots contain within. The colonies from the pond showed a yellowish colored colonies with a slight pink tint to them. These colonies also presented a distinc odor when the plate lid was lifted. Microscopic analysis of the the gram stained cultures showd great similarity between to two cultures. Both cultures were gram positive bacillus with dipolar red spots at each end. The cells from the pond were in collections forming chains and also individual cells. The rock pit cells were only presented as individual cells. For pure culture isolation the two samples were inoculated to nutrient broth and incubated. The culture from the pond was placed at 37 degrees celsius and the rock pit culture was placed at 30 degrees celsius. The rock pit sample was initialy grown at 37 degree celsius and due to decreased growth and the cool environment that it was isolated from it was decided to

place this culture at a lower temperature for further incubation in hopes of collecting a greater quantity of cells.

Because the samples were taken from two completely different ecosystems, with different human interaction levels, any homology in the V2 site on the 16S rRNA could be due to polution. With the source of any polution being different for both samples, the homology would suggest that yes polution could be man made, natural, or even a combination of both.

Notes

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APPENDIX

C

Sample Tests

SAMPLE TEST FOR THIS BOOK

Question 1 (50 points)

The DNA synthesis facility sends you a 20-nucleotide primer as a dry powder; this oligonucleotide has a molecular weight of 6600. You dissolve the oligonucleotide in 1 ml of sterile distilled water; this is your "concentrated primer stock." You pipette 5 μl of the "concentrated primer stock" into 495 μl of water and measure the absorbance at 260 nm; the reading is 0.61. A spectrophotometer reading of 1 absorbance (OD) unit at 260 nm indicates a concentration of 33 μg/ml for a short single-stranded oligonucleotide.

What is the concentration of the "concentrated primer stock"?

Please show your calculations. You may express the answer as μg/ml, or you may give the μM concentration.

How much must you dilute the "concentrated primer stock" to make a 10 μM solution for use in PCR?

Please show your calculations.

Question 2 (30 points)

You have a PCR primer 25 nucleotides long that contains 50% G + C. This primer is 100% complementary to the template DNA, and your PCR reaction contains 100 mM NaCl. What is the melting temperature (T_m) of the duplex DNA formed between this primer oligonucleotide and the template DNA under these conditions?

$T_m = 16.6 \log [Na] + 0.41 (\%G + C) + 81.5 - 500/bp$, where % G + C = percentage expressed as a whole number (for example, *50*, not *0.5*, indicates 50%), [Na] = **molar** salt concentration, and bp indicates length of DNA:DNA hybrid in base pairs.

Question 3 (70 points)

Restriction endonuclease digests of plasmid pMB311 produced these fragments:

*Eco*RI: 6.0 kb
*Pst*I: 3.5, 2.0, 0.5 kb
*Eco*RI and *Pst*I: 2.5, 2.0, 1.0, 0.5 kb
*Sal*I: 3.8, 2.2 kb
*Sal*I and *Pst*I: 1.8, 1.7, 1.5, 0.5, 0.5 kb (two 0.5 kb fragments)

Use this information to draw a circular restriction map of pMB311. Put the cleavage sites for *Eco*RI, *Pst*I, and *Sal*I on a single map.

Question 4 (25 points)

Improve the following sentences:

Both the crude and purified PCR products were determined to be 560 base pairs in length.

It was estimated that the crude product was two times brighter than the ladder.

Identification of the unknown environmental organism was identified as being *Escherichia coli*.

DNA was extracted as described for *Agrobacterium* above.

Ends of restriction fragments produced by *Pst*I cleavage contain four unpaired bases that can hydrogen bond to the complimentary bases on the end of another *Pst*I fragment.

Question 5 (10 points)

$T_m = 16.6 \log [Na] + 0.41 (\%G + C) + 81.5 - 500/bp$, where % G + C = percentage expressed as a whole number (for example, *50*, not *0.5*, indicates 50%), [Na] = **molar** salt concentration, and bp indicates length of DNA:DNA hybrid in base pairs. You have a radiolabeled single-stranded DNA probe 250 nucleotides long that contains 50% G + C. You hybridize this probe to a Southern blot in a hybridization solution that contains 2 × SSC; note that 20 × SSC contains 3M NaCl. Assume that the probe is 100% complementary to the target DNA on the Southern blot. What is the melting temperature (T_m) of the hybrid formed between this probe and the target DNA under these conditions?

Question 6 (10 points)

In experiment 3, you cultured unidentified bacteria, isolated genomic DNA from the cultured cells, and used this DNA as template for PCR. The authors of the paper on bacterial diversity in the Amazon isolated bacterial DNA directly from soil samples without growing the bacteria in culture. In one sentence, please explain why the authors chose this approach.

Question 7 (15 points)

List three parameters that affect the melting temperature (T_m) of annealed primer:template duplex DNA in a PCR reaction.

Notes

Notes

STUDY QUESTIONS FROM DAY AND GASTEL

The instructor will include some of these questions on the test. Read the chapters in *How to Write and Publish a Scientific Paper* to find the answers.

Chapter 1

"The best English is that which

Chapter 2

Define IMRAD.

Chapter 3

List ten questions you should ask when revising your writing.

a.

b.

c.

d.

e.

f.

g.

h.

i.

j.

Chapter 4

What is the difference between a scientific paper and a review paper?
A scientific paper must enable peers to

a.

b.

c.

Chapter 5

What does the term "salami science" mean?

If your paper contains ideas that are not your own, you must

Chapter 6

What is the "impact factor" of a journal?

Chapter 7

Day and Gastel define a good title as

What is wrong with this title: "Action of Antibiotics on Bacteria"?

Chapter 8

A scientific paper should list as authors those who

Chapter 9

What word limit do many journals set for abstracts?

The abstract should

a.

b.

c.

d.

What is the shortest abstract ever written?

Chapter 10

A good introduction should

a.

b.

c.

d.

e.

Chapter 11

What is the one rule for a properly written materials and methods section of a paper?

Chapter 12

What tense should you use to write the results section?

Two ingredients of the results section are

a.

b.

Chapter 13

A well-structured discussion may

a.

b.

c.

Chapter 14

Should an author obtain permission before mentioning someone in the acknowledgment?

Chapter 15

Two rules for preparing the references are

a.

b.

Chapter 16

Should a table present data vertically or horizontally?

Chapter 17

When should you use a graph instead of a table?

Chapter 24

Should an editorial acknowledge other viewpoints?

Chapter 26

When you write for the public you must

a.

b.

Chapter 30

List the ten commandments of good writing.

a.

b.

c.

d.

e.

f.

g.

h.

i.

j.

Which voice is usually better, active or passive?

What tense should you use to state previously published findings?

When you describe your work in a paper, what tense should you use?

Chapter 31

Define jargon.

a.

b.

c.

Chapter 33

Which sentence is better?

"There is another method that is gaining acceptance."
"Another method is gaining acceptance."

Chapter 37

A grant proposal must convince the funding agency that

a.

b.

c.

d.

e.

f.

Chapter 40

If you review an early draft of a paper, focus on

and

If you review a nearly final draft of a paper, focus on

Appendix C.A

Supply preferred words for the following jargon:

Jargon:	**Preferred Terms:**
a considerable number of	_____
a decreased amount of	_____
a majority of	_____
a great deal of	_____
absolutely essential	_____
accounted for by the fact that	_____
count the number of	_____
employ	_____
for the purpose of	_____
is defined as	_____
is similar to	_____
it has been shown that	_____
prior to	_____
red in color	_____
the vast majority of	_____
utilize	_____

Notes

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APPENDIX

D

Guidance for Presentations

On the last day of class, each team presents its results from experiments 3 and 4. Each presentation should last ~10–15 minutes. At least three team members must speak.

Estimate the time required by the number of slides. Allow ~1 minute per slide, except for the title slide and simple images. Speak slowly and clearly. Project your voice so that all can hear.

Practice your talk.

Slides must be clear; keep them simple. Use a legible font (at least 32 point). Do not use fancy fonts. Use simple images that are large enough to see. Do **not** use distracting backgrounds. The background must contrast with the font.

Divide the talk into three sections; each student should present one section: Introduction, Results, and Discussion.

Begin with an **informative** title slide. Indicate the environment sampled, and summarize the results.

Word the title concisely, and list the authors below the title.

The Introduction should address these questions:

What do you know about the system?
Why is the system interesting?
What question did you address?

The Results should answer these questions:

What did you do?
What did you find?

Show the data required to tell the story. Omit nonessential data and the results of failed procedures.

The Discussion should answer these questions:

What do the results mean?
Did they answer the question?
What will you do next?

Most talks include Acknowledgments that recognize others directly involved in the work, colleagues who helped, and funding sources. Your talks do not need Acknowledgments.

APPENDIX

E

Revised Paragraph from *J. Biol. Chem.* 266: 3811–3814 (1991)

The original version has 198 words, 1222 characters.

"The biochemical characterization of the elements involved in the translocation of proteins across endoplasmic reticulum membranes would be greatly facilitated by the ability to purify chemical amounts of those substrates which can be transferred across the membrane as full-length precursors, i.e. posttranslationally. Currently, study of the mechanism by which proteins are transported across the endoplasmic reticulum depends upon radiochemical amounts of proteins translated in a cell-free system (1). Amounts are so limiting that basic biochemical studies, such as an analysis of saturation of translocation sites, cannot be undertaken. Moreover, in eukaryotic systems the translocation of most proteins is coupled to their translation, i.e. transfer across the membrane is obligatorily cotranslational. A major exception to this cotranlational requirement is found in the case of the *Saccharomyces cerevisiae* mating pheromone prepro-α-factor (2–4)." It has been shown that prepro-α-factor even maintains its translocation competence when denatured in and diluted out of 8 M urea (5). This ability of prepro-α-factor to be posttranslationally translocated makes it an ideal substrate for study as translocation can effectively be uncoupled from translation. Were chemical quantities of this preprotein available, it would then represent an ideal probe in the biochemical dissection of the process of translocation.

The revised version has 93 words, 642 characters.

Study of protein translocation across endoplasmic reticulum membranes depends on the ability to purify chemical amounts of substrates transferred posttranslationally across the membrane as full-length precursors. Current studies of protein transport across the endoplasmic reticulum depend on radiochemical amounts of proteins translated in a cell-free system (1). Limited yields of these proteins preclude saturation of translocation sites. Eukaryotes couple translocation of most proteins with their translation. *Saccharomyces cerevisiae* mating pheromone prepro-α-factor is an exception (2–4). Prepro-α-factor is an ideal substrate for studying in vitro protein translocation because its translocation is uncoupled from translation.

This sentence disrupts the flow of the paragraph:

"It has been shown that prepro-α-factor even maintains its translocation competence when denatured in and diluted out of 8 M urea (5)."

Move it elsewhere and revise it as follows:

Prepro-α-factor denatured in 8 M urea regains translocation competence when the urea is removed by dialysis (5).

APPENDIX

F

Abstract from *Appl. Environ. Microbiol.* 63: 2647–2653 (1997)

Although the Amazon Basin is well known for its diversity of flora and fauna, this report represents the first description of the microbial diversity in Amazonian soils involving a culture-independent approach. Among the 100 sequences of genes coding for small-subunit rRNA obtained by PCR amplification with universal small-subunit rRNA primers, 98 were bacterial and 2 were archaeal. No duplicate sequences were found, and none of the sequences had been previously described. Eighteen percent of the bacterial sequences could not be classified in any known bacterial kingdom. Two sequences may represent a unique branch between the vast majority of bacteria and the deeply branching, predominantly thermophilic bacteria. Five sequences formed a clade that may represent a novel group within the class Proteobacteria. In addition, rRNA intergenic spacer analysis was used to show significant microbial population differences between a mature forest soil and an adjacent pasture soil.

APPENDIX

G

Sequence Editing Tutorial

This tutorial outlines the steps required to analyze sequences of 16S rRNA amplicons cloned in pCR4 during experiment 4. Sequences in this example were generated using the M13R and T7 sequencing primers (Fig. 4.7). Locate the PCR primers (8-27F and 926-907F) and flanking vector sequences. Next, locate the *Msp*I and *Rsa*I restriction sites closest to the 8-27F PCR primer, and measure the number of bases between the cleavage site and the 5′ end of the Fam-labeled 8-27F primer. The example demonstrates how to use Word to perform this analysis.

8-27F primer: 5′- AGAGTTTGATC(A/C)TGGCTCAG -3′

Complement: 3′- TCTCAAACTAG(T/G)ACCGAGTC -5′

Reverse complement: 5′- CTGAGCCA(G/T)GATCAAACTCT -3′

926-907R primer: 5′- CCGTCAATTCCTTT(A/G)AGTTT -3′

926-907 reverse complement: 5′- AAACT(C/T)AAAGGAATTGACGG -3′

926-907R primer (3′ to 5′): 3′- TTTGA(G/A)TTTCCTTAACTGCC -5′

Vector sequences are underlined. *Eco*RI sites in the vector are orange. The 8−27F primer is red. The reverse complement of the 926-907R primer is pink. *Msp*I (C/CGG) and *Rsa*I (GT/AC) sites are pink. Bases used for the BLAST search are ***italicized and bold***.

SEQUENCE FROM THE M13R PRIMER

GGACTAGTCCTGCAGGTTTAAACGAATTCGCCCTTAGAGTTTGATCCTGGCTCAG*GACGAACGC*
TGGCGGCGTGCCTAATACATGCAAGTAGAACGCTGAAGGAGGAGCTTGCTTCTCTGGATGAGTT
GCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTGGTAGCGGGGGATAACTATTGGAAACGATA
GCTAATACCGCATAAGAGTAGATGTTGCATGACATTTGCTTAAAAGGTGCAATTGCACCACTAC
CAGATGGACCTGCGTTGTATTAGCTAGTTGGTGGGGTAACGGCTCACCAAGGCGACGATACATA
GCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACG
*GCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAAGAAGCGAAAGTGACG**GTAC**CTGCAGAAGAAG*
*CG**CCGG**CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTAT*
TGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACTCCGG
*ATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACTG**GAATTC**CTGGTGTAGCGGT*
GAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGGCGC
TGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTT
GGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGC
*CTGGGGAGTACGGCCGCAAGGCTA*AAACTCAAAGGAATTGACGGAAGGGCGAATTCGCGGCCGC
TAAATTCAATTCGCCCTATAGTGAGTCGTA

Notes

Notes

STEPS IN ANALYSIS

1. Use Ctrl-F to search for *Eco*RI sites (**G/AATTC**) in the vector; some amplicons may contain an internal *Eco*RI site; this one does.
2. Identify the vector sequences (GCCCTT and AAGGGC) that lie between the *Eco*RI sites in the vector and the PCR primers.
3. Use Ctrl-F to search for the 5′ end of the 8-27F primer (AGAGTTTGATC); highlight the entire 8-27F primer (AGAGTTTGATCCTGGCTCAG). Ctrl-F will **not** find a sequence that spans a line break. If you do not find the 8-27F primer in the M13R-primed sequence, the amplicon may have inserted in the opposite orientation. Search the sequence for the 5′ end of the 926-907R primer (CCGTCAATTCCTTT); highlight the entire 926-907R primer (CCGTCAATTCCTTTAAGTTT).
4. Place the cursor at the 3′ end of the 8-27F primer, and use Ctrl-F to find the nearest *Msp*I site (**C/CGG**) in the amplicon. Place the cursor at the 5′ end of the 8-27F primer, and highlight the sequence from this position through the first base (**C**) of the *Msp*I site:

AGAGTTTGATCCTGGCTCAG*GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACGCTG*
AAGGAGGAGCTTGCTTCTCTGGATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTG
GTAGCGGGGGATAACTATTGGAAACGATAGCTAATACCGCATAAGAGTAGATGTTGCATGACAT
TTGCTTAAAAGGTGCAATTGCACCACTACCAGATGGACCTGCGTTGTATTAGCTAGTTGGTGGG
GTAACGGCTCACCAAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACT
GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCT
GATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAAGA
AGCGAAAGTGACGGTACCTGCAGAAGAAGCGC

5. Use the "word count" tool to count the number of characters (bases) in the highlighted sequence. In this case, the highlighted text contains 480 characters, indicating that the *Msp*I T-RFLP fragment for this amplicon is 480 bp long. This fragment extends from the 5′ end of the Fam-labeled 8-27F primer through the 3′ end (C) of the cut in the nearest *Msp*I site.
6. Place the cursor at the 3′ end of the 8-27F primer, and use Ctrl-F to find the nearest *Rsa*I site (**GT/AC**) in the amplicon. Place the cursor at the 5′ end of the 8-27F primer and highlight the sequence from this position to the middle (**GT**) of the *Rsa*I site:

AGAGTTTGATCCTGGCTCAG*GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACGCTG*
AAGGAGGAGCTTGCTTCTCTGGATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTG
GTAGCGGGGGATAACTATTGGAAACGATAGCTAATACCGCATAAGAGTAGATGTTGCATGACAT
TTGCTTAAAAGGTGCAATTGCACCACTACCAGATGGACCTGCGTTGTATTAGCTAGTTGGTGGG
GTAACGGCTCACCAAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACT
GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCT
GATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAAGA
AGCGAAAGTGACGGT

7. Use the "word count" tool to count the number of characters (bases) in the highlighted sequence. In this case, the highlighted text contains 463 characters, indicating that the *Rsa*I T-RFLP fragment for this amplicon is 463 bp long. This fragment extends from the 5′ end of the Fam-labeled 8-27F primer through the 3′ end (T) of the cut in the nearest *Rsa*I site.

8. Identify peaks in the T-RFLP profiles that correspond to the sizes of the T-RFLP fragments predicted from the sequence.
9. Use the sequence that lies between the two primers (*italicized)* for a BLAST search.

GACGAACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACGCTGAAGGAGGAGCTTGCTTCTCT
GGATGAGTTGCGAACGGGTGAGTAACGCGTAGGTAACCTGCCTGGTAGCGGGGGATAACTATTG
GAAACGATAGCTAATACCGCATAAGAGTAGATGTTGCATGACATTTGCTTAAAAGGTGCAATTG
CACCACTACCAGATGGACCTGCGTTGTATTAGCTAGTTGGTGGGGTAACGGCTCACCAAGGCGA
CGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGA
GGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAAGAAGCGAAAGTGACGGTACCTG
CAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATC
CGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCT
CAACTCCGGATCTGCGGTGGGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGG
TGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATT
AACTGGCGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCC
GTAAACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAA
GTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTA

Notes

Notes

ANALYSIS OF SEQUENCES THAT CONTAIN THE REVERSE COMPLEMENT OF THE 8–27F PRIMER

We use the T7-primed sequence of the same amplicon to illustrate how to locate the T-RFLP fragments if the amplicon inserted in the opposite orientation relative to the vector. You do **not** need to do this if you have a sequence that contains the 8-27F primer.

Notes

Notes

SEQUENCE FROM THE T7 PRIMER

TTCGCCCTTCCGTCAATTCCTTTGAGTTT*TAGCCTTGCGGCCGTACTCCCCAGGCGGGGCACTT*
AATGCGTTAGCTACGGCGCGGAAAACGTGGAATGTCCCCCACACCTAGTGCCCAACGTTTACGG
CATGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCATGCTTTCGCTCCTCAGCGCCAGTTAA
TGCCCAGAGACCTGCCTTCGCCATCGGTGTTCCTCCTGATATCTGCGCATTTCACCGCTACACC
AGGAATTCCAGTCTCCCCTACATCACTCTAGTCTGCCCGTACCCACCGCAGATCCGGAGTTGAG
CCCCGGACTTTCACGGCAGACGCGACAAACCGCCTACGAGCTCTTTACGCCCAATAATTCCGGA
TAACGCTTGCGCCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGCGCTTCTTCTGCA
GGTACCGTCACTTTCGCTTCTTCCCTACTGAAAGAGGTTTACAACCCGAAGGCCGTCATCCCTC
ACGCGGCGTCGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAG
GAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTATGTATCGTC
GCCTTGGTGAGCCGTTACCCCACCAACTAGCTAATACAACGCAGGTCCATCTGGTAGTGGTGCA
ATTGCACCTTTTAAGCAAATGTCATGCAACATCTACTCTTATGCGGTATTAGCTATCGTTTCCA
ATAGTTATCCCCCGCTACCAGGCANGTTACCTACGCGTTACTCACCCGTTCGCAACTCATCCAG
AGAAGCAAGCTCCTCCTTCAGCGTTCTACTTGCATGTATTANGCACGCCGCCAGCGTTCGTCCT
GAGCCAGGATCAAACTCTAAGGGCGAATTCGTTTAAACCTGCNNACTAGTCCCTTTAGTGAGGG
TTAATTCTGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTATCCGCTCAC

8-27F primer: 5′- AGAGTTTGATC(A/C)TGGCTCAG -3′

Complement: 3′- TCTCAAACTAG(T/G)ACCGAGTC -5′

Reverse complement: 5′- CTGAGCCA(G/T)GATCAAACTCT -3′

926-907R primer: 5′- CCGTCAATTCCTTT(A/G)AGTTT -3′

926-907 reverse complement: 5′- AAACT(C/T)AAAGGAATTGACGG -3′

926-907R primer (3′ to 5′): 3′- TTTGA(G/A)TTTCCTTAACTGCC -5′

Vector sequences are underlined. *Eco*RI sites in the vector are orange. The 8–27F primer is red. The reverse complement of the 926-907R primer is pink. *Msp*I (C/CGG) and *Rsa*I (GT/AC) sites are pink. Bases used for the BLAST search are ***italicized and bold***.

Notes

Notes

STEPS IN ANALYSIS

1. Use Ctrl-F to search for *Eco*RI sites (**G/AATTC**) in the vector; some amplicons may contain an internal *Eco*RI site; this one does. The first *Eco*RI site in the vector is not present in this sequence.
2. Identify the vector sequences (GCCCTT and AAGGGC) that lie between the *Eco*RI sites in the vector and the PCR primers.
3. Use Ctrl-F to search for the 5′ end of the 926-907R primer (CCGTCAATTCCTTT); highlight the entire 926-907R primer (CCGTCAATTCCTTTGAGTTT).
4. Use Ctrl-F to search for the 5′ end of the reverse complement of the 8-27F primer (CTGAGCCA); highlight the entire 8-27F primer (CTGAGCCAGGATCAAACTCT).
5. Place the cursor at the 3′ end of the 926-907R primer, and use Ctrl-F to find **all** *Msp*I (C/CGG) and *Rsa*I (GT/AC) sites in the amplicon. Place the cursor at the 3′ end of the reverse complement of the 8-27F primer, and highlight the sequence from this position to the middle (**AC**) of the **nearest** *Rsa*I site **upstream** in the amplicon:

*AC*CGTCACTTTCGCTTCTTCCCTACTGAAAGAGGTTTACAACCCGAAGGCCGTCATCCCTCACG
CGGCGTCGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAG
TCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTATGTATCGTCGCC
TTGGTGAGCCGTTACCCCACCAACTAGCTAATACAACGCAGGTCCATCTGGTAGTGGTGCAATT
GCACCTTTTAAGCAAATGTCATGCAACATCTACTCTTATGCGGTATTAGCTATCGTTTCCAATA
GTTATCCCCCGCTACCAGGCANGTTACCTACGCGTTACTCACCCGTTCGCAACTCATCCAGAGA
AGCAAGCTCCTCCTTCAGCGTTCTACTTGCATGTATTA*N*GCACGCCGCCAGCGTTCGTCCTGAG
CCAGGATCAAACTCT

6. Use the "word count" tool to count the number of characters (bases) in the highlighted sequence. In this case, the highlighted text contains 463 characters, indicating that the *Rsa*I T-RFLP fragment for this amplicon is 463 bp long.
7. Place the cursor at the 3′ end of the reverse complement of the 8-27F primer, and highlight the sequence from this position through the last base (G) of the **nearest** *Msp*I site (**C/CGG**) **upstream** in the amplicon.

*G*CGCTTCTTCTGCAG*GTAC*CGTCACTTTCGCTTCTTCCCTACTGAAAGAGGTTTACAACCCGAA
GGCCGTCATCCCTCACGCGGCGTCGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACT
GCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTC
GGCTATGTATCGTCGCCTTGGTGAGCCGTTACCCCACCAACTAGCTAATACAACGCAGGTCCAT
CTGGTAGTGGTGCAATTGCACCTTTTAAGCAAATGTCATGCAACATCTACTCTTATGCGGTATT
AGCTATCGTTTCCAATAGTTATCCCCCGCTACCAGGCANGTTACCTACGCGTTACTCACCCGTT
CGCAACTCATCCAGAGAAGCAAGCTCCTCCTTCAGCGTTCTACTTGCATGTATTANGCACGCCG
CCAGCGTTCGTCCTGAGCCAGGATCAAACTCT

8. Use the "word count" tool to count the number of characters (bases) in the highlighted sequence. In this case, the highlighted text contains 480 characters, indicating that the *Msp*I T-RFLP fragment for this amplicon is 480 bp long.

9. Identify peaks in the *Rsa*I and *Msp*I T-RFLP profiles that correspond to the sizes of the T-RFLP fragments predicted from the sequence.
10. Use the sequence that lies between the two primers (see step 7) for a BLAST search. The following table and alignment will appear on your screen.

Accession	Description	Max Score	Total Score	Query Coverage	E Value	Max Ident
DQ157995.1	Arthrobacter sp. J3.33 16S ribosomal RNA gene, partial sequence	1136	1136	99%	0.0	90%
DQ157993.1	Arthrobacter sp. J3.16 16S ribosomal RNA gene, partial sequence	1136	1136	99%	0.0	90%
GU377126.1	Arthrobacter sp. sptzw42 16S ribosomal RNA gene, partial sequence	1134	1134	74%	0.0	98%

```
>  gb|DQ157995.1| Arthrobacter sp. J3.33 16S ribosomal RNA gene, partial
sequence
Length=1517
Score = 1136 bits (615), Expect = 0.0
Identities = 789/870 (90%), Gaps = 24/870 (2%)
Strand=Plus/Plus
Query 4 GAACGCTGGCGGCGTGCCTAATACATGCAAGTAGAACGCTGAAGGAGGAGCTTGCTTCTC  63
        |||||||||||||||| ||| |||||||||| ||||| |||| |   ||||||| |
Sbjct 24 GAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAA-G-CCAGCTTGC-T-GG  79
Query 64 TGGATGAGTTGCGAACGGGTGAGTAACGCGTAG-GTAACCTG-CCTGGTAGC-GGGGGAT  120
         ||||| ||| ||||||||||||||||| ||| | |||||||| |||  || |   |||||
Sbjct 80  TGGATTAGTGGCGAACGGGTGAGTAACACGT-GAGTAACCTGCCCT--TAACTCTGGGAT  136
Query 121 AA-CTATTGGAAACGAT-AG-CTAATACCGCATAAGAGTAGAT-GTTGCATGACATTTGC  176
          || |  | ||||||  |  | |||||||| ||| || |   |  | |||||      |
Sbjct 137 AAGC-CTGGGAAAC--TGGGTCTAATACCGGATATGACT-CCTCATCGCATGGTGGGGGG  192
Query 177 TTAAAAGGTGCAATTGCACCACTACCAGATGGACCT-GCGTTGTATTAGCTAGTTGGTGG  235
          |  |||| |   ||||      |    |||||| || |||   ||| |||| |||||||
Sbjct 193 TGGAAAGCT-TTATTG---TGGTTTTGGATGGA-CTCGCGGCCTATCAGCTTGTTGGTGA  247
Query 236 GGTAACGGCTCACCAAGGCGACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTG  295
          ||||| ||||||||||||||||    |||||| |||||||||||| ||||||||||
Sbjct 248 GGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTG  307
Query 296 GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG  355
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 308 GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG  367
Query 356 CGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTT  415
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 368 CGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTT  427
Query 416 TCAGTAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGC  475
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 428 TCAGTAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGC  487
Query 476 CAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTGGGCGTAAAGAGC  535
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 488 CAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTGGGCGTAAAGAGC  547
Query 536 TCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACTCCGGATCTGCGGTG 595
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 548 TCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACTCCGGATCTGCGGTG 607
Query 596 GGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATG 655
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 608 GGTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATG 667
Query 656 CGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGGCGCTG 715
          |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct 668 CGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTG 727
Query 716 AGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACG 775
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 728 AGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACG 787
Query 776 TTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTG 835
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 788 TTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTG 847
Query 836 CCCCGCCTGGGGAGTACGGCCGCAAGGCTA 865
          |||||||||||||||||||||||||||||
Sbjct 848 CCCCGCCTGGGGAGTACGGCCGCAAGGCTA 877
```

Notes

APPENDIX

H

Peak Scanner Tutorial

Peak Scanner (v1.0) is a free program supplied by Applied Biosystems (http://www.appliedbiosystems.com/absite/us/en/home.html). You use this program to analyze T-RFLP data. Open Peak Scanner and proceed as follows.

1. Select "Start New Project" (Figs. H.1 and H.2).

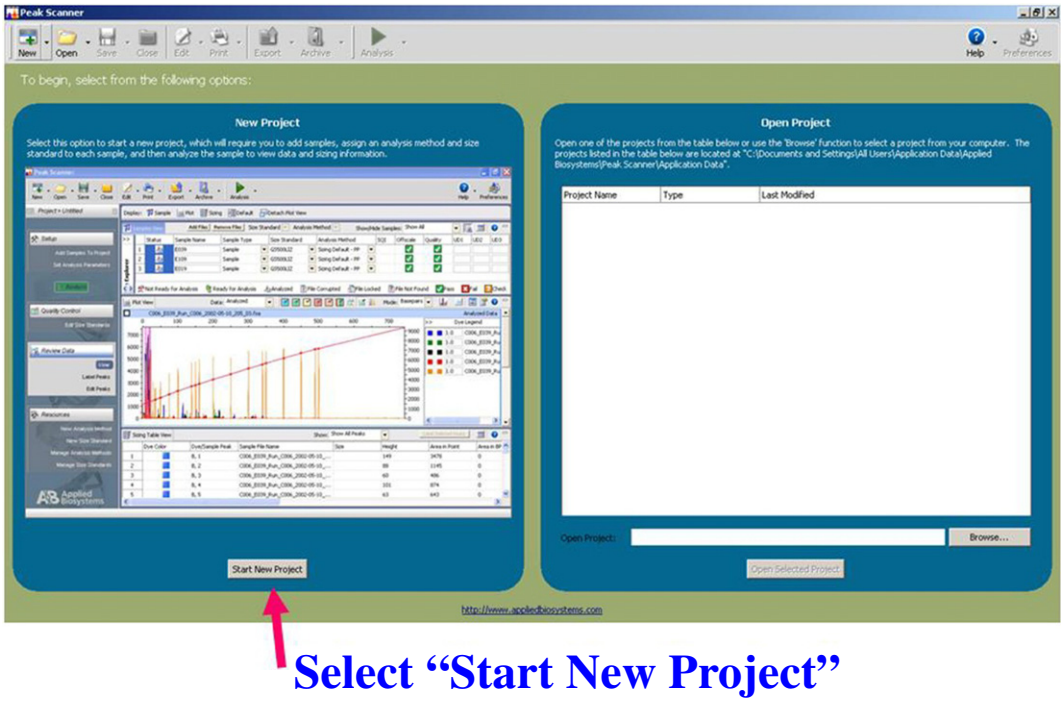


FIGURE H.1

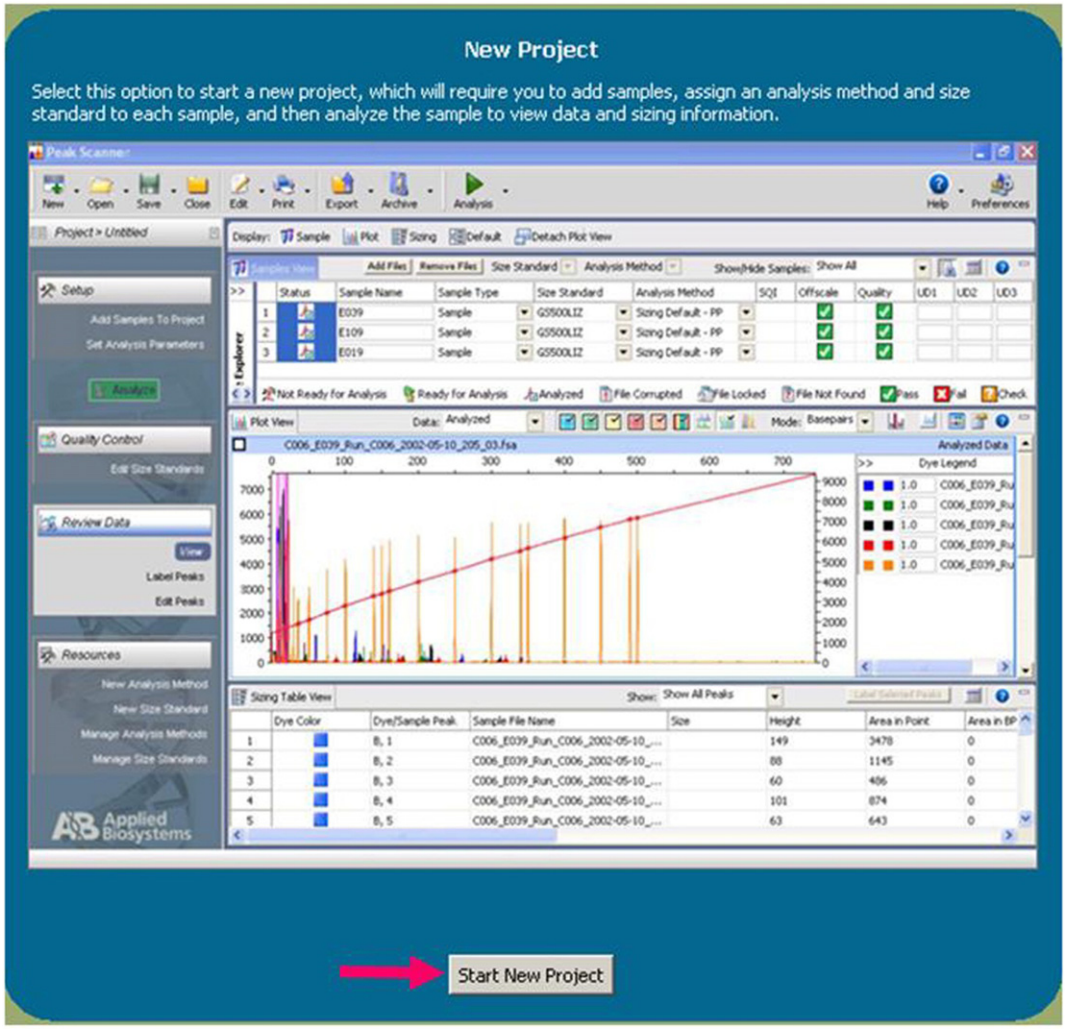

New Project
Select this option to start a new project, which will require you to add samples, assign an analysis method and size standard to each sample, and then analyze the sample to view data and sizing information.
Peak Scanner
New
Open
Save
Close
Edit
Print
Export
Archive
Analysis
Help
Preferences
Project > Untitled
Setup
Add Samples To Project
Set Analysis Parameters
Analyze
Quality Control
Edit Size Standards
Review Data
View
Label Peaks
Edit Peaks
Resources
New Analysis Method
New Size Standard
Manage Analysis Methods
Manage Size Standards
Applied Biosystems
Display:
Sample
Plot
Sizing
Default
Detach Plot View
Add Files
Remove Files
Size Standard
Analysis Method
Show/Hide Samples:
Show All
Status
Sample Name
Sample Type
Size Standard
Analysis Method
SQI
Offscale
Quality
UD1
UD2
UD3
E039
E109
E019
Sample
GS500LIZ
Sizing Default - PP
Not Ready for Analysis
Ready for Analysis
Analyzed
File Corrupted
File Locked
File Not Found
Pass
Fail
Check
Plot View
Data:
Analyzed
Mode:
Basepairs
C006_E039_Run_C006_2002-05-10_205_03.fsa
Analyzed Data
Dye Legend
Sizing Table View
Show:
Show All Peaks
Dye Color
Dye/Sample Peak
Sample File Name
Size
Height
Area in Point
Area in BP
Start New Project

FIGURE H.2

2. Select "Add Files" (Fig. H.3).

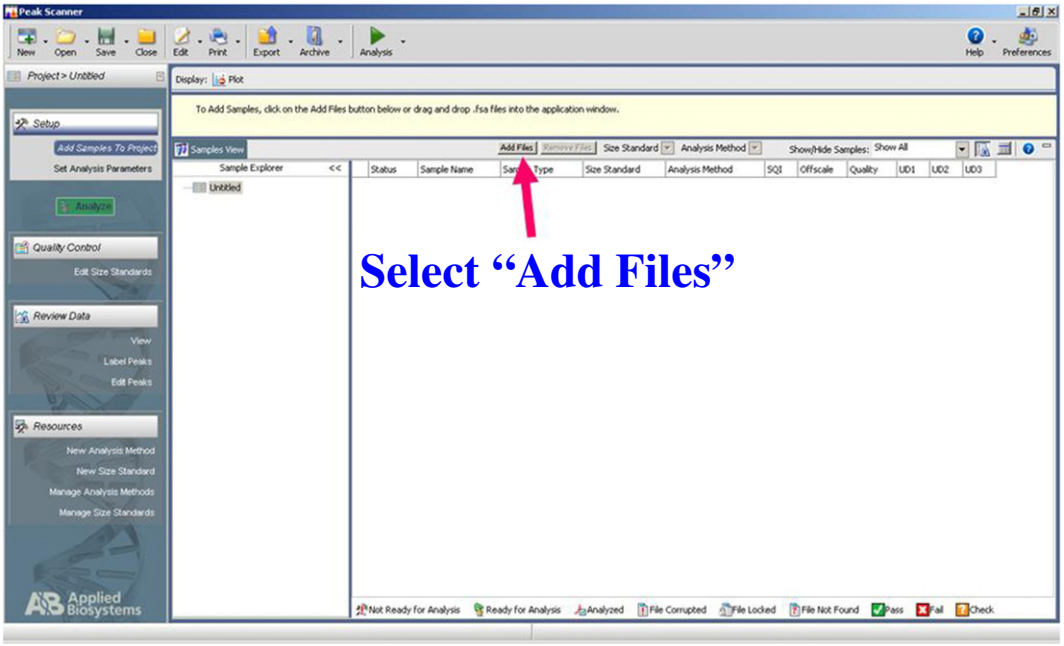


FIGURE H.3

3. Browse and highlight .fsa files (Fig. H.4).
4. Select "Add Selected Files" (Fig. H.4).
5. Select "OK" (Fig. H.4).

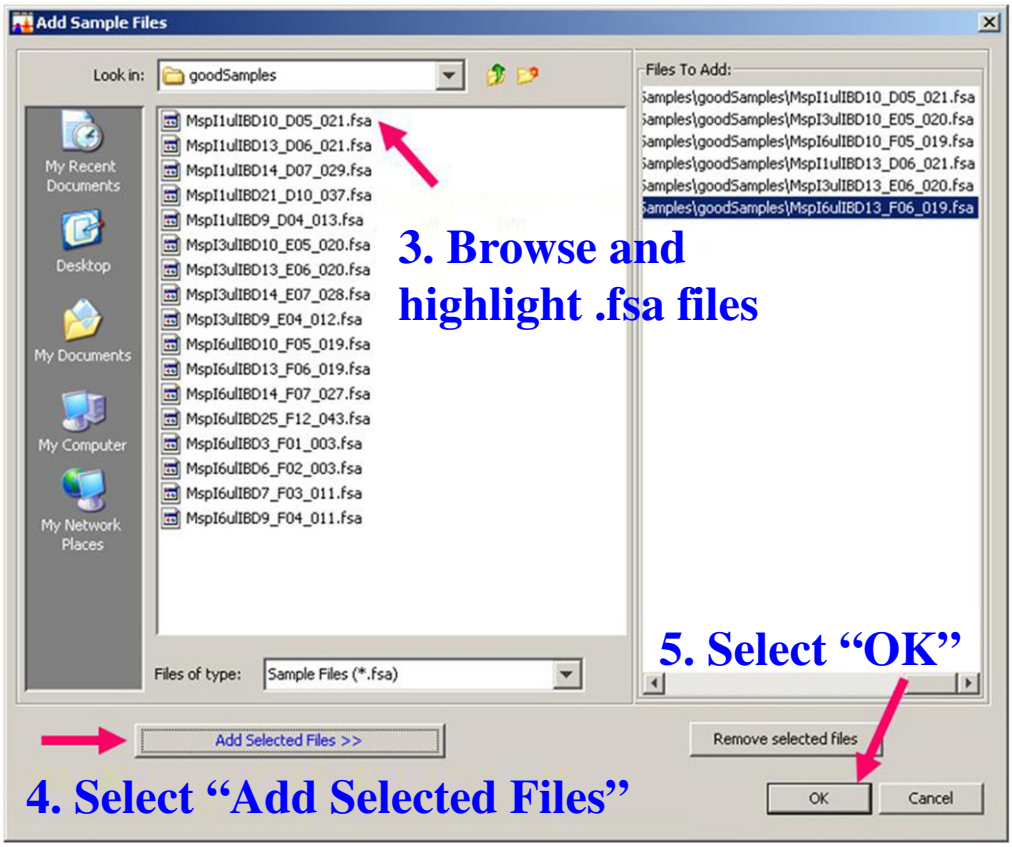


FIGURE H.4

6. Select "Map Marker 1000" or "1200 LIZ" under the "Size Standard" menu (Fig. H.5). Choose the size standard the service laboratory used to analyze your samples. Although either standard is appropriate for the amplicons generated in experiment 4, we prefer Map Marker 1000. T-RFLP fragment lengths estimated using the Map Marker 1000 size standard agree with those predicted based on DNA sequences of the amplicons. Fragment lengths estimated using the 1200 LIZ size standard were six or seven bases shorter than the lengths predicted from DNA sequences. The Peak Scanner software does not contain these size standards as built-in options. To add these standards, proceed as directed in Appendix I.
7. Select "Sizing Default – NPP" under the "Analysis Method" menu (Fig. H.5). NPP indicates "no primer present" in the samples. We removed the PCR primers from the samples we submitted for T-RFLP analysis.
8. Select "Analyze" (Fig. H.5).
9. Select "View" (Fig. H.5).

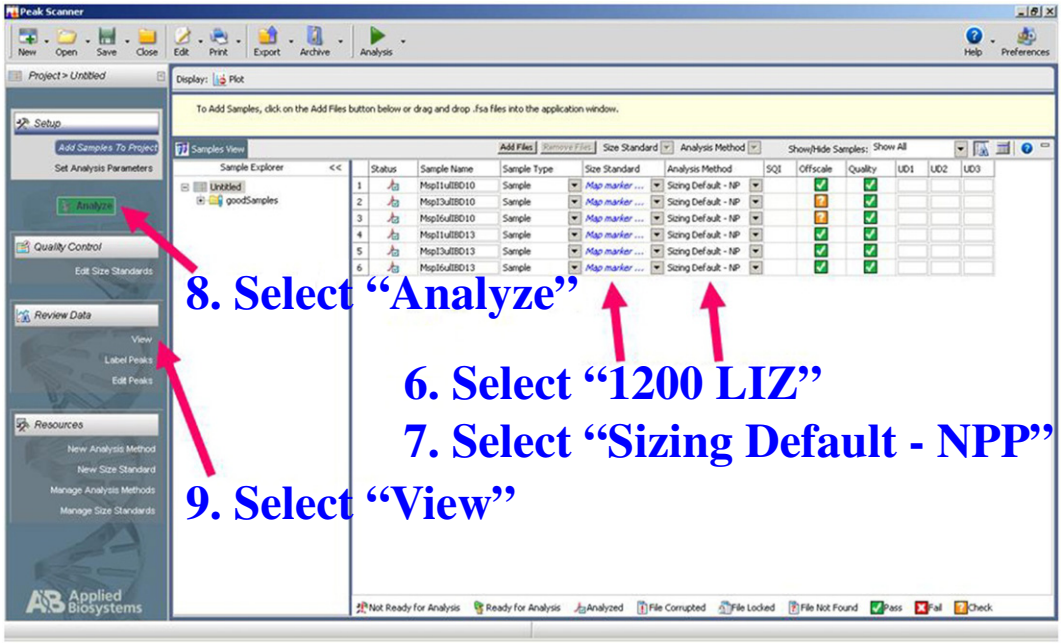


FIGURE H.5

10. Uncheck all peak color boxes except blue (Fig. H.6).
11. Select "Edit Plot Settings" (Fig. H.6).

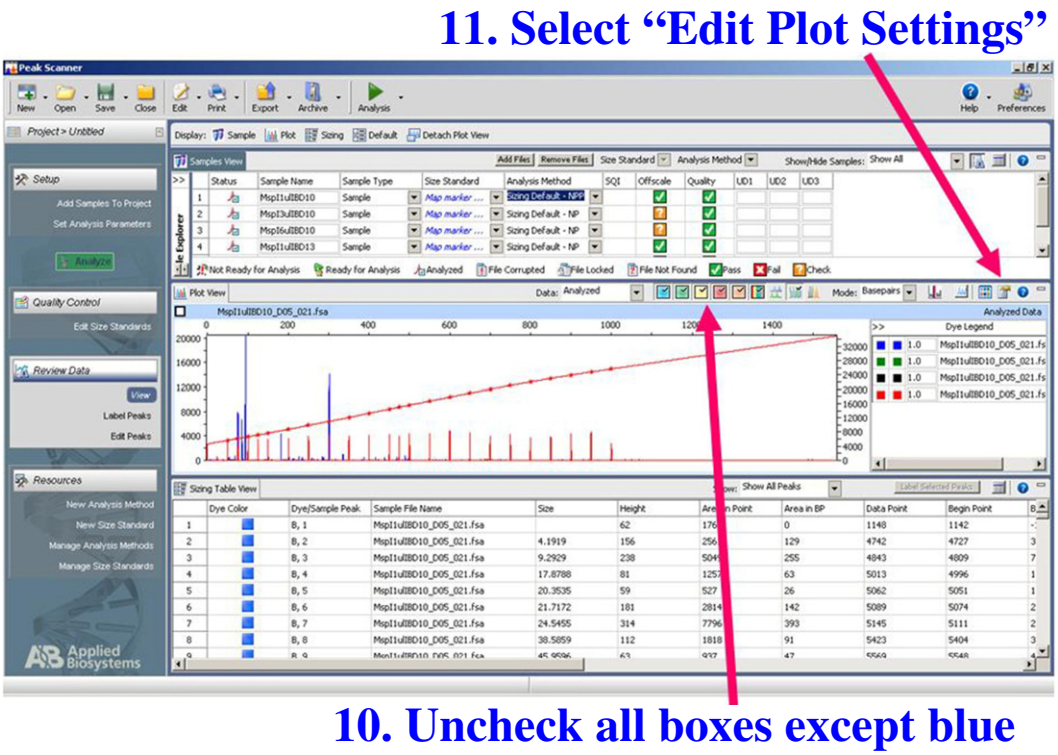


FIGURE H.6

12. Uncheck "Overlay Sizing Curve" (Fig. H.7).
13. Close the window (Fig. H.7).

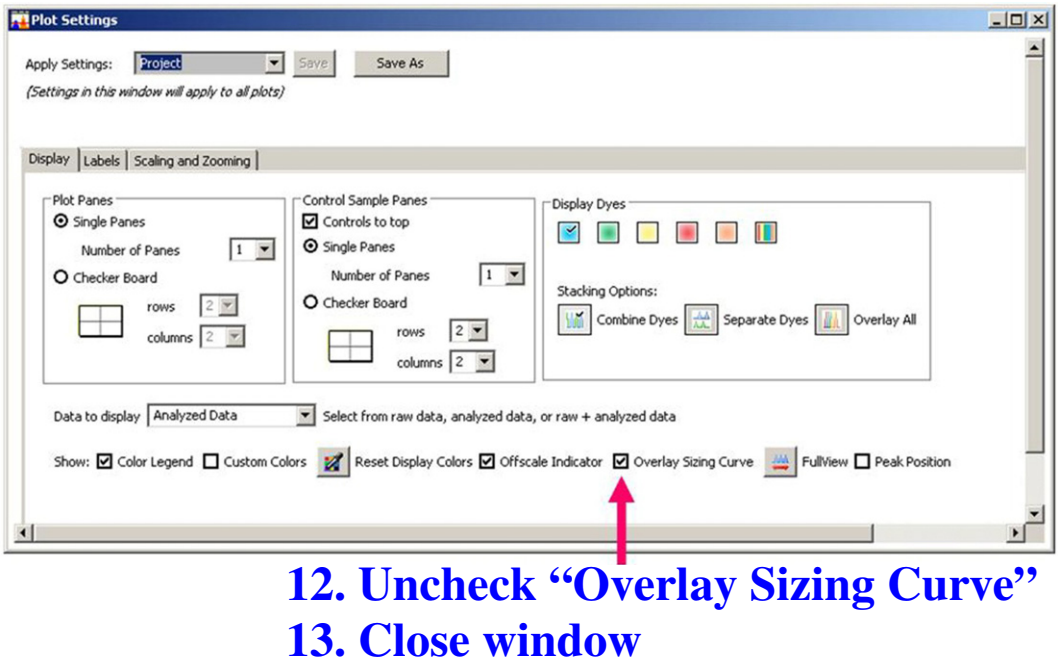


FIGURE H.7

14. Move the cursor arrow under "1000." A magnifying glass will appear (Fig. H.8).
15. Drag the magnifying glass left to "0." This resets the X-axis to extend from 0 to 1000 (Fig. H.8).

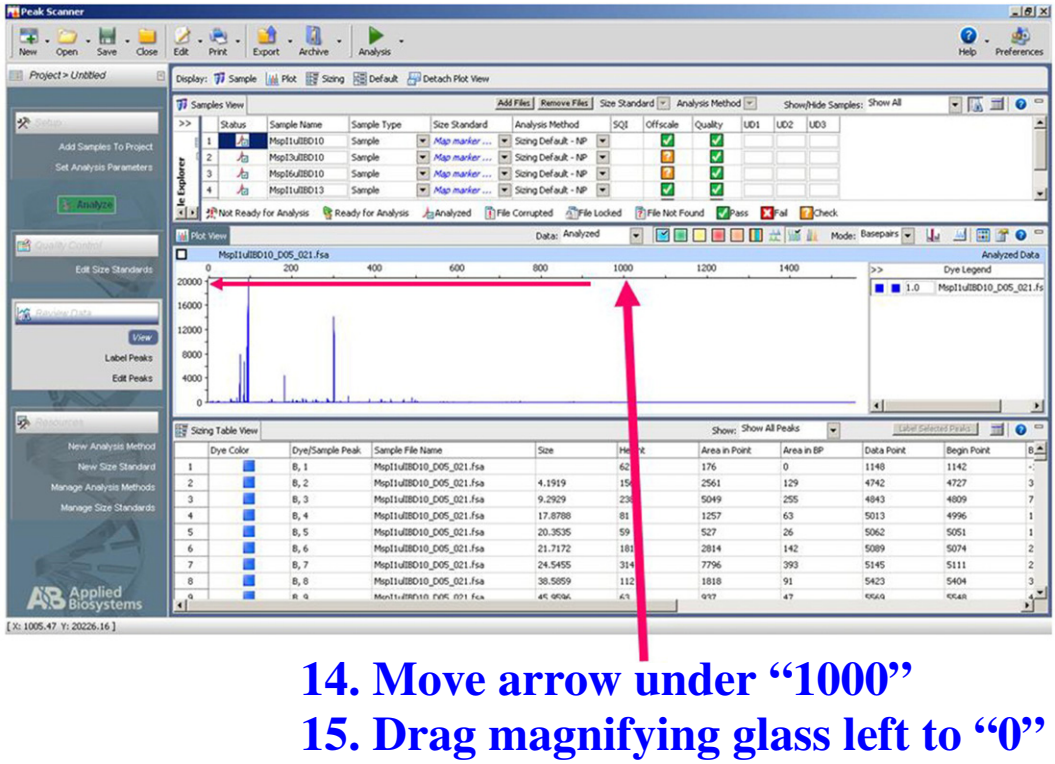


FIGURE H.8

16. Select "Print" (Fig. H.9).

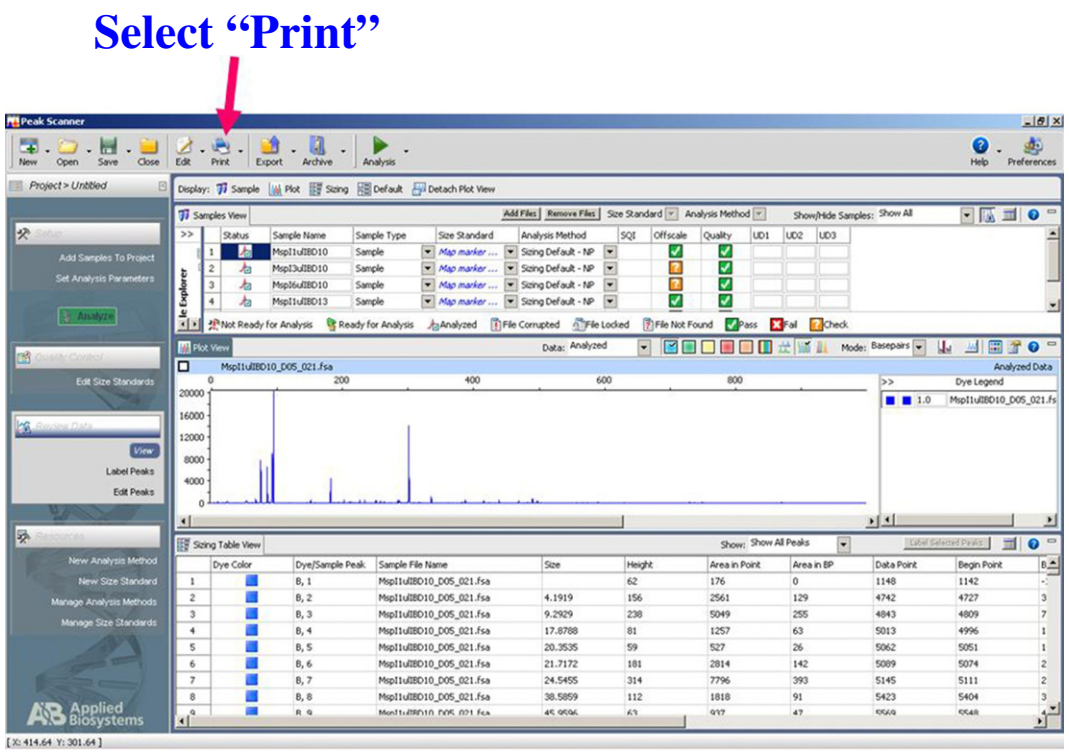


FIGURE H.9

17. Select "Adobe PDF" (Fig. H.10).
18. Select "Preferences" (Fig. H.10).

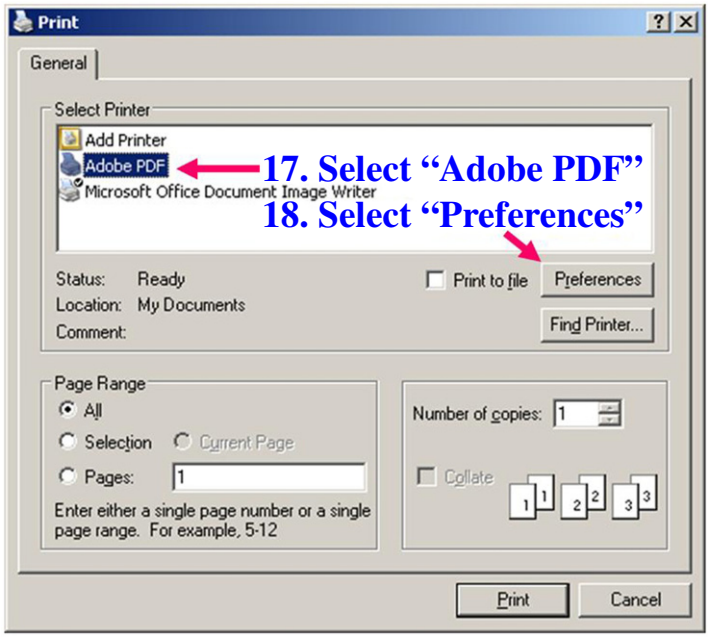


FIGURE H.10

19. Select "Landscape" (Fig. H.11).
20. Select "OK" (Fig. H.11).

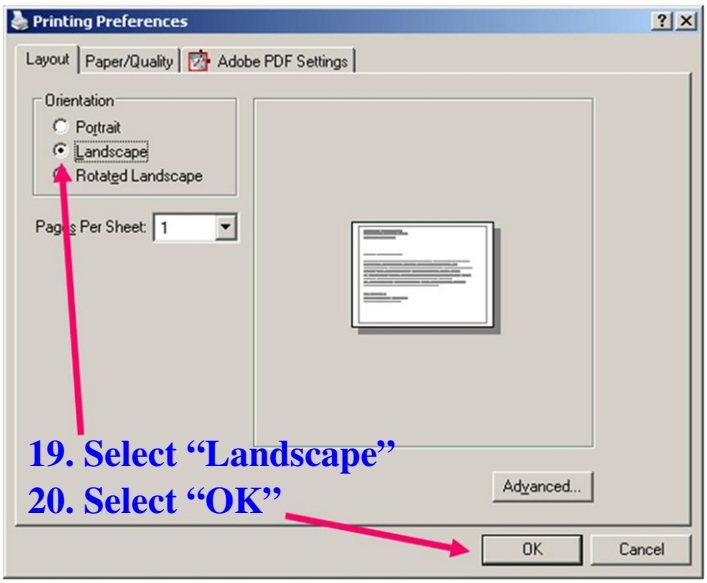


FIGURE H.11

21. Press Ctrl + P or select "Print" in the "File" menu (Fig. H.12).

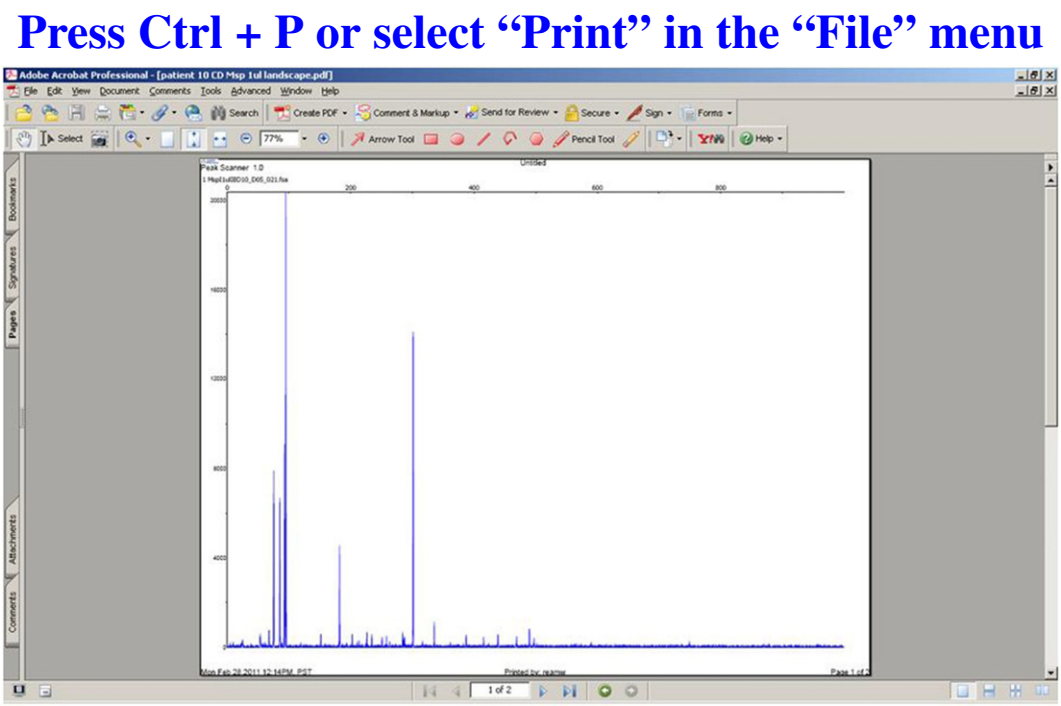


FIGURE H.12

22. Select "Microsoft Office Document Image Writer" and Current view" (Fig. H.13).
23. Select "OK" (Fig. H.13).

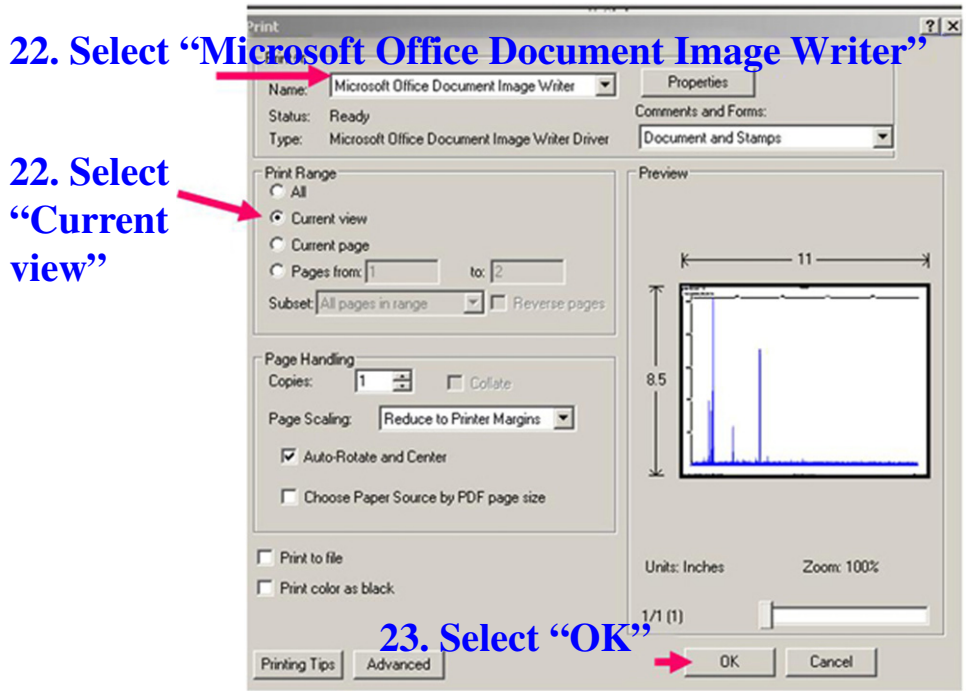


FIGURE H.13

24. Press Ctrl + A and Ctrl + I to select and copy the image (Fig. H.14).
25. Press Ctrl + V to paste the image into a PowerPoint file (Fig. H.14).

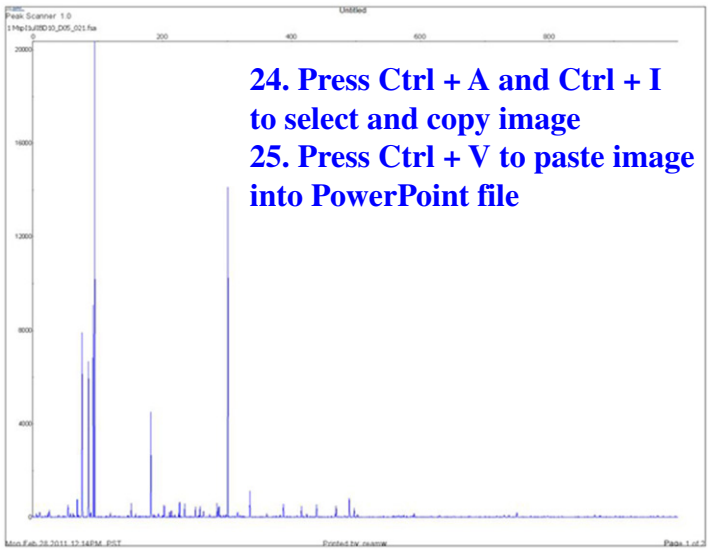


FIGURE H.14

26. Select "Sizing Table View" and "Edit Settings" (Fig. H.15).

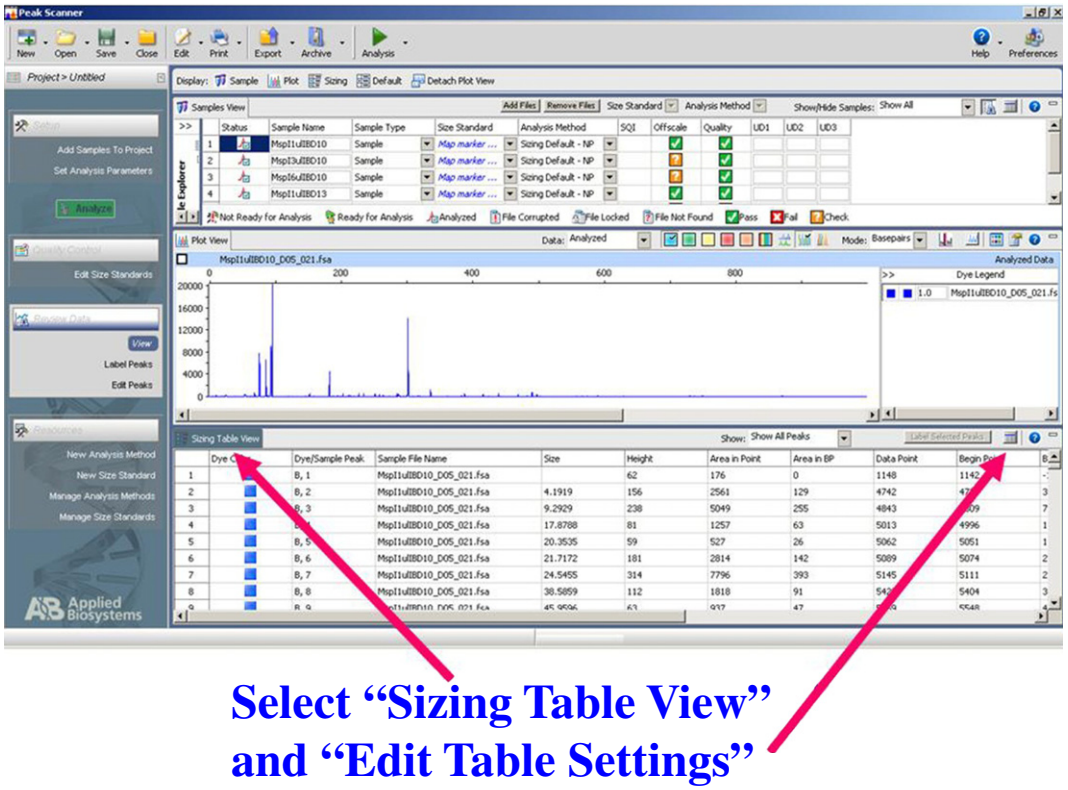


FIGURE H.15

27. Highlight items 7–10 and select "Remove Selected" (Fig. H.16).

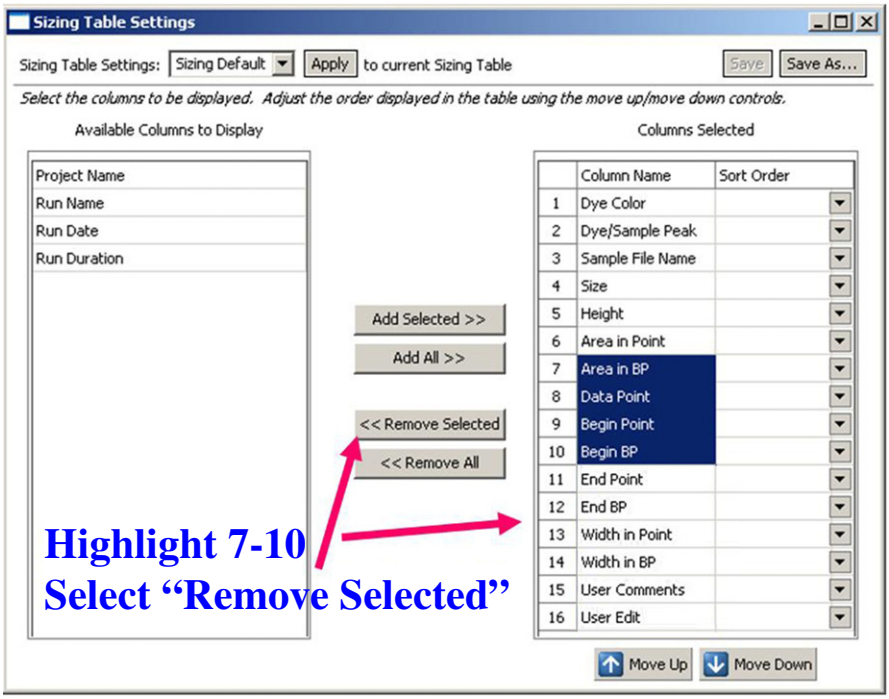


FIGURE H.16

28. Highlight item 1 and select "Remove Selected" (Fig. H.17).
29. Select "Apply" and close the window (Fig. H.17).

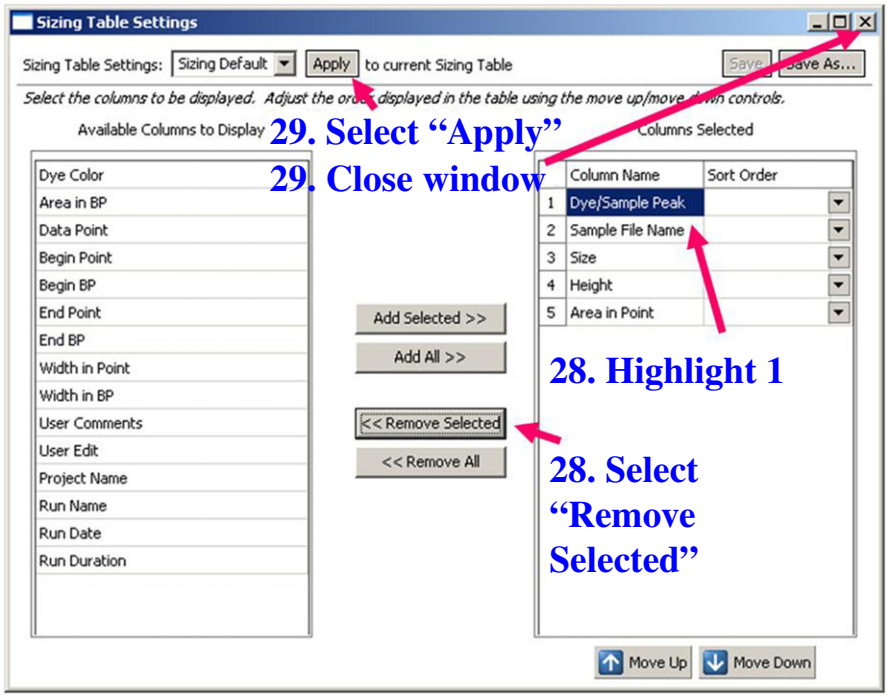


FIGURE H.17

30. Select "Export" (Fig. H.18).

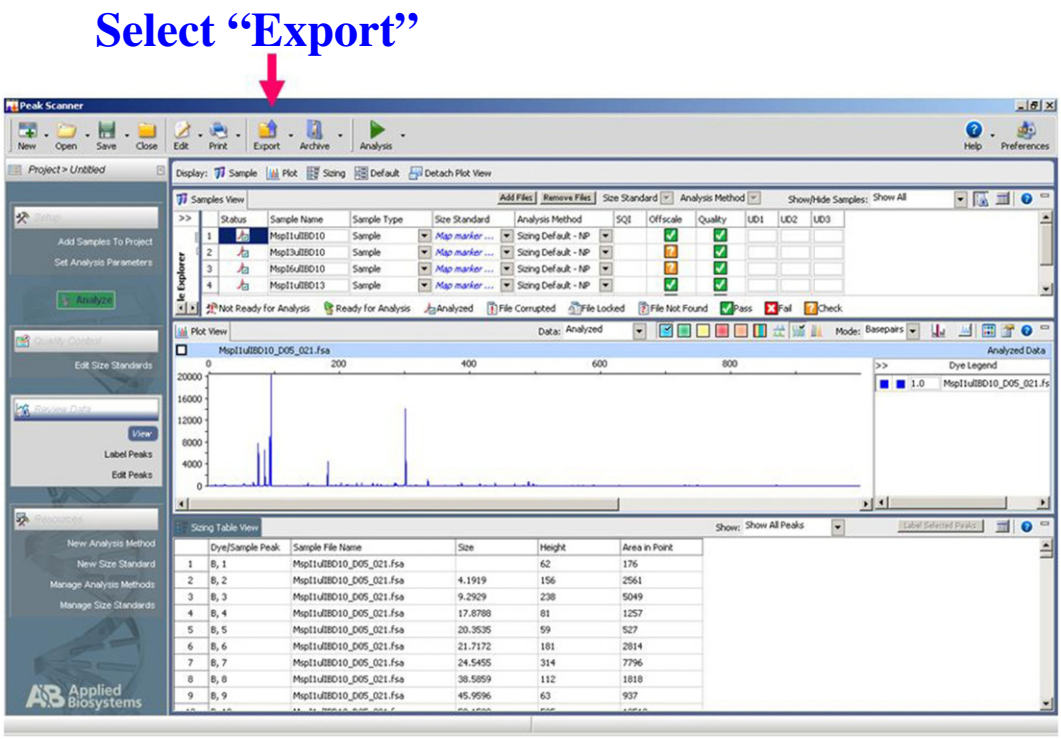


FIGURE H.18

31. Open the .txt file. Press Ctrl + A and Ctrl + C to select and copy the data (Fig. H.19).

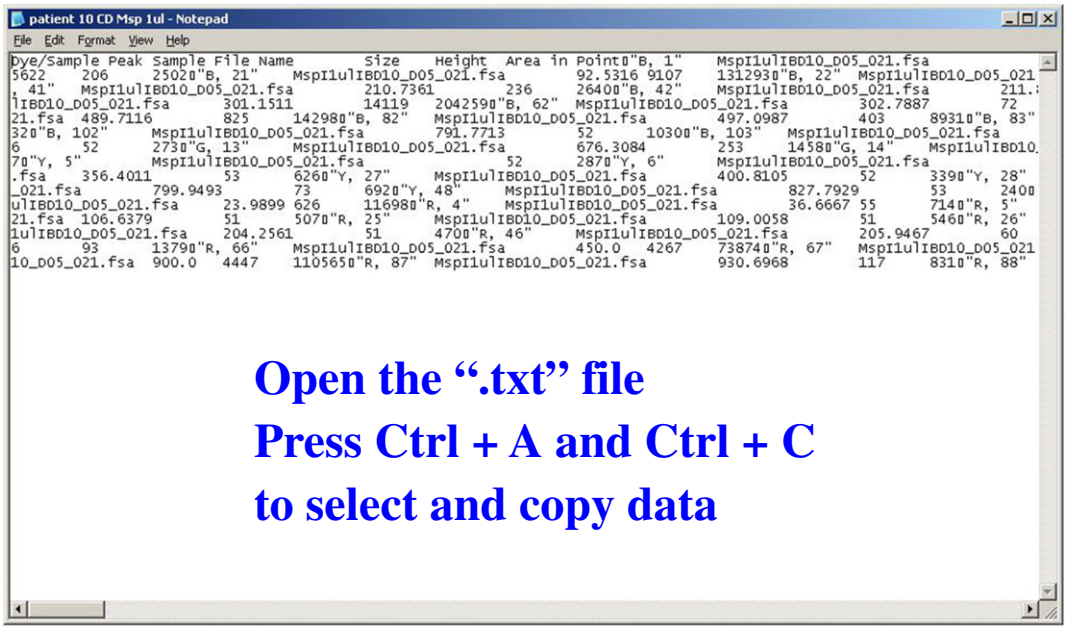
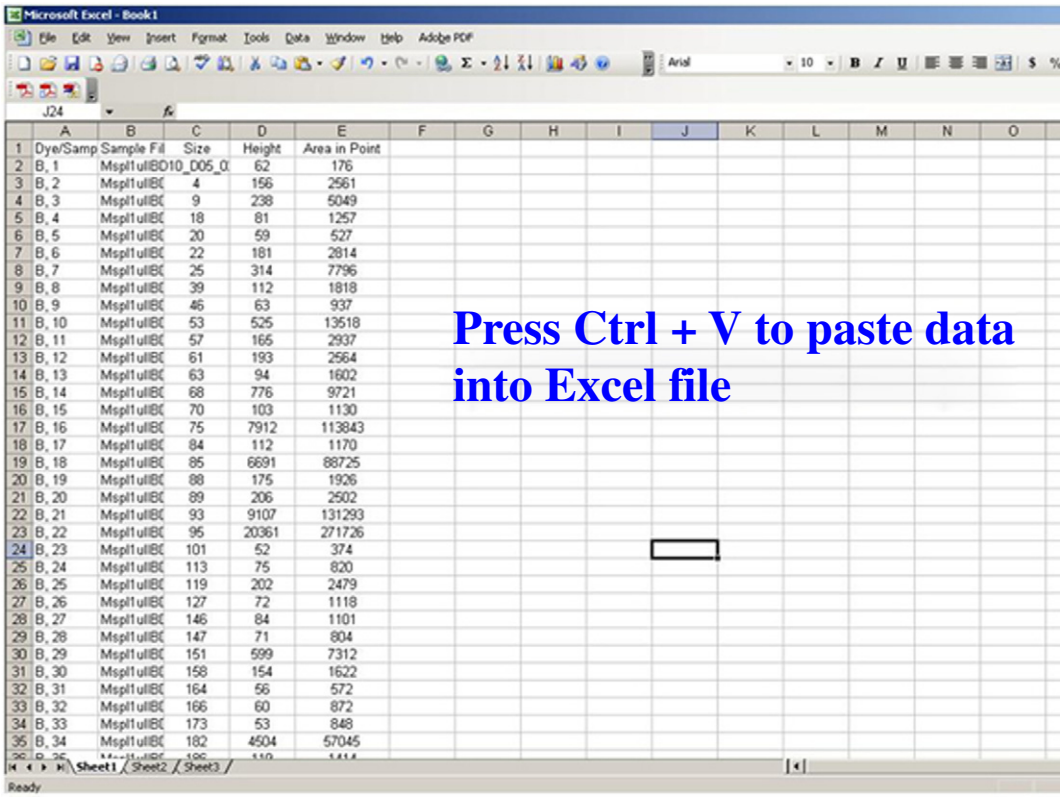


FIGURE H.19

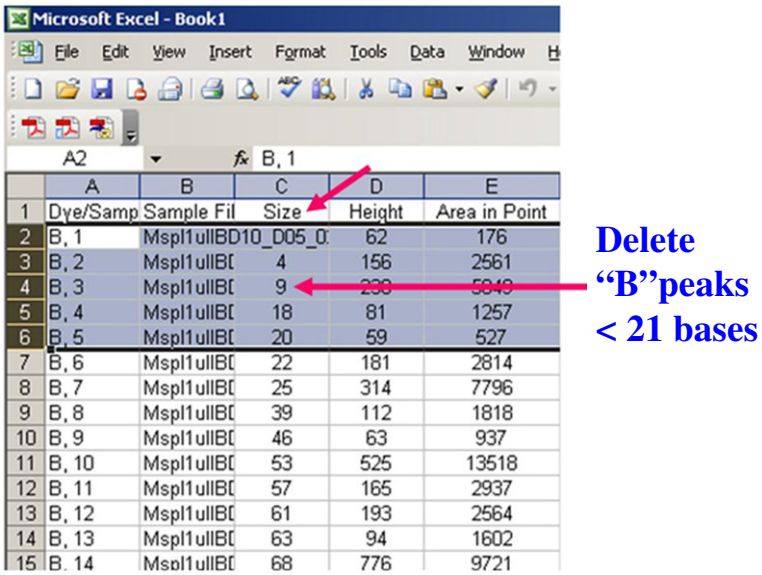
32. Press Ctrl + V to paste the data into an Excel file (Fig. H.20).



	A	B	C	D	E
1	Dye/Samp	Sample Fil	Size	Height	Area in Point
2	B, 1	MspI1ulB	D10_D05_0	62	176
3	B, 2	MspI1ulB	4	156	2561
4	B, 3	MspI1ulB	9	238	5049
5	B, 4	MspI1ulB	18	81	1257
6	B, 5	MspI1ulB	20	59	527
7	B, 6	MspI1ulB	22	181	2814
8	B, 7	MspI1ulB	25	314	7796
9	B, 8	MspI1ulB	39	112	1818
10	B, 9	MspI1ulB	46	63	937
11	B, 10	MspI1ulB	53	525	13518
12	B, 11	MspI1ulB	57	165	2937
13	B, 12	MspI1ulB	61	193	2564
14	B, 13	MspI1ulB	63	94	1602
15	B, 14	MspI1ulB	68	776	9721
16	B, 15	MspI1ulB	70	103	1130
17	B, 16	MspI1ulB	75	7912	113843
18	B, 17	MspI1ulB	84	112	1170
19	B, 18	MspI1ulB	85	6691	88725
20	B, 19	MspI1ulB	88	175	1926
21	B, 20	MspI1ulB	89	206	2502
22	B, 21	MspI1ulB	93	9107	131293
23	B, 22	MspI1ulB	95	20361	271726
24	B, 23	MspI1ulB	101	52	374
25	B, 24	MspI1ulB	113	75	820
26	B, 25	MspI1ulB	119	202	2479
27	B, 26	MspI1ulB	127	72	1118
28	B, 27	MspI1ulB	146	84	1101
29	B, 28	MspI1ulB	147	71	804
30	B, 29	MspI1ulB	151	599	7312
31	B, 30	MspI1ulB	158	154	1622
32	B, 31	MspI1ulB	164	56	572
33	B, 32	MspI1ulB	166	60	872
34	B, 33	MspI1ulB	173	53	848
35	B, 34	MspI1ulB	182	4504	57045

FIGURE H.20

33. Delete "B" peaks < 21 bases. These peaks are smaller than the PCR primer and cannot represent genuine T-RFLP fragments (Fig. H.21).



	A	B	C	D	E
1	Dye/Samp	Sample Fil	Size	Height	Area in Point
2	B, 1	MspI1ullBD10_D05_0		62	176
3	B, 2	MspI1ullB[	4	156	2561
4	B, 3	MspI1ullB[	9	[illegible]	[illegible]
5	B, 4	MspI1ullB[	18	81	1257
6	B, 5	MspI1ullB[	20	59	527
7	B, 6	MspI1ullB[	22	181	2814
8	B, 7	MspI1ullB[	25	314	7796
9	B, 8	MspI1ullB[	39	112	1818
10	B, 9	MspI1ullB[	46	63	937
11	B, 10	MspI1ullB[	53	525	13518
12	B, 11	MspI1ullB[	57	165	2937
13	B, 12	MspI1ullB[	61	193	2564
14	B, 13	MspI1ullB[	63	94	1602
15	B, 14	MspI1ullB[	68	776	9721

FIGURE H.21

34. Delete "B" peaks > 950 bases (Fig. H.22). Most 16S rDNA-derived amplicons produced using the 8-27F/926-907R primer set lie in the 880–939 bp range. Genuine 16S rDNA amplicons larger than 950 bp are unlikely; most amplicons larger than 950 bp result from amplification of nontarget sequences. In T-RFLP studies, full-length amplicons are rare, because most 16S rDNA amplicons contain sites for *Msp*I and *Rsa*I. Incomplete digestion may explain the presence of full-length amplicons.

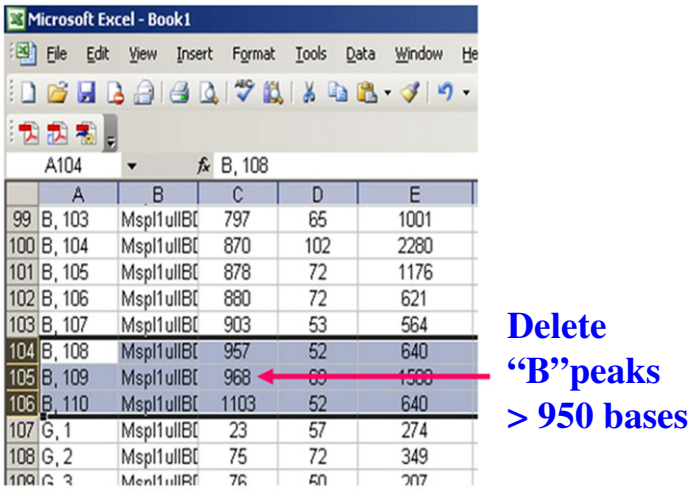


FIGURE H.22

35. Delete "G," "Y," and "R" peaks (Fig. H.23).

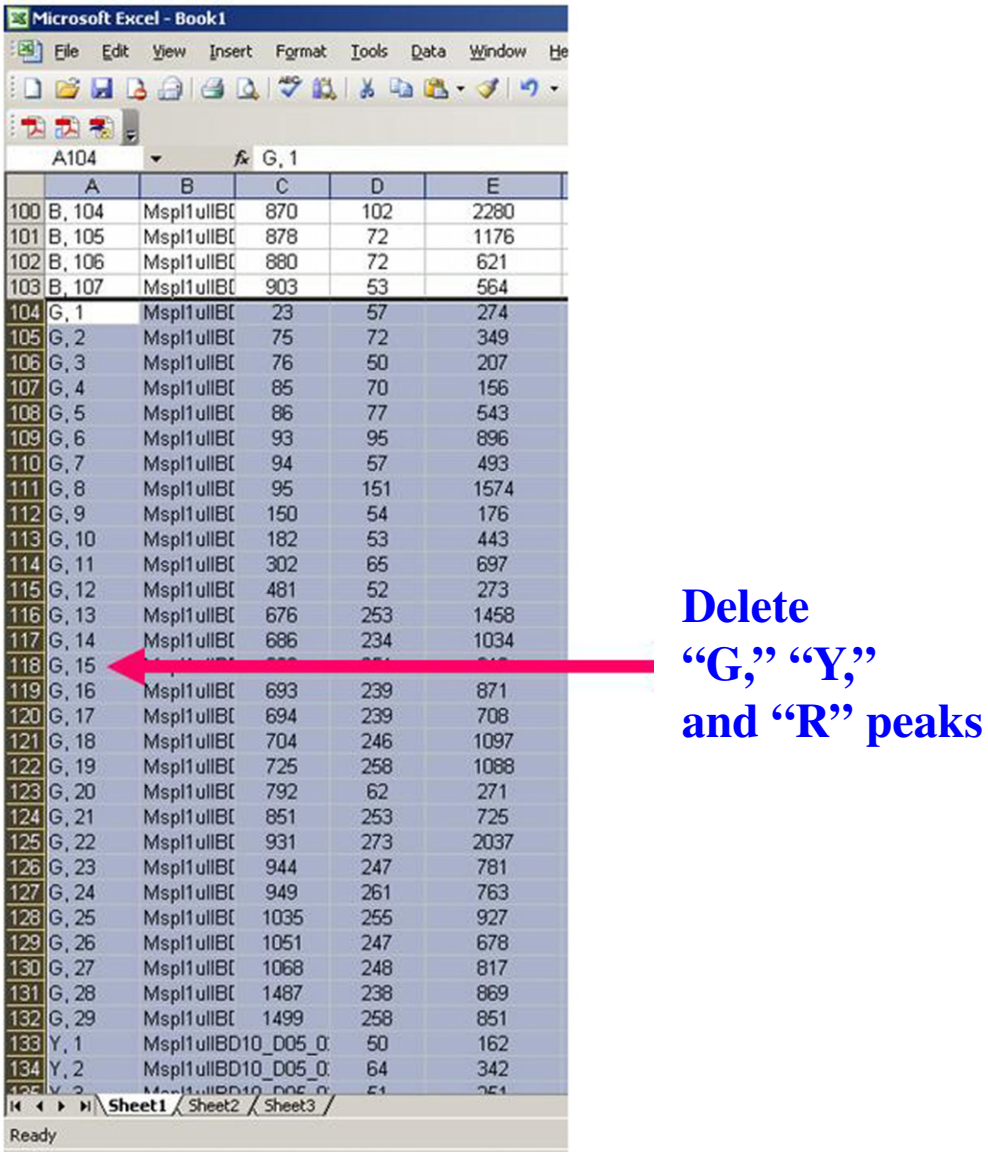
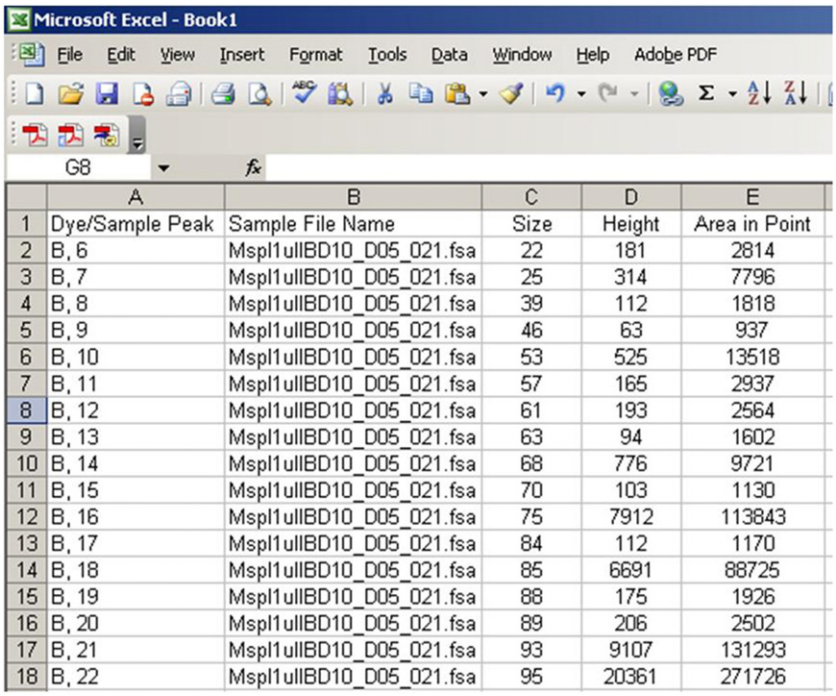


FIGURE H.23

36. Save the Excel and Peak Scanner (.pjc) files in your folder (Fig. H.24).



	A	B	C	D	E
1	Dye/Sample Peak	Sample File Name	Size	Height	Area in Point
2	B, 6	MspI1ullBD10_D05_021.fsa	22	181	2814
3	B, 7	MspI1ullBD10_D05_021.fsa	25	314	7796
4	B, 8	MspI1ullBD10_D05_021.fsa	39	112	1818
5	B, 9	MspI1ullBD10_D05_021.fsa	46	63	937
6	B, 10	MspI1ullBD10_D05_021.fsa	53	525	13518
7	B, 11	MspI1ullBD10_D05_021.fsa	57	165	2937
8	B, 12	MspI1ullBD10_D05_021.fsa	61	193	2564
9	B, 13	MspI1ullBD10_D05_021.fsa	63	94	1602
10	B, 14	MspI1ullBD10_D05_021.fsa	68	776	9721
11	B, 15	MspI1ullBD10_D05_021.fsa	70	103	1130
12	B, 16	MspI1ullBD10_D05_021.fsa	75	7912	113843
13	B, 17	MspI1ullBD10_D05_021.fsa	84	112	1170
14	B, 18	MspI1ullBD10_D05_021.fsa	85	6691	88725
15	B, 19	MspI1ullBD10_D05_021.fsa	88	175	1926
16	B, 20	MspI1ullBD10_D05_021.fsa	89	206	2502
17	B, 21	MspI1ullBD10_D05_021.fsa	93	9107	131293
18	B, 22	MspI1ullBD10_D05_021.fsa	95	20361	271726

FIGURE H.24

APPENDIX

I

Adding Size Standards to Peak Scanner

We can use either of two size standards for the T-RFLP analysis in experiment 4.

Map Marker 1000 contains DNA fragments labeled with a red dye; the fragment sizes are 50, 75, 100, 125, 150, 200, 250, 300, 350, 400, 450, 475, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, and 1000 bases.

The 1200 LIZ size standard contains DNA fragments labeled with an orange dye (LIZ); the fragment sizes are 20, 30, 40, 60, 80, 100, 114, 120, 140, 160, 180, 200, 214, 220, 240, 250, 260, 280, 300, 314, 320, 340, 360, 380, 400, 414, 420, 440, 460, 480, 500, 514, 520, 540, 560, 580, 600, 614, 620, 640, 660, 680, 700, 714, 720, 740, 760, 780, 800, 820, 840, 850, 860, 880, 900, 920, 940, 960, 980, 1000, 1020, 1040, 1060, 1080, 1100, 1120, 1160, and 1200 bases.

To add size standards, open Peak Scanner and proceed as follows.

1. Select "Start New Project" (Figs. I.1 and I.2).

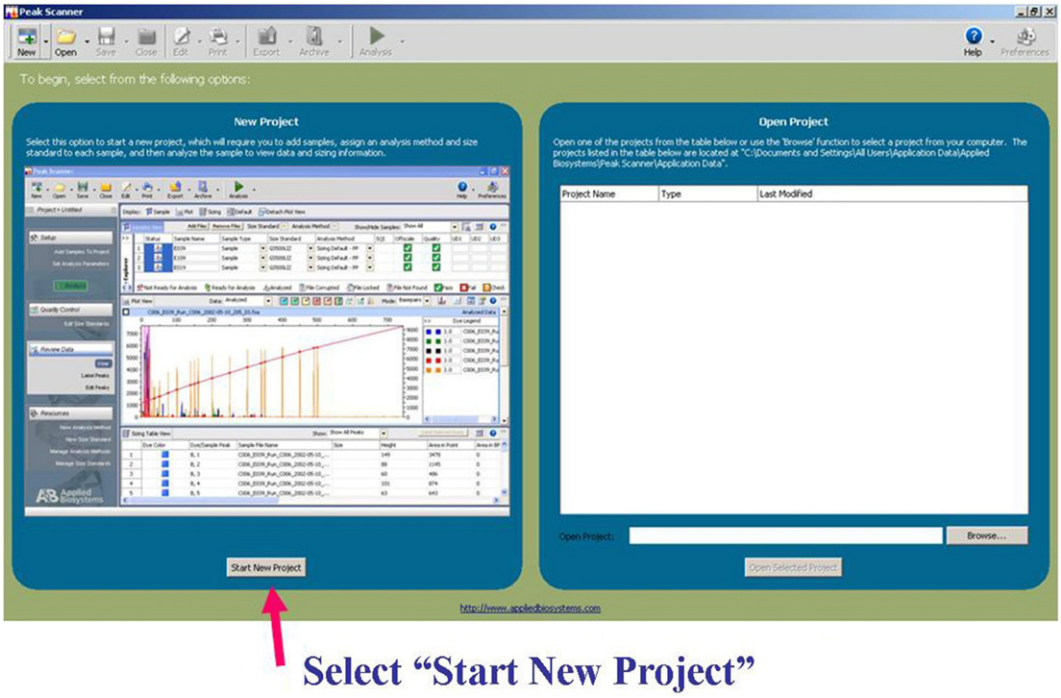


FIGURE I.1

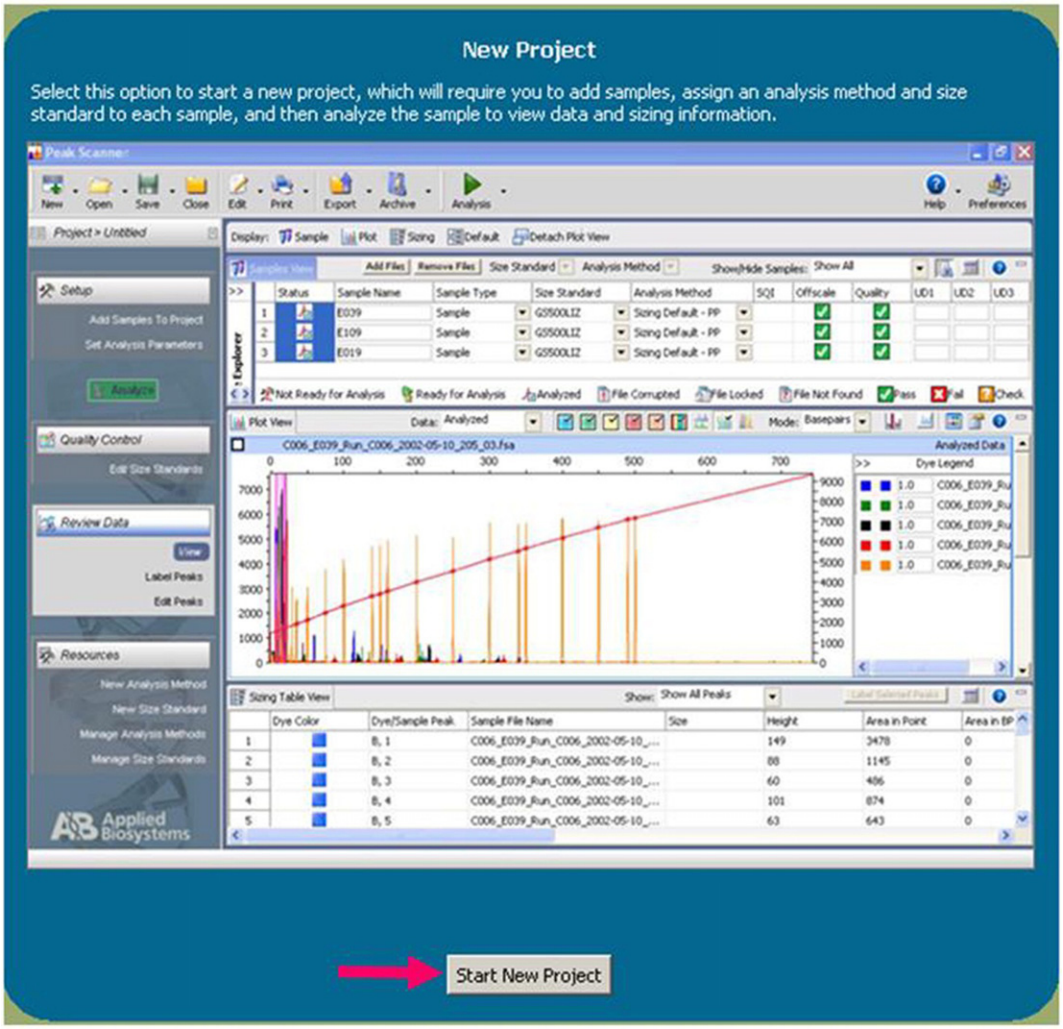

New Project
Select this option to start a new project, which will require you to add samples, assign an analysis method and size standard to each sample, and then analyze the sample to view data and sizing information.
Start New Project

FIGURE I.2

2. In the "*Resources*" area, select "New Size Standard" (Fig. I.3).

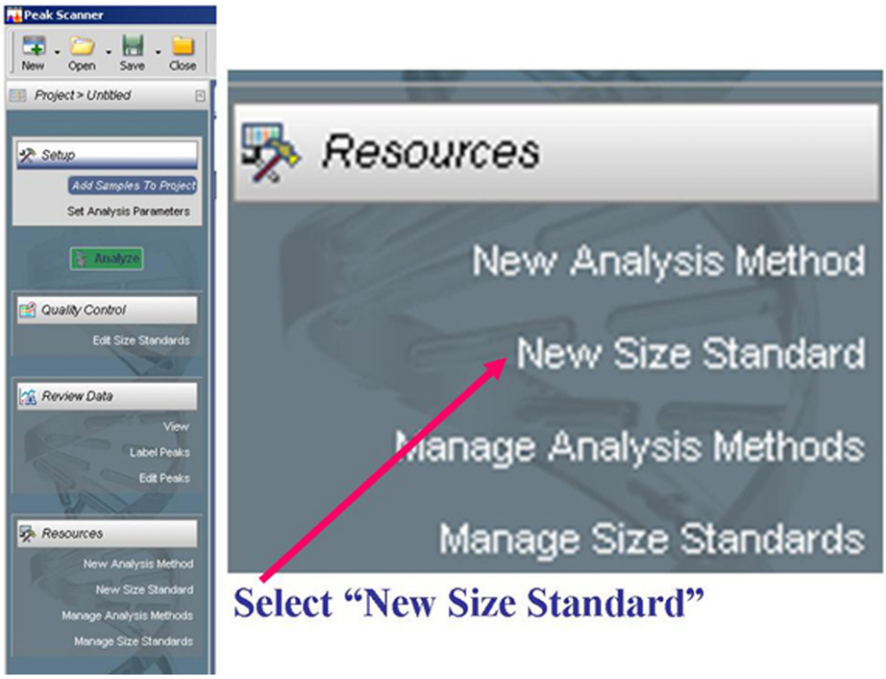


FIGURE I.3

3. In the "Size Standard Editor" window, enter the name of the marker (Map Marker 1000 or 1200 LIZ) in the "Size Standard Name" box (Fig. I.4).
4. Set the "Dye Color" menu to "Red" for Map Marker 1000 or "Orange" for 1200 LIZ. **The correct color is essential.**
5. Enter the sizes of the DNA molecules in the size standard in the "Enter new Size Standard definition" box (Fig. I.4).

   For Map Marker, enter 50, 75, 100, 125, 150, 200, 250, 300, 350, 400, 450, 475, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000.

   For 1200 LIZ, enter 20, 30, 40, 60, 80, 100, 114, 120, 140, 160, 180, 200, 214, 220, 240, 250, 260, 280, 300, 314, 320, 340, 360, 380, 400, 414, 420, 440, 460, 480, 500, 514, 520, 540, 560, 580, 600, 614, 620, 640, 660, 680, 700, 714, 720, 740, 760, 780, 800, 820, 840, 850, 860, 880, 900, 920, 940, 960, 980, 1000, 1020, 1040, 1060, 1080, 1100, 1120, 1160, 1200.
6. Select "Add Sizes" (Fig. I.5).
7. Select "Save" (Fig. I.6).

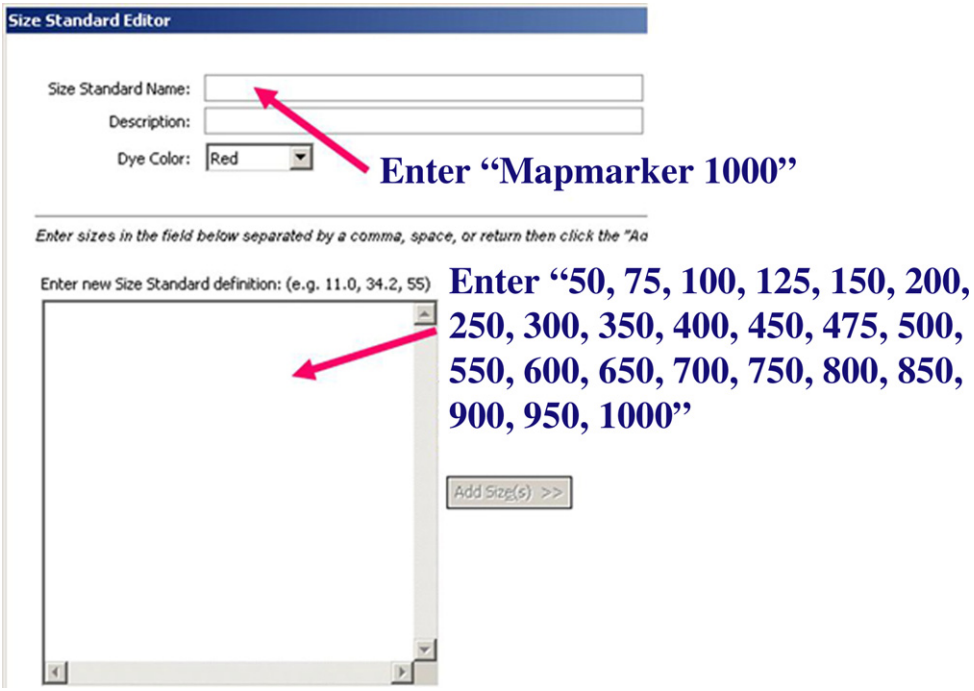


FIGURE I.4

FIGURE I.5

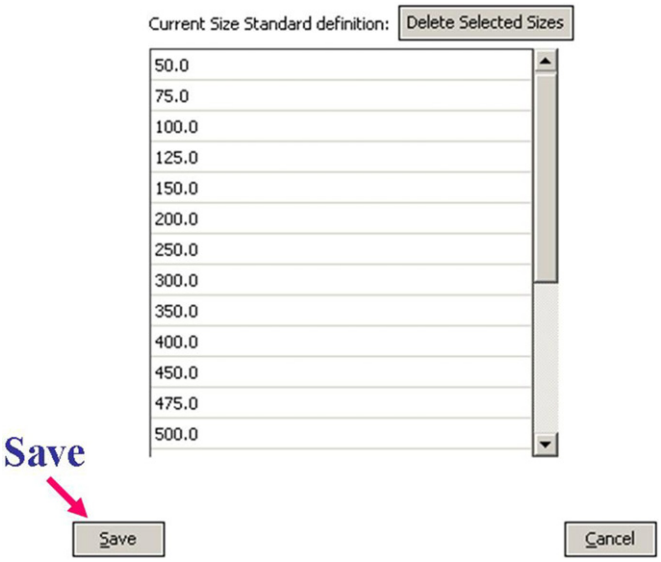


FIGURE I.6

APPENDIX

J

PRIMER Tutorial

NONMETRIC MULTIDIMENSIONAL SCALING USING PRIMER

Nonmetric multidimensional scaling (NMDS) compares populations present in multiple samples. The PRIMER (Plymouth Routines in Multivariate Ecological Research) program (http://www.primer-e.com) calculates the similarity of each pair of samples in a data set. PRIMER plots each sample as a point in a two- or three-dimensional format, such that samples with similar populations lie closer together than samples with dissimilar populations. The axes do not have units, hence the term *nonmetric*.

PRIMER uses the Bray-Curtis similarity coefficient to compare samples.

$$S_{ab} = 100[1 - \text{sum}_{s=1\ldots n}|y_{sa} - y_{sb}|/\text{sum}_{s=1\ldots n}(y_{sa} + y_{sb})]$$

S_{ab} = similarity between samples a and b
y_{sa} = abundance of species s in sample a
y_{sb} = abundance of species s in sample b

For each species in the data set, the program determines the difference in abundance between two samples, and it divides this number by the total abundance of this species in both samples. To determine the similarity coefficient, PRIMER performs this calculation for all species in the data set. The program calculates a similarity coefficient for each pair of samples in the data set.

To use PRIMER, create an Excel file in the following format (Table J.1):

Row 1 = title (or leave empty).
Row 2, column 2 → last column = sample names.
Column 1, row 2 = column header (or leave blank).
Column 1, row 3 → last data row = species names (or T-RFLP fragment sizes).
Column 2 → last column, row 3 → last data row = species abundance (or T-RFLP peak area).

Enter the peak area for each T-RFLP peak in these cells. If you sequence enough cloned 16S rDNA amplicons, you can enter species abundance as a percentage of the total population.

TABLE J.1 Bacterial Genera in Gastric Biopsies from Patients with Inflammatory Bowel Disease (IBD) and IBD-Free Controls

Seqence Percentages																		
Genus	**2 NL**	**3 NL**	**4 NL**	**5 NL**	**6 NL**	**8 NL**	**38 NL**	**13 NL**	**16 NL**	**14 CD**	**21 CD**	**25 CD**	**30 UC**	**37 UC**	**33 CD**	**32 UC**	**35 UC**	**34 CD**
Actinomyces	8	0	0	3	1	4	1	16	1	2	0	4	0	0	1	2	0	3
Atopobium	0	0	0	3	0	0	1	1	0	0	0	2	0	0	0	1	0	0
Brachybacterium	0	0	0	1	0	0	0	0	0	0	11	0	0	0	0	0	0	0
Capnocytophaga	1	0	0	0	1	0	0	2	1	0	0	0	0	0	0	0	0	1
Catonella	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Corynebacterium	0	0	0	0	1	2	0	2	0	0	0	0	0	0	0	0	0	0
Eubacterium	0	0	0	1	0	1	0	1	2	0	0	4	0	0	1	4	0	0
Fusobaterium	9	0	3	0	0	4	0	2	7	0	0	0	0	4	1	0	3	3
Gemella	5	1	3	0	10	6	4	1	0	0	2	2	10	3	1	3	0	0
Granulicatella	4	1	6	0	0	3	3	1	0	4	2	2	1	0	0	2	0	0
Kocuria	0	0	0	0	0	0	0	1	1	0	11	0	0	0	0	0	0	1
Lachnospiraceae	0	0	0	0	0	2	0	0	1	0	0	2	0	0	0	0	0	0
Lactobacillus	0	0	0	0	1	0	0	0	0	2	0	0	0	0	3	0	0	0
Leptotrichia	13	0	0	0	0	2	0	1	4	0	0	8	0	0	1	2	5	0
Megasphaera	4	0	0	0	0	0	0	1	0	0	0	0	0	0	0	5	0	0
Neisseria	4	0	3	0	10	1	0	0	7	0	0	0	0	0	0	1	8	1
Poryphyromonas	14	0	9	0	0	0	0	4	18	2	0	0	0	0	3	0	5	1
Prevotella	22	23	9	23	0	6	33	50	37	15	16	8	0	8	34	30	60	10
Propionibacterium	0	0	0	0	0	0	0	0	0	2	2	14	1	0	0	0	0	0
Streptococcus	12	52	46	63	66	42	43	9	13	65	37	43	85	80	41	22	14	73
Veillonella	4	9	2	4	0	18	5	1	3	5	7	6	0	0	8	14	2	1
status	n	n	n	n	n	n	n	n	n	c	c	c	u	u	c	u	u	c
severity	n	n	n	n	n	n	n	n	n	i	i	i	i	i	i	i	a	a
gender	m	m	m	f	m	m	m	m	m	f	m	m	m	m	m	f	m	m

Note: Numbers indicate the percent of the bacterial population comprising each genus. Letters following patient identification numbers indicate disease status: NL, IBD-free; CD, Crohn's disease; UC, ulcerative colitis. Letters in "status" row indicate IBD-free (n), Crohn's (c), and ulcerative colitis (u) patients. Letters in "severity" row indicate IBD-free (n), inactive IBD (i), or active IBD (a). Letters in "gender" row indicate male (m) and female (f) patients.

Leave an empty row below the last data row. In the rows beneath this empty row, enter sample properties (for example, the source) for each sample. Describe the property in column 1 (e.g., gender, disease status, or severity), and enter the information in each sample column.

To create NMDS plots using PRIMER:

1. Select "Open" in the "File" menu.
2. Set "File type" to "Excel."
3. Select an Excel file.
4. Select "Open."
5. Select "Sample data."
6. Select "Next."
7. Select "Samples as columns."
8. Set "Blank" to "Zero."
9. Select "Finish."
10. Select "Pre-treatment" in the "Analyze" menu.
11. Select "Transform" in the "Pre-treatment" menu.
12. Select the desired transformation method. You have five choices: "None," "Square root," "Fourth root," "Log (X + 1)," and "Presence/absence."
    a. "None" gives the greatest emphasis to the most abundant species and effectively ignores minor components of the population (e.g., 1000 = 1000; 100 = 100; 10 = 10; 1 = 1).
    b. "Square root" takes the square root of the data (e.g., 81 → 9; 9 → 3; 1 → 1).
    c. "Fourth root" takes the square root of the square root of the data (e.g., 81 → 3; 1 → 1).
    d. "Log (X + 1)" takes the log of the data (e.g., 1000 → 3; 100 → 2; 10 → 1; 1 → 0.3).
    e. "Presence/absence" gives equal emphasis to species regardless of their abundance (e.g., 1,000 → 1; 100 → 1; 1 → 1).
13. Select "Resemblance" in the "Analyze" menu.
14. Select "Samples."
15. Select "Bray-Curtis similarity" in the "Resemblance" menu.
16. Select "OK" to proceed.
17. Select "MDS" in the "Analysis" menu.
18. Select "OK" to accept the default settings in the "MDS:" menu.
19. Two- and three-dimensional plots appear. Check the "Spin" box to rotate the 3D image.

Samples with similar bacterial populations lie closer to each other than samples with dissimilar bacterial populations.

Notes

APPENDIX

K

Checklist for Grading Laboratory Reports

Section/Items Scored	Points	Maximum Score	Comments
Name, Title, Date:			
informative, brief, correct		10	
Purpose:			
clear and concise		10	
correct		10	
grammar and spelling		10	
sentence structure		10	
Methods:			
clear and concise		10	
correct		10	
complete; proper references		10	
grammar and spelling		10	
sentence structure		10	
Results:			
overall organization		10	
paragraph structure		10	
clear and concise		10	
grammar and spelling		10	
sentence structure		10	

(*Continued*)

(*Continued*)

Section/Items Scored	Points	Maximum Score	Comments
accurate		10	
thorough		10	
figures labeled and neat		10	
no methods or discussion		10	
appropriate style; elegance		10	
Discussion:			
overall organization		10	
paragraph structure		10	
clear and concise		10	
sentence structure		10	
grammar and spelling		10	
logical, supported by data		10	
thorough		10	
integrates theory with results		10	
explains unexpected results		10	
Conclusion:			
clear and concise		10	
sentence structure		10	
grammar and spelling		10	
logical, supported by data		10	
Questions		70	
Total		400	

APPENDIX

L

Peer Review Checklist

Reviewer_____ Experiment #_____

Author _____ Date _____

1. Look for awkward sentences, poorly organized paragraphs, incorrect grammar, and misspelled words. Even grammatically correct sentences can be awkward and difficult to read. Sentences should be simple and straightforward. Use the active voice instead of the passive voice, and eliminate redundant words. Replace vague, qualitative adjectives such as *large* or *small* with numbers. For example, *a tenfold increase* is much more informative than *a large increase*. Explain new terms clearly, and clarify ambiguous statements.
2. Check that the name, title, and date are present. Is the title informative and concise?
3. The purpose statement should be clear, concise, complete, and correct.
4. The methods section should describe the procedures completely, clearly, and concisely. It should cite references properly and indicate modifications made to the procedure.
5. **The results section should begin with an overview of the work.** After reading the results section, a scientist who has not read the methods section should understand the experiment, but it should not include details from the methods section. Strike a balance between too little and too much information by describing the procedures well enough to understand the experiment. Do not include details required to repeat the experiment, which belong in the methods section. For example, do not describe the composition of buffers or media in the results; the methods section should contain this information or reference a publication that does.
6. **Present the results in an order that leads the reader through the experiment.** Is the organization of the data logical? Is the presentation clear? Would changing the organization make the report easier to understand?
7. Figures and tables must have appropriate titles, legends, and citations in the text. Each lane of a gel photograph must have a label, and the figure legend must list the contents of each lane.

8. Does the text accurately describe the figures? The data must support statements made in the text.
9. **The discussion should begin with a brief introduction that makes the purpose of the experiment clear.** This section should flow logically from an introduction through the results to a sound conclusion. The discussion may include background material that puts the need for the experiments in perspective. A good discussion does not merely reiterate information presented in the results section. After a brief recap of the question asked and the work done to answer it, the author should state the meaning of the data and indicate how the findings affect our current understanding of the field. In other words, what was the answer to the question, and what does that answer mean?

    Experiments often produce inconclusive results with more than one interpretation. The discussion should present all possibilities and suggest which one the author believes is correct. If the experiment produced unexpected results, such as "extra" bands on a gel, the discussion must address results that do not fit the author's expectations. The discussion should provide a plausible explanation of such data.
10. The conclusion should summarize the experiment in two or three sentences. The data must support the conclusions.
11. What is best about this writing?
12. What needs improvement?

Notes

Notes

APPENDIX

M

Career Advice

Pursue a career that you find interesting, enjoyable, and worthwhile. Spend as much time as possible doing what you enjoy. Do what is meaningful to you. Do not waste time. Spend as little time as possible doing things you do not enjoy. You do not have forever.

Consider these words of wisdom from William Rehnquist, former Chief Justice of the U.S. Supreme Court:

> "Another way to look at life is to see it as a different kind of department store.
>
> A store where such things as worldly success, love of music, enjoyment of painting, a six-handicap golf game, a close relationship with your son or daughter, and many other similar things are for sale.
>
> But the commodity with which they are purchased is not money but time.
>
> And quite the contrary to the way the capitalist system works with money and goods, every one of us is given exactly the same amount of time in each hour, in each day and in each year.
>
> It is a limited amount, and it is impossible for anyone to be so rich in time that he can enjoy every single one of the things which time may buy.
>
> So, there is a choice to be made. . ."
>
> ***—William Rehnquist, former Chief Justice, U.S. Supreme Court***

APPENDIX

N

Molecular Microbiology Laboratory Preparation Manual

This preparation manual is for a class of 72 students. In experiments 1 and 2, students work in pairs, whereas during experiments 3 and 4 they work individually. Accordingly, we listed quantities of reagents required per pair of students for the first two units. The manual indicates materials needed by each student for the final two experiments. For each gel electrophoresis experiment, we indicate the number of lanes per pair (or per student). Each student (or pair) should have a set of pipettors as well as safety glasses, a lab coat, and gloves. We list the equipment required for the entire class, but we do not indicate the precise number of microcentrifuges, vortexers, and other instruments. If possible, no more than six students (or three pairs) should share one piece of equipment. We routinely prepare 10% more reagents than necessary.

MEDIA ROOM PREPARATION MANUAL

Experiment 1: Plasmid Purification and Restriction Mapping

One Week before Class 2

Strain curator needs to obtain and complete the following:

2 LB ampicillin (50 μg/ml) agar plates; note: please pour "thick" plates, 25 ml/plate.
2 sterile 250-ml flasks with 30 ml LB ampicillin broth.
2 sterile 50-ml centrifuge tubes.
36 sterile 1.5-ml microcentrifuge tubes.
4 LB ampicillin (50 μg/ml) agar stabs.
2 LB ampicillin (50 μg/ml) + kanamycin (25 μg/ml) agar stabs.
100 ml lysis buffer: 50 mM glucose + 10 mM EDTA + 25 mM Tris, pH 8. Mix 4.5 ml of a 20% sterile glucose solution. Add 4 ml of sterile 0.25 M EDTA. Add 2.5 ml of sterile 1 M Tris, pH 8. Add 89 ml of sterile distilled water; adjust pH to 8.

Filter sterilize. Store in refrigerator.
Rinse all glassware with distilled water.
Use sterile distilled water to prepare all reagents.

Week 1

CLASS 2: PLASMID PURIFICATION AND RESTRICTION

Items per pair of students (plan for 36 pairs):

0.15 ml 5 M potassium acetate: Dissolve 49.1 g potassium acetate in 100 ml of sterile distilled water. Take 60 ml of this solution and add 11.5 ml of glacial acetic acid and 28.5 ml of sterile distilled water. The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Store at room temperature.
0.25 ml phenol[*] equilibrated in 1 M Tris, pH 8.0: Store at 4°C. Note: Phenol must be **colorless**, not pink.
0.25 ml isoamyl alcohol:chloroform[*]: Add 1 ml isoamyl alcohol to 24 ml chloroform.
0.01 ml 3.5 M sodium acetate. Store at room temperature. If you have anhydrous sodium acetate, add 28.7 g to 50 ml sterile distilled water, then adjust pH to 5.2 with glacial acetic acid and bring to a final volume of 100 ml with sterile distilled water. If you have sodium acetate trihydrate, add 47.6 g to 50 ml sterile distilled water, then adjust the pH to 5.2 with glacial acetic acid and bring to a final volume of 100 ml with sterile distilled water.
1 ml TE, pH 8: 10 mM Tris + 1 mM EDTA, pH 8: Mix 0.121 g Tris base + 0.0372 g disodium EDTA; add 50 ml sterile distilled water, then bring pH to 8.0 with HCl. Bring final volume to 100 ml with sterile distilled water. Autoclave. Store at room temperature.
0.003 ml 10X *Pst*I reaction buffer: Obtain from enzyme supplier (Fermentas or Gibco). Store frozen.
0.002 ml *Pst*I enzyme (10−20 units/μl): Obtain from Fermentas or Gibco freezer. Store at −20°C in a nondefrosting freezer.
0.5 ml 95% ethanol: (chill on ice on **class day 2**).
0.5 ml sterile distilled water.
18 sterile 1.5-ml microcentrifuge tubes.
36 sterile yellow tips for P20 and P200 pipetman.
21 sterile blue tips for P1000 pipetman.
0.15 ml resuspended NM522 (pKN800) pellet (stored in the freezer).
0.06 ml lysozyme solution: 80 mg lysozyme dissolved in 1 ml sterile distilled water. **Prepare 1 hour before class 2**; store on ice.
0.2 ml SDS-NaOH solution: 8.8 ml sterile distilled water + 0.2 ml 10 N NaOH + 1 ml of 10% SDS solution. **Mix 1 hour before class 2** and store at room temperature.
10 ul RNase A (5 mg/ml). Heat stock to 65°C for 15 minutes, then store frozen.
Safety goggles and lab coats are required; students should supply their own coats.

[*]Phenol, isoamyl alcohol, and chloroform may be combined (25:1:24) or ordered as a mixture from Gibco/BRL (catalog number 15593-031).

Week 2

CLASS 3: AGAROSE GEL ELECTROPHORESIS AND TRANSFORMATION

Materials for entire class (72 students = 36 pairs):

600 ml of 0.8% agarose: Dissolve 4.8 g of agarose in 600 ml of 1 × TAE electrophoresis buffer. Use a microwave or hotplate to melt the agarose before class.
1 liter of 20 × TAE electrophoresis buffer: 96.8 g Tris base + 22.87 ml glacial acetic acid + 0.75 g EDTA. Add distilled water to 1 liter. Mix well. Store at room temperature.
6 liters of 1 × TAE: Prepare 250 ml of 1 × TAE to fill each electrophoresis apparatus × 12 minigels + 1 liter/large apparatus (**estimate**) x 2 large gels. In addition you will need 600 ml of 1 × TAE to prepare enough agarose for 12 minigels (30 ml each) and 2 large gels (**estimate** 100 ml each).
Loading solution: 36 microcentrifuge tubes, each with 10 μl of 0.25% bromophenol blue + 30% glycerol dissolved in sterile distilled water. Store at room temperature.
5 ml ethidium bromide (10 mg/ml): Dissolve 50 mg of ethidium bromide in 5 ml of sterile distilled water. Store in the refrigerator and protect from light. Do not aliquot. Caution: Ethidium bromide is a carcinogen.
100 μl Fermentas GeneRuler 1 kb DNA ladder molecular weight standard: Load 5 μl/lane (0.5 μg/lane). Ladder is supplied ready-to-use at 0.1 μg/μl.

Items per pair of students (plan for 36 pairs):

12 sterile 1.5-ml microcentrifuge tubes.
20 sterile yellow tips.
5 sterile blue tips.
4 pairs of disposable gloves.
1 can sterile 1-ml pipettes (at least 5 pipettes/pair).
12 ml sterile LB broth.
8 LB agar plates containing 50 μg/ml ampicillin.
4 LB agar plates (no antibiotics).
1 alcohol bottle (for sterilizing glass spreading rods).
2 glass rods for spreading plates.
175 μl frozen competent *E. coli* DH5α cells (Stratagene or GIBCO/BRL).

BEGINNING OF WEEK 2

Strain curator needs the following (for experiment 2):

1 LB kanamycin (25 μg/ml) + ampicillin (50 μg/ml) plate (fresh).
10 ml LB kanamycin/ampicillin broth (fresh).
150 ml LB kanamycin/ampicillin broth (fresh).
1 ml 0.1 M IPTG (isopropylthiogalactoside; 28.2 mg/ml in water). Filter, sterilize, and store frozen.

ORDERING INFORMATION

United States Biochemical Company (800-321-9322)

Item	Catalogue No.	Unit	Price
Lysozyme	18645	1 gm	$10.00
SDS	21651	25 gm	$10.00
Phenol	20083	100 ml	$20.00
Sodium acetate	21608	500 gm	$20.50
*Pst*I (order 2)	70595	10,000 units	$44.50
Agarose-LE	32802	25 gm	$30.50
Ethidium bromide	32813	1 gm	$5.50
Bromophenol blue	12370	59 gm	$5.40

Sigma Chemical Company (800-325-3010)

Item	Catalogue No.	Unit	Price
Potassium acetate	P3542	100 gm	$8.60
Isoamyl alcohol	I1885	100 ml	$8.00

VWR (800-225-0440)

Item	Catalogue No.	Unit	Price
Chloroform	IB05040	500 ml	$17.00

Gibco/BRL (800-828-6686)

Item	Catalogue No.	Unit	Price
E. coli DH5α, competent, subcloning grade (or Stratagene)	18265-017	2 × 2 ml	$49.00 × 2

Fermentas (800-340-9026)

Item	Catalogue No.	Unit	Price
GeneRuler, 1 kb DNA ladder, ready-to-use	SM0313	250 μg	$140.00

Week 3

Experiment 2: Affinity Purification of a Histidine-Tagged Protein

CLASS 5: LYSE CELLS AND BIND HIS-TAGGED PROTEIN TO Ni RESIN

Items per pair of students (plan for 36 pairs):

10 μl PMSF solution (200 mM in isopropanol, stored in freezer).
20 μl lysozyme (30 mg/ml) in water (made fresh; keep on ice).
2 ml B-PER buffered detergent reagent, Pierce Chemical Co.
5 ml 1:10 diluted B-PER buffered detergent reagent (Pierce).
250 μl nickel-chelated agarose (50% suspension).
500 μl wash buffer (Pierce).
250 μl elution buffer (Pierce).
1 box yellow tips for P-20 and P-200.
1 box blue tips for P-1000.
50 sterile microcentrifuge tubes.
1 ml sterile distilled water.

CLASS 6: SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Items per group of three students (plan for 25 groups of three):

50 μl 3 × sample buffer (see recipe that follows).
25 sterile microcentrifuge tubes.
1 box yellow tips for P-20.
16 15% polyacrylamide precast mini-gel (Bio-Rad) (need 1 gel/2 groups of 3 = 12 gels/72 students). We have 8 gel boxes; each box holds 2 gels. We use 16 gels.

Each group of students has eight samples: eluates 1 and 2, nonadsorbed proteins, and total cellular protein from both induced (IPTG-treated) and uninduced cultures. Each gel must accommodate two groups of students. Because the Bio-Rad gels (#161-0938) have 15 wells, each pair must omit one sample (nonadsorbed proteins from uninduced cells). Thus, each pair loads seven samples on shared gels; molecular weight standards are loaded in the 15th lane.

400 ml 1 × Tris/glycine/SDS electrophoresis buffer (per four groups; plan for 16 gels in eight gel rigs = 400 ml × 8 = 3200 ml).
5 μl protein MW standards, low range (Gibco/BRL).
25 ml Gel Code Blue G-250 stain (Bio-Rad).
1 25-ml glass pipette.

Recipe: 3 × sample buffer:

Reagent	Volume (μl)
1 M Tris-HCl, pH 6.8	100
Glycerol	400
10% SDS	320
1% bromophenol blue	20
β-mercaptoethanol	80

ORDERING INFORMATION

Pierce

Item	Catalogue No.	Unit	Price
Nickel agarose kit; includes B-PER detergent, wash buffer, elution buffer, nickel agarose	78300VE	1	$295.00
Gel Code Blue stain reagent	24590GV	500 ml	$29.00
PMSF; phenylmethylsulfonyl fluoride	36978	5 g	$62.00
IPTG	34060	1 g	$42.00

Bio-Rad

Item	Catalogue No.	Unit	Price
Precast 15% polyacrylamide gels, 15-well; 10/pkg	161-0938	2 pkg	$82.00
10 × Tris/glycine/SDS electrophoresis buffer	161-0732	1 l	$20.00

Gibco/BRL

Item	Catalogue No.	Unit	Price
Protein MW standards, Mark 12	LC5677	500 μl	$69.00

Rainin

Item	Catalogue No.	Unit	Price
Microcapillary pipette tips, round gel-well style	GT-250-6	2 × 200/rack	$29.00

Week 4

Experiment 3: PCR and DNA Sequence Analysis of Bacterial RNA Genes, and Experiment 4: Extract Metagenomic DNA from Environmental Samples

CLASS 7: ISOLATE BACTERIA FROM ENVIRONMENT (UNIT 3) AND EXTRACT DNA FROM ENVIRONMENTAL SAMPLES (UNIT 4)

Note: Students work individually not in pairs for experiments 3 and 4. Plan for 72 students.

Class 7 materials to isolate bacteria:

3 LB agar plates.
1 sterile centrifuge tube containing 1 ml of sterile distilled water.
1 Mo Bio Bead Solution tube from Ultra Clean Soil DNA Isolation kit.

Items from TAs, per student (plan for 72 students):

1 loop for streaking agar plates.
1 Bunsen burner and striker.
1 set of Pipetmen (P20, P200, P1000).

For entire class:

6 boxes to hold inoculated agar plates.
3 vortex mixers equipped with Mo Bio Vortex Adapter tube holders.
6 variable-speed fixed-angle microcentrifuges set for 10,000 × g (**not** 10,000 rpm).
3 (or more) plate streaking stations in each warm room; provide sterile toothpicks (clearly labeled), empty containers for used toothpicks (clearly labeled), clean work surface, and two LB agar plates/student.

Items from media room, per student (plan for 72 students):

3 LB agar plates.
1 sterile centrifuge tube containing 1 ml sterile distilled water.
1 sterile BD BBL Culture Swab EZ.
20 sterile toothpicks; **1 bottle/student.**
1 box aerosol-resistant (ART) tips for P20.
1 box aerosol-resistant (ART) tips for P200.
1 box aerosol-resistant (ART) tips for P1000.
Gloves.
1 Mo Bio Bead Solution tube from Ultra Clean Soil DNA Isolation kit.
1 Mo Bio Bead spin filter cartridge from Ultra Clean Soil DNA Isolation kit.
60 μl solution S1 (from Mo Bio Soil kit).
200 μl solution IRS (from Mo Bio Soil kit).
250 μl solution S2 (from Mo Bio Soil kit).
1.3 ml solution S3 (from Mo Bio Soil kit); supply this in a tube from the Mo Bio kit.
600 μl solution S4 (from Mo Bio Soil kit) (order extra, in addition to the amount in the kit).
50 μl solution S5 (from Mo Bio Soil kit).
3 clean 2-ml centrifuge tubes (from Mo Bio Soil kit) (one tube with 1.3 ml solution S3).

TA's role: Verify that all items are present and in place when class 6 begins.

Label the boxes 25°C, 30°C, and 37°C. Transfer boxes to warm rooms after class. The **next day (class 7 + 1 day)**, students examine their plates, and they pick and streak two isolated colonies onto separate LB plates. **They streak their plates in the warm rooms, not the teaching lab.** They place the streaked plates in the appropriate incubator. The following day, the TAs examine the streaked plates for growth and move plates with adequate growth to the cold room. TAs bring the streaked plates to class 7.

Class 7 + 2 days, students examine plates in warm rooms and streak for single colonies. Make certain that warm rooms are unlocked and sterile toothpicks (clearly labeled), LB agar plates (two per student), containers (clearly labeled) for used toothpicks, and clean work surfaces are available. Students do **not** have access to the teaching lab for streaking plates. They must streak plates in the cold room. Make certain they have the materials required.

CLASS 8: GRAM STAIN/MICROSCOPY AND INOCULATE BROTH

Items per student (plan for 72 students):

1 rack for culture tubes.
1 wire inoculating loop.

1 microscope.
2 glass microscope slides and cover slips.
1 Bunsen burner and striker.
1 250-ml beaker.
1 tripod with screen (to hold beaker over burner).
1 wash bottle filled with distilled water.
1 reagents for Gram stain (crystal violet, Gram's iodine, 95% alcohol, safranin).
1 reagents for spore stain (5% malachite green, safranin).

Items from media room, per student (plan for 72 students):

2 sterile culture tubes containing 3 ml LB broth (each).
2 sterile inoculating sticks.

Equipment required for class 8:

3 racks for incubating culture tubes at 25°C, 30°C, 37°C.
3 shaker/incubators; one each at 25°C, 30°C, and 37°C.

TA's role: Verify that all items are present and that incubators are at proper temperatures. Bring streaked plates to class 8.

Class 8 + 1 day, TAs remove culture tubes from the incubators (provided they show adequate growth) and store them in the refrigerator until class 9. TAs bring cultures to class 9.

Week 5

CLASS 9: ISOLATE GENOMIC DNA AND FREEZE CULTURES

Items from TAs, per student (plan for 72 students):

2 3-ml LB broth cultures of unknown bacteria (isolated from the environment during class 7 and inoculated from streaks during class 8).
Rack for microcentrifuge tubes.
Floats for microcentrifuge tubes.
1 box yellow pipette tips.
1 box blue pipette tips.
1 P20 pipetman.
1 P200 pipetman.
1 P1000 pipetman.
Ice bucket.
Safety goggles and lab coat; students should supply their own coats.

Items from media room, per student (plan for 72 students):

1 sterile 1-dram screw-cap freezer vial containing 0.2 ml DMSO. Distribute dimethylsulfoxide to vials, replace caps, and autoclave.
1 ml 25 mM Tris + 10 mM EDTA, pH 8 (sterile). Mix 3 g Tris base + 3.36 g EDTA; add 950 ml distilled water, adjust to pH 8 with HCl, and bring to 1 liter with distilled water. Autoclave. Store at room temperature.
40 μl lysozyme (30 mg/ml). Dissolve in 25 mM Tris pH 8 **immediately before use**. Keep on ice.

20 μl proteinase K (50 mg/ml). Dissolve in sterile distilled water **immediately before use**. Keep on ice.
40 μl 25% SDS. Dissolve 25 g SDS in sterile distilled water. Store at room temperature.
120 μl 5 M NaCl. Dissolve 29.2 g NaCl in distilled water and bring to 100 ml. Autoclave. Store at room temperature.
1 ml phenol:chloroform:isoamyl alcohol (25:24:1). Equilibrate with Tris, pH 8. Store at 4°C.
1 ml chloroform:isoamyl alcohol (24:1).
4 ml 95% ethanol. **Place on ice before class**.
1 ml 70% ethanol. **Place on ice before class**.
1 ml DNA buffer = 10 mM Tris + 0.1 mM EDTA, pH 8. Mix 1.21 g Tris base with 1 ml of 100 mM EDTA, pH 8. Bring volume to 950 ml, adjust pH to 8 with HCl, then bring to 1 liter with distilled water. Autoclave and store at room temperature. To make 100 mM EDTA, dissolve 3.36 g EDTA in 100 ml distilled water, adjust pH to 8 with NaOH.
4 sterile 1.5 ml microcentrifuge tubes.

Equipment required for class 9:

8 (or more) microcentrifuges and 12 (or more) vortex mixers.
Water baths at 37°C, 50°C, and 68°C.
Floating racks for 1.5-ml microcentrifuge tubes.
Freezer box to hold 48 1-dram vials.
Speed Vac centrifuge/concentrator.
Portable fume hoods; container for organic waste.
Autoclave bags for plates, microcentrifuge tubes, and tips; autoclave container for supernatant solutions; autoclave rack for culture tubes.

CLASS 10: PCR

Items per student (students work individually, plan for 72 students):

190 μl PCR reaction mixture (TA will prepare just before class. Store on ice.)	
Mixture (190 μl/student) contains:	**For 50 students:**
20 μl 10 × PCR buffer (Perkin Elmer)	1000 μl
20 μl 2 mM (each) dNTP mix	1000 μl
12 μl 25 mM $MgCl_2$	600 μl
20 μl 50% acetamide	1000 μl
4 μl of 10 μM 27 F primer	200 μl
4 μl of 10 μM 519R primer	200 μl
1 μl *Taq* DNA polymerase (5 units/μl)	50 μl
109 μl distilled water	5450 μl
190 μl total volume	9500 μl

The 10 × PCR buffer, 2 mM dNTP mix, 25 mM $MgCl_2$, *Taq* DNA polymerase, distilled water, and 0.2 ml thin-wall reaction tubes are supplied by Perkin Elmer and are contained in the GeneAmp kit with AmpliTaq (N801-0055), the AmpliTaq 250 + Buffer II kit (N808-0161), the GeneAmp PCR core kit (N808-0009), the GeneAmp dNTPs kit (N808-0007), and the MicroAmp (0.2 ml) reaction tube with cap (N801-0540). One each of these products is sufficient for 40 students. Order acetamide separately (Sigma).

Order PCR primers from Invitrogen:

Primer 27F: 5′-AGA GTT TGA TCM TGG CTC AG-3′
Primer 519R: 5′-GWA TTA CCG CGG CKG CTG-3′

10 μl sterile distilled water (DNA free).
2 yellow BARRIER (aerosol-resistant) tips for P20.
2 yellow BARRIER tips for P200.
2 0.2-ml MicroAmp PCR reaction tubes, with cap (Perkin Elmer product #N801-0540).
6 1.5-ml microcentrifuge tubes (sterile).
16 blue tips for pipetman (sterile).
16 yellow tips for pipetman (sterile).
1 P20 pipetman.
1 P200 pipetman.
1 P1000 pipetman.
2 pairs of disposable gloves.
1 ice bucket + ice.
1 rack for microcentrifuge tubes.
1 rack for PCR tubes.
1 plastic beaker for discarding used tips.
1 forceps.
1 thermal cycler with hot top.

TA's role: Prepare PCR mix just before class 10. Check the concentrations of each primer before adding to the mix. Remove PCR reactions from thermal cycler and store them at −20°C until class 11. Help set up the thermal cycler.

Week 6

CLASS 11: PURIFY PCR PRODUCT

Items per student (students work individually, plan for 72 students):

1 QiaQuick PCR Purification cartridge (Qiagen).
0.5 ml QiaQuick buffer PBI.
0.75 ml QiaQuick buffer PE (contains 70% ethanol).
150 μl sterile distilled water.
5 1.5-ml microcentrifuge tubes (sterile).
Rack for microcentrifuge tubes.
Rack for PCR tubes.
10 yellow tips (sterile).

4 blue tips (sterile).
P20, P200, and P1000 pipettors.
1 pair disposable gloves.

Equipment required for class 11:

8 (or more) microcentrifuges.

TA's role: Provide racks to hold tubes containing purified and unpurified PCR products and no-template control reactions. Collect these samples after class and store frozen until class 12.

CLASS 12: AGAROSE GEL ELECTROPHORESIS AND TEMPLATE PREPARATION

Items per student (students work individually, plan for 72 students):

12 pmol primer oligonucleotide 27F in 1 μl of sterile distilled water.
20 μl sterile distilled water.
6 μl agarose gel loading solution (50% glycerol + 0.05% bromophenol blue).
4 1.5-ml microcentrifuge tubes (sterile).
Rack for microcentrifuge tubes.
10 yellow tips (sterile).
P20 pipettor.
1 pair disposable gloves.

Materials for entire class:

10 μg Gibco/BRL low MW DNA mass ladder (500 ng/gel × 14 gels).

We have 15- and 16-well combs for the 12 mini-gels. Use 20-well combs for the two large gels. We need 3 lanes/student × 4 students/mini-gel (15 wells) + 3 markers, or 3 lanes/student × 5 students/mini-gel (16 wells) + 1 marker = 16 wells/gel, or 3 lanes/student × 6 students + 2 markers for large gels. Use all available gel boxes. Each student will have 3 samples: PCR product before and after purification and no-template control reaction.

10.5 g **NuSeive 3:1 agarose** (0.5 g/25 ml of 2% gel × 13 mini-gels + 2 g/100 ml **estimate** for large gels).
10 liters 1 × TAE electrophoresis buffer: 40 mM Tris-acetate + 2 mM EDTA (25 ml/gel + 400 ml/tank × 12) (1 liter of **50 × TAE**: 242 g Tris base + 57.1 ml glacial acetic acid + 100 ml of 0.5 M EDTA, pH 8.0).
20 μl of 10 mg/ml ethidium bromide/gel (1.5 μl/25 ml gel × 12 mini-gels + 6 μl/100 ml gel × 2 large gels).

The class (72 students) pours 12 2.0% 3:1 **NuSeive agarose** mini-gels (25 ml each) and 2 large gels (**estimate 100 ml/gel**). Each mini-gel contains 15 or 16 sample wells. Each large gel contains 20 wells.

Equipment required for class 12:

1 hot plate or microwave to melt agarose.
13 125-ml flasks for agarose.
12 agarose mini-gel boxes + casting trays and combs; 2 large gel boxes + casting trays and combs.
8 dual-outlet power supplies and 14 sets of leads.

1 UV transilluminator, camera, film, face shield.
Speed Vac centrifuge/concentrator.

TA's role: Place frozen PCR reactions at instructors' bench at front of lab. Set out gel casting trays with combs, electrophoresis apparatus, and power supplies. Supervise preparation of purified PCR products for sequencing; ensure that samples contain 25 ng of PCR product and 12 pmol of primer 27F in a total volume of 12 μl. Concentrate dilute samples in the Speed Vac, if necessary. Collect samples and submit to lab for sequencing.

Week 7

CLASS 13: NO LAB EXERCISE; MIDTERM EXAM. FREE DAY FOR TAS

Experiment 4: PCR Amplification of 16S rRNA Genes for Terminal Restriction Fragment Length Polymorphism Analysis.

Week 7

CLASS 14: PCR FOR T-RFLP

Items per student (students work individually, plan for 72 students):

190 μl PCR reaction mixture (TA will prepare just before class. Store on ice.)	
Mixture (190 μl/student) contains:	**For 50 students:**
20 μl 10 × PCR buffer (Perkin Elmer II)	1000 μl
4 μl 10 mM (each) dNTP mix	200 μl
28 μl 25 mM $MgCl_2$	1400 μl
4 μl of 10 μM 8–27F "Fam"-labeled primer	200 μl
4 μl of 10 μM 926–907R primer	200 μl
1 μl *Taq* DNA polymerase (5 units/μl)	50 μl
129 μl distilled water	6450 μl

190 μl total volume	**9500 μl**
Mixture (190 μl/student) contains:	**For 75 students:**
20 μl 10 × PCR buffer (Perkin Elmer II)	1500 μl
4 μl 10 mM (each) dNTP mix	300 μl
28 μl 25 mM $MgCl_2$	2100 μl
4 μl of 10 μM 8–27F "Fam"-labeled primer	300 μl
4 μl of 10 μM 926–907R primer	300 μl
1 μl *Taq* DNA polymerase (5 Units/μl)	75 μl
129 μl distilled water	9675 μl
190 μl total volume	14250 μl

The 10 × PCR buffer, 2 mM dNTP mix, 25 mM $MgCl_2$, *Taq* DNA polymerase, distilled water, and 0.2-ml thin-wall reaction tubes are supplied by Perkin Elmer and are contained in the GeneAmp kit with AmpliTaq (N801-0055), the AmpliTaq 250 + Buffer II kit (N808-0161), the GeneAmp PCR core kit (N808-0009), the GeneAmp dNTPs kit (N808-0007), and the MicroAmp (0.2 ml) reaction tube with cap (N801-0540). Each one of these products is sufficient for 40 students.

Order PCR primers from Invitrogen:

Primer 8–27 F "Fam"-labeled: 5′- AGA GTT TGA TCM TGG CTC AG -3′
(select "Fam" label at 5′ end for the 8–27F primer)
Primer 926–907 R: 5′- CCG TCA ATT CCT TTR AGT TT -3′

10 μl sterile distilled water (DNA free).
2 yellow BARRIER (aerosol-resistant) tips for P20.
2 yellow BARRIER tips for P200.
2 0.2-ml MicroAmp PCR reaction tubes, with cap (Perkin Elmer product # N801-0540).
6 1.5-ml microcentrifuge tubes (sterile).
16 blue tips for Pipetman (sterile).
16 yellow tips for Pipetman (sterile).
1 P20 pipetman.
1 P200 pipetman.
1 P1000 pipetman.
2 pairs of disposable gloves.
1 ice bucket + ice.
1 rack for microcentrifuge tubes.
1 rack for PCR tubes.
1 plastic beaker for discarding used tips.
1 forceps.
1 thermal cycler with hot top.

TA's role: Prepare PCR mix just before **class 14**. Check the concentrations of each primer before adding to the mix. Remove PCR reactions from the thermal cycler and store them at −20°C until **class 15**. Help set up thermal cycler.

Week 8

CLASS 15: PURIFY T-RFLP PCR PRODUCT; TOPOISOMERASE-MEDIATED LIGATION TO PLASMID pCR4-TOPO AND TRANSFORMATION; 1% AGAROSE GEL; "NANODROP" SPECTROPHOTOMETER

Items per student (students work individually) **for amplicon purification**:

1 QiaQuick PCR Purification cartridge (Qiagen).
0.5 ml QiaQuick buffer PBI.
0.75 ml QiaQuick buffer PE (contains 70% ethanol).
150 μl sterile distilled water.
5 1.5-ml microcentrifuge tubes (sterile).
Rack for microcentrifuge tubes.
Rack for PCR tubes.

10 yellow tips (sterile).
4 blue tips (sterile).
P20, P200, and P1000 pipettors.
1 pair disposable gloves.

Items per student for ligation:

1 0.5-ml centrifuge tube containing 1 μl of pCR4-TOPO vector + 1 μl of salt solution (1.2 M NaCl + 60 mM $MgCl_2$; supplied with vector DNA kit). Prepare immediately before class; hold on ice.

Items per student (students work individually) **for transformation**:

1 vial One Shot TOP10 chemically competent *E. coli* cells; thaw on ice immediately before use.
Ligation mixture (from previous step, class 15).
250 μl SOC broth (supplied with cells) warmed to 37°C.
2 LB agar plates containing ampicillin (50 μg/ml), IPTG (40 μM), and X-gal (0.004%). Prepare 2 LB ampicillin/IPTG/X-gal plates per student; they use two plates in class 15 (make 0.1 M stock of IPTG in sterile distilled water; add 10 μl/25 ml plate) (make 2% w/v stock of X-gal in dimethylformamide; add 50 μl/25 ml plate).
1 culture tube (per student) containing 2 ml LB broth + ampicillin (50 μg/ml).
1 glass "hockey stick" spreader (to spread transformed cells).
1 beaker ethanol (95%) to sterilize hockey stick.
1 Bunsen burner.
1 vial of sterile toothpicks; distribute to students to streak white colonies the next day.
1 P20 pipetman.
1 P200 pipetman.
1 P1000 pipetman.
1 box yellow tips, sterile.
1 box blue tips, sterile.
1 ice bucket.
Water bath at 42°C.
Floats to hold tubes.
Shaking incubator set to 37°C; tape to secure tubes to clamps.
Box to transport plates to 37°C incubator.

Items per student for agarose gel:

6 μl agarose gel loading solution (50% glycerol + 0.05% bromophenol blue).
4 1.5-ml microcentrifuge tubes (sterile).
Rack for microcentrifuge tubes.
10 yellow tips (sterile).
P20 pipettor.
1 pair disposable gloves.

Materials for entire class:

10 μg Invitrogen low MW DNA mass ladder (500 ng/gel × 14 gels).

Need 2 lanes/student × 4 students/gel + 2 markers = 10 wells/gel.
Each student has 2 samples: PCR product after purification and no-template control reaction. Use all 14 available apparatus. Mini-gels have 15 or 16 wells; large gels have 20 wells.

5 g agarose (0.25 g/25 ml of 1% gel × 12 gels + 1 g/100 ml × 2 large gels).
10 liters 1 × TAE electrophoresis buffer: 40 mM Tris-acetate + 2 mM EDTA (25 ml/gel + 400 ml/tank × 12 + 1 liter/tank × 2) (1 liter of **50 × TAE**: 242 g Tris base + 57.1 ml glacial acetic acid + 100 ml of 0.5 M EDTA, pH 8.0).
20 μl of 10 mg/ml ethidium bromide/gel (1.5 μl/25 ml gel × 12 mini-gels + 6 μl/100 ml gel × 2 large gels).

The class (72 students) will pour 12 1% agarose mini-gels (25 ml each) and 2 large gels (**estimate** 100 ml/gel). Each mini-gel contains 15–16 sample wells; each large gel contains 20 wells.

Equipment required for class 14, agarose gel:

1 hot plate or microwave to melt agarose.
12 125-ml flasks for agarose + 2 large flasks.
12 mini-gel boxes + casting trays and combs; 2 large gel boxes + casting trays and combs.
8 dual-outlet power supplies and 14 sets of leads.
1 UV transilluminator, camera, face shield.

TA's role: Place frozen PCR reactions at instructors' bench at front of lab; use separate racks for each bench. Set out gel casting trays with combs, electrophoresis apparatus, and power supplies. Supervise preparation of purified PCR products for sequencing; ensure that samples contain 25 ng of PCR product and 12 pmol of primer 27F in a total volume of 12 μl. Concentrate dilute samples in the Speed Vac, if necessary. Collect samples and submit to lab for sequencing.

Equipment required for class 15, amplicon purification:

8 (or more) microcentrifuges.
Storage racks or boxes to hold purified amplicons in freezer storage racks or boxes to hold ligated plasmid DNA in refrigerator.

TA's role: Provide racks to hold tubes containing purified and unpurified PCR products and no-template control reactions. Reserve 1 hour on nanodrop spectrophotometer; read OD of purified PCR products; write concentration (in ng/μl) on each tube and report data to students. Collect purified PCR product and no-template control after class and store frozen until class 16.

Week 8

CLASS 15 + 1 DAY: INOCULATE BROTH WITH TRANSFORMANT (WHITE COLONY)

Items per student (students work individually) for inoculation: Make certain students have access to their plates in the 37°C incubator, sterile toothpicks, and 1 culture tube (per student) containing 2 ml LB broth + ampicillin (50 μg/ml).

Students examine plates in warm rooms and inoculate broth with a white colony. Make certain that warm rooms are unlocked and that sterile toothpicks (clearly labeled), culture tubes with 2 ml LB broth + ampicillin (50 μg/ml) (one per student), containers (clearly labeled) for used toothpicks, and clean work surfaces are available. Make plates containing transformants available in 37°C room. Students do **not** have access to the teaching lab for inoculating tubes. They must streak plates in the cold room. Make certain they have the materials required. Make certain students have access to a shaker at 37°C; put racks in the shaker to hold their tubes. Prepare sufficient shaking incubators set to 37°C and racks to hold 72 tubes.

Class 15 + 2 days, put broth cultures in cold room. Hold until class 16; bring to class 16.

CLASS 16: RESTRICT AMPLICON; INOCULATE BROTH WITH COLONY

Items per student for restriction (class 16):

1 0.5-ml tube containing 1 μl (10 units) of *Rsa*I + 2 μl of 10x buffer + 1 μl of BSA (1 mg/ml).
1 0.5-ml tube containing 1 μl (10 units) of *Msp*I + 2 μl of 10x buffer + 1 μl of BSA (1 mg/ml).

Prepare tubes with restriction enzymes just prior to use and hold on ice.

1 0.5-ml tube containing 50 μl sterile distilled water.
6 empty 0.5-ml tubes.
1 P20 pipetman.
1 box yellow tips.
Water bath set at 37°C.
Floats to hold tubes.

CLASS 16: PLASMID DNA PURIFICATION

Items per student (students work individually) **for plasmid isolation**:

Gloves.
P20 pipetman.
P200 pipetman.
P1000 pipetman.
1 box yellow tips.
1 box blue tips.
1.7-ml centrifuge tubes.
Microcentrifuges.
Waste containers for supernatant solutions (autoclave after class).
1 Qiaprep spin column (from Qiagen Spin Miniprep kit; Cat. No. 27106).
250 μl Qiagen buffer P1 + RNaseA.

250 μl Qiagen buffer P2.
350 μl Qiagen buffer N3.
500 μl Qiagen buffer PB.
750 μl Qiagen buffer PE.
50 μl Qiagen buffer EB (10 mM Tris, pH 8).

Retrieve students' broth cultures from cold room and distribute by seat number.

The TAs take the purified plasmid DNAs to the services laboratory and measure the concentration (OD_{260} = optical density at 260 nm) of each sample using the "nanodrop" spectrophotometer. They record the concentration (in ng/μl).

TEACHING ASSISTANT PREPARATION MANUAL

General duties:

Verify that the media kitchen and strain curator have prepared the materials needed.
Ensure that equipment, strains, and materials are present by the date indicated.
Set up the laboratory at least 1 hour before class.
Anticipate and minimize bottlenecks—
Distribute reagents so that only two students (or teams) share a stock.
Reagents are in color-coded (unlabeled) tubes; write the color code on both boards.
Use every gel apparatus available.
Place equipment so that students can load all gels simultaneously.
Provide freezer boxes to each bench to store samples; use a separate box for each experiment.
Have students put samples for DNA sequence or T-RFLP analysis **in order** in freezer boxes. We do not have time to sort these samples.
Encourage students to stop talking when the instructor is ready to begin class.
Check flowcharts during prelab lecture, but remain silent; do not talk while the instructor is talking.
Take attendance for your lab benches.
Assign each student (or team) specific lanes on gels; provide gel-loading sheets.
Stay in the laboratory for the entire class period.
Put away equipment, samples, and reagents at the end of class. Make certain the laboratory is clean, and autoclave discarded cultures.
Grade lab reports, and check in-class writing assignments.
Students submit lab reports via e-mail. Use "track changes" and "insert comments" as you grade their reports. Return the graded report and Excel grade sheet to the student via e-mail. Send me an Excel sheet with the grades.

Four Days before Class 2

Confirm that strains, reagents, and equipment are ready for Class 2.

Experiment 1: Plasmid Purification and Restriction Mapping

Week 1

CLASS 1: MEET STUDENTS IN LAB AT START OF CLASS 1

CLASS 2: PLASMID PURIFICATION AND RESTRICTION

Items per pair of students (plan for 36 pairs):

1 microcentrifuge with tube holders.
1 P20 pipetman.
1 P200 pipetman.
1 P1000 pipetman.
2 pairs of disposable gloves.
2 goggles.

1 ice bucket with ice.
1 rack to hold microcentrifuge tubes.
2 plastic beakers for discarded tips.
1 forceps for handling sterile microcentrifuge tubes.
Safety goggles and lab coat; students should bring their own coats.

Items from media room, per pair of students:

0.15 ml 5 M potassium acetate: Dissolve 49.1 g potassium acetate in 100 ml of sterile distilled water. Take 60 ml of this solution and add 11.5 ml of glacial acetic acid and 28.5 ml of sterile distilled water. The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Store at room temperature.
0.25 ml phenol* **equilibrated in 1 M Tris, pH 8.0**: Store at 4°C. Note: Phenol must be **colorless**, not pink.
0.25 ml isoamyl alcohol:chloroform:* Add 1 ml isoamyl alcohol to 24 ml chloroform.
0.01 ml 3.5 M sodium acetate. If you have anhydrous sodium acetate, add 28.7 g to 50 ml sterile distilled water, then adjust pH to 5.2 with glacial acetic acid and bring to a final volume of 100 ml with sterile distilled water. If you have sodium acetate trihydrate, add 47.6 g to 50 ml sterile distilled water, then adjust the pH to 5.2 with glacial acetic acid and bring to a final volume of 100 ml with sterile distilled water. Store at room temperature.
1 ml TE, pH 8: 10 mM Tris + 1 mM EDTA, pH 8: Mix 0.121 g Tris base + 0.0372 g disodium EDTA; add 50 ml sterile distilled water, then bring pH to 8.0 with HCl. Bring final volume to 100 ml with sterile distilled water. Autoclave. Store at room temperature.
0.003 ml 10X *Pst*I reaction buffer: Obtain from enzyme supplier (Fermentas or Gibco). Store frozen.
0.002 ml *Pst*I enzyme: (10−20 units/μl). Obtain from Fermentas or Gibco freezer. Store at −20°C in a nondefrosting freezer.
0.5 ml 95% ethanol: (chill on ice on **class day 2**).
0.5 ml sterile distilled water:
0.06 ml lysozyme solution 80: mg lysozyme dissolved in 1 ml sterile distilled water. **Prepare one hour before class 2**; store on ice.
0.2 ml SDS-NaOH solution: 8.8 ml sterile distilled water + 0.2 ml 10 N NaOH + 1 ml of **10% SDS solution**: **Mix one hour before class 2** and store at room temperature.
10 μl RNaseA (5 mg/ml). Heat stock to 65°C for 15 minutes, then store frozen.
18 sterile 1.5-ml microcentrifuge tubes.
36 sterile yellow tips for P20 and P200 pipetman.
21 sterile blue tips for P1000 pipetman.

Items from strain curator, per pair of students:

1 tube (per pair of students) containing 0.15 ml of the resuspended pellets of NM522 (pKN800) in labeled 1.5-ml microcentrifuge tubes (stored in the freezer).

*Phenol, isoamyl alcohol, and chloroform may be combined (25:1:24) or ordered as a mixture from Gibco/BRL (catalog number 15593-031).

Equipment required for class 2:

1 37°C incubator.
1 37°C water bath.
1 70°C water bath or heating block.
6 floating racks for microcentrifuge tubes in water baths.
4 glass bottle for phenol waste.
12 vortexers (or more).
1 Speed Vac concentrator.
Portable fume hoods.
6 freezer boxes to store plasmid DNA (1 box/bench of 12 students).

Week 2

CLASS 3: AGAROSE GEL ELECTROPHORESIS AND TRANSFORMATION

Verify that all items are present by the start of class. Retrieve freezer boxes containing plasmid DNA and distribute to correct benches.

Assign each student (or team) specific lanes on gels; provide gel-loading sheets.

The class (72 students = 36 pairs) uses 12 0.8% agarose mini-gels (30 ml each) and 2 large gels (**estimate** 100 ml/gel). Each mini-gel contains 15–16 sample wells. Each large gel contains 20 wells. (2 lanes/pair × 3 pairs/gel + 1 marker = 7 lanes/gel; 3 pairs/gel × 12 gels = 36 pairs).

Equipment required for class 3:

Place power supplies (7), agarose gel apparatus (14 = 12 mini-gels + 2 large gels) with combs and casting trays, UV transilluminator, microcentrifuges (all available) with racks, and 14 glass Pyrex dishes in the classroom.

Materials for entire class from media room (72 students = 36 pairs):

600 ml of 0.8% agarose: Dissolve 4.8 g of agarose in 600 ml of 1 × TAE electrophoresis buffer. Use a microwave or hotplate to melt the agarose before class.
1 liter of 20 × TAE electrophoresis buffer: 96.8 g Tris base + 22.87 ml glacial acetic acid + 0.75 g EDTA. Add distilled water to 1 liter. Mix well. Store at room temperature.
6 liters of 1 × TAE: Prepare 250 ml of 1 × TAE to fill each electrophoresis apparatus × 12 mini-gels + 1 liter/large apparatus (**estimate**) × 2 large gels. In addition, you need 600 ml of 1 × TAE to prepare enough agarose for 12 mini-gels (30 ml each) and 2 large gels (**estimate** 100 ml each).
Loading solution: 36 microcentrifuge tubes, each with 10 μl of 0.25% bromophenol blue + 30% glycerol dissolved in sterile distilled water. Store at room temperature.
5 ml ethidium bromide (10 mg/ml): Dissolve 50 mg of ethidium bromide in 5 ml of sterile distilled water. Store in the refrigerator and protect from light. Do not aliquot. Caution: ethidium bromide is a carcinogen.
100 μl Fermentas GeneRuler 1 kb DNA ladder molecular weight standard: Load 5 μl/lane (0.5 μg/lane). Ladder is supplied ready-to-use at 0.1 μg/μl.

Items from TAs, per pair of students (plan for 36 pairs):

1 ice bucket filled with ice.
1 P20 pipetman.
1 P200 pipetman.
1 P1000 pipetman.
2 safety glasses or goggles.

Items from media room, per pair of students:

12 sterile 1.5-ml microcentrifuge tubes.
20 sterile yellow tips.
5 sterile blue tips.
4 pairs of disposable gloves.
1 can sterile 1-ml pipettes (at least 5 pipettes/pair).
12 ml sterile LB broth.
8 LB agar plates containing 50 μg/ml ampicillin.
4 LB agar plates (no antibiotics).
1 alcohol bottle (for sterilizing glass spreading rods).
2 glass rods for spreading plates.
175 μl frozen competent *E. coli* DH5αcells.

CLASS 3 + 1 DAY

Make certain that the 30°C room is open so that students can view their plates.

CLASS 4: NO LABORATORY EXERCISE. FREE DAY FOR TAS

Experiment 2: Affinity Purification of a Histidine-Tagged Protein.

Week 3

CLASS 5: LYSE CELLS AND BIND HIS-TAGGED PROTEIN TO NI RESIN

Items from TAs, per group of three students (plan for 25 groups of 3):

1 micropipettor, 2–20 μl (P-20).
1 micropipettor, 20–200 μl (P-200).
1 micropipettor, 200–1000 μl (P-1000).
1 box yellow tips for P-20 and P-200.
1 box blue tips for P-1000.
50 sterile microcentrifuge tubes.
1 microcentrifuge tube rack.
1 Styrofoam container of ice.

Items from media room, per group of three students:

10 μl PMSF solution (200 mM in isopropanol, stored in freezer).
2 ml B-PER buffered detergent reagent, Pierce Chemical Co.
5 ml 1:10 diluted B-PER buffered detergent reagent (Pierce).
20 μl lysozyme (30 mg/ml) in water (made fresh; keep on ice).

250 μl nickel-chelated agarose (50% suspension).
500 μl wash buffer (Pierce).
250 μl elution buffer (Pierce).
1 ml sterile distilled water.

Items from strain curator, per group of three students:

1.5 ml IPTG-induced culture of *E. coli* (pQE-FnbA) in 1.5-ml microcentrifuge tube.
1.5 ml uninduced culture of *E. coli* (pQE-FnbA) in 1.5-ml microcentrifuge tube.

Equipment needed for class 5:

8 (or more) microcentrifuges.
12 (or more) vortexers.
6 freezer boxes to store protein samples (1 box/bench of 12 students).

CLASS 6: SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Retrieve freezer boxes containing protein samples and distribute to correct benches.
Items from TAs, per group of three students (plan for 25 groups of 3):

25 sterile microcentrifuge tubes.
1 micropipettor, 2−20 μl (P-20).
1 box yellow tips for P-20.
7 microcapillary tips for P-20.
1 small spatula.
1 razor blade.
1 25-ml glass pipette.
1 250 or 500-ml graduated cylinder.
1 gel staining tray.
1 polyacrylamide gel electrophoresis apparatus (need 1 Bio-Rad mini-protean apparatus/4 groups of 3 = 6 apparatus/72 students). Each apparatus holds two gels. **We have eight rigs; use them all.**
1 microcentrifuge tube rack.
1 styrofoam container of ice.

Items from media room, per group of three students:

50 μl 3 × sample buffer.
1 15% polyacrylamide pre-cast mini gel (Bio-Rad) (need 1 gel/2 groups of 3 = 12 gels/72 students). **We have 8 rigs; use 16 gels.**

Each group of three students has eight samples: eluates 1 and 2, nonadsorbed proteins, and total cellular protein from both induced (IPTG-treated) and uninduced cultures. Each gel must accommodate two groups of three students. Because the Bio-Rad gels (#161-0938) have 15 wells, each pair (on shared gels) must omit one sample (nonadsorbed proteins from uninduced cells). Each group loads seven samples; molecular weight standards are loaded in the 15th lane.

5 μl protein MW standards, Invitrogen Mark 12 (catalogue no. LC5677).
400 ml 1 × Tris/glycine/SDS electrophoresis buffer (per four groups of three).
25 ml Gel Code Blue G-250 stain (Bio-Rad).

Equipment needed for class 6:

8 (or more) microcentrifuges.
4 double-outlet power supplies for SDS-PAGE.
1 95°C water bath.
1 light table (white light), camera, film.

TA's role: Verify that all materials are present. Set out items for lab, including the protein samples from class 5. Adjust the water bath to 95°C, and see that it is at the correct temperature by class time. Monitor use of the light source and camera. Assign each student (or team) specific lanes on gels; provide gel-loading sheets.

Experiment 3: PCR and DNA Sequence Analysis of Bacterial rRNA Genes, and Experiment 4: Extract Metagenomic DNA from Environmental Samples

Note: Students work individually, **not** in pairs for experiments 3 and 4.

Week 4

CLASS 7: ISOLATE BACTERIA FROM ENVIRONMENT (UNIT 3) AND EXTRACT DNA FROM ENVIRONMENTAL SAMPLES (UNIT 4)

Items from TAs, per student (plan for 72 students):

1 loop for streaking agar plates
1 Bunsen burner and striker
1 set of Pipetmen (P20, P200, P1000)

For entire class:

6 boxes to hold inoculated agar plates.
Vortex mixers equipped with Mo Bio Vortex Adapter tube holders.
Variable-speed fixed-angle microcentrifuges set for 10,000 × g (**not** 10,000 rpm).
3 (or more) plate streaking stations in each warm room; provide sterile toothpicks (clearly labeled), empty containers for used toothpicks (clearly labeled), clean work surface, and two LB agar plates/student.

Items from media room, per student (plan for 72 students):

3 LB agar plates.
1 sterile test tube containing 1 ml LB broth.
1 sterile centrifuge tube containing 1 ml sterile distilled water.
2 sterile BD BBL Culture Swabs EZ.
20 sterile toothpicks; **1 bottle/student**.
1 box aerosol-resistant (ART) tips for P20.
1 box aerosol-resistant (ART) tips for P200.

1 box aerosol-resistant (ART) tips for P1000.
Gloves.
1 Mo Bio Bead Solution tube from Ultra Clean Soil DNA Isolation kit.
1 Mo Bio Bead spin filter cartridge from Ultra Clean Soil DNA Isolation kit.
60 μl solution S1 (from Mo Bio Soil kit).
200 μl solution IRS (from Mo Bio Soil kit).
250 μl solution S2 (from Mo Bio Soil kit).
1.3 ml solution S3 (from Mo Bio Soil kit); supply this in a tube from the Mo Bio kit.
600 μl solution S4 (from Mo Bio Soil kit) (order extra, in addition to the amount in the kit).
50 μl solution S5 (from Mo Bio Soil kit).
3 clean 2-ml centrifuge tubes (from Mo Bio Soil kit) (one tube with 1.3 ml solution S3).

TA's role: Verify that all items are present and in place when class 7 begins.
Label the boxes 25°C, 30°C, and 37°C. Transfer boxes to warm rooms after class.

The next day **(Class 7 + 1 day)**, students examine their plates, and they pick and streak two isolated colonies onto separate LB plates. They streak their plates in the warm rooms, not the teaching lab. Make certain that warm rooms are unlocked and that sterile toothpicks (clearly labeled), LB agar plates (two per student), containers (clearly labeled) for used toothpicks, and clean work surfaces are available. Students do **not** have access to the teaching lab for streaking plates. They place the streaked plates in the appropriate incubator. The following day the **TAs** examine the streaked plates for growth and move plates with adequate growth to the cold room. TAs bring the streaked plates to class 8.

Week 4

CLASS 8: GRAM STAIN/MICROSCOPY AND INOCULATE BROTH

Items per student (plan for 72 students):

1 rack for culture tubes.
1 wire inoculating loop.
1 microscope.
2 glass microscope slides and cover slips.
1 Bunsen burner and striker.
1 250-ml beaker.
1 tripod with screen (to hold beaker over burner).
1 wash bottle filled with distilled water.
1 reagents for Gram stain (crystal violet, Gram's iodine, 95% alcohol, safranin).
1 reagents for spore stain (5% malachite green, safranin).

Items from media room, per student (plan for 72 students):

2 sterile culture tubes containing 3 ml LB broth (each).
2 sterile inoculating sticks.

Equipment required for class 8:

3 racks for incubating culture tubes at 25°C, 30°C, 37°C.
3 shaker/incubators; one each at 25°C, 30°C, and 37°C.

TA's role: Verify that all items are present and that incubators are at proper temperatures.

Bring streaked plates to class 8.

CLASS 8 + 1 DAY

On the day after class 8, TAs remove culture tubes from the incubators (provided they show adequate growth) and store them in the refrigerator until class 9. TAs bring cultures to **class 9.**

Week 5

CLASS 9: ISOLATE GENOMIC DNA AND FREEZE CULTURES

Items from TAs, per student (plan for 72 students):

2 3-ml LB broth cultures of unknown bacteria (isolated from the environment during class 7 and inoculated from streaks during class 8).
Rack for microcentrifuge tubes.
Floats for microcentrifuge tubes.
1 box yellow pipet tips.
1 box blue pipet tips.
1 P20 pipetman.
1 P200 pipetman.
1 P1000 pipetman.
Ice bucket.
Lab coat; student should supply their own coats.
Safety goggles.

Items from media room, per student (plan for 72 students):

1 sterile 1-dram screw-cap freezer vial containing 0.2 ml DMSO. Distribute dimethylsulfoxide to vials, replace caps, and autoclave.
1 ml 25 mM Tris + 10 mM EDTA, pH 8 (sterile). Mix 3 gm Tris base + 3.36 gm EDTA; add 950 ml distilled water, adjust to pH 8 with HCl, and bring to 1 liter with distilled water. Autoclave. Store at room temperature.
40 μl lysozyme (30 mg/ml). Dissolve in 25 mM Tris pH 8 **immediately before use**. Keep on ice.
20 μl proteinase K (50 mg/ml). Dissolve in sterile distilled water **immediately before use**. Keep on ice.
40 μl 25% SDS. Dissolve 25 g SDS in sterile distilled water. Store at room temperature.
120 μl 5 M NaCl. Dissolve 29.2 gm NaCl in distilled water and bring to 100 ml. Autoclave. Store at room temperature.
1 ml phenol:chloroform:isoamyl alcohol (25:24:1). Equilibrate with Tris, pH 8. Store at 4°C.
1 ml chloroform:isoamyl alcohol (24:1).
4 ml 95% ethanol. **Place on ice before class**.
1 ml 70% ethanol. **Place on ice before class**.

1 ml DNA buffer = 10 mM Tris + 0.1 mM EDTA, pH 8. Mix 1.21 g Tris base with 1 ml of 100 mM EDTA, pH 8. Bring volume to 950 ml, adjust pH to 8 with HCl, then bring to 1 liter with distilled water. Autoclave and store at room temperature. To make 100 mM EDTA, dissolve 3.36 g EDTA in 100 ml distilled water, adjust pH to 8 with NaOH.
4 sterile 1.5-ml microcentrifuge tubes.

Equipment required for class 9:

8 (or more) microcentrifuges and 12 (or more) vortex mixers.
Water baths at 37°C, 50°C, and 68°C.
Floating racks for 1.5-ml microcentrifuge tubes.
2 freezer boxes to hold 2 × 48 1-dram vials.
Speed Vac centrifuge/concentrator.
Portable fume hoods (**in addition to** fume hood in lab).
Containers for organic waste.
Autoclave bags for plates, microcentrifuge tubes, and tips.
Autoclave container for supernatant solutions.
Autoclave rack for culture tubes.

CLASS 10: PCR

Items per student (students work individually, plan for 72 students):

190 μl PCR reaction mixture.

The TA will prepare the mixture just before class; use bleached dedicated pipets and ART tips in a clean bleached hood to exclude extraneous DNA. Store on ice. Do a test PCR with a smaller master mix **before** class to ensure the reagents work; include positive and no-template controls.

Mixture (190 μl/student) will contain:	For 50 students:	For 75 students:
20 μl 10 × PCR buffer (Perkin Elmer)	1000 μl	1500 μl
20 μl 2 mM (each) dNTP mix	1000 μl	1500 μl
28 μl 25 mM $MgCl_2$	1400 μl	2100 μl
20 μl 50% acetamide	1000 μl	1500 μl
4 μl of 10 μM 27 F primer	200 μl	300 μl
4 μl of 10 μM 519R primer	200 μl	300 μl
1 μl *Taq* DNA polymerase (5 Units/μl)	50 μl	75 μl
93 μl distilled water	4650 μl	6975 μl
190 μl total volume	9500 μl	14250 μl

The 10 × PCR buffer, 2 mM dNTP mix, 25 mM $MgCl_2$, *Taq* DNA polymerase, distilled water, and 0.2-ml thin-wall reaction tubes are supplied by Perkin Elmer and are contained in the GeneAmp kit with AmpliTaq (N801-0055), the AmpliTaq 250 + Buffer II kit

(N808-0161), the GeneAmp PCR core kit (N808-0009), the GeneAmp dNTPs kit (N808-0007), and the MicroAmp (0.2 ml) reaction tube with cap (N801-0540). Each one of these products is sufficient for 40 students.

Order acetamide separately (Sigma).

Order PCR primers from Invitrogen:

Primer 27F: 5′-AGA GTT TGA TCM TGG CTC AG-3′
Primer 519R: 5′-GWA TTA CCG CGG CKG CTG-3′

10 μl sterile distilled water (DNA free).
2 yellow BARRIER (aerosol-resistant) tips for P20.
2 yellow BARRIER tips for P200.
2 0.2-ml MicroAmp PCR reaction tubes, with cap (Perkin Elmer product # N801-0540).
6 1.5-ml microcentrifuge tubes (sterile).
16 blue tips for pipetman (sterile).
16 yellow tips for pipetman (sterile).
1 P20 pipetman.
1 P200 pipetman.
1 P1000 pipetman.
2 pairs of disposable gloves.
1 ice bucket + ice.
1 rack for microcentrifuge tubes.
1 rack for PCR tubes.
1 plastic beaker for discarding used tips.
1 forceps.
1 thermal cycler with hot top.

TA's role: Prepare PCR mix just before **class 10**. Check the concentrations of each primer before adding to the mix. Remove PCR reactions from the thermal cycler and store them at −20°C until **class 11**. Help set up thermal cycler.

Week 6

CLASS 11: PURIFY PCR PRODUCT

Items per student (students work individually, plan for 72 students):

1 QiaQuick PCR Purification cartridge (Qiagen).
0.5 ml QiaQuick buffer PBI.
0.75 ml QiaQuick buffer PE (contains 70% ethanol).
150 μl sterile distilled water.
5 1.5-ml microcentrifuge tubes (sterile).
Rack for microcentrifuge tubes.
Rack for PCR tubes.
10 yellow tips (sterile).
4 blue tips (sterile).
P20, P200, and P1000 pipettors.
1 pair disposable gloves.

Equipment required for class 11:

8 (or more) microcentrifuges.

TA's role: Provide racks to hold tubes containing purified and unpurified PCR products and no-template control reactions. Collect these samples after class and store frozen until class 12.

CLASS 12: AGAROSE GEL ELECTROPHORESIS AND TEMPLATE PREPARATION

Items per student (students work individually, plan for 72 students):

12 pmol primer oligonucleotide 27F in 1 μl of sterile distilled water.
20 μl sterile distilled water.
6 μl agarose gel loading solution (50% glycerol + 0.05% bromophenol blue).
4 1.5-ml microcentrifuge tubes (sterile).
Rack for microcentrifuge tubes.
10 yellow tips (sterile).
P20 pipettor.
1 pair disposable gloves.

Materials for entire class:

10 μg Gibco/BRL low MW DNA mass ladder (500 ng/gel × 14 gels).

We have 15- and 16-well combs for the 12 mini-gels. Use 20-well combs for the two large gels. We need 3 lanes/student × 4 students/mini-gel (15 wells) + 3 markers, or 3 lanes/student × 5 students/mini-gel (16 wells) + 1 marker = 16 wells/gel, or 3 lanes/student × 6 students + 2 markers for large gels. Use all available gel boxes.

Each student has three samples: PCR product before and after purification and no-template control reaction.

10.5 g NuSeive 3:1 agarose (0.5 g/25 ml of 2% gel x 12 mini-gels + 2 g/100 ml **estimate** for large gels).
10 liters 1X TAE electrophoresis buffer: 40 mM Tris-acetate + 2 mM EDTA (25 ml/gel + 400 ml/tank x 12) (1 liter of **50 × TAE**: 242 g Tris base + 57.1 ml glacial acetic acid + 100 ml of 0.5 M EDTA, pH 8.0)
20 μl of 10 mg/ml ethidium bromide/gel. (1.5 μl/25 ml gel x 13 mini-gels + 6 μl/100 ml gel × 2 large gels)

The class (72 students) pours 14 2.0% 3:1 NuSeive agarose mini-gels (25 ml each). Each mini-gel contains 15 or 16 sample wells. Each large gel contains 20 wells.

Equipment required for class 12:

1 hot plate or microwave to melt agarose.
12 125-ml flasks for agarose.
12 mini-gel boxes + casting trays and combs + 2 large gel boxes + casting trays and combs.
8 dual-outlet power supplies and 14 sets of leads.
1 UV transilluminator, camera, film, face shield.
Speed Vac centrifuge/concentrator.

TA's role: Place frozen PCR reactions at instructors' bench at front of lab. Set out gel casting trays with combs, electrophoresis apparatus, and power supplies. Supervise preparation of purified PCR products for sequencing; ensure that samples contain 25 ng of PCR product and 12 pmol of primer 27F in a total volume of 12 μl. Concentrate dilute samples in the Speed Vac, if necessary. Assign each student (or team) specific lanes on gels; provide gel-loading sheets. Collect samples and submit to lab (e.g, Functional Biosciences) for sequencing.

TAs prepare plasmid DNA for sequence analysis and ship labeled samples to lab. For those who wish to use Functional Biosciences, the following is the shipping address:

Functional Biosciences Inc.
MGE Innovation Center
505 South Rosa Road, Suite 17
Madison, Wisconsin 53719
Phone: 608-441-8125
FAX: 608-441-8127
e-mail: sequence@functionalbio.com

PREPARING PURIFIED DNA

The quality of the DNA sequencing reaction depends on the cleanliness of the PCR product. To avoid degradation of the ends of the PCR products, we recommend cleaning the samples immediately after the PCR reaction. Provide 10 μl of purified PCR product at 50 ng/μl.

CUSTOMER PROVIDED PRIMERS

Provide primers in nuclease-free water at a concentration of 10 μM (picomoles/μl). For a 96-well format, provide at least 120 μl of primer for each plate. For individual samples, provide 10 μl per sample. By default, the sequencing laboratory performs sequencing reactions at an annealing temperature of 50°C.

CLASS 13: NO LAB EXERCISE; MIDTERM EXAM. TAS NEEDED TO MONITOR SMALL LAB

Experiment 4: PCR Amplification of 16S rRNA Genes for Terminal Restriction Fragment Length Polymorphism Analysis.

Week 7

CLASS 14: PCR FOR T-RFLP

Items per student (students work individually, plan for 72 students):

190 μl PCR reaction mixture.

TA will prepare just before class. Store on ice. Do a test PCR with a smaller master mix **before** class to ensure the reagents work; include positive and no-template controls.

Mixture (190 μl/student) will contain:	For 75 students:	For 50 students:
20 μl 10X PCR buffer (Perkin Elmer II)	1500 μl	1000 μl
4 μl 10 mM (each) dNTP mix	300 μl	200 μl
28 μl 25 mM $MgCl_2$	2100 μl	1400 μl
3 μl of 10 μM 8–27 F "Fam"-labeled primer	225 μl	150 μl
1 μl of 10 μM 8–27 F unlabeled primer	75 μl	50 μl
4 μl of 10 μM 926–907 R primer	300 μl	200 μl
1 μl *Taq* DNA polymerase (5 units/μl)	75 μl	50 μl
129 μl distilled water	9675 μl	6450 μl
190 μl total volume	14250 μl	9500 μl

The 10 × PCR buffer, 2 mM dNTP mix, 25 mM $MgCl_2$, *Taq* DNA polymerase, distilled water, and 0.2-ml thin-wall reaction tubes are supplied by Perkin Elmer and are contained in the GeneAmp kit with AmpliTaq (N801-0055), the AmpliTaq 250 + Buffer II kit (N808-0161), the GeneAmp PCR core kit (N808-0009), the GeneAmp dNTPs kit (N808-0007), and the MicroAmp (0.2 ml) reaction tube with cap (N801-0540). Each one of these products is sufficient for 40 students.

Order PCR primers from Invitrogen:

Primer 8–27 F "Fam"-labeled: 5′- AGA GTT TGA TCM TGG CTC AG -3′
(select "Fam" label at 5′ end for the 8–27 F primer)
Primer 926–907 R: 5′- CCG TCA ATT CCT TTR AGT TT -3′

10 μl sterile distilled water (DNA free).
2 yellow BARRIER (aerosol-resistant) tips for P20.
2 yellow BARRIER tips for P200.
2 0.2-ml MicroAmp PCR reaction tubes, with cap (Perkin Elmer product # N801-0540).
6 1.5-ml microcentrifuge tubes (sterile).
16 blue tips for pipetman (sterile).
16 yellow tips for pipetman (sterile).
1 P20 pipetman.
1 P200 pipetman.
1 P1000 pipetman.
2 pairs of disposable gloves.
1 ice bucket + ice.
1 rack for microcentrifuge tubes.
1 rack for PCR tubes.
1 plastic beaker for discarding used tips.
1 forceps.
1 thermal cycler with hot top.

TA's role: Prepare PCR mix just before **class 14**. Check the concentrations of each primer before adding to the mix. Help set up the thermal cycler. Remove PCR reactions from thermal cycler and store them at −20°C until **class 15**.

Week 8

CLASS 15: PURIFY T-RFLP PCR PRODUCT; TOPOISOMERASE-MEDIATED LIGATION TO PLASMID pCR4-TOPO AND TRANSFORM LIGATION; 1% AGAROSE GEL; "NANODROP" SPECTROPHOTOMETER

Items per student (students work individually) **for amplicon purification:**

1 QiaQuick PCR Purification cartridge (Qiagen).
0.5 ml QiaQuick buffer PBI.
0.75 ml QiaQuick buffer PE (contains 70% ethanol).
150 μl sterile distilled water.
5 1.5-ml microcentrifuge tubes (sterile).
Rack for microcentrifuge tubes.
Rack for PCR tubes.
10 yellow tips (sterile).
4 blue tips (sterile)
P20, P200, and P1000 pipettors.
1 pair disposable gloves.

Items per student (students work individually) **for ligation:**

1 0.5-ml centrifuge tube containing 1 μl of pCR4-TOPO vector + 1 μl of salt solution (1.2 M NaCl + 60 mM $MgCl_2$; supplied with vector DNA kit). Prepare immediately before class; hold on ice.

Items per student (students work individually) **for transformation:**

1 vial One Shot TOP10 chemically competent *E. coli* cells; thaw on ice immediately before use.
Ligation mixture (from previous step this class).
250 μl SOC broth (supplied with cells) warmed to 37°C.
2 LB agar plates containing ampicillin (50 ug/ml), IPTG (40 μM), and X-gal (0.004%).
1 glass "hockey stick" spreader (to spread transformed cells).
1 beaker ethanol (95%) to sterilize hockey stick.
1 Bunsen burner.
1 vial of sterile toothpicks; distribute to students to streak white colonies the next day.
1 P20 pipetman.
1 P200 pipetman.
1 P1000 pipetman.
1 box yellow tips, sterile.
1 box blue tips, sterile.
1 ice bucket.
Water bath at 42°C.
Floats to hold tubes.

Shaking incubator set to 37°C; tape to secure tubes to clamps.
Box to transport plates to 37°C incubator.

Items per student for agarose gel:

6 μl agarose gel loading solution (50% glycerol + 0.05% bromophenol blue).
4 1.5-ml microcentrifuge tubes (sterile).
Rack for microcentrifuge tubes.
10 yellow tips (sterile).
P20 pipettor.
1 pair disposable gloves.

Materials for entire class:

10 μg Invitrogen low MW DNA mass ladder (500 ng/gel × 14 gels).

Need 2 lanes/student × 4 students/gel + 2 markers = 10 wells/gel.
Each student has two samples: PCR product after purification and no-template control reaction. Use all 14 available apparatus. Mini-gels have 15 or 16 wells; large gels have 20 wells.

5 g agarose (0.25 g/25 ml of 1% gel × 12 gels + 1 g/100 ml × 2 large gels).
10 liters 1X TAE electrophoresis buffer: 40 mM Tris-acetate + 2 mM EDTA (25 ml/gel + 400 ml/tank × 12 + 1 liter/tank × 2) (1 liter of **50 × TAE**: 242 g Tris base + 57.1 ml glacial acetic acid + 100 ml of 0.5 M EDTA, pH 8.0).
20 μl of 10 mg/ml ethidium bromide/gel (1.5 μl/25 ml gel × 12 mini-gels + 6 μl/100 ml gel × 2 large gels).

The class (72 students) pours 12 1% agarose mini-gels (25 ml each) and 2 large gels (**estimate** 100 ml/gel). Each mini-gel contains 15–16 sample wells; each large gel contains 20 wells.

Equipment required for class 15, agarose gel:

1 hot plate or microwave to melt agarose.
12 125-ml flasks for agarose + 2 large flasks.
14 agarose gel electrophoresis boxes + casting trays and combs.
8 dual-outlet power supplies and 14 sets of leads.
1 UV transilluminator, camera, face shield.

TA's role: Place frozen PCR reactions at instructors' bench at front of lab; use separate racks for each bench. Set out gel casting trays with combs, electrophoresis apparatus, and power supplies. Supervise preparation of purified PCR products for topoisomerase-mediated cloning. Reserve time on nanodrop spectrophotometer and measure concentration of purified amplicons.

Equipment required for class 15, amplicon purification:

8 (or more) microcentrifuges.
Storage racks or boxes to hold purified amplicons in freezer.
Storage racks or boxes to hold ligated plasmid DNA in refrigerator.

TA's role: Provide racks with tubes containing purified and unpurified PCR products and no-template control reactions. Reserve 1 hour on nanodrop spectrophotometer; read OD of purified PCR products; record concentration (in ng/μl) and report data to students. Assign each student specific lanes on gels; provide gel-loading sheet.

CLASS 15 + 1 DAY: INOCULATE BROTH WITH TRANSFORMANT (WHITE COLONY)

Items per student (students work individually) **for inoculation**:

Make plates containing transformants available (in 37°C room).
1 culture tube containing 2 ml LB broth + ampicillin (50 μg/ml).
Shaking incubators set to 37°C and racks sufficient to hold 72 tubes.

The next day (**Class 15 + 2 days**) inspect tubes for growth and bring to class 16.

Make certain students have access to their plates in the 37°C incubator and a 37°C shaker, sterile toothpicks, and 1 tube of LB broth containing ampicillin (50 μg/ml).

Students examine plates in warm rooms and inoculate L broth + ampicillin with a single white colony. Make certain that warm rooms are unlocked and that sterile toothpicks (clearly labeled), tubes of LB broth + ampicillin (one per student), containers (clearly labeled) for used toothpicks, and clean work surfaces are available. Make certain students have access to a shaker at 37°C; put racks in the shaker to hold their tubes.

Students do **not** have access to the teaching lab for inoculating cultures. They must inoculate broth cultures in the warm room. Make certain they have the materials required and access to a 37°C shaker with racks to hold tubes.

CLASS 16: RESTRICT AMPLICON; PREPARE PLASMID DNA FROM BROTH CULTURE

Items per student for restriction (class 16):

1 0.5-ml tube containing 1 μl (10 units) of *Rsa*I + 2 μl of 10x buffer + 1 μl of BSA (1 mg/ml).
1 0.5-ml tube containing 1 μl (10 units) of *Msp*I + 2 μl of 10x buffer + 1 μl of BSA (1 mg/ml).

Prepare tubes with restriction enzymes just prior to use and hold on ice.

1 0.5-ml tube containing 50 μl sterile distilled water.
6 empty 0.5-ml tubes.
1 P20 pipetman.
1 box yellow tips.
Water bath set at 37°C.
Floats to hold tubes.

CLASS 16: PLASMID DNA PURIFICATION

Items per student (students work individually) **for plasmid isolation**:

Gloves.
P20 pipetman.
P200 pipetman.
P1000 pipetman.
1 box yellow tips.
1 box blue tips.

1.7-ml centrifuge tubes.
Microcentrifuges.
Waste containers for supernatant solutions (autoclave after class).
1 Qiaprep spin column (from Qiagen Spin Miniprep kit; Cat. No. 27106).
250 μl Qiagen buffer P1 + RNaseA.
250 μl Qiagen buffer P2.
350 μl Qiagen buffer N3.
500 μl Qiagen buffer PB.
750 μl Qiagen buffer PE.
50 μl Qiagen buffer EB (10 mM Tris, pH 8).

Retrieve students' broth cultures from the 37°C shaker and distribute by seat number.

The TAs take the purified plasmid DNAs to the services laboratory and measure the concentration (OD_{260} = optical density at 260 nm) of each sample using the "nanodrop" spectrophotometer. They record the concentration (in ng/μl) and report the data to students. TAs prepare plasmid DNA for sequence analysis and ship labeled samples to Functional Biosciences. TAs submit restricted amplicons to services laboratory for cleanup and T-RFLP analysis.

Functional Biosciences Inc.
MGE Innovation Center
505 South Rosa Road, Suite 17
Madison, Wisconsin 53719
Phone: 608-441-8125
FAX: 608-441-8127
e-mail: sequence@functionalbio.com

PREPARING PURIFIED DNA

The quality of the DNA sequencing reaction depends on the quality of the purified DNA. Most DNA purification kits should provide DNA at a quality sufficient for sequencing. Provide DNA at a concentration of 100 ng/μl in a volume of 10 μl.

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STRAIN CURATOR PREPARATION MANUAL

Experiment 1: Plasmid Purification and Restriction Mapping

SIX DAYS BEFORE CLASS 2

Streak both isolates (A and B) of *E. coli* strain NM522 (pKN800) for single colonies on LB ampicillin agar. Incubate overnight at 37°C.

Prepare two sets of LB agar stabs for NM522 (pKN800-A) and NM522 (pKN800-B) (on LB + ampicillin) and *E. coli* (pQE-FnbA) (on LB + ampicillin and kanamycin).

FOUR DAYS BEFORE CLASS 2

Remove plates from 37°C incubator, seal with parafilm, and store at 4°C.

Week 1

THREE DAYS BEFORE CLASS 2

Inoculate both strains (A and B) of glow-in-the-dark NM522 (pKN800) from the LB-AMP plates into 30 ml LB-ampicillin broth. Incubate cultures in 250-ml flasks with aeration (200 rpm shaking) at 37°C overnight.

TWO DAYS BEFORE CLASS 2

1. Centrifuge each culture at 5,000 × g for 10 minutes at 4°C.
2. Discard the supernatant solutions.
3. Thoroughly suspend each pellet in 3 ml of cold lysis buffer (50 mM glucose + 10 mM EDTA + 25 mM Tris, pH 8).
4. Label 36 sterile 1.5-ml microcentrifuge tubes with numbers from 1 to 36.
5. Dispense 0.15 ml of the resuspended pellets into the labeled 1.5-ml microcentrifuge tubes. Put NM522 (pKN800-A) in the odd-numbered tubes and NM522 (pKN800-B) in the even-numbered tubes.
6. Store the aliquots in a nondefrosting freezer until **class 2.**

CLASS 2: PLASMID PURIFICATION AND RESTRICTION

Provide resuspended pellets to teaching assistants at start of c**lass 2**.

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Bibliography

Required and Suggested Readings

Required Writing Manuals

Day, R.A., Gastel, B., 2011. How to Write and Publish a Scientific Paper, seventh ed. Greenwood, Santa Barbara, CA.
This book is required reading for students in this class. Day and Gastel provide instructions for writing a scientific paper, and they discuss common errors that occur in scientific writing. Their prose is lively, making this book a pleasure to read.

Bruslind, L., Burke, M., and Ream, W. Scientific Writing for Microbiology Majors. 2001. <http://microbiology.science.oregonstate.edu/files/micro/WIC_WritingManual.pdf>.

Recommended Resources for Writers

Day, R.A., Sakaduski, N.D., 2011. Scientific English: A Guide for Scientists and other Professionals, third ed. ABC-CLIO, Santa Barbara, CA.
Scientific English is an excellent companion for *How to Write and Publish a Scientific Paper*. In this book, Day and Sakaduski discuss parts of speech, grammar, phrases, clauses, sentences, and paragraphs. It is not as tedious as it sounds, due to their straightforward, readable prose. Most students benefit from reading this book.

Kanare, H.M., 1985. Writing the Laboratory Notebook. American Chemical Society, Washington, DC.
Kanare discusses how and why to write and organize lab notebooks. He includes a chapter on patents and a photograph of the notebook pages where Alexander Fleming recorded the discovery of penicillin.

Reif-Lehrer, L., 1982. Writing a Successful Grant Application. Science Books International, Inc., Boston.
This book is indispensable for those students who intend to embark on a career that will require them to write grant proposals. Grant writing differs from other types of scientific writing; this book helps improve your chances of success.

Cook, C.K., 1985. Line by Line: How to Improve Your Own Writing. Houghton Mifflin Co., Boston.
Line by Line contains examples of poorly written sentences together with improved versions. Cook illustrates a number of common mistakes and shows how a copy editor would fix them.

Strunk W., Jr., White, E.B., 1979. The Elements of Style, third ed. Macmillan Publishing Co., New York.
Generations of writers have studied Strunk and White, which has become a classic. It is short and packed with good advice.

Truss, L., 2003. Eats, Shoots and Leaves. Gotham Books, New York.
This lively book covers punctuation comprehensively.

Three additional books belong on the desk of any writer.

1. Webster's Dictionary.
2. Roget's Thesaurus. "A Thesaurus is the opposite of a dictionary. You turn to it when you have the meaning already but don't yet have the word" (I.A. Richards).
3. Burchfield, R.W., 1996. The New Fowler's Modern English Usage, third ed. Oxford University Press, Oxford, UK. This comprehensive guide to the proper use of words is replete with examples. Do you want to know when you may end a sentence with a preposition? This book tells you.

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Bush, G.L., Tassin, A.M., Friden, H., Meyer, D.I., 1991. Secretion in yeast: Purification and *in vitro* translocation of chemical amounts of prepro-α-factor. J. Biol. Chem. 266, 3811–3814.

Borneman, J., Triplett, E.W., 1997. Molecular microbial diversity in soils from eastern Amazonia: Evidence for unusual microorganisms and microbial population shifts associated with deforestation. Appl. Environ. Microbiol. 63, 2647–2653.

Suggested Background Reading

Engebrecht, J., Nealson, K., Silverman, M., 1983. Bacterial bioluminescence: Isolation and genetic analysis of functions from Vibrio fischeri. Cell 32, 773–781.

Signas, C., Raucci, G., Jonsson, K., Lindgren, P.-E., Anantharamaiah, G.M., Hook, M., et al., 1989. Nucleotide sequence of the gene for a fibronectin-binding protein from *Staphylococcus aureus*: Use of this peptide sequence in the synthesis of biologically active peptides. Proc. Natl. Acad. Sci. USA 86, 699–703.

Leonard, J.A., Shanks, O.C., Hodges, L., Ream, W., Hofreiter, M., Wayne, R.K., et al., 2007. DNA everywhere: Ancient DNA studies identify extraneous DNA in PCR reagents. J. Archaeol. Sci. 34, 1361–1366.

Rappe, M.S., Suzuki, M.T., Vergin, K.L., Giovannoni, S.J., 1998. Phylogenetic diversity of ultraplankton plastid small-subunit rRNA genes recovered in environmental nucleic acid samples from the Pacific and Atlantic Coasts of the United States. Appl. Environ. Microbiol. 64, 294–303.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., et al., 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239, 487–91.

Shanks, O.C., Hodges, L., Tilley, L., Kornfeld, M., Larson, M.L., et al., 2005. DNA from ancient stone tools and bones excavated at Bugas-Holding, Wyoming. J. Archaeol. Sci. 32, 27–38.

Hunter, P.J., Hand, P., Pink, D., Whipps, J.M., Bending, G.D., 2010. Both leaf properties and microbe-microbe interactions influence within-species variation in bacterial population diversity and structure in the lettuce (*Lactuca*species) phyllosphere. Appl. Environ. Microbiol 76, 8117–8125.

Required Editorials

Crouch, M.L., 1990. Debating the responsibilities of plant scientists in the decade of the environment. *Plant Cell* 2, 275–277.

Editorial, 1996. Distrust in genetically altered foods. Nature 383, 559.

Salyers, A., 1996. The real threat from antibiotics. Nature 384, 304.

Bengtsson, B.O., 1997. Pros and cons of foreign genes in crops. Nature 385, 290.

Borlaug, N., 2000. We need biotech to feed the world (editorial). Wall St J (December 6).

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