SECOND EDITION

MANIPULATION AND EXPRESSION OF RECOMBINANT DNA

A LABORATORY MANUAL

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Manipulation and Expression of Recombinant DNA

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Second Edition

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Fluorescence images of plant epidermal and root hair cells expressing Green Fluorescent Protein (GFP) fused with microtubule associated protein, MAP4 (left-hand panel) and actin binding protein, Talin (right-hand panel). These constructs provide an excellent system to monitor cytoskeletal dynamics in living cells. New evidence confirms that root knot nematodes and rhizobia produce an essentially identical response in cytoskeletal dynamics. See article by Weerasinghe, Bird, and Allen. *PNAS,* Feb. 22, 2005.

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Preface

The manipulation and expression of recombinant DNA is deceptively simple: cut and paste DNA together and new proteins can be made. But to really understand the power of biotechnology, there is no adequate substitute for sequential, wet lab experiments that lead students through the process from isolating DNA to making recombinant protein. Eight years have passed since the first edition of this manual was published. The second edition provides updated experiments and techniques but the core principles remain the same: combining nucleic acids from different organisms to produce a recombinant product. The experiments described in this manual continue to provide a solid conceptual basis for understanding the power and promise of biotechnology, even as the field evolves.

In our experience, students from many different disciplines, including life sciences, agriculture and veterinary medicine, chemical engineering, and physics have benefited from these experiments. Each of these disciplines uses biotechnology differently but the principles are the same. One of the most rewarding parts of working with students is watching them transition from knowing about biotechnology to "*Yes*, I see the applications of biotechnology." Once the power of recombining nucleic acids to produce novel structures is demonstrated, advanced biotechnologies in health care, agriculture, and nanotechnology become apparent.

When this manual was first written (1997), the human genome was not yet sequenced and products developed through biotechnology were just beginning to be marketed. Now, most of the corn, soybean, and cotton grown in the United States have been genetically altered to the benefit of farmers. Consumer benefits are in development and plants have been modified to detoxify methyl mercury, detect gunpowder, and express vaccines. Our knowledge of the biological basis of human genetic disease has skyrocketed, and noninvasive methods for treating cancer and other diseases are beginning to emerge. There is no longer any doubt that the quality of life can be improved by biotechnology.

Mistakes have been made, such as the contamination of tacos meant for human consumption by transgenic maize approved only for animal feed in 2001. However, the political and social consequences of such mistakes are severe and Aventis no longer produces transgenic plants. While the caveat remains that any technology can be used for good or bad, we must ask if we can afford not to use biotechnology to address quality of life issues. The continued safeguarding of our food supply and environment will require scientists, corporations, and governments to work together to formulate policies that promote technological innovations. Long-term initiatives that address global demands, restoration of natural resources, and international cooperation can and should include biotechnology. It is our hope that years from now, biotechnology will be seen as a highly refined tool to fight disease, promote a healthy, ecologically robust planet, and ensure quality of life for everyone.

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Note to Instructors

These laboratory exercises were developed in the context of the curriculum offered by the North Carolina State University Biotechnology Program (http://www.ncsu.edu/biotechnology). Students take the Manipulation and Expression of Recombinant DNA course as a prerequisite to more specialized laboratory courses, including Fermentation of Recombinant Microorganisms, Protein Purification, Animal Cell Culture, Microarray Technology, PCR and DNA Fingerprinting, RNA Purification and Analysis, and others. The laboratories in this course prepare students well both for these specialized courses, and for independent research in a molecular biology laboratory, both at the undergraduate and graduate student levels.

Our Manipulation and Expression of Recombinant DNA course is a 4-credit lecture/lab course. We meet for 2 hours once per week for lecture, and allot 5 hours for one lab period per week. We recommend this schedule because for several of the labs, students must inoculate cultures or perform other short activities prior to their lab day. It works out well for them to do so at the end of their lecture period (these activities are listed as "interim laboratory sessions" in the Contents). The majority of the laboratories do not require the full 5 hours, but a few of them do. Additionally, there are a few labs for which incubation times are simply too long for them to be included in the exercises. In these cases, the steps are included in the protocols with a note that the instructor will perform that particular part of the experiment for the class (for example, the induction of the fusion protein using IPTG, necessary for several laboratories, takes 3 hours). For this reason, we recommend offering the laboratory as an afternoon course so that the instructor can begin the incubations in the morning, rather than in the middle of the night.

If your semester is too short to accommodate all 14 lab sessions, the exercise described in Lab Session 5 can be omitted. It is included as an inexpensive alternative to commercially available competent cells. Frozen competent cells can be purchased from a variety of vendors. Alternatively, competent cells can be prepared by the prep staff in bulk and stored at −80°C. The commercial cells have the advantage that they often have a higher transformation efficiency than the home-made cells.

In this manual we often refer to "lab stations." This course was designed for students to work in pairs. Each pair of students is assigned a particular bench that they use every week. We number the benches and refer to them as lab stations. Students label all of their experiments, cultures, and so forth with their station number rather than their initials.

All antibodies and plasmids described in this manual are available commercially, with the exception of the positive control, pAD1. A small quantity of this plasmid is available at no cost (other than shipping) from Dr. Sue Carson at NCSU. Contact Dr. Carson at Bit_Minor@ncsu.edu, and include in the subject heading

"pAD1 request." Appendix 2 lists company and catalog numbers of plasmids and *E. coli* host strains used in this manual.

Instrumentation

Certain lab sessions provide detailed instructions for using a particular brand-name apparatus. Similar equipment from other vendors can be substituted. Appropriate instrument-specific instructions should be substituted to minimize confusion. This is especially true of the DNA agarose gel electorphoresis units, the protein polyacrylamide gel electrophoresis units, the transfer apparatus for Western blotting, and the sonicators. Essential laboratory equipment is listed in Appendix 1.

Nomenclature

In the literature, the nomenclature for the abbreviations of the enhanced green fluorescent protein gene and its gene product has been inconsistent at best, and downright confusing at worst. In this publication, we will use "*egfp*" to refer to the gene and "EGFP" to refer to the gene product. Likewise, we will use "*gst*" for the glutathione-S-transferase gene and "GST" for its gene product. Bacterial genes discussed in this book will use standard bacterial nomenclature with the gene name lowercase and italicized, and the gene product with a capitalized first letter and not italicized. For example, the gene for the *lac* repressor is "*lac*I" and its gene product is "LacI."

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PART II
Screening Transformants

PART III
Expression, Detection, and Purification of Recombinant Proteins from Bacteria

Introduction

CONCEPTUAL OUTLINE FOR EXPERIMENTS

Goal: . Make a fusion protein by splicing genes from two organisms: one from *Escherichia coli (gst)* and an enhanced gene derived from the green fluorescent jellyfish *Aequoria victoria (egfp)*. Expression of the fused gene will produce a single protein in bacteria. The *E. coli* part of the fusion protein will be used as a tag to purify the fusion protein. The *A. victoria* portion of the fusion protein can then be characterized or used for antibody production.

I. EXPERIMENTAL PROCEDURES

(See Figure 1 for a diagrammatic representation.)

- Isolate plasmid DNA using large-scale cultures of bacteria containing either the cloned *A. victoria* gene or the *E. coli* expression vector.
- Use restriction enzymes to cut the vector (containing the *E. coli gst* gene) and the insert (*A. victoria egfp* DNA). Use DNA ligase to "paste" the vector and insert DNA together.
- Introduce the ligated DNA into *E. coli*.
- Identify bacterial transformants having *A. victoria* DNA inserts by colony hybridization using purified, labeled *A. victoria* DNA as a probe.
- Identify bacterial transformants that correctly express the *A. victoria* DNA in a fusion protein by immunoassays.
- Confirm positive clones by Polymerase Chain Reaction analysis.
- Isolate DNA from bacterial clones positive for both the DNA and monoclonal antibody probes. Digest DNA with restriction enzymes to further confirm the presence of the *A. victoria* gene and verify that only one copy was inserted.
- Perform final confirmation of *egfp*-positive clones by fluorescence.
- Use sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of bacterial cell preps to determine the level of expression of the fusion protein in different transformants. (This is a pilot experiment for large-scale isolation of fusion protein.)
- Transfer protein from SDS-PAGE onto nitrocellulose membrane to perform Western blot analysis to confirm the stability of the fusion protein.
- Induce a large-scale culture of the transformed bacteria with isopropyl-β-D-thiogalactopyranoside (IPTG) to make large amounts of the fusion protein.

- Purify the fusion protein on a substrate affinity column.
- Perform protein quantification of eluted fractions.
- Use SDS-PAGE of intermediates in the purification procedure and final eluates to check for purity and degradation.

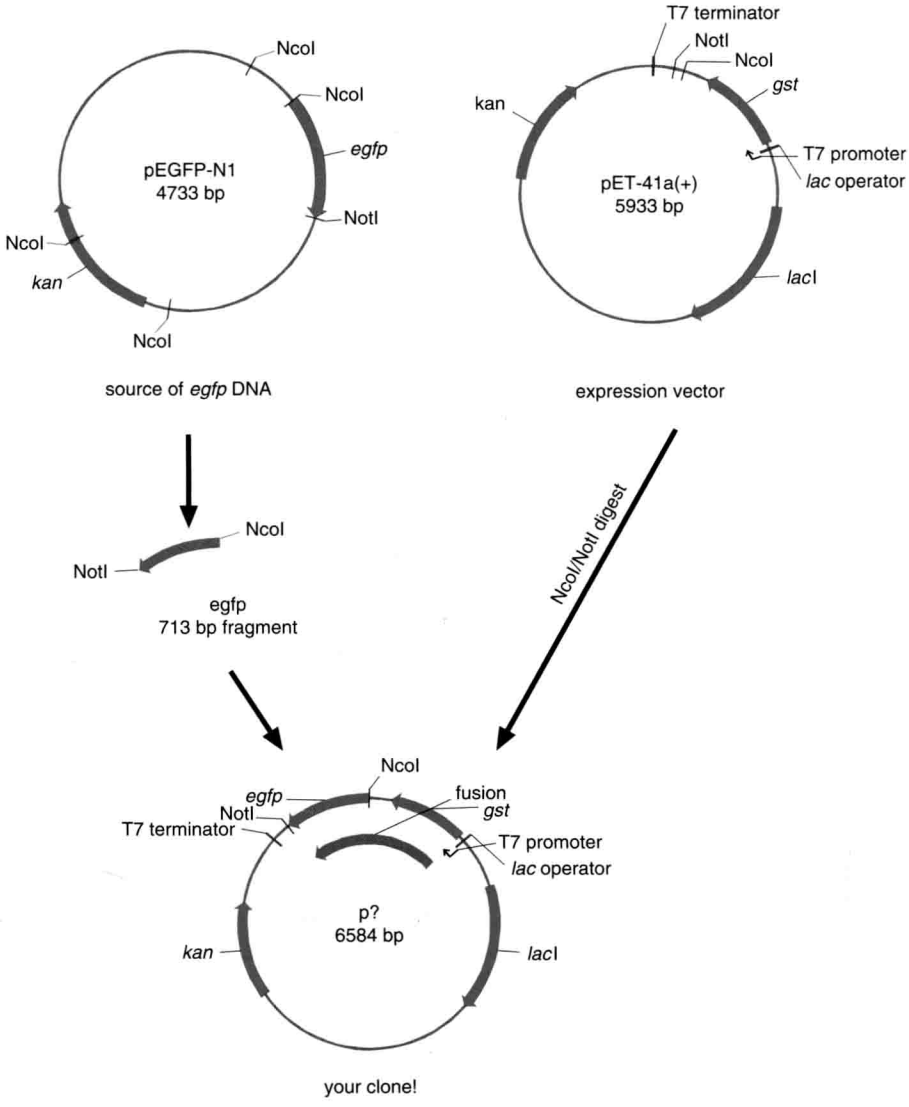

Fig. 1 Experimental procedure diagram

II. LABORATORY SAFETY

Hazards that you may be exposed to during the course of the laboratory exercises include working with toxic compounds and ultraviolet (UV) irradiation. Special precautions must be taken when working with recombinant DNA. To ensure the safety and well-being of students and support staff, the following rules will be strictly enforced.

A reckless attitude about the use of equipment or the safety of others will cause you to be dropped from the course.

The following rules *must* be observed at all times in the laboratory:

1. No smoking, drinking, or eating is allowed in the laboratory. Never store food in laboratory freezers or refrigerators.

2. You are responsible for providing a lab coat. Wear your lab coat, gloves, and safety glasses when working in the fume hood or in the presence of dangerous or potentially dangerous substances. No sandals or other open-toed shoes are allowed in the laboratory at any time.

3. Always wear gloves when working with ethidium bromide, phenol, or any hazardous or potentially hazardous substance. Remove your gloves before leaving the laboratory and wash the outsides of your gloves frequently to prevent contamination of equipment, etc., with caustic agents.

4. Long hair must be tied back at all times, and avoid loose-fitting clothing to avoid hazards associated with open flames, sterile cultures, and hazardous chemicals.

5. Dispose of microorganisms, including the tubes used for their growth, in orange bags marked "BIOHAZARD" for autoclaving. Liquid medium and plastic pipettes used to transfer microorganisms will be collected in specially marked flasks containing bleach.

6. Dispose of glass *only* in properly marked blue boxes designated for glass disposal. This includes glass culture tubes, pipettes, and broken glass. Do not put glass or any sharp object in the orange autoclavable bags marked BIOHAZARD.

7. Keep your lab bench free of unnecessary clutter. Use cabinets and drawers for storing personal items and extra supplies, not for food. At the end of the day, your bench should be clean. Micropipettes and gel rigs have been known to wander from their home station. The best way to ensure that your equipment is there the next day is to store it out of sight when you leave.

8. Wear ear protection when working with the sonicator.

9. Always wear a UV-protective full-face shield when using the transilluminator. Your safety glasses are *not* UV protective. Do not try to analyze your gel on the transilluminator. Take a picture and analyze the picture. If you need to excise a band from a gel on the transilluminator, make sure you are wearing a UV-protective full-face shield.

10. Wash your hands thoroughly before you leave the laboratory.

11. Spills should be cleaned up immediately. Make sure you have an adequate supply of paper towels at your station. If you need more, see an instructor. Spill control pillows are located on top of the yellow cabinet for flammable chemicals. Notify an instructor if you spill a chemical that is marked "poison" or "caustic." If you spill liquid containing live microorganisms, notify an instructor and pour disinfectant on the spill.

12. Immediately report all accidents such as spills, cuts, burns, or other injuries to an instructor.

13. Know the location of the fire extinguisher, eye wash station, emergency shower, and emergency exits.

14. If you have trouble with a power supply or the leads to a gel, report it to an instructor. If you see someone receiving an electrical shock, use a nonconducting object, such as a plastic beaker, to break the circuit or you may receive the shock as well.

15. Leave all laboratory facilities and equipment in good condition at the end of the class. Before leaving the laboratory, check to make sure that all electrical equipment is turned off and that the gas to the Bunsen burner is turned off.

16. No pets are allowed in the laboratory.

17. Dispose of hazardous chemicals, such as chloroform, methanol, and ethidium bromide, only in designated containers. Do not pour them down the sink. Each hazardous chemical will have its own waste container. If in doubt, ask an instructor before dumping questionable reagents down the sink.