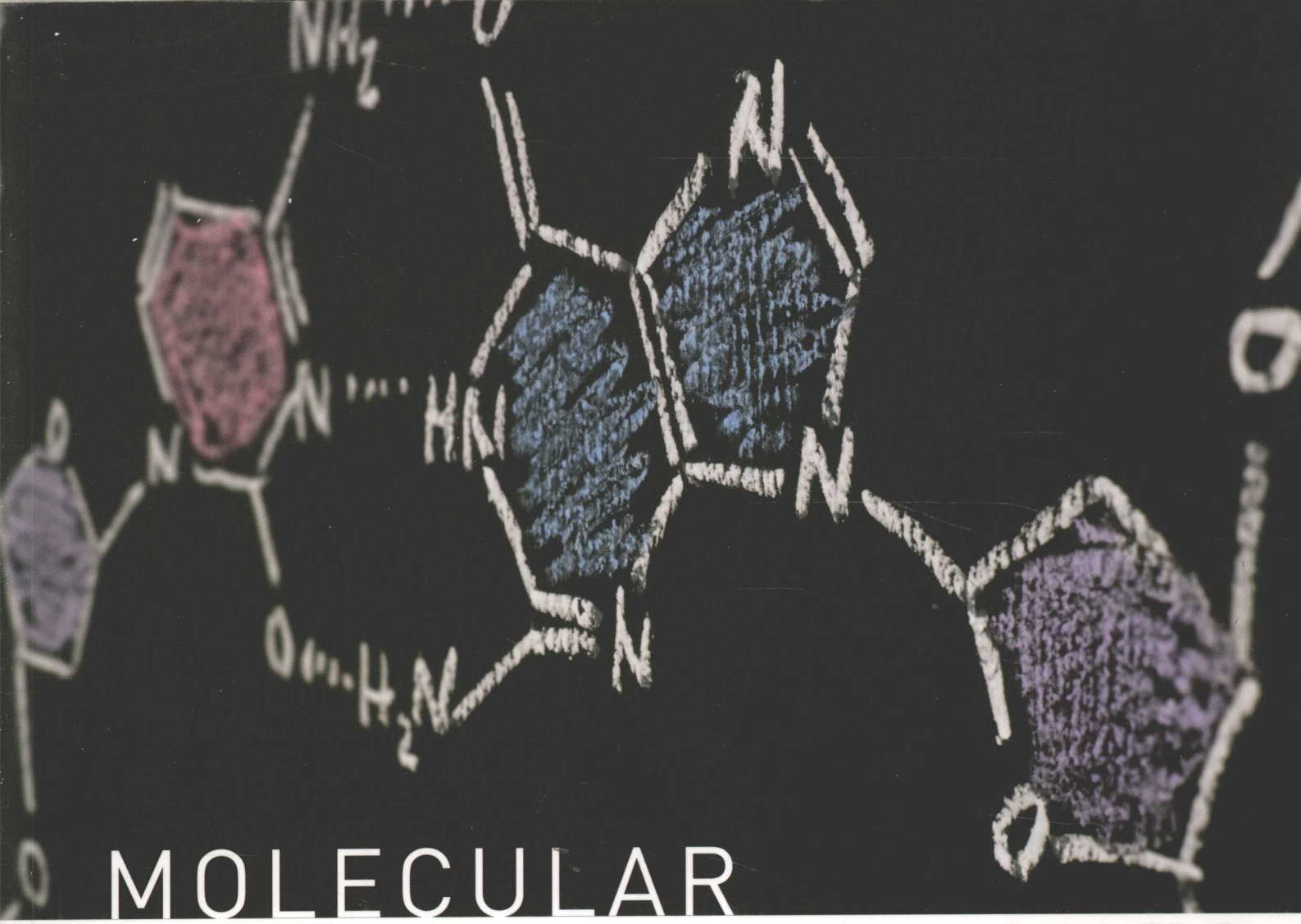

MOLECULAR BIOLOGY TECHNIQUES

A CLASSROOM LABORATORY MANUAL

SUSAN CARSON
HEATHER B. MILLER
D. SCOTT WITHEROW

Molecular Biology Techniques: A Classroom Laboratory Manual

THIRD EDITION

Susan Carson
Heather B. Miller
D. Scott Witherow

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Molecular Biology Techniques: A Classroom Laboratory Manual

Preface

Recombinant DNA technology touches many aspects of our lives. From drought-tolerant crops to biofuels to pharmaceuticals, genetically modified organisms surround us. Throughout its history, biotechnology has relied heavily on DNA cloning to produce protein products more efficiently or to make modified genes or combinations of genes. *E. coli* or other hosts serve as a cellular "factories" to churn out large amounts of protein. Human insulin, for example, is produced recombinantly using many of the same techniques described in this book.

In the seven years since the most recent edition of *Manipulation and Expression of Recombinant DNA*: *A Laboratory Manual* was published, molecular biology research and its tools have greatly advanced. Genomic sequences of thousands of organisms are now known. Cloning by PCR has all but replaced traditional cloning methods. DNA sequencing is cheap and accessible. Tools to study that elusive nucleic acid, RNA, are now established as mainstream molecular biology techniques.

Emerging evidence suggests that the central dogma of molecular biology, "DNA makes RNA makes protein," is more complex and has more exceptions than we ever imagined. The transition of a human gene from DNA to RNA may not be as faithful as we once thought. Recently, more research has focused on RNA, and this has led to groundbreaking findings. These discoveries draw an even more complex picture of gene expression. Not only can this multifaceted nucleic acid serve as a "messenger," but it can silence genes, perform catalysis, and generate protein diversity!

This new classroom laboratory manual uses the semester-long project concept introduced in *Manipulation and Expression of Recombinant DNA*: *A Laboratory Manual*, but the laboratory sessions are updated to reflect current cloning techniques. We also added a new section (five laboratory sessions) on measuring mRNA levels to underscore our conviction that today's molecular biology students need to be confident working with RNA. The laboratory sessions in this new manual *Molecular Biology Techniques*: *A Classroom Laboratory Manual* reflect the nature of modern science; that is, scientists need the experience to work with macromolecules at each level of gene expression: DNA, RNA and protein.

What will we learn in the next seven years? Students taking this course right now will be at the leading edge of exciting new discoveries. We can't wait to find out!

About the Authors

Left to right: Sue Carson, Heather Miller, Scott Witherow

Dr. Sue Carson is the Academic Coordinator of the Biotechnology Program, Teaching Associate Professor of Plant Biology, and the Director of the NSF-funded Synthetic Biology Research Experience for Undergraduates at North Carolina State University. She graduated from Rutgers University (New Brunswick, NJ) with a B.S. in Biotechnology, and from The University of North Carolina (Chapel Hill, NC) with a Ph.D. in Microbiology. Her area of scientific expertise is in molecular mechanisms of bacterial pathogenesis. Dr. Carson's current work focuses on college-level science education. She has received multiple awards for teaching excellence and innovation and is a member of the Howard Hughes Medical Institute Science Education Alliance, promoting inquiry-guided learning in the college classroom laboratory. She co-authored the molecular biology lab manual *Manipulation and Expression of Recombinant DNA: A Laboratory Manual 2e* (Academic Press, 2006), and has published numerous peer-reviewed papers in the area of course and curriculum development.

Dr. Heather Miller is a teaching postdoctoral fellow in the Biotechnology Program at North Carolina State University. She graduated from Clarion University of Pennsylvania (Clarion, PA) with a B.S. in Molecular Biology/Biotechnology, and from Duke University (Durham, NC) with a Ph.D. in Molecular Genetics and Microbiology. Her area of scientific expertise is RNA biology. Her research has focused on HIV-1 gene expression and the coupling of transcription and splicing in mammalian systems. She has developed and taught multiple biotechnology courses and is engaged in the scholarship of teaching and learning.

Dr. D. Scott Witherow is an Assistant Professor of Chemistry and Biochemistry at the University of Tampa. He graduated from Rollins College (Winter Park, FL) with an A.B. in Chemistry, and from the University of Miami (Miami, FL) with a Ph.D. in Molecular and Cellular Pharmacology. His research has focused primarily on G protein-mediated signal transduction processes in mammalian systems. Following two research postdoctoral fellowships, Dr. Witherow served as a teaching postdoctoral fellow at North Carolina State University, where he published and presented multiple papers in the field of science education and developed a passion for teaching.

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We would like to thank the many people who contributed to this manual. Melissa Cox was instrumental in piloting the experiments and in critically editing the prep lists. We also thank the North Carolina State University Colleges of Agriculture and Life Sciences, Engineering, Veterinary Medicine, Physical and Mathematical Sciences and Natural Resources, and the Office of the Provost. Their support for the Biotechnology Program led to the development of this manual.

Note to Instructors

These laboratory exercises were developed in the context of the curriculum offered by the North Carolina State University Biotechnology Program (http://biotech.ncsu.edu). Students take our biotechnology core course "Manipulation and Expression of Recombinant DNA" as a prerequisite to more specialized laboratory courses including Real-Time PCR, RNA Interference and Model Organisms, mRNA Transcription and Processing, Protein Purification, Animal Cell Culture, Microarray Technology, Plant Genetic Engineering, Protein–Protein Interactions, Deep Sequencing and others. The laboratories in the core course prepare students well for these specialized courses, and for independent research in a molecular biology laboratory, both at the undergraduate and graduate student levels.

We use the first 14 lab sessions of this manual in our core course, and then a subset of students go on to pursue advanced courses that focus on working with RNA. We chose to include a section on analyzing mRNA levels in this manual. Programs may choose to substitute some of the earlier screening labs with an RNA lab at the end, or add the RNA modules into another course.

Our biotechnology core course is a four-credit lecture/lab course. We meet for two hours once per week for a lecture, and allot four 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 referred to as "*interim laboratory sessions*"). The majority of the laboratories do not require the full four hours, but a few of them do (notably Lab Session 6). Additionally, there are a few labs for which incubation times are simply too long to reasonably include 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 two to four 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 the middle of the night.

We use commercially prepared competent cells in our course. If your budget does not permit this, it is possible to prepare "home-made" competent cells and store them in a −80°C freezer. We have included the protocol for preparation of competent cells as Appendix 3.

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 to a numbered lab station. Students label all of their experiments, cultures, etc., with their station number, rather than initials.

All antibodies described in this manual are available commercially. The pBIT and pEGFP-N1 plasmids are available at no cost (other than shipping) to institutions of higher education for educational purposes from Dr. Sue Carson at NCSU. Contact Dr. Carson at bit_minor@ncsu.edu, and include in the subject heading "pBIT request." pET-41a is available commercially, and we are not licensed to distribute it.

Instrumentation

Certain lab sessions provide detailed instructions for using a particular brand-name apparatus. Similar equipment from other vendors can be easily substituted. Appropriate instrument-specific instructions should be substituted to minimize student confusion. This is especially true of the DNA agarose gel electrophoresis units, the protein polyacrylamide gel electrophoresis units, the transfer apparatus for western blotting and real-time-capable thermal cycler.

We highly recommend the use of a Nanodrop spectrophotometer. Only 1–2 μl of sample is needed to quantify DNA and RNA. Standard spectrophotometers that use traditional quartz cuvettes require the use of much greater quantities of sample, and in some cases, the sample cannot be diluted for readings because the concentration would be too low. If you do not have access to a Nanodrop spectrophotometer, in some cases where sample amount is limiting, running a small amount of sample on a gel and estimating the quantity is the best method.

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 (either DNA or mRNA) 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 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."

Introduction

Conceptual Outline for Experiments

Goal: Make a fusion protein by joining 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 visualized by fluorescence.

Experimental Procedures

Part I: Manipulation of DNA

(See Figure 1 for a diagrammatic representation.)

- Isolate plasmid DNA using cultures of bacteria containing the *E. coli* expression vector pET-41a.
- Use restriction enzymes to cut the pET-41a vector.
- Use PCR to amplify the insert (*A. victoria egfp* DNA) from pEGFP-N1, and restriction digest to form sticky ends.
- Use DNA ligase to "paste" the vector and insert DNA together.
- Introduce the ligated DNA into *E. coli*.

Part II: Screening Transformants

- Identify bacterial transformants that correctly express the *A. victoria* DNA in a fusion protein by probing with a monoclonal antibody.
- Confirm positive clones by polymerase chain reaction.
- Isolate DNA from transformants and digest with restriction enzymes to further validate the presence of the *A. victoria* gene.
- Final confirmation of *egfp*-positive clones by fluorescence.
- Verify that single nucleotide errors did not occur in the *egfp* gene during PCR cloning by DNA sequencing.

Part III: Expression, Detection, and Purification of Recombinant Proteins from Bacteria

- Use sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis to confirm the expression of the fusion protein.

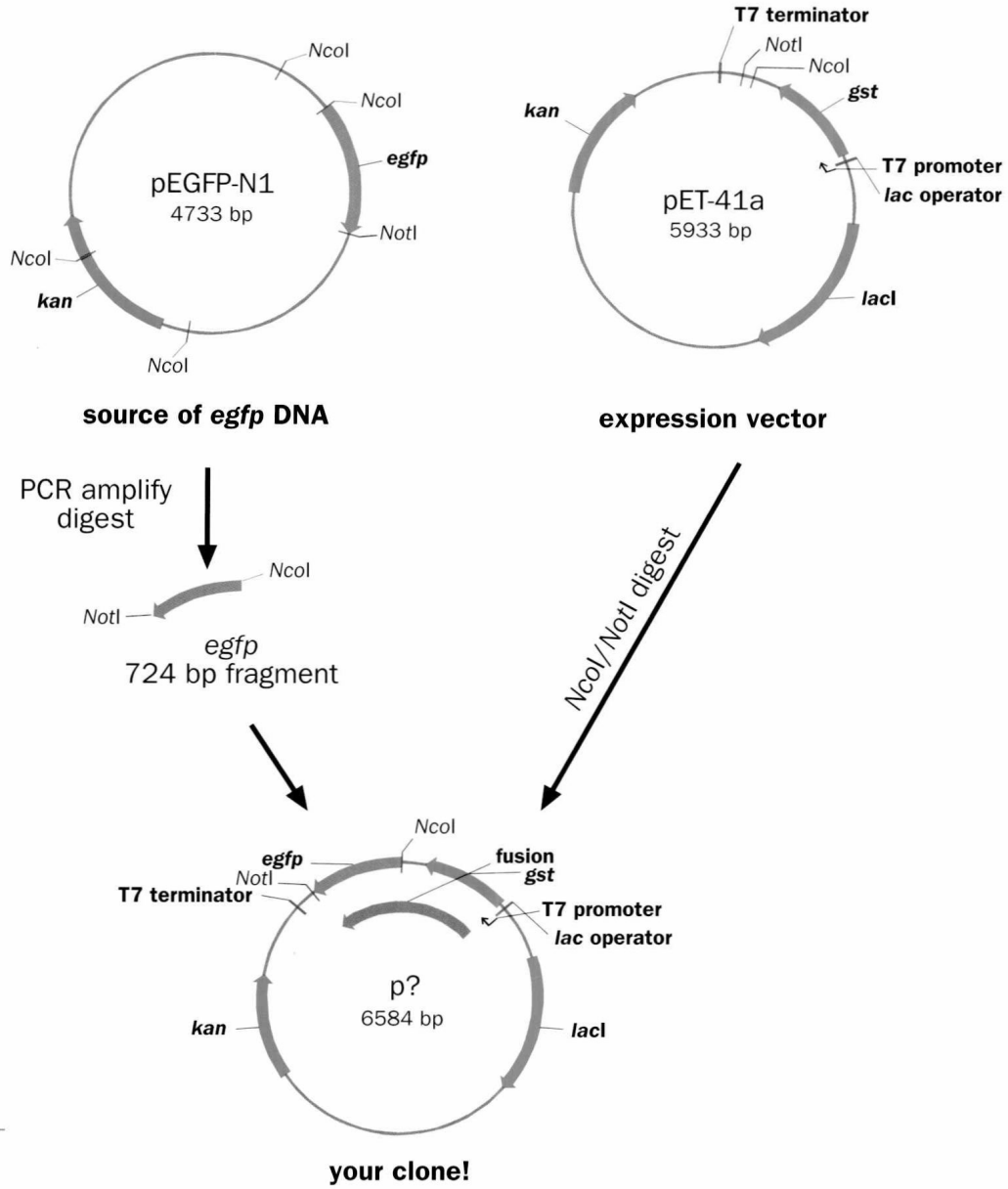

FIG. 1
Cloning procedure diagram

- 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 purification fractions to check for purity and degradation.

Part IV: Analysis of mRNA Levels

- Purify total RNA from a positive bacterial clone.
- Perform reverse transcription polymerase chain reaction to quantify mRNA levels after induction with IPTG and/or lactose.

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 drinking or eating is allowed in the laboratory. No food or drinks should be brought into the laboratory.
2. Safety glasses provided must be worn at all times in the laboratory. No sandals or other open-toed shoes are allowed in the laboratory at any time.
3. Always wear gloves when working with any hazardous or potentially hazardous substance. Remove your gloves before leaving the laboratory and change 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 bags marked "BIOHAZARD" for autoclaving. Liquid medium containing microorganisms will be collected in specially marked containers containing bleach.
6. Dispose of glass *only* in properly marked boxes designated for glass disposal. Do not put glass or any sharp object in the autoclavable bags marked biohazard!
7. Keep your lab bench free of unnecessary clutter. Use cabinets and drawers for storing personal items and extra supplies. At the end of the day, your bench should be clean and equipment put away.
8. Wear ear protection when working with the sonicator.
9. Always wear a UV-protective full-face shield when using the transilluminator if it is not in a safety cabinet. Your safety glasses are *not* UV protective. Do not try to analyze your gel on the transilluminator. Observe the gel on the monitor if you are using a digital imager, or take a photograph and analyze the photograph if you do not have a digital imager.
10. Wash your hands thoroughly before you leave the laboratory.
11. All spills should be cleaned up immediately. Notify an instructor if you spill a potentially hazardous chemical or liquid containing live microorganisms.
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

non-conducting object, such as a plastic beaker, to break the circuit or you may also receive a shock.

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 only in designated containers. Do not pour them down the sink.
18. An up-to-date immunization against tetanus is strongly recommended.
19. For a tutorial on general laboratory safety, visit the website http://www.ncsu.edu/project/ungradreshhmi/evaluationModule/login.php (login is free).

General Operating Procedures

- Reagents: Aliquoting reagents and supplies for everyone in the course at the same time is difficult. Your patience and cooperation are greatly appreciated. If you run out of a reagent or enzyme and cannot find it on a lab cart, please ask a technician or TA for more.
- Enzymes: Enzymes are very expensive and can be ruined by prolonged exposure to room temperature or by contamination. Always use a fresh, sterile pipette tip for each enzyme. Keep the enzyme on ice; never put it in a microcentrifuge rack at room temperature. Always add enzymes to your reactions as the last component; addition of enzyme to unbuffered solutions can kill its activity.
- Pipette tips: You will be provided with three boxes of pipette tips: P10, P20/P200 and P1000. When you run out of tips, notify your instructor.
- Repairs: A list of supplies and equipment at your station will be made available to each pair of students at the first lab session. You are responsible for the equipment at your station. If you encounter any difficulties with operation, please ask for assistance. If equipment is damaged in any way, please report it so that we can have it repaired. Barring willful destructiveness, you will not have to pay for repairs.
- Equipment: Please make sure that you understand the proper use of this equipment before attempting to operate it. If in doubt, ask an instructor, not another student. In addition to costly repairs, improper use of this equipment can be very dangerous.

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

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