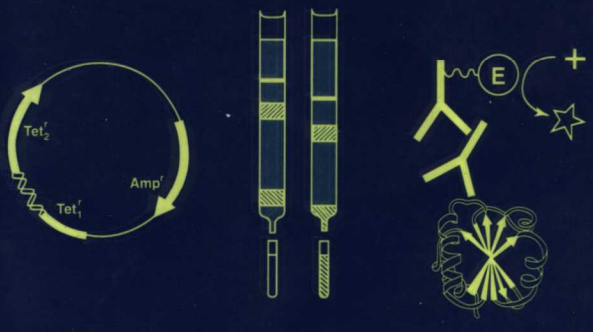

Cloning, Gene Expression, and Protein Purification

Experimental Procedures and Process Rationale

Charles Hardin
Jennifer Edwards
Andrew Riell
David Presutti
William Miller
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PREFACE

This manual evolved to train advanced undergraduate and introductory graduate students to enter high-tech jobs in health care diagnoses and treatments, pharmaceutical industries, and graduate studies across a broad set of disciplines. Our goal was to produce a broadly encompassing tool that could help these students develop first-rate laboratory skills. Since we focus on quantitative analyses of biological macromolecules and reactions, students should have taken at least one year of quantitative and organic chemistry courses prior to this training. Additional experiences with common laboratory tools (pipets, quantitative spectroscopy, electrophoresis, centrifugation) and some microbiology training are all preferable, but not strictly essential if appropriately trained instructors work interactively with the students to correct the deficiencies. This book cannot teach the information alone, but it attempts to do the next best thing, teaching students how to teach themselves. Students who acquire these skills should be able to compete successfully for jobs in biochemistry and biotechnology laboratories.

The laboratory exercises and supplementary information were designed to peel back the surface of the experiments and expose their inner workings. They are also meant to be informative and yet be relatively easy to read by students from a variety of scientific backgrounds. The desired outcome is a fully trained student ready to perform truly independent research, understanding what it means to pursue a fully integrated program of laboratory techniques, experimental planning, and both pre- and post-process troubleshooting. Concepts and terms are readily accessed in an extensive index. The purposes of techniques are usually described in detail, as are the consequences of the various types of manipulations. Supplemental literature explains a variety of theoretical explanations designed to foster a better intuitive sense of the experimental outcome as a result of the molecular behavior of the participants.

The approach used in the manual is distinctive in the following ways.

1. The plasmid, cDNAs, and antibody probes are all well characterized in the research literature and are available from an easily accessible source, the American Type Culture Collection (ATCC). Instructors are generally familiar with the techniques used in this manual; we facilitate their expertise by providing these tested materials on a worldwide basis.
2. Information is provided to accompany the lectures and labs from the product and research literature, including primary sources and reviews of both practical and theoretical utility.
3. The relevant parts of original methods and reagent literature are reproduced as seen in the original sources as a means of introducing students to the interwoven nature of the linkage between literature sources and laboratory use.
4. An alphabetized list of defined terms is provided for each of the two parts, allowing the student to rapidly define unfamiliar phraseology or discipline-specific jargon. New terminology and important summary statements are italicized in the text to help the students identify important phrases or conclusions.
5. An alphabetized reagents list is provided in the appendices, allowing the student to rapidly reference common reagents used in the laboratory protocols.
6. All topics and subtopics are organized in a format consistent throughout the manual to assist ease of reading and organizing thought processes. Main topics are numbered and in bold, subtopics are italicized and numbered with numerical extensions, and subtopics are subdivided into categories that are italicized and numbered with two number extensions.
7. A flow chart accompanies each part to facilitate understanding the order and progress of the course experiments.
8. Five types of articles (Innovation/Insight, Theory/Principles/Principles, Process Rationale, Vendor Literature, and Alternative Approach) are included in this manual. These titles have been chosen to accompany each one of the articles to clarify the type of supplemental literature the section provides.
9. Symbols are located at the top outer corner of each new section to indicate the section type (Introductory Material - Compass rose, Flow Charts - Direction coordinates, Appendices - Reference books, Labs - Pipet in Eppendorf tube, lectures (Part I) – Vector, lectures (Part II) - Folded protein, Original literature (Part I) - DNA helix, Original literature (Part II) - ELISA schematic, and Index - Lighthouse).
10. This laboratory manual contains an introductory unit and two main parts. The introductory unit provides practice with many basic skills that are used in the biotechnology laboratory. Part 1 focuses on nucleic acids, including DNA and RNA. Part 2 focuses on proteins, including antibodies and combinatorial technology.
11. Students are encouraged to prepare laboratory writeups in the same format as primary literature manuscripts. This helps the student learn how to clearly and succinctly define the immediate and overall problems, the approach to solving the problem, the nature of the results, and the importance of the results to the overall goal of the project.

The ultimate goal is to make students aware of how to approach multistep projects by ushering them through the learning stages involved in proceeding from the initial cloning experiments, through the gene expression procedures, and finally to the protein purification and analysis techniques.

NOTE TO INSTRUCTORS

The plasmids and antibody used in this manual are available from Dominique Robertson at NCSU (niki_robertson@ncsu.edu). Although the plasmids are available at no cost, there is a small processing charge for the monoclonal antibody.

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We dedicate this book to Derek, Hong, and my parents (by Chuck), Steven, my husband (by Jennifer), Mo, and my family (by Andy), Elizabeth, Madelene, and my parents (by Dave), Laura, my wife (by Bill), Robert and Monica (by Niki).

C. H., J. P., A. R., D. P., W. M., and D. R.

NCSU
Raleigh, NC
April 7, 2000

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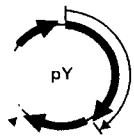
INTRODUCTORY UNIT: INTRODUCTION TO THE BIOCHEMICAL LABORATORY



1. Pipets

The laboratories, lectures, and appendices in this section emphasize the critical nature of proper pipeting. They provide practice in preparing nucleotide and amino acid samples and then characterizing them using ultraviolet absorbance spectrophotometry. Pipeting errors are characterized and the concept of propagated error is explained and illustrated. Protein concentration is measured next, illustrating a simple assay of the total peptide group concentration using a coupled redox-activated dye technique, the BCA assay. All enzyme activity assays (see section 2) require determining protein concentrations, so accomplishing the key goal of monitoring improvements in protein activities requires multiple applications of this fundamental measurement.

2. Introduction/Overview

A majority of the scientific activities done to date under the heading "molecular biology" have involved studying (*1*) production of proteins, (*2*) functions catalyzed by them, or (*3*) controls mediated by them. Most typical projects proceed by the fundamental steps: (*1*) the gene that encodes the protein is captured in an expression plasmid, (*2*) the DNA sequences are determined, and (*3*) the protein is produced via transcription and translation by a virus, bacterium, yeast, or higher eukaryotic cell. Thus, we will focus on the pathway of processes that lead from the DNA to its RNA transcript to the translated protein, with emphasis on how biomolecular structures and the conditions of the surrounding medium work together to produce functionality.

The laboratories and lectures in this section are intended to teach the points to consider in the course of this type of research. Doing reliable experiments is necessary to obtain patents, move a product through early R & D stages, on to clinical trials and finally to sale. This requires careful use of analytical techniques and statistical analyses of the data. Reproducibility is the central tenet of responsible science. Doing otherwise can constitute production of unreliable results and possibly committing fraud!

A remarkably diverse set of factors can affect biomolecular functions. The dependence of functional characterization protocols on isolation, storage, reconstitution, and function assay conditions must be characterized and standardized in order to claim that one has developed a trustable material. This book is designed to explain how one thinks when working to purify, preserve, and assay DNAs, RNAs, and proteins.

3. Sample Preparation

Molecular biological experiments typically involve dissociating one or a few of the macromolecular components of a particular functional assembly, recovering the individual components in as pure a form as possible, then reconstituting the system under conditions "simple enough" to control variables sufficiently well so one can claim reproducibility. For many multistep experimental schemes, this involves obtaining pure components and one or a set of enzymes, DNAs, or RNAs, for use as analytical reagents (e.g., catalysts, redox reagents, labeling or linking materials, internal standards, control substrates). As a result, a huge "biotechnology" industry has evolved to supply verified supporting reagents, thereby reducing the difficulty of assembling the required reagents; the key problem becomes obtaining the biomolecules and specialized small molecules that aren't commercially available. The labs, lectures, and appendices in this book are primarily concerned with preparing pure samples and understanding the factors that will allow one to reproducibly maintain their functions. Mastering these skills, and understanding how to generalize the concepts and apply them to a previously uninvestigated problem, is the key to becoming a working experimental molecular biologist.

A tip. Try to avoid the very worrisome situation we call "kit mentality." This is practiced among students who have not been adequately taught or don't care to make the effort to understand how the chemistry of the situation determines the chances for success, especially when troubleshooting is required. The "kit mentality" practitioner treats the experimental protocol like a "black box" recipe that need not be understood explicitly. For example, they'll transfer "2 µl from the reagent in the green tube" while having no idea what the material is or does.

4. Buffers and Other Variables Considered to Preserve Functional Activities

Proper use of buffers is essential if a researcher is to claim that he/she has developed a reproducible molecular biological method. Since reproducibility is

the hallmark of conscientious science, it is imperative that we discuss detailed analytical chemical aspects of *buffers* and *factors one must control* to ensure that they function properly in the experimental milieu of the reaction studied. Norman Good elaborated this subject in his classical papers.

Consider the difference between the chemical *concentrations* and the *activities* of the relevant functional groups on all of the chemical species. Molar specific activities of components in a sample might change with variations in any or all of the following factors: (*i*) pH, (*ii*) one or more buffer components, (*iii*) binding and/or enzymatic substrates, (*iv*) activity-modifying "spectator" molecules, (*v*) mono-, di- and polyatomic ions, (*v*) the enzyme(s), protein(s), nucleic acid(s), carbohydrate(s), and/or polysaccharide(s), (*vi*) lipids, (*vii*) other nonpolar or amphipathic molecules, and many other possibilities, depending on the situation under study.

Temperature changes usually affect the activities of buffer components as well as the other factors listed above. Some buffers (e.g., phosphate) have pK_as that are relatively stable over a range of temperatures. In contrast, the pH of the ubiquitous buffer Tris(hydroxymethyl)aminomethane ("Tris") changes by several tenths of a unit as the temperature is varied across the ranges encountered in typical protocols. This "pH drift" can dramatically affect biochemical activities. For example, preserving the activity of a protein in a particular DNA binding and cleavage reaction sometimes requires that one maintain the pH in a particular range. Moving outside that range can decrease the activity of the protein, sometimes irreversibly. Using a protocol that requires changing temperature, which shifts the pH of Tris, might fail to yield products because the pH change inactivates DNA recognition.

Troubleshooting problems with your reactions can require some subtle considerations. One must sometimes untangle the chemical consequences of interactions between synergistically linked chemical species, energy sources, forces, constraints, entropies, etc. In the case cited above, one should look up the temperature-dependence pK_a coefficient of the pK_a of Tris and other buffers, and select one with a less temperature-dependent pK_a. This consideration is reasonably subtle to the novice troubleshooter.

Our hope is that students will read such analyses and try to learn how to generalize the learned lesson(s) to analogous situations. More importantly, this approach will benefit diligent and mastery-minded students in future pursuits, as they work to develop new protocols and use those obtained from others in the "real world," both privately communicated and commercial. All seasoned molecular biologists have spent time working through "snags" that developed because they don't understand how the chemistry of the situation leads to products, or why the process is "screwed up" by unsuspected interactions between components, constraints, etc. "Paying one's dues" involves undergoing enough such encounters to learn how to minimize one's time in failed processes. Success involves learning how to preserve sufficient materials and activities from one reaction for the next stage of the process.

Reduction of theoretical principles to practical demonstrations is always necessary to prove a new fact. The truly capable experimentalist produces more materials and facts (i.e., they're more successful) because they spend less time repeating flawed protocols. This is often accomplished by taking the time and effort to think about the problem carefully before merely "trying again because I must have screwed something up last time." Maybe the process is not working because some subtle problem(s) lurks beneath the "recipe" of the protocol. This is one potential danger of "recipe mentality," a cousin of the "kit mentality" concept described above. We discourage student and practicing researchers from making up 'recipe cards' with buffer preparation protocols for general distribution to novices.

Make your solutions and samples according to the general procedure summarized below. (Standard brackets notation indicates concentrations throughout this book.) A specific example is used to illustrate.

Note that the units and decimal places are aligned carefully. It may seem overbearing to ask that the information be provided this way. However, when troubleshooting, it can be very enlightening to have this clear exposition of all of the components,

Targeted [Component] in Final Sample/Solution	[Stock Solution]	Volume of Stock Solution Added	Fold Dilution
20 mM Tris-HCl, pH 7.4	200.0 mM	2.0 µl	10.0x
100 mM NaCl	2.0 M	2.0 µl	10.0x
4 mM $MgCl_2$	0.1 mM	0.5 µl	25.0x
10 % ethanol	95 %	2.1 µl	9.5x
H_2O		+13.4 µl	
		Total volume = 20 µl	

stocks intended for use, and final intended concentrations, along with intended volume changes to be used to achieve the final concentrations (typically dilutions). Having the student's (or employee's) notebook with this information for use as the basis of discussions between professor (manager) and student (employee) provides good discipline and can pay off when troubleshooting a problematic experiment. This level of organization will also help when preparing to expand a "pilot protocol" to a more fully realized experiment, in which a range of solutions must be prepared in parallel.

When making stock solutions, be certain that they've been adjusted to the proper pH and volume and kept clean and "microbe free." Most labs are equipped with H_2O "polishing" facilities, which pass the solvent through several stages of ion exchange and organic chemical-removing resin materials, achieving H_2O that is generally clean enough to use in molecular biological bench experiments. However, if organisms are to be grown, e.g., in food-source containing media, solutions should be treated by passage through syringe filtration devices, such as those sold by Amicon (Centricon®), or by autoclaving. Solutions will not 'keep' forever on the shelf in a lab at ca. 22°C. Withholding Mg^{2+} for storage, and only combining it with the other components for the reaction or process when needed, can help limit bacterial contamination. Going one step further, placing the chelator *ethylenediamine-tetraacetic acid* (really Na_2EDTA^{2-}; usually called "EDTA") in the solution will ensure that trace metal or cation contaminants are scavenged. Conversely, it makes little sense to add a required metal (e.g., Mg^{2+}) to a reaction mixture that contains a lot of EDTA, since it will render the metal unavailable for its role in the reaction.

5. pH Meters and Adjustment Techniques

A pH meter is used to determine and adjust the pH of a solution. Two general approaches exist. In the first, one places the calibrated pH probe directly in the buffer solution *while* one adjusts the pH. Of course, one must be particularly careful to avoid transferring contaminants, including chemicals, ions, or microbes, along with the pH probe. In the second approach, one makes concentrated stock solutions, composed of pure protonated and deprotonated buffer species whose pK_a values bracket the targeted pH. One then makes the target buffer by mixing appropriate amounts of the buffer components (taken from a previously developed table) and adjusting the volume to the final value by adding H_2O.

The first technique is superior to the second because it is less restrictive. Adjusting the pH, while monitoring the approach to the target value directly, ensures that the proper buffer is produced. In contrast, the stock-mixing procedure requires the table or doing calculations using the Henderson-Hasselbach equation

$$pH = pK_a + \log \frac{[A^-]}{[HA]} \qquad (1)$$

where A^- is the less protonated conjugate base species and HA is the more protonated conjugate acid, the two species surrounding the target pH.

Four considerations will help ensure that the buffer is prepared correctly. First, the pK_a must be within one unit of the target pH in order to ensure that the buffer has sufficient buffer capacity to truly limit pH changes. Second, solutions must be equilibrated *at* the temperature used *in* the experiment (see above). Third, one must fine-tune the pH when large volumes of H_2O are added to dilute stock components to their final concentrations. Lastly, when preparing stock solutions of pH-dependent solution components (e.g., EDTA) one must adjust the pH using an immersed pH probe *while* dissolving the minimally soluble tetraprotonated powder. It will dissolve only as it becomes partly deprotonated. If not careful, one risks large errors in achieving the desired concentration.

6. Biotechnology Notes

It is instructive to place the concept of pH measurements in a broader context. It can be very important to determine the concentrations of other types of cations *in vivo* (e.g., Ca^{2+}). Some clever methods have been developed to accomplish this goal, e.g., the now classical technology developed by Roger Tsien and others during the 1980s. When a Ca^{2+} chelator, which is molecularly tethered to a partially inactivated fluorophore, binds cation, the fluorescent molecule becomes activated to fluoresce. As a result, the fluorescence is proportional to the $[Ca^{2+}]$ and one can study their fluctuations. These reagents have been used to follow progressions in cell cycle stages, development, diseased states, etc. Similar reagents have also been developed to determine $[K^+]$, $[Na^+]$, $[H^+]$ and others.

A related set of techniques has also been developed to study enzymatic reactions. These reagents consist of a molecular "cage" that carries an enzymatic reactant or ion cofactor and can be triggered with a flash of light of the appropriate

wavelength to release the contents of the cage, thereby initiating the reaction to be studied.

7. Spectroscopy

The *absorbance spectrum* is used in many, many protocols to determine concentrations of nucleic acids, proteins, and many important substrates and cofactors. Spectroscopic approaches have one key advantage over wet lab techniques: they are relatively noninvasive. In fact, materials sometimes emerge from the analysis sufficiently unscathed for further use.

Analytes, the subject of the spectroscopic experiment, are typically scanned in scaled-down reactions in quartz cuvettes. Spectra are also obtained using diluted analytical samples that have been prepared from "stock samples." This stock is also used to execute several reactions, in parallel, allowing use of the same characterized reactant, without requiring analysis of each individual sample.

The *spectrum* presents a two-dimensional plot of absorbance as a function of wavelength, usually scanned across the ultraviolet (UV) range (200 to 350 nm). Proteins containing one or more of the aromatic chromophores – phenylalanine, tyrosine, and tryptophan – will have reasonably intense absorbance. For example, 1 mg of an immunoglobulin G (M_r ca. 150 kDa) produces, very roughly, an absorbance at 280 nm (A_{280}) of 1.0. Nucleic acids always contain a large complement of their chromophoric nucleobases, producing more intense net absorbance than proteins. For example, a ca. 25 µg/ml transfer RNA solution (M_r 25 kDa) produces an A_{260} of about 1.0. These figures are approximations. The exact absorbance at a stipulated wavelength per mole of material is embedded in the "molar extinction coefficient" (ε_{280}), the key to the "Beer-Lambert equation" (Beer's Law).

Introductory lab 2 demonstrates the use of Beer's Law and how to acquire absorbance spectra with amino acids and nucleotide solutions. The student is exposed only to a very small sampling of the range of incredibly powerful spectroscopic techniques. Fluorescence, circular dichroism, Raman spectroscopy, and nuclear magnetic resonance are not covered in detail here, but are very important methods in biochemistry. Consult a text such as *Physical Biochemistry* by van Holde, Johnson, and Ho (Prentice-Hall, 1998) for detailed descriptions. Any current issue of *Biochemistry*, *Journal of Molecular Biology*, or even *Cell* will give illustrations of relevant.

8. Protein Concentration Determination Assays

The BCA method is used in Introductory lab 2. The basis of the technique builds on its popular predecessors the Bradford and Lowry methods. The reagents in the BCA reaction include bicinchonic acid and copper hydroxide. This technique begins with the Biuret reaction, which involves protein-induced reduction of Cu^{2+} to Cu^{1+}. In this reaction, Cu^{2+} atoms are reduced to Cu^{1+} by peptide groups in the protein backbone in a redox-coupled reaction. Second, a redox-active dye reacts with the Cu^{1+} that was produced by the peptide-Cu^{1+} redox reaction. Then the dye bicinchonic acid (BCA) is added, which produces an intense purple color by binding to and amplifying the Cu^{1+} signal. The intense purple color is proportional to the amount of peptide groups in the protein. The technique is preferred for two reasons, because it is less sensitive to inhibitory effects caused by other components in the samples (e.g., buffers, cations, substrates) and because all proteins react in proportion to the number of peptide groups. In contrast, the inferior A_{280} method requires knowledge of the number of aromatic amino acids per mole of protein, which differs from protein to protein, resulting in a semiquantitative analysis at best.

Bovine serum albumin (BSA) concentration standards are typically used to calibrate the measurements. A large range of BSA products are listed in a typical source catalog (e.g., Sigma), and products differ in lipid content, purity, and other factors. The researcher must make their choice carefully since assay standardization depends critically on the assumption that BSA acts analogously to the protein of interest. One should generalize this mindset to include all comparison-oriented experiments. It's very common to do so in

BCA redox-coupled reaction scheme:

Step 1 - Biuret reaction:

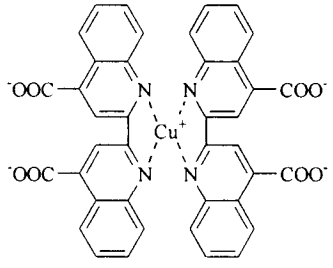
$1\ Cu^{2+}$ + 1 protein $\xrightarrow{\text{BCA reagent}}$ $1\ Cu^{1+}$ + 1 protein

(reduced peptide bond) OH^- (pale blue) (oxidized)

Step 2 - $BCA_2\ Cu^{1+}$ complex formation:

$1\ Cu^{1+}$ + 2 BCA $\longrightarrow$ BCA_2Cu^{1+} complex

(dark purple)



molecular biology. For example, one typically assumes proteins electrophorese in linear proportionality to log M_r in sodium dodecyl sufate (SDS) gel experiments. Unfortunately, this is not always the case in certain applications (e.g., when they are glycosylated).

9. An Aside

The point of view of the physicist typically limits study to a very small number of interacting species; simplicity rules. This is done to create a controlled situation that can be modeled mathematically and where statistical variations can be characterized thoroughly. A biological cell is exceedingly complicated, whether studied intact, as chemically treated ("fixed") preparations, as excised tissues, or as soluble biomolecules immersed in a reaction mixture. Problems associated with controlling variables increase dramatically as the size, number of interacting components, and complexity of relations increase. While our ability to probe these factors *in vivo* has advanced dramatically during our lives, especially with the advent of isotopic, tomographic, and nuclear magnetic resonance imaging methods, in most cases we're far from being able to pass a Star Trek-like medical instrument over a diseased or flawed body part in order to decipher detailed genetic problems or even fix them.

THEORY/PRINCIPLES
COURSE DESCRIPTION

1. Overview

The sets of techniques in Parts 1 and 2 are performed as part of a multistepped experimental chain designed to "subclone" a fragment of DNA and then verify that each substep was successful. This overall process involves purifying a defined sequence from a biological source, preparing its ends enzymatically, and then splicing it to a second larger fragment to create a replaceable circular plasmid. The location of this inserted fragment is designed to create a defined circular DNA sequence that supports the RNA synthesis process (transcription). The RNA product is designed to support use by the ribosome to make a specified protein (via translation).

In Part 1, students perform a project composed of restriction enzyme digestion, DNA ligation, DNA separation by cloning procedures such as restriction enzyme digestion, DNA ligation, DNA separation by agarose gel electrophoresis, plasmid isolation, and Southern blot analyses of DNA. A flowchart that shows the detailed connection between the subprojects is shown at the beginning of Unit 1.

In Part 2, students learn how to design expression vector constructs, and perform bacterial transformation, polyacrylamide electrophoresis, and immunoblot analysis of proteins, respectively. A flowchart is shown at the beginning of Unit 7. Researchers often resolve biomolecules that differ with respect to size, charge, polarity (electroneutrality), solubility (hydrophobicity), or in terms of substrate- or ligand-binding properties, using an appropriately selected chromatography method. Structural and solution factors control protein purification by affecting its binding capacity and catalytic function(s). Utilities that have been created by taking advantage of these functional groups are described, from a variety of viewpoints, in accompanying literature supplements. Procedures used to make plasmid derivatives that encode fused protein sequences are described. Ligand affinity chromatography is used to specifically purify the "fusion protein." Purification fractionates are analyzed to determine how quantitative functional activities distribute across the chromatography profile. As a general lesson, students learn how to make "tailored" proteins with the possibility of producing dual functions, or even introducing brand new biochemical capabilities.

An interconnected set of chromatography experiments is performed to help the students learn how to optimize their ability to confront less successful experimental outcomes – a common real-life situation. This challenge helps students develop an understanding of the types of questions to ask when you have to weigh the relative utilities of two different approaches, each of which produces marginal success. This touch of realism simulates a typical juncture in decision making. It is often necessary to proceed down both roads and then assess the relative merits of each path. The best path is chosen after measuring quantitative specific activities of samples from the four types of chromatography and the initial cellular extracts, and making direct comparisons. More knowledge regarding the specific identity of the proteins is obtained using protein immunoblotting experiments, after resolving them according to size by gel electrophoresis. We emphasize the utility and generalizability of protein- and antibody-enzyme conjugate technologies.

Students learn to think about exactly why molecules bind a set of surfaces with distinctly different compositions, including carbohydrate-based gel filtration resins and matrices containing either cation-exchange sites, alkane groups, or specified bioligands. The results show how solution conditions affect biomolecule-resin binding interactions and thereby the relative purities of the biochemical products

Putting this set of processes in terms that are defined in the glossary:

a. Subclone a DNA fragment of the *myo*-3 gene and express the encoded protein as a β-galactosidase-*myo*-3 fusion protein. *Myo*-3 is a gene that encodes one of four myosin heavy chain isoforms in the nematode *Caenorhabditis elegans*. The gene was obtained by heterologous hybridization selection from a genomic DNA library prepared from *C. elegans*.
b. Use antibodies generated to bind a β-galactosidase-*myo*-3 fusion protein to identify *E. coli* cells that translate protein from the cloned *myo*-3 expression vector.
c. Purify and analyze the purity of the fusion protein obtained from this expression.

2. Procedures

2.1 Part 1.

1. Prepare master plates of the cloned *myo*-3 gene vector and "empty" protein expression vector.
2. Large scale "plasmid preps" of the *myo*-3 gene-containing vectors (pW and p2D) and the β-galactosidase expression plasmid (pUR288).
3. Restriction digestion and gel electrophoresis of the *myo*-3 cloning vectors and expression vector with *Hin*dIII.
4. Isolate purified *myo*-3 DNA fragment and digested expression vector from excised agarose gel pieces.
5. Clone the 480-bp *myo*-3 *Hin*dIII fragment into pUR288.

6. Polymerase chain reaction amplification of *myo*-3 fragment from the expression vector to test for correct insert orientation and reading frame.
7. Prepare competent *E. coli* cells and transform them with the recombinant expression vector.
8. Identify, by immunoassay, colonies that were transformed with the *myo*-3-containing protein expression vector and translate the protein encoded by *myo*-3 correctly.
9. Isolate genomic DNA from 2 strains of *C. elegans* (CB1407 and N2) and perform *Hin*dIII restriction digestion.
10. Construct a *myo*-3 digoxigenin-labeled probe from the cloned *myo*-3 gene fragment.
11. Southern blot the *Hin*dIII-digested *C. elegans* genomic DNA and probe it with the digoxigenin-labeled DNA to verify the duplication of the *myo*-3 gene in one strain of genomic DNA.

2.2 Part 2.

12. Isolate colonies derived from a single *E. coli* colony and induce cultures with IPTG to stimulate the β-gal-*myo*-3 fusion protein promoter.
13. Harvest and disrupt *E. coli* cell walls to release the expressed fusion protein.
14. Purify fusion proteins by gel filtration column chromatography and quantitate protein concentration and perform β-galactosidase enzyme activity assays with eluted fractions.
16. Purify the fusion protein by ion exchange column chromatography and quantitate the protein concentration and perform β-galactosidase enzyme activity assays with eluted fractions.
17. Purify the fusion protein by substrate (galactose) affinity column chromatography and quantitate the protein concentration and perform β-galactosidase enzyme activity assays with eluted fractions.
18. Quantitate the protein concentration and perform β-galactosidase enzyme assays of eluted affinity chromatography fractions.
19. Construct an enzyme assay activity purification table.
20. Perform discontinuous SDS polyacrylamide gel electrophoresis (PAGE) to characterize the size and purity of the purified β-gal-*myo*-3 fusion protein.
21. Perform 'Western' immunoblot assay to verify correct protein folding and the extent of purification of materials at different stages of purification.

3. Useful Literature

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3.4 Ancillary Subjects.

1. Jack, R. C. (1995) *Basic Biochemical Laboratory Procedures and Computing*, Oxford University Press, N.Y.
2. Ravel, R. (1995) *Clinical Laboratory Medicine*, 6th Ed., Mosby-Year Book, Inc., Saint Louis, MO.
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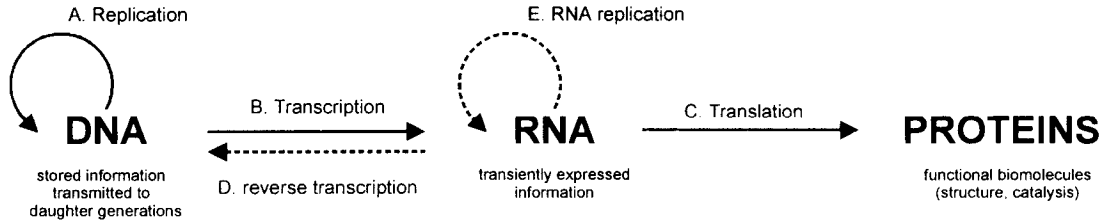
THEORY/PRINCIPLES

"CENTRAL DOGMA OF MOLECULAR BIOLOGY"



This key statement encompasses the essence of the field that has been named—for better or worse—"molecular biology." It outlines mechanisms, essential requirements, and information flow used to regenerate and propagate the genetic material of a cell. Pivotal roles are played by the following key biomolecules:

(A) DNA polymerase, template DNA, protein "factors" (helicases, single-strand binding proteins)

(B) RNA polymerase, mRNA transcription factors, repressors, activators

(C) ribosomes, aminoacyl tRNA synthetases, transfer RNA, amino acids, translational factors

Some life forms reproduce via two other important processes that illustrate nucleic acid metabolism that falls outside the limits of the traditional dogma. Nature is not dogmatic.

(D) reverse transcriptase – transcription catalyzed by this enzyme proceeds in the reverse direction, from RNA template to produce DNA. RNA tumor viruses (retroviruses) produce their DNA life-cycle form this way.

(E) Qβ replicase – with some viruses (e.g., Qβ) the genome is a RNA and replication is catalyzed by and RNA-dependent RNA; RNA is used to make replicated RNA.