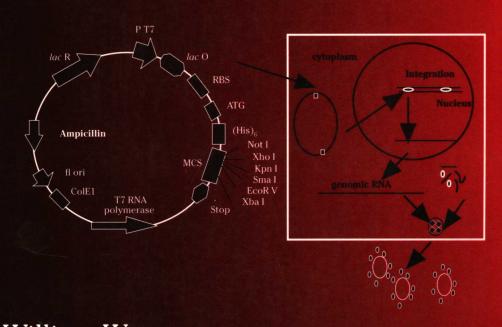
Methods in Gene Biotechnology



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Preface

We are living in an era of a biotechnology revolution. To many molecular and cellular biologists, the flood of information on new methodologies is particularly overwhelming because biotechnology is forging ahead and bringing about rapid changes day after day. Because every single organism depends on molecular actions for survival, molecular biology research has become more and more dominant in multiple disciplines. There appears to be a general tendency for NIH and other funding agencies to award grants with high priority to those research proposals that employ molecular biology approaches. How can one update one's knowledge of biotechnology and use the most recent, proven techniques for novel research? One of the aims of this book is to provide investigators with the tools needed for modern molecular and cellular biology research.

Another aim of this book is to guide graduate students in their thesis research. In our experience, good graduate training mandates independent performance with minimum advice from a mentor. How does one select a novel research project for a thesis? What are the hypotheses, objectives, and experimental designs? How does one grasp technical problems and master current techniques? Where does one begin and what are the predicted results? As a matter of fact, a graduate student needs some help with these questions. This book will provide the clues.

This book covers a wide range of current biotechnology methods that have been developed and are widely used in molecular biology, biochemistry, cell biology, and immunology. The methods and protocols described in the appropriate chapters include the following: approaches to novel research, rapid isolation of specific cDNAs or genes by PCR, construction and screening of cDNA and genomic DNA libraries, preparation of DNA constructs, nonisotopic and isotopic DNA or RNA sequencing, information superhighway and computer databases of nucleic acids and proteins, characterization of DNA, RNA, or proteins by Southern, northern, or western blot hybridization, gene overexpression, gene underexpression and gene knockout in mammalian systems, analysis of cellular DNA or abundance of mRNA by radioactive in situ hybridization (RISH), localization of DNA or abundance mRNA by fluorescence in situ hybridization (FISH), in situ PCR hybridization of low copy genes and in situ RT-PCR detection of low abundance mRNAs, new strategies for gene knockout, and large-scale expression and purification of recombinant proteins in cultured cells. Each chapter covers the principle(s), underlying methods, and techniques, in a detailed step-by-step description of each protocol, notes, tips, and troubleshooting guide. We have found that many of the currently available books in molecular biology contain only protocol recipes. Unfortunately, many fail to explain the principles and concepts behind the methods outlined or to inform the reader of possible pitfalls in the methods described. We intend to fill in these gaps.

For the information of the reader, Chapter 1 was written by William Wu, Peter B. Kaufman, and Michael J. Welsh. Chapters 2, 3, 6, 10, 12 to 14 were written by William Wu.

Chapters 8, 9, 16, and 17 were written by William Wu and Michael J. Welsh. Chapters 5, 7, and 15 were written by William Wu and Peter B. Kaufman. Chapters 4, 11, and 18 were written by William Wu and Helen Zhang. Nonetheless, all four authors have worked as a team.

W. Wu M.J. Welsh P.B. Kaufman H. Zhang

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Chapter 1

Approaches to Novel Research

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Introduction

We are living in the era of technological revolution. To many molecular biologists the flood of new information is particularly overwhelming, because biotechnology is forging ahead and bringing about changes day after day. How can one catch up with the new biotechnology, and use the most recent, proven techniques for novel research? As indicated in the preface, one of the aims of this book is to provide investigators with tools for molecular biology research. Perhaps because every single organism depends on molecular actions for survival, molecular biology research becomes more and more dominant in multiple disciplines. ¹⁻²¹ In fact, there is a general tendency for federal and private funding agencies to increasingly award grants to those research proposals that use molecular biology approaches. Given the fact that

research funding resources are quite limited, that the funding budget is decreasing, and that the number of research proposals rapidly increases year after year, the fundamental question is how can one bring funding to his research to still survive? One strategy is to write an excellent proposal using molecular biology tools. However, what catches the eyes in light of research projects and funding resources? How does one grasp research problems and march towards a new direction? How does one write an important and scientifically sound research proposal with new ideas, comprehensive designs, and methodologies? What is your research plan and where do you start? This chapter can provide some insights and ideas that may help novel research projects to obtain potential funding.

Another aim of this chapter is to guide graduate students in their thesis research. In our experience, good graduate training mandates independent performance with minimum advice from a mentor. How does one select a novel research project for a thesis? What are the hypotheses, objectives, and experimental designs? How does one grasp technical problems and master current techniques? Where does one begin and what are the predicted results? How does one interpret research data and decide on the next experiments? Assuredly, a graduate student needs some help with these questions. This chapter will provide some answers.

The major objective of this book is to help the reader pursue research in molecular biology. The present chapter serves as a guide to the use of current strategies and techniques in the book in order to develop novel research strategies. Several examples of research proposals of 2 to 5 years, with specific aims, strategic designs, and methods, are illustrated below. It should be noted that the following examples are not the actual proposal formats. It is hoped, however, that these examples will be a valuable guide for your novel research, proposal funding, and/or thesis research. The examples can also be adapted and applied to other appropriate novel research projects.

Proposal 1. Identification of a New Drug-Targeting Protein(s) and Isolation of a Novel Gene(s)

Drug discovery is one of the most interesting research goals to public health and is certainly invaluable to pharmaceutical companies. Once a new drug is produced, it will open up broad areas for new research. The fundamental question concerns its cure mechanism. Many kinds of research can be conducted using the drug. In our view, the most promising research proposal offers identification of the drug-targeting protein(s) and isolation of a novel gene(s). Because molecular interaction is the basis for the cure of a disease with a new drug, it is reasonable to hypothesize that there may be one or more proteins or genes that are potentially targeted by the drug. Because the discovery of a new drug is of great concern to the public, this plan would most likely get funded by NIH, pharmaceutical companies, or other private sector groups. Figure 1.1 illustrates the strategies, research design, and molecular biology methodologies that one can use, along with references to the appropriate chapters.

Proposal 2. Exploration of the Functions or Roles for the Expression of a Gene Targeted by a New Drug

Once a novel protein or gene targeted by a new drug or an important chemical has been identified, further research needs to be done. One logical and promising funding project is determining the function of the targeted protein or gene. The information from the research

Specific Aim 1. Identification of a new drug-targeting protein

- 1 Radio-labeling of the drug.
- 2. Radio-binding assay of the drug to a protein mixture from cell- or tissue-type.
- Identification of drug-binding protein(s) by SDS-PAGE and/or 2-D gel electrophoresis (Chapter 11).
- 5 Digestion of the protein(s) with trypsin and/or cyanogen Purification of the bound protein(s) (Chapter 11). 4

direction.

Searching of GenBank for similarity between the drug-targeted bromide (CNBr) and sequencing of peptide fragments of the protein(s) (Protein Sequencer's Instructions) protein(s) and other known proteins (Chapter 6).

CONGRATULATIONS ON YOUR SUCCESS IN THE **IDENTIFICATION OF A NEW DRUG-**TARGETED PROTEIN(S)!!!

encoding the drug-binding protein 2. Rapid isolation and sequencing of partial-length the amino acid sequence (Chapter 2). protein, you are heading in the right For a new

1 Design and synthesis of oligonucleotides based on

Specific Aim 2. Cloning and isolation of the novel gene

- Isolation and sequencing of the full-length cDNA cDNA by PCR (Chapters 2 & 5)
 - GenBank searching for potential novelty of from a cDNA library (Chapters 3 & 5) the cDNA (Chapter 6).
- from a genomic DNA library (Chapters 15, 5 & 6) 5 Isolation and characterization of the genomic gene

CONGRATULATIONS ON YOUR SUCCESS IN **ISOLATING A NOVEL GENE TARGETED** BY A NEW DRUG!!!

FIGURE 1.1

Research design and methodologies for the identification of a novel gene(s) targeted by a new drug.

will provide crucial evidence for the cure mechanism of a disease by the drug. This involves sophisticated skills, including generation and use of transgenic mice as an animal model (Figure 1.2).

Proposal 3. Verification of Potential Function of a Specific Gene by the Gene Knockout Approach

To verify the potential role of a novel important gene, the best strategy is to knock out the expression of the gene. For example, if it is reasonable to believe that a gene plays a key role in heart development, a smart approach is to target the gene *in vivo* by knockout. If the gene becomes null, diseases such as failure of heart development would be predicted to occur based on the hypothesis. This is certainly a very sound proposal with promising funding for 3 to 4 years, assuming that no previous grants have been awarded for this type of proposal to other laboratories. The general approaches and methods are diagrammed in Figure 1.3.

Proposal 4. Identification of the Functional Domain of a Protein by Site Specific Mutagenesis

Very often, the functions of a novel protein have been demonstrated but no one knows which specific fragment of the protein is the active domain or binding site. If the protein or enzyme is very important, there is a good reason to write a 2-year proposal on the identification of the functional domain of the novel protein or enzyme. The strategies for doing this are outlined in Figure 1.4.

Proposal 5. Identification of Toxicant-Binding Protein(s) and Isolation of a Novel Gene that Is Related to the Toxicant and Heart Hypertrophy Using an Animal Model

The heart is the first organ formed in animals, and diseases such as heart hypertrophy, heart attack, or failure are of great concern to the public. Additionally, toxicity mediated by toxicants becomes more and more of an environmental concern. If there is good reason to hypothesize that a toxicant (e.g., chemicals, proteins, drugs, carbohydrates) may be an inducer of heart hypertrophy, a 4- to 5-year proposal would be profound and promising for funding. This is a long-term research project that may need collaboration between laboratories. Specific aims, strategies, designs, expected results, and methodologies, as found in the appropriate chapters, are outlined in Figure 1.5.

Proposal 6. Rescue of an Immune-Deficient System Via Gene Therapy

It is well known that an immune deficiency disease, such as HIV, is of great concern to the public at the present time. Many important proteins, such as the CD families, have been discovered to play a significant role in the deficient immune system. In order to increase the

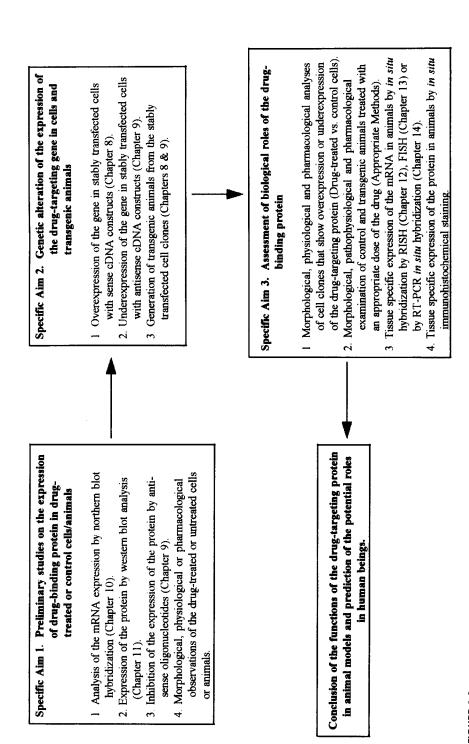
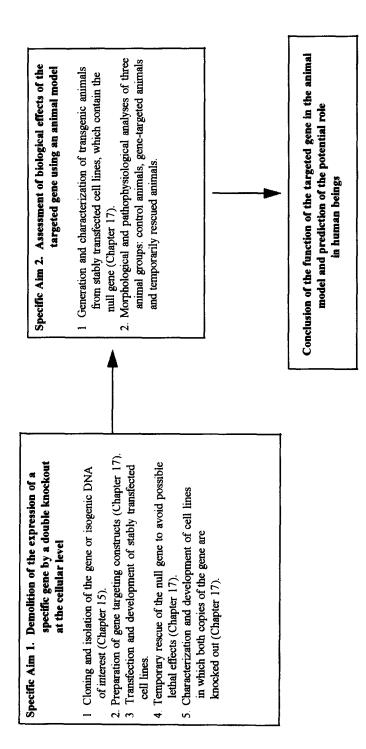


FIGURE 1.2

Research approaches and methods for exploration of the potential functions of a new drug-targeting protein using cultured cells and animal models.



Research approaches and methods for exploration of the potential functions of a new drug-targeting protein using cultured cells and animal models. FIGURE 1.3

Specific Aim 1. Mutagenesis in vitro of the cDNA coding for

- the functional protein of interest
- 1. Isolation of the cDNA encoding the target protein (Chapter 3) 2. Preparation of cDNA mutations by site-specific mutagenesis or by serial deletions (Chapter 5).
 - Subcloning and isolation of mutant cDNA constructs for functional analysis (Chapter 4).

Specific Aim 2. Functional analysis of the mutant protein

- 1 In vitro assay of the mutant proteins (e.g., phosphorylation, dephosphorylation, protein-protein interaction and identification of the specific functional domain or region.
- Transfection of cells with the mutant cDNA constructs and analysis of the potential roles of the mutant proteins at the cellular level (Chapters $8 \ \& 9$)
 - 3. Functional assessment of the mutant proteins *in vivo* by generation of transgenic animals from the transfected cell clones, which will be used as an animal model to study the function of the protein in depth.

Conclusion of the identification of the functional domain of the candidate protein and future research highlights.

FIGURE 1.4

Design and methods for identification of a functional domain(s) for a given protein by mutagenesis.

Specific Aim 1. Preliminary studies on potential heart hypertrophy-inducible toxicants in treated- and untreated-mice or rats

- Morphological, toxicological and cardiovascular observations of the heart tissues from animals treated versus untreated with the toxicant of concern (Appropriate methods).
- Identification of the toxicant-induced or -repressed protein(s) by 2-D gel electrophoresis (Chapter 11) in normal heart and hypertrophy tissues.

Ьį

 Labeling of the toxicant and identification of the toxicantbinding protein(s) by 2-D gel electrophoresis (Chapter 11).

Specific Alm 4. Assessment of biological roles of the drugbinding protein

- Morphological, physiological and toxicological analyses of cell clones that show overexpression or underexpression of the targeting protein (treated vs. control cells).
 - Morphological, cardiovascular and toxicological examination of control and transgenic animals treated with an appropriate dose of the toxicant (Appropriate Methods)
- 3. Tissue specific expression of the targeting mRNA in animals by *in situ* hybridization by RISH (Chapter 12), FISH (Chapter 13) or by *in situ* RT-PCR (Chapter 14)
- 4. Tissue-specific expression of the targeting proteins in animals by in situ immunohistochemical staining.

Specific Aim 2. Characterization of the toxicant-binding protein(s) and identification of novel genes

- 1 Purification of the targeted protein(s) (Chapter 11).
- 2. Digestion of the protein(s) with trypsin and/or cyanogen bromide (CNBr) and sequencing of peptide fragments of the protein(s) (Protein Sequencer's Instructions).
 - Searching of GenBank to identify the targeted, novel or known protein(s) (Chapter 6).
- 4. Design and synthesis of oligonucleotide primers based on the amino acid sequence of the targeted protein(s) identified in Aim 2 and isolation of partial-length cDNA by PCR or 5 '-RACE (Chapter 2).
- Identification of the toxicant-induced or -repressed mRNA species by subtractive cDNA cloning (Chapter 3).
 - 5. Sequencing of cDNA and searching of GenBank for novel gene(s) (Chapters 5 & 6).

Specific Aim 3. Genetic alteration of the expression of the toxicant-targeting gene in cells and transgenic animals

- 1. Overexpression of the gene in stably transfected cells with sense cDNA constructs (Chapter 8).
- Underexpression of the gene in stably transfected cells with antisense cDNA constructs (Chapter 9).
 - 3. Generation of transgenic animals from the stably transfected cell clones (Chapters 8 & 9).

FIGURE 1.5

Comprehensive research design and methods for identification of a novel protein(s) and gene(s) in heart hypertophy induced by a toxicant using an animal model.