LABORATORY MANUAL

Molecular Techniques: Protein Chemistry and Molecular Cloning

August 20 - October 2, 2002

Interdisciplinary Center for Biotechnology Research
University of Florida

TABLE OF CONTENTS

Acknowledgments

In the true spirit of interdisciplinary collaboration, many have worked together in the organization of this course. Our thanks to all for their generosity of time and willingness to share their expertise.

Special thanks to:

Dr. Phil Laipis, Dr. David Silverman and Dr. Gino vanHeeke for providing us with the plasmid construct used for the *in vitro* expression of CAII.

Dr. John Aris for designing the immunostaining exercise
All faculty members who have kindly agreed to lecture in this course.

ICBR Education and Training Core staff

David Moraga Amador, Ph.D., Scientific Director
Nancy Denslow, Ph.D., Protein Chemistry Instructor
Bill Farmerie, Ph.D., Scientific Advisor
Sharon Norton, B.S., Senior Biological Scientist
Larbi Gallagher, M.S., Biological Scientist
James Smith, Student Assistant
Teresa Benton, Registration Officer

Laboratory Instructors

Bill Farmerie, Ph.D., ICBR Molecular Services Core
David Moraga Amador, Ph.D., ICBR Education and Training Core
Nancy Denslow, Ph.D., ICBR Protein Chemistry Core
Patrick Larkin, Ph.D., Protein Chemistry Core
Scott McMillen, ICBR Protein Chemistry Core

Lecturers

Mavis Agbandje-McKenna, Ph.D., Department of Biochemistry and Molecular Biology, COM
Arthur Edison, Ph.D., Department of Biochemistry and Molecular Biology, COM.
Nancy Denslow, Ph.D., ICBR Protein Chemistry Core
Greg Erdos, Ph.D., ICBR Electron Microscopy Core
Bill Farmerie, Ph.D., ICBR Molecular Services Core
Dan Purich, Ph.D., Department of Biochemistry and Molecular Biology, COM
Susan Frost, Ph.D., Department of Biochemistry and Molecular Biology, COM
John Aris, Ph.D., Department of Anatomy & Cell Biology
Phil Laipis, Ph.D., Department of Biochemistry and Molecular Biology
Patrick Larkin, Ph.D., Department of Biochemistry and Molecular Biology
David Moraga Amador, Ph.D., ICBR Education and Training Core
Paul Klein, Ph.D., Pathology, Immunology and Laboratory Medicine, COM
Brian Cain, Ph.D., Department of Biochemistry and Molecular Biology
Maurice Swanson, Department of Molecular Genetics and Microbiology
David Powell, Ph.D., Chemistry Department
Sheldon Schuster, Ph. D., ICBR Director, Department of Biochemistry & Molecular Biology
Henry Baker, Ph.D., Department of Molecular Genetics and Microbiology
Paul Gulig, Ph.D., Department of Molecular Genetics and Microbiology
Ben Dunn, Ph.D., Department of Biochemistry and Molecular Biology
Chris West, Ph.D., Department of Anatomy & Cell Biology

This manual was prepared by David Moraga Amador, Patrick Larkin and Scott McMillen. Many of the procedures have been adapted from previous IDP Laboratory manuals and other ICBR workshops (Protein Chemistry, Recombinant DNA Analysis, and Differential Gene Expression).

The content of the manual also reflects the valuable contribution of Bill Farmerie and Nancy Denslow. Several of the illustrations used in this manual were adapted from the Bio-Rad® (Hercules, CA) Mini-Protean III and PROTEAN® IEF Cell instruction manuals.

LABORATORY SCHEDULE

Tuesday, August 20

8:30	-	8:45	**Laboratory Course Objectives** (Dr. David Moraga) Room C1-7
8:45	-	9:30	**Lecture:** Carbonic Anhydrase Model (Dr. David Silverman) Room C1-7
9:30	-	10:30	**Lecture:** Introduction to Protein Structure (Dr. Nancy Denslow) Room C1-7
10:45	-	11:15	**Introduction to the lab:** Dr. David Moraga. Room MDL-6 (CG-29)
11:15	-	12:45	**Exp. 2**: Lysis of *E.coli* .
12:45	-	1:15	**Lunch (on your own).**
1:15	-	2:00	**Lab:** Bioinformatics I: Introduction to Web-based tools (Bill Farmerie/Li Liu)
2:00	-	3:15	**Exp. 4:** Affinity purification.
3:15	-	4:15	**Exp 3A,B,C:** SDS-PAGE Analysis of bacterial lysate fractions.
4:15	-	5:30	**Exp. 4 cont. and Exp. 5:** Affinity purification and Gel filtration. **Exp. 3D:** gel staining/destaining

Lab assistants: Exp. 5, concentrate samples O/N using Amicon filters.

Wednesday, August 21

Lab assistants: Put gels from Exp. 3 in 2% glycerol.

8:30	-	9:30	**Lecture:** Protein Purification (Dr. Nancy Denslow) Room C1-7
9:30		10:30	**Lecture:** Theory of Electrophoresis (Dr. Nancy Denslow) Room C1-7 **Exp. 8**: Minigel apparatus. Pour separating gel. Tris-glycine system.
10:30	-	11:30	**Exp. 6:** Protein concentration assay (Excel help, Patrick Larkin)
11:30	-	12:00	**Exp. 3E:** Gel Drying **Exp. 8:** Pour stacking gel
12:00	-	12:30	**Lunch**
12:30	-	1:30	**Exp. 8:** Prepare, load samples, electrophorese.
1:30	-	2:15	**Exp. 11A:** Set up O/N rehydration of Two-D gels (Marjorie Chow)
2:15	-	3:15	**Exp. 9A:** Set-up protein transfer.
3:30	-	5:00	**Graduate Students Reception: Founders Gallery**

Lab Assistants: Exp. 9B,C: 4:15-5:00 Stop Western Transfer. Block O/N

Thursday, August 22

8:30	-	9:30	**Lecture:** Post-translational Modifications of Proteins (Dr. Chris West) Room C1-7
9:30	-	10:30	**Lecture:** Chromatography of Proteins (Dr. Dan Purich) Room C1-7
10:30	-	11:00	**Exp. 9C:** Wash and add 1^{o} Ab to immunoblots. (MDL-6)
11:00	-	12:00	**Lab lecture:** Bioinformatics II: Introduction to BioTools (Gene and Peptools) (Bill Farmerie/Li Liu)
12:00	-	12:30	**Exp. 9C:** Wash PVDF/Add 2^{o} Ab.
12:30	-	1:00	**Lunch**
1:00	-	1:45	**Exp. 11B:** Start first dimension of Two-di electrophoresis.
1:45	-	2:30	**Exp. 9C:** Finish Western blots (wash, color substrate addition).
2:30	-	4:30	**Exp. 12A:** Introduction and set up enzyme digestions for Mass Spec.
4:30	-	5:00	**Lab lecture:** Bioinformatics III: Assignment of projects (Bill Farmerie/Li Liu)

Lab assistants: Exp. 12: If necessary, terminate trypsin digestion for every one at 5:30 PM and dry in speed vac O/N

Friday, August 23

8:30	-	9:30	**Lecture:** Membrane Proteins Characterization (Dr. Susan Frost) Room C1-7
9:30	-	10:30	**Lecture:** Mass Spectrometry of Proteins (Dr. David Powell) Room C1-7
10:30	-	11:30	**Exp. 11C,D**: Equilibration of IEF strips and begin 2^{o} dimension (Room MDL-6)
11:30	-	12:30	**Lecture:** Computer Modeling of Proteins (Dr. Ben Dunn) Room MDL-6
12:30	-	1:00	**Lunch**
1:00	-	1:30	**Exp. 11D, E:** Disassemble two-dimensional gel (2^{o} dimension), fix and stain O/N
1:30	-	3:30	**Exp. 12A,B,C:** Mass Spectrometry, sample spotting on plates.
3:30	-	5:30	Structural Analysis of Proteins: Tour (Dr. Agbandje-M., Dr. McKenna, Dr. Edison)

Monday, August 26

2:00	-	3:00	**Lecture:** Antibodies (Linda Green) Room C1-9
3:00	-	4:00	**Lecture:** Confocal and Electron Microscopy (Dr. Greg Erdos) Room C1-9

Tuesday, August 27

10:30	-	11:45	**Experiment 13:** Fixing, blocking, addition of primary Ab.
11:45	-	12:45	**Lecture**: Immunocytochemistry: Principles and Applications (Dr. Jon Aris) MDL-6
12:45	-	1:15	**Lunch**
1:15	-	1:45	**Experiment 13:** Washing and addition of secondary Ab.
2:45	-	3:30	**Experiment 13:** Washing and mounting of slides
3:30	-	5:30	**Exp. 12: Mass Spec Experiment** Groups 21, 22, 23 (room CG-22): 3:30-4:30 Groups 18, 19, 20 (room CG-22): 4:30-5:30 **Exp. 13: Immunocytochemistry slides viewing** (Room MDL-6) Groups 1, 2 (3:30-4:00 PM) Groups 3, 4 (4:00-4:30 PM) Groups 5, 6 (4:30-5:00 PM) Groups 7, 8 (5:00-5:30 PM)

Wednesday, August 28

10:30	-	4:00	**Exp. 13: Immunocytochemistry slides viewing** (Room MDL-6) Groups 9,10 (10:30-11:00 AM) Groups 11, 12 (11:00-11:30) Groups 13, 14 (11:30-12:00) Groups 15, 16 (12:00-12:30 PM) Groups 17, 18 (12:30-1:00) Groups 19, 20 (1:00-1:30) Groups 21, 22 (1:30-2:00) Groups 23, 24 (2:00-2:30)
10:30	-	5:30	**Exp. 12: Mass Spec Experiment** (room CG-22) Groups 15, 16, 17 (10:30-11:30 AM) Groups 1, 2, 3 (11:30-12:30 PM) Groups 4, 5, 6 (1:00-2:00) Groups 7, 8, 9 (2:00-3:00) Groups 10, 11, 12 (3:00-4:00) Groups 13, 14 (4:00-5:00)
11:00	-	12:00	**Lab Test 1: First offer**
4: 00	-	5:00	**Lab Test 1: Second offer**

Thursday, August 29		
1:00-3:00 PM	Radioisotopes Use short course	Rm C1-3
Friday, August 30		
1:00-3:00 PM	Radioisotopes Use short course	Rm C1-17

Tuesday, September 3

10:30	-	12:30	**Exp. 14A:** RNA Isolation.
12:30	-	1:00	**Lunch**

1:00	-	2:15	**Exp. 14B:** Cleaning apparatus, pouring gel, sample prep, loading gel
2:15		3:30	**Lecture:** PCR Theory (Dr. Bill Farmerie)
3:30	-	4:00	**Exp. 14B**: Stain, Destain, Picture
4:00	-	5:00	**Lecture**: Bioinformatics IV-BioTools for Primer design (Bill Farmerie/Li Liu).

Lab assistants: Exp. 114A: RNA Spectrophotometric assessment

Wednesday, September 4

10:30	-	11:00	**Exp. 14B:** Interpreting RNA gels. Quantifying RNA from gels
11:00		12:00	**Exp. 15B:** Designing your own primers for RT-PCR of mouseCAII (computers!)
12:00	-	12:45	**Lunch**
12:45	-	2:00	**Exp. 15A**: Designing your own optimization experiment for the RT-PCR of mouse CAII using your own primers and set-up reactions.
2:00	-	3:00	**Exp. 15A:** Set up cDNA synthesis and PCR amplifications
3:00	-	3:45	**Lecture**: Microarray-based approaches to Environmental Research (Dr. Patrick Larkin) Room MDL-6
3:45	-	5:15	**Exp. 19B:** Plasmid minipreps/ Set up restriction enzyme digestions of cDNA clones.

Monday, September 9

2:00	-	3:00	**Lecture:** Bacterial Physiology and Genetics (Dr. Paul Gulig) Room C1-9
3:00	-	4:00	**Lecture:** Cloning Vectors (plasmids, etc.) (Dr. Paul Gulig) Room C1-9

Tuesday, September 10

10:30	-	11:15	**Exp. 16A:** Gel electrophoresis of RT-PCR samples.
11:15	-	12:00	**Exp. 19C:** Set up PCR of diluted plasmid minipreps of cDNA clones
12:00	-	12:45	**Lunch**
12:45	-	1:45	**Exp. 19D:** QIAquick clean up of PCR products/ concentrating.
1:45	-	2:00	**Exp. 16A:** Stop electrophoresis/ stain/ Lab assistants de-stain and photograph.
2:30	-	4:00	**Exp. 16A,B,C:** Stain and excise bands. Purify DNA from gel slice. Pour gel.
4:00	-	5:15	**Exp. 16C**: Analytical gel electrophoresis of pure PCR products.

Lab assistants: Stain and take pictures for Exp. 16C.

Wednesday, September 11

10:30	-	11:00	**Exp. 19E:** Agarose gel analysis of restriction digests and purified PCR products
11:00	-	12:00	**Lecture:** Practical aspects of membrane macroarray design and analysis (Dr. Patrick Larkin)
12:00	-	12:45	**Lunch.**
12:45	-	1:30	**Exp. 16D:** Start TOPO ligation system (bacterial transformation)
1:30	-	2:00	**Exp. 19E:** Staining of agarose gel/ photodocumentation
2:00	-	2:15	**Exp. 16D:** Continue TOPO cloning.
2:15	-	3:15	**Lab Discussion:** Quantifying DNA from gel picture (3 pictures: Macroarray clones -adjust conc., Genomic DNA gel-provided, Dig probe gel-provided)
3:15	-	3:45	**Exp. 16D**: Spread X-gal on agar plates. Plate cells.
3:45	-	5:00	**Exp. 19F:** Spotting of nylon membranes- Macroarrays.

Monday, September 16

2:00	-	3:00	**Lecture:** Critical Parameters for PCR (Dr. Bill Farmerie) Room C1-9
3:00	-	4:00	**Lecture:** DNA Chips (Dr. Henry Baker) Room C1-9

Wednesday, September 18

10:30	-	11:15	**Exp. 17D:** Agarose electrophoresis of pre-digested DNA for Southerns (given)
11:15	-	11:45	**Exp. 16E**: Begin overnight cultures from individual bacterial colonies.

11:45	-	12:30	**Lunch**
12:30	-	1:30	**Lecture**: Bioinformatics V-*In Silico* cloning (Bill Farmerie/Li Liu).
1:30	-	2:30	**Lab: Exp. 16F.1:** Computer time for in *In Silico* cloning
2:15	-	2:30	E**xp. 17D:** Bring gels to staining station. Lab assistant will stain and take
2:30	-	4:00	**Exp. 17E:** Set up Southern Transfer
4:00	-	5:00	**Lab talks:** Southern Blotting, Blue-white screening (David Moraga)

Monday, September 23

2:00	-	3:00	**Lecture:** Site-Directed Mutagenesis (Brian Cain), Room # C1-9
3:00	-	4:00	**Lecture:** Yeast two-hybrid System (Maurice Swanson), Room # C1-9

Tuesday, September 24

8:30	-	10:30	**Library Skills (First 1/2 class)**
10:45	-	12:00	**Exp. 16E2-E3, F, and G:** Plasmid miniprep, set up restriction enzyme digestions, and pour gel.
12:00	-	12:45	**Lunch**
12:45	-	1:15	**Exp. 16G:** Load gels for insert orientation determination.
1:15	-	1:45	**Exp. 17E:** Dismantle Southern, UV-crosslink.
1:45	-	2:15	**Exp. 17E-F1:** Begin Southern hybridization.
2:15	-	2:30	**Exp. 16G:** Stain gels and photograph.
2:30	-	3:30	**Lab discussion:** Probe amounts, denaturation, etc. **Exp. 17F1**: Denature Dig-labeled probe and begin hybridization O/N
3:30	-	4:45	**Lab lecture:** Methods for quantitating mRNA (Northerns/RPA/RT-PCR/Taqman) Dr. Bill Farmerie

Wednesday, September 25

10:30	-	12:30	**Analysis of Macroarray Data**: Dr. Patrick Larkin
12:30	-	1:15	**Lunch**
1:15	-	4:00	**Exp. 17F1, F2:** Finish Southern hybridization.
4:00	-	5:00	**Final Lab discussion**

Thursday (September 26)
1:00-3:00 PM both days Biosafety Short Course Rm Shands 6120
Friday (September 27)
1:00-3:00 PM Biosafety Short Course Rm C1-15

Monday, September 30

2:00	-	3:00	**Lab Test** (Room MDL-6)

Tuesday, October 1 (Bioinformatics presentations: 20 minutes each) Room MDL-6

8:30	-	10:30	**Library Skills (Second 1/2 class)**
10:30	-	12:00	Bioinformatics presentation help session
12:00	-	1:00	**Lunch**
1:00	-	5:00	**Bioinformatics Project Presentations:** Groups 1-10

Wednesday, October 2 (CONTINUE PRESENTATIONS)

10:50	-	12:30	Groups 11-15
12:30	-	1:00	**Lunch**
1:00	-	5:00	Groups 15-23

Section 1: Protein Chemistry: Protein Extraction, Protein Purification and Enzyme Assay

Experiments 1-7

Experiment 1: Expression of Recombinant Carbonic Anhydrase in *E. coli.*

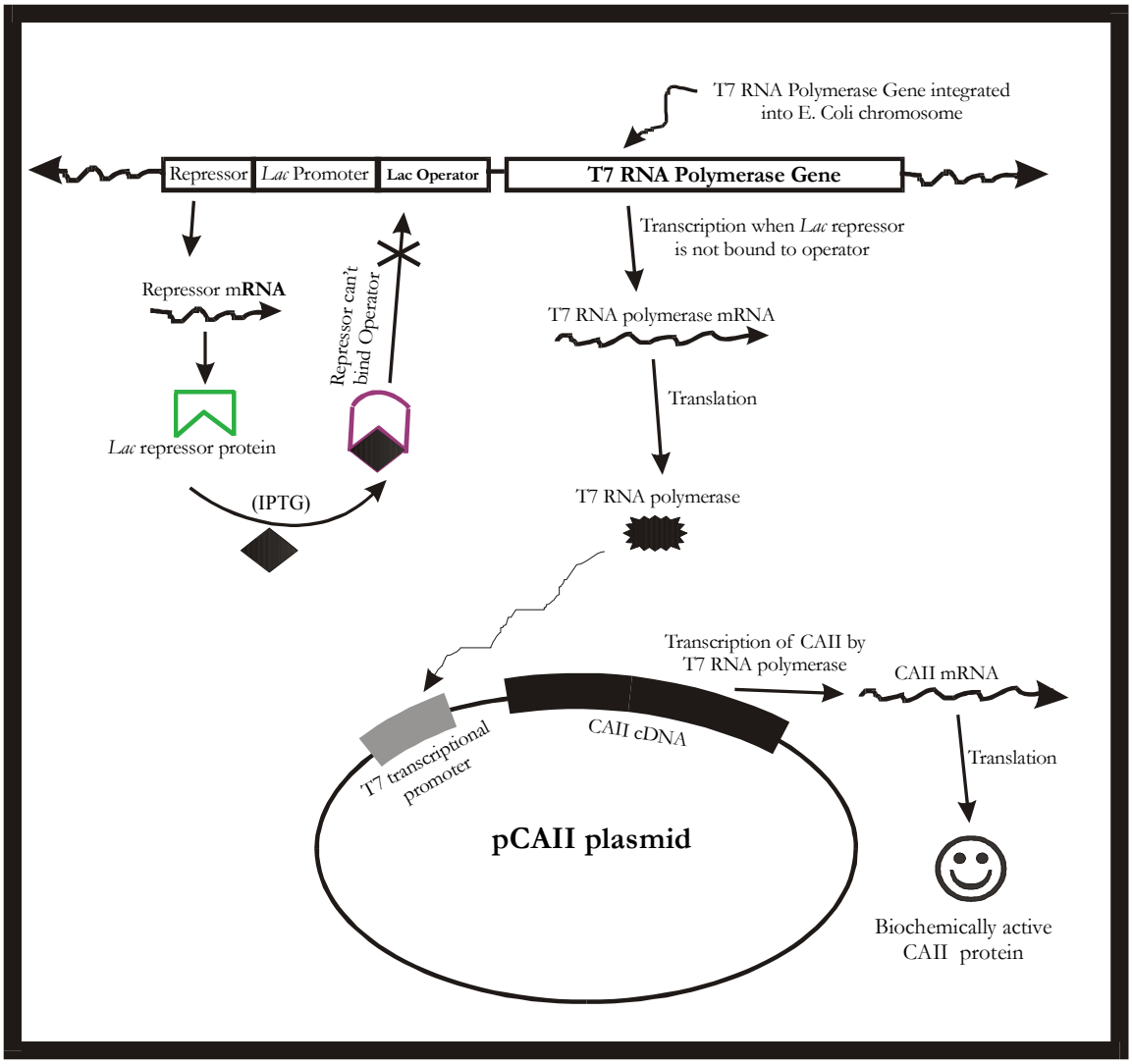
This Experiment utilizes BL21(DE3), an *Escherichia coli* strain transformed with a plasmid vector called pCA. This plasmid contains a recombinant human gene coding for carbonic anhydrase (hCAII). In this vector, the expression of hCAII is controlled by a bacteriophage T7 RNA polymerase promoter element. These promoter sequences are not recognized by endogenous *E. coli* RNA polymerase. However, BL21(DE3) also contains bacteriophage T7 RNA polymerase in its chromosome, downstream from a promoter and operator derived from the lactose operon. Bacterial synthesis of T7 RNA polymerase is induced by the addition of an inhibitor of the lac repressor protein.

Such cascade of events is a common strategy in the bacterial expression of recombinant proteins because many exogenous proteins may be "toxic" to *E. coli* (i.e., cell growth is inhibited by the overexpression of the exogenous protein). In practice, cells are allowed to grow undisturbed under optimum conditions until they reach the upper portion of the logarithmic growth phase. At this point, the inducer of T7 RNA polymerase expression is added thus precipitating the series of events that ultimately result in the expression of hCAII.

A large amount of the recombinant protein is obtained largely due to two factors: 1) The T7 RNA polymerase promoter is a "strong" promoter element, and 2) The huge number of bacterial cells in the culture that are induced to synthesize the foreign protein. The point at which cells are induced and the length of the induction period must be optimized for every experiment.

In summary, bacterial expression of hCAII in this experiment is accomplished through the following cascade of events:

- Isopropyl thiogalactoside (IPTG), a non-hydrolyzable analog of lactose, is added to the cell mixture, inactivating the *lac* repressor protein that is normally bound at the *lac* operator site in the bacterial chromosome.
- Dislodging of the lac repressor from the lac operator (derepression of the lac operator) allows bacterial RNA polymerase to recognize the lac promoter thus resulting in the transcription of the gene for T7 RNA polymerase.
- The T7 RNA polymerase in turn transcribes the hCAII cDNA gene on the plasmid, resulting in the production of hCAII.



Background on Protein Isolation Techniques

There are many reasons why one may choose to isolate a protein. Often, the goal is simply to determine the level of expression of a given protein in a tissue or cell type. However, if the goal is to isolate and purify the largest possible quantity of a protein, it is advantageous to identify a tissue where the desired protein is highly expressed. Many of the classical approaches to protein characterization have been simplified by the advent of gene cloning and the development of heterologous protein expression systems. In general, however, protein purification issues remain the same regardless of the protein source. In the case of recombinant proteins expressed in prokaryotes (e.g. the protein used in this workshop), the purified protein yield and level of biological activity is influenced by many factors, including but not limited to the following:

a) strain differences
b) choice of growth media
c) whether the cells are processed immediately or stored frozen before use
d) the choice of buffers
e) the presence of protease inhibitors in the isolation buffers
f) resuspension densities
g) osmolarity of the resuspension buffer
h) growth phase at which the cells are harvested

Optimal isolation conditions, i.e., conditions that allow the highest possible level of biologically active protein, are determined by trial and error.

Several books review extensively protein purification strategies and procedures, two of these sources are provided below:

1. Deutscher, M.P. (ed.) 1990. Guide to Protein Purification. *Methods in Enzymology* **182**, Academic Press, San Diego.
2. Scopes, R.K. 1994. Protein Purification Principles and Practice. Third Edition. Springer-Verlag, New York.

Parts A, B and C have been completed by the laboratory staff prior to today. The procedure is included here for discussion.

Part A: Growth of cells containing a CAII expression plasmid

1. In a sterile 500 ml Erlenmeyer flask, prepare 100 ml of Luria Broth supplemented with 100 µl of a 12.5 mg/ml tetracycline solution in 50% (v/v) ethanol/water (LB^{tet}). In a separate flask, prepare the same solution for the control.
2. Inoculate the LB^{tet} with a single colony from a fresh plate of BL21(DE3)/pCA (These are *E. coli* cells that have been transformed with a plasmid into which CAII has been cloned). Also, inoculate the control with a single colony.
3. Grow both cultures at 37°C with moderate agitation (300 rpm) for 3-4 hr. Monitor the growth of the culture by measuring the optical density at 550 nm. The cells are ready for induction when the optical density reaches 0.6 to 0.8.

Part B: Induction of carbonic anhydrase synthesis

1. Add 250 µl of 5 mM $ZnCl_2$ to the bacterial culture to make the final $ZnCl_2$ concentration ~12.5 µM. Repeat for the control bacterial culture.
2. Add 400 µl of 0.1 M IPTG to the bacterial culture to induce the synthesis of recombinant carbonic anhydrase (CAII). Leave out the 0.1 M IPTG in the control.
3. Incubate the cells at 30°C an additional 4-5 hr in a gyrotory shaker (300 rpm).

Part C: Harvesting and washing bacterial cells

Important: For all remaining steps, keep the cells and solutions on ice as much as possible.

1. Decant 50 ml of cells into a clean 50 ml conical centrifuge tube. Perform the same procedure for the control.
2. Harvest cells by centrifugation at 3000 rpm, 4°C, for 10 min in a Sorvall RT6000B centrifuge.
3. Pour off supernatant into waste container with disinfectant solution.
4. Decant an additional 50 ml of cells into the same 50 ml conical centrifuge tube and harvest as before, so that every tube contains a cell pellet that corresponds to a 100 ml culture.

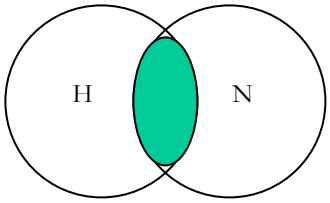
5. Rinse cells as follows. Resuspend bacterial cell pellet in 10 ml of 50 mM Tris, pH 7.6. Centrifuge at 3000 rpm for 3 min to pellet cells.
6. Pour off supernatant into waste container.
7. Repeat steps 5 and 6
8. Allow pellet to drain and store frozen at -20°C if necessary before proceeding to the cell lysis step. Remember, you should have one cell pellet for cells that were induced and one for the control (cells that were not induced). The latter pellet will be provided to you by the lab instructors.

Part D: Molecular biology databases as research tools

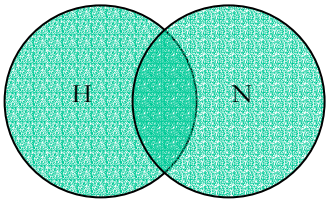
Finding a DNA sequence has become easier in the past few years. No longer are sequences published in journals, taking up space and making it necessary for extensive library searches and interlibrary loans. Anyone with an Internet connection has access to databases for finding just about any sequence that has been analyzed to date. Some of the difficulties include, knowing where to find the databases, knowing how to search the databases, and wading through all of that data that are available.

Once you know where some of the databases are (see appendix), your next step is learning some basic search techniques. Most databases are based on something called BOOLEAN logic. These databases allow for phrases such as 'and' 'or' 'butnot' and *, where * is used as a wild card. These phrases allow you to restrict or expand your searches to focus on the field of choice that hopefully contains only the few pieces of information that are of interest to you. For example, if you want to search for transcription factors for the human disease NIDDM you might start by entering *transcription factors* 'and' *human* to find all the citations with transcription factors and human in the entry. You could then expand this search to include mouse sequence by adding *'or mouse'*. If you want all the citations for the carbonic anhydrase add the * wildcard and it will search plurals or any other citations where the word carbonic anhydrase is contained as a 'root'. If you want all the citations for human carbonic anhydrase but not mouse carbonic anhydrase you can search carbonic anhydrase 'and' human 'butnot' mouse. All of these techniques will help you to either narrow the number of superfluous sequences that your search returns or expand your search. This will reduce the amount of time it takes you to review the sequences that your search has returned.

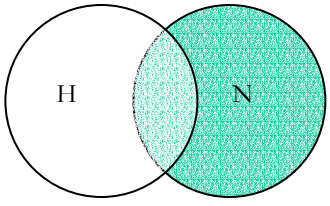
BASIC BOOLEAN LOGIC USED IN DATABASE SEARCHING



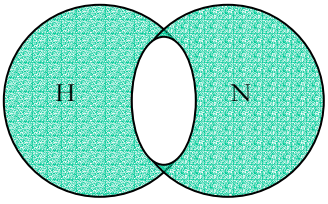
Citations that contain the descriptors H **'AND'** N only



Citations that contain the descriptors H **'OR'** N



Citations that contain the descriptors N only, **'BUT NOT'**, H **'AND'**N



Citations that contain the descriptors H **'OR'** N, **'BUT NOT'** H and N

Exercise 1: Introduction to database searching.

For the following exercises you will want to use the instructions below:

1. Connect to the internet
2. Go to the following site http://www.ncbi.nlm.nih.gov/
3. To search for a sequence:
    - Choose GenBank from the drop down menu
    - Type in the name of your sequence
    - Click go
4. To search for journal articles
    - Choose PubMed from the drop down menu
    - Type in the name of your sequence
    - Click go
5. To search for location of a gene
    - Choose Locus Links from the drop down menu
    - Type in the name of your sequence
    - Click go
6. To search for similarities
    - From the Locus Links page choose U from the Links
    - This will take you to Unigene
    - One of the pieces of information is similar sequences
    - OR
    - From the Locus Links page choose H from the Links
    - This will take you to Homologene
    - Click more next to the organism sequence that you are interested in
    - One of the pieces of information is the similarity of two organismal sequences
7. To search for SNPs
    - From the Locus Links page choose V from the Links
    - This will take you to the SNP database for your gene of interest
    - Click on the Ref SNP ID
    - Click on the Assay ID
    - Find the sequence
    - All the SNPs recorded on the first page are not all different SNPs
8. To search for disease information
    - Choose OMIM from the drop down menu
    - Type in the name of your sequence
    - Click go

1. Find and save the genomic cDNA (mRNA) sequence of human carbonic anhydrase II (hCAII) and mouse carbonic anhydrase II (MusCAIIm).

2. Find at least 3 journal articles about the protein structure of hCAII and give the citations.

3. Find out where hCAII is located in the human genome. Where in the mouse genome is MusCAIIm located?

4. How similar are the mouse and human proteins for carbonic anhydrase II?

5. Are there any SNPs in the carbonic anhydrase II gene?

6. Are there any diseases associated with mutations of the carbonic anhydrase II gene?

7. What are the two major roles of CAII in the body?

Experiment 2: Lysis of *E. coli* and Preparation of Carbonic Anhydrase Extract

After an appropriate source for a desired protein has been identified, the purification process begins with tissue homogenization or cellular lysis. The amount of protein recovered and its chemical integrity are strongly influenced by the extent of lysis and the conditions in which it is carried out. Which lysis method is appropriate depends on several factors, including the tissue or cell type, that is, whether a cell wall is present; and where the protein is localized in the cell. For instance, the protein may be localized in one the cellular organelles that itself has to be isolated and further processed.
Lysis methods include the following (Deutscher, 1990):

- enzymatic lysis, the digestion of the cell wall by lysozyme;
- high pressure shearing (Manton-Gaulin press, French press);
- sonic disruption ;
- freeze-thaw cycles;
- agitation in a glass bead mill (Dyno-Mill).

These methods may be used independently or in combination, with the best results determined empirically for each case.

A serious complication for protein isolation from *E. coli* may arise when a cell sequesters the expressed heterologous protein in insoluble fractions called "inclusion bodies." This potential difficulty may be avoided by including a transport or secretion sequence at the N-terminus of the recombinant gene. As a consequence of this modification, the recombinant protein is either (1) exported to the periplasmic space, to be released into the medium through simple osmotic shock (rapid changes in salt concentration), or (2) secreted into the medium. In either case, full cell disruption is unnecessary. This simplifies the purification process. The solution is centrifuged to separate the essentially intact cells from the supernatant containing the protein.

For a bacterial strain such as BL21(DE3)pLysS, two to three cycles of freezing and thawing are often sufficient for lysis. In this experiment a frozen cell pellet will be thawed and lysed with lysozyme in a suitable buffer. This type of enzymatic lysis is very effective and results in the release of all the cellular content, including high molecular mass nucleic acids. The cell lysate is highly viscous, thus making pipetting and phase separation difficult. The presence of nucleic acids may interfere with chromatographic steps in the purification process and sometime make flow rates prohibitively slow. Digestion of the cell lysate with DNase decreases viscosity and makes possible the efficient separation of insoluble cellular material from the supernatant fraction. Mechanical cell lysis methods are usually sufficient to disrupt the cell wall and membrane and to shear, thus yielding cell lysate that is suitable for chromatography. However, such harsh treatments may adversely affect the protein of interest.

Regardless of the cell lysis method, one might choose to eliminate nucleic acids (along with other insoluble material) by treating lysate with complexing agents such as spermidine, polyethyleneimine, streptomycin sulfate, or protamine sulfate. All of these compounds are very efficient for nucleic acid removal but in some cases they may also bind and precipitate the desired protein. Therefore, if one wishes to use nucleic acid complexing agents, the choice has to be made carefully in order to ensure optimum protein recovery (Scopes, 1994).
Cell lysis also liberates proteases from their compartments. Therefore it is often essential to include several protease inhibitors (e.g. EDTA, EGTA, PMSF) in the lysis buffer in order to minimize proteolysis. If any other compounds are known to enhance the stability of the recombinant protein, these can also be included at this step (e.g. $ZnCl_2$ for CAII).

References

1. Deutscher, M.P. (ed.) 1990. Guide to Protein Purification. *Methods in Enzymology* **182.**, Academic Press, San Diego.

2. Scopes, R.K. 1994. Protein Purification Principles and Practice. Third Edition. Springer-Verlag, New York.

Procedure

Wear gloves! PMSF is toxic!

Lab instructors will provide frozen bacterial cells prepared as in Experiment 1. You will not need to process uninduced cells.

1. Thaw and resuspend bacterial pellet in 10 ml ($1/10^{th}$ of the original culture volume) of ice-cold lysis buffer (recipe provided below). Vortex and transfer into a 15 ml conical tube. (This is done to facilitate centrifuging of tubes from the entire class in a single centrifuge). Place on ice.
2. Add to the cell pellet 100 µl of 100 mM PMSF (1 mM final concentration). Add 250 µl of a 10 mg/ml solution of lysozyme (made fresh that day). Incubate at room temperature for 15 min.
3. Add 100 µl of 5 mM $ZnCl_2$ to a 50 µM final concentration. Leave on ice for 10 min.
4. Using a wide bore tip, pipet 70 µl of the lysed cells into a 1.5 ml microcentrifuge tube and label **#2.1**/Group No. ("induced, crude, total" cell extract). This sample will be used in experiment 8.
5. Again, using a wide bore tip, transfer 120 µl into another 1.5 ml microcentrifuge tube.
6. Inspect the protamine sulfate solution provided in microcentrifuge tube. Pipet in and out and vortex repeatedly until translucent pellet is dissolved (**very important**!). Add 18 µl of 3.0% protamine sulfate solution (30 mg/ml) to the microcentrifuge tube that contains 120 µl cell lysate. Add 1 ml of the 3.0% protamine sulfate solution to the remaining cell lysate in the 15 ml tube from step 3.

Note: Failure to dissolve the protamine sulfate solution will result in incomplete elimination of nucleic acids. This will cause clogging of the affinity column in Experiment 4.

7. Mix content in <u>microcentrifuge tube</u> by pipetting in and out for 1 min and centrifuge at 14000 rpm for 5 min at 4°C.
8. Mix content in the <u>15 ml</u> tube by manual agitation for 2 min and then spin at 3000 rpm in the RT6000B centrifuge for 10 min at 4°C.
9. Retrieve microcentrifuge tube from step 7 and pipet supernatant into a clean tube labeled **#2.2**/Group No. (induced crude sup). This fraction will be used in Experiments 8 and 11. Save also the pellet and label tube **#2.3**/Group No. (induced crude pellet). This fraction will be used in Experiment 8.
10. Retrieve sample from step 8 and check the viscosity of the solution with a one ml pippette. If still viscous, add more 3.0% protamine sulfate (0.5 ml). Again, mix well and centrifuge as before. Following centrifugation, transfer 250 µl into a fresh microcentrifuge tube labeled **#2.4**/Group No. (Sup. Exp. 6). This sample will be used later in Exps. 6, 7 and 10.
11. Transfer the rest of the sample from step 8 into a clean 15 ml conical tube, that has been labeled **#2.5**/Group No. (Sup. Exp. 4). This sample will be used later in Exps. 4, 5, 6, 7, 8 and 10.

All samples intended for use in later experiments will be collected by the lab staff. Make sure their label clearly differentiate them from the rest of the samples. You should have collected the following samples:

Sample Label	Content and use
#2.1/Group No	Induced, crude, total cell extract.
#2.2/Group No.	Induced crude sup.
#2.3/Group No.	Induced crude pellet.
#2.4/Group No.	Sup. Exp. 6

#2.5/Group No.	Sup. Exp. 4

Solutions

Lysis buffer: (20 mM Tris/0.5 mM EDTA/0.5 mM EGTA/pH 8.7)

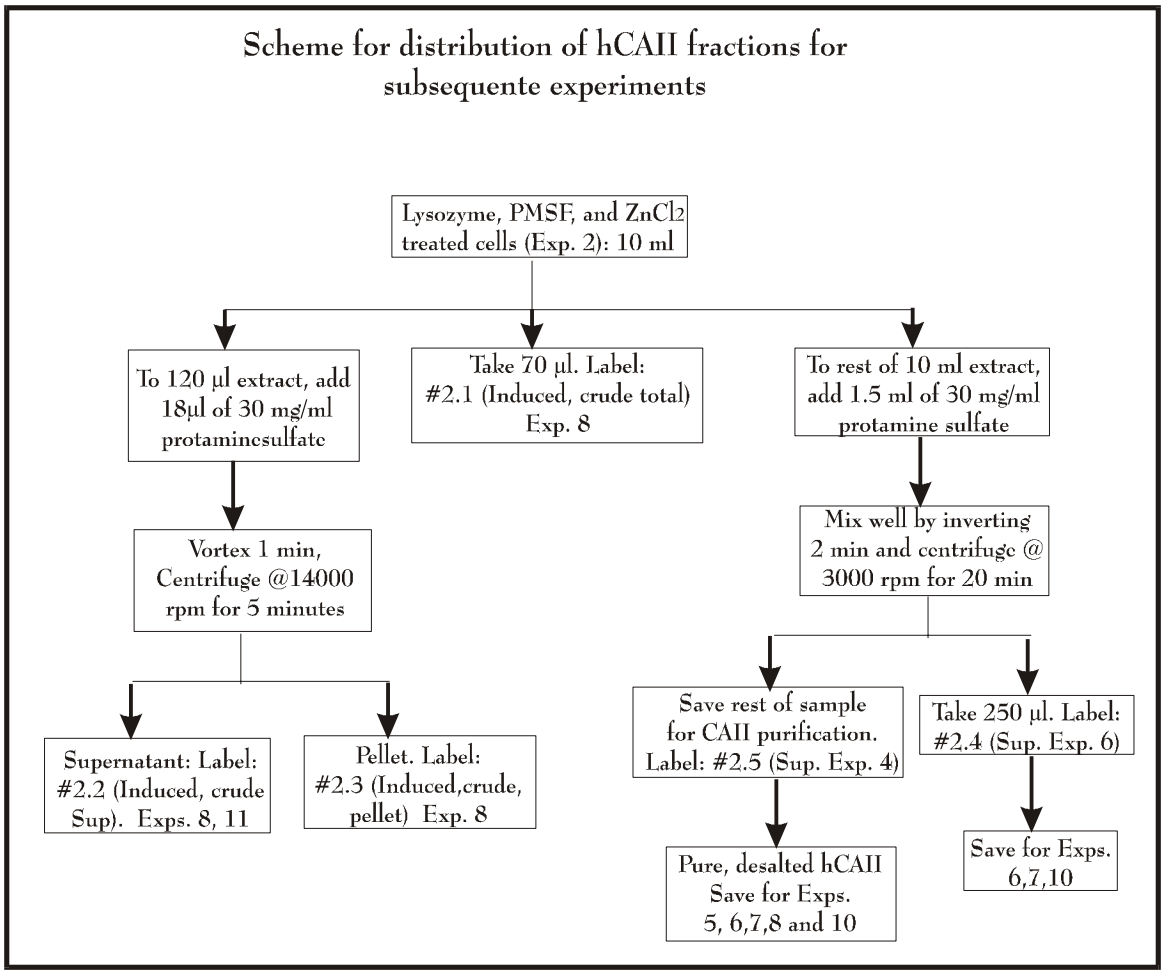
Reagent	Amount
dH_2O	800.0 ml
Trizma	2.44 g
0.5 M EDTA	1.0 ml
0.5 M EGTA	1.0 ml

Adjust pH to 8.7 with HCl
Add dH_2O to 1.0 L
Store at 4°C.

100 mM Phenylmethylsulfonylfluoride: (=100X, PMSF stock solution) (Kodak #9432):

Reagent	Amount
2-propanol	10.0 ml
PMSF	174.0 mg

Store at -20°C.


Scheme for distribution of hCAII fractions for subsequente experiments
Lysozyme, PMSF, and ZnCl2 treated cells (Exp. 2): 10 ml
To 120 µl extract, add 18µl of 30 mg/ml protaminesulfate
Take 70 µl. Label: #2.1 (Induced, crude total) Exp. 8
To rest of 10 ml extract, add 1.5 ml of 30 mg/ml protamine sulfate
Vortex 1 min, Centrifuge @14000 rpm for 5 minutes
Mix well by inverting 2 min and centrifuge @ 3000 rpm for 20 min
Supernatant: Label: #2.2 (Induced, crude Sup). Exps. 8, 11
Pellet. Label: #2.3 (Induced,crude, pellet) Exp. 8
Save rest of sample for CAII purification. Label: #2.5 (Sup. Exp. 4)
Take 250 µl. Label: #2.4 (Sup. Exp. 6)
Pure, desalted hCAII Save for Exps. 5, 6,7,8 and 10
Save for Exps. 6,7,10

Experiment 3: SDS-PAGE, Tris/Glycine System

In Experiment 3, you will use SDS-PAGE to analyze the various fractions from your protein expression experiment. Such analysis should be performed at this stage in order to make sure that induction of recombinant protein expression yielded the "desired results". For example, this could mean that a large band, presumably corresponding to CAII, is observed in the induced sample and not in the control sample. Moreover, CAII should be mostly present in the soluble cell extract of induced cells and not in the insoluble cell pellet.

Moreover, this experiment illustrates how electrophoresis is used to characterize proteins. Our working materials are bacterial extracts from Experiment 2. Proteins may be characterized rapidly and reproducibly using SDS polyacrylamide gel electrophoresis, which separates proteins based primarily on their molecular mass. SDS (Sodium Dodecyl Sulfate or Sodium Lauryl Sulfate) binds along polypeptide chains through the hydrophobic tail at a ratio of about 1.4 g SDS per 1 g protein. It generally denatures the proteins' three-dimensional conformation and renders them biologically inactive (with some exceptions, e.g. proteases). Because the negatively-charged heads of the SDS molecules are hydrophilic, facing the solvent (water), proteins in the solution are effectively solubilized. The mass of the negatively-charged SDS-protein complex is proportional to the molecular mass of the protein, so all the proteins present, regardless of their charge distribution, can be separated by electrophoresis on a polyacrylamide gel.

The widely-used Laemmli gel method uses tris/glycine as the electrode buffer. This method was modified by Schägger and von Jagow (1987) by replacing try/glycine for tris/tricine. Such modification substantially improves the resolution of proteins in the low molecular mass range (2-20 kd). The tris/glycine method is employed by the protocol presented here.

Much of the resolving power of SDS gel electrophoresis for resolving proteins relies primarily on the use of a stacking gel, which concentrates protein samples into small zones prior to their entry into the separating gel. In the stacking gel, as the negatively-charged complexes move toward the positive electrode, the Cl^- ions move fastest and the tricine ions move slowest. The protein-SDS complexes, moving at an intermediate speed, are concentrated into a small zone between the two.

In the separating gel, two phenomena occur. First, glycine is rendered more negative in the altered pH, and it speeds ahead of the proteins. Second, the proteins begin their progress at essentially the same point, and therefore their electrophoretic mobility (i.e., different bands at various positions of the lane) accurately reflect their molecular masses.

References

Bollag, D. M. and S. J. Edelstein. 1991. Protein Methods. Wiley-Liss, New York.

Bradford, M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-252.

Matsudaira, P. T. 1989. A Practical Guide to Protein and Peptide Purification for Microsequencing. Academic Press, San Diego.

Schägger, H. and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 10 kDA. *Anal. Biochem.* **166**: 368-379.

Walker, J. M. 1984. Methods in Molecular Biology Volume 1: Proteins. Humana Press, New Jersey.

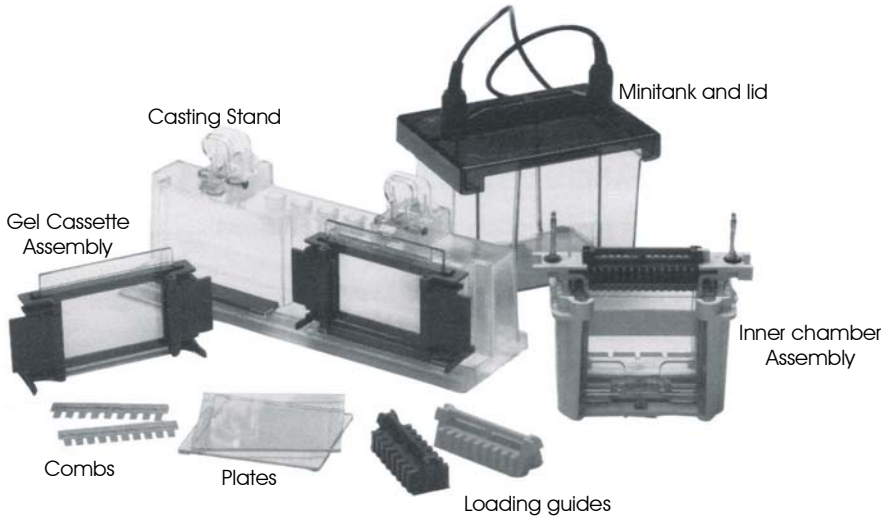
Wray, W., T. Boulikas, V. P. Wray, and R. Hancock. 1981. Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* **118**: 197-203.

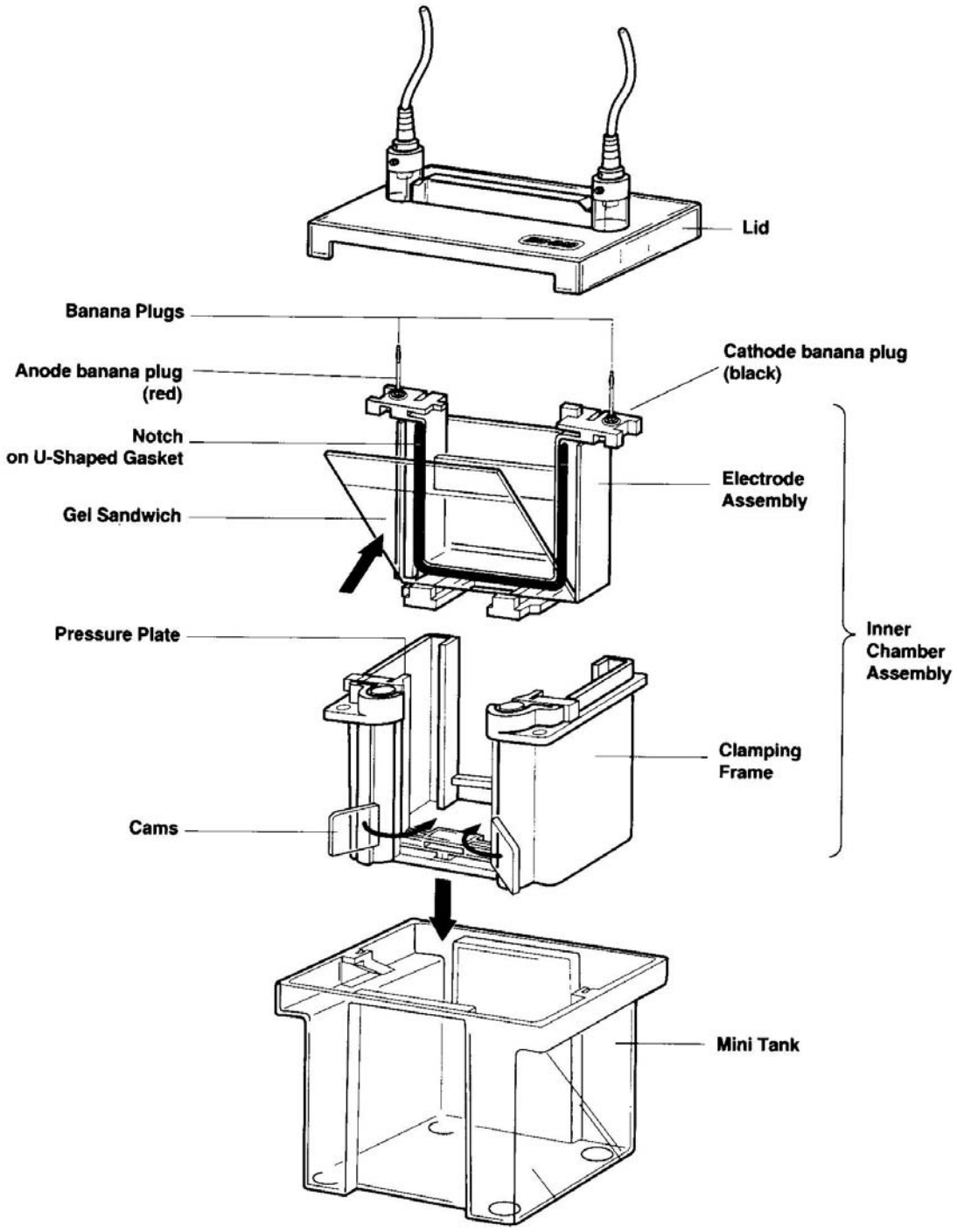
Apparatus and Setup

The gel apparatus we are using is available commercially from BioRad (Mini Protean 3). It consists of two buffer reservoirs separated by a gel sandwiched between two glass plates. The sample wells are formed by a template "comb" placed within the stacking gel. Gels are run with the negative electrode on the top (or inner chamber) and the positive electrode on the bottom (or outside chamber).

First, the separating gel is poured between the two glass plates and allowed to polymerize. The stacking gel is poured on top of the polymerized separating gel. For the Tris-tricine system, the top and the bottom electrode buffers are different in composition. See appendix 1.

The Mini-PROTEAN 3 system includes a casting stand and glass plates with permanently bonded gel spacers that simplify hand casting. The cell can run one or two gels, and the mini tank is compatible with other BioRad electrode modules for western blotting and two-dimensional electrophoresis.




Lid
Banana Plugs
Anode banana plug (red)
Cathode banana plug (black)
Notch on U-Shaped Gasket
Electrode Assembly
Gel Sandwich
Pressure Plate
Inner Chamber Assembly
Clamping Frame
Cams
Mini Tank

Part A: Setting up the Mini Protean 3 Gel Electrophoresis Apparatus

A pre-cast Laemmli gel (10% Tris/Glycine, SDS-PAGE) will be provided for this experiment. You will be using Mini Protean 3 BioRad gel apparatus. Please refer to the procedure below for a detailed description of the correct use of the apparatus. For this experiment you will be using precast gels. You will be casting your own gel (separating and stacking) later in the workshop (Experiment 8). Note that in Laemmli gels the same buffer (electrode buffer) is added to the cathode and the anode (top and outer chambers).

Gel Cassette Preparation Using Pre-cast Gels

1. Remove the pre-cast gel from the storage pouch.
2. Gently remove the comb and rinse the wells thoroughly with distilled water or running buffer.
3. Cut along the dotted line at the bottom of the pre-cast gel cassette with a razor blade.
4. Pull the clear tape at the bottom of the pre-cast gel cassette to expose the bottom edge of the gel.

Module Assembly

Note: You will be sharing an apparatus with the group next to you.

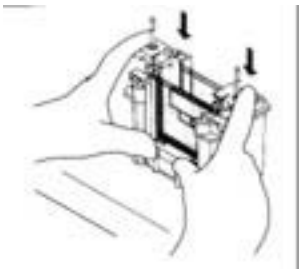
1. Place the Gel Cassettes into the slots at the bottom of each side of the Electrode Assembly. Be sure the Short plate of the Gel Cassette faces inward toward the notches of the U-shaped gaskets.

Notes:

The group with which you will be sharing the gel apparatus either will have done this step or will do it next, using the other side of the Electrode Assembly. If only one gel is to be run, use the mini cell buffer dam to create the upper (inner) chamber.

2. Lift the Gel Cassettes into place against the green gaskets and slide into the Clamping frame.

3. Press down on the Electrode Assembly while closing the two cam levers of the Clamping Frame to from the Inner the Chamber and insure proper seal of short plate to notch of the gasket.



4. Lower the Inner Chamber Assembly into the Mini Tank. Fill the inner chamber with about 130 ml of electrode buffer. Check for leaks and reassemble if necessary. Do not overfill the Inner Chamber Assembly as this will cause siphoning of buffer which can result in buffer loss and interruption of electrophoresis.

Note: In Tris tricine gels the inner (top) chamber uses cathode buffer, whereas the lower chamberr uses anode buffer.

5. Pour 200 ml electrode buffer in the mini tank (outer chamber).
6. Flush the sample wells with electrode buffer using a 1 ml Pipetman to remove un-polymerized acrylamide and any other contaminants.
7. The gel is now ready for sample loading.

Note: Separating gel can be poured one day in advance and stored at room temperature. The stacking gel should be poured immediately before needed.

Part B: Sample Preparation

1. To the tube labeled #2.3 (induced, crude pellet, from exp. 2) add 10µl ddH_2O and 10 µl 2x LSB and resuspend the pellet. Also prepare samples for lanes #3 and #4 according to the following table. The other samples to be loaded onto the gel will be provided for you.

> Note: "Induced crude, total" sample (Exp. 2, step 4) will also be used in Experiments 8 and 11. Store frozen until needed.

Lane #	Name	Volume of sample (µl)	2X LSB (µl)
1	Blank	-	-
2	Non induced, crude, total extract (provided)	15	15
3	Induced, crude, total extract (#2.1)	15	15
4	Induced, crude supernatant (#2.2)	10	10
5	Induced, crude pellet (#2.3)	10 (water)	10
6	Carbonic anhydrase standard (0.5 mg/ml)	10	10
7	Low Range MW markers (ready to load)	10	-
8	Blank	-	-

2. Heat samples to 85°C for 5 min.
3. Pulse spin samples in microcentrifuge for 5 sec.
4. Load samples (entire volume) into the wells as indicated in the Table above using a sample loading tip. Be careful not to poke the gel well. Avoid introducing air bubbles.

Part C: Running the gel

1. Place the lid on the SDS-PAGE apparatus and attach the electrode plugs to the proper electrodes. (-) black to black and (+) red to red.
2. Set the voltage to 125 V, and continue electrophoresis until the dark blue dye migrates to 1 cm from the bottom of the gel. Total time for the electrophoresis will be about 1 hr.
3. Turn off power supply and disconnect electrode plugs.

Wear gloves!

4. Remove inner core/gel sandwich assembly from gel tank and discard buffer from inner chamber in to the sink. Remove the gels.
5. For pre-cast gels, cut tape along each side of the gel which holds small and large plates together and lift off the small plate. The gel will stick to one of the two glass plates.
6. Remember to wear gloves to handle gel. This is most important when the gel is to be stained with a sensitive method such as silver stain. The gel is now ready to be stained or prepared for western transfer.

Part D: Coomassie blue staining/destaining

This method of staining can detect as little as 0.1 µg protein in a single band.

1. Wash gel three times with 20 ml water, 5 minutes each in sandwich container.
2. Pour a small amount of Biosafe Coomassie stain (20 ml) in a clean sandwich container. Place the gel into the dish and gently agitate for 30-60 min at room temp on top of an orbital shaker. Pour off stain into the sink.
3. Wash gel twice with 50 ml of water, 15 minutes each to de-stain and gently agitate gel on top of an orbital shaker.
4. Add 25 ml of 2% v/v glycerol (in water) and leave gel in this solution O/N to prepare for drying.

Solutions

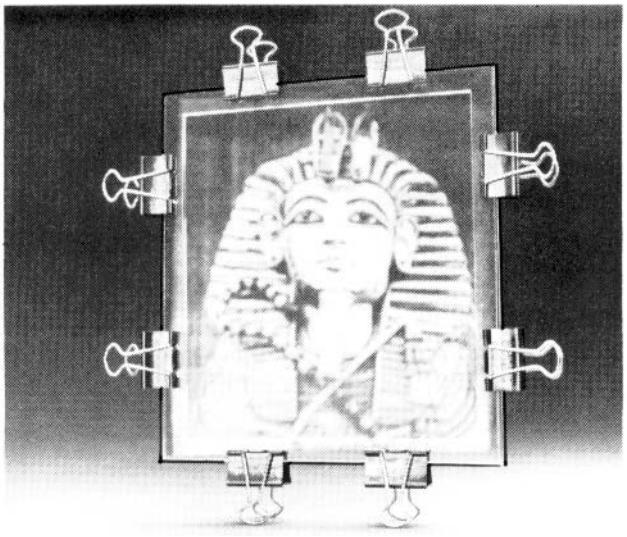
Biosafe Coomassie Stain (BioRad Catalog # 161-0786)

Regular Coomassie Blue Stain

Coomassie blue gel stain: (1 L)	Coomassie gel de-stain: (1 L)
0.02% (w/v)0.2 g Coomassie Blue R-250	50 ml methanol
400 ml 40% (v/v) methanol	100 ml glacial acetic acid
550 ml H_2O	850 ml H_2O
50 ml 5% (v/v) glacial acetic acid	

Part E: Drying SDS-PAGE gels

1. Cut stacking gel from separating gel with knife and discard stacking gel.
2. Locate the plexiglass frame and two pieces of gel-drying membranes (Biogel wrap) cut to size slightly larger than plexiglass frame. Pre-wet the gel-drying membranes in 2% glycerol.
3. Place one sheet of wet membrane on the solid Plexiglas base. Place gel on membrane, making sure to remove air bubbles that are trapped between the gel and the membrane.
4. Lay the second sheet of wet membrane across the gel; again remove air bubbles. Position the upper frame and clamp edges with four binder clips. Let sit overnight in a well ventilated area to dry.



Stock solutions for Tris-glycine SDS-PAGE Laemmli gels

30% Acrylamide mix: (100 ml)

Acrylamide	29.2 g
Bisacrylamide	0.8 g

Add ddH_2O to a final volume of 100 ml filter and store dark and cool.

4X Separating buffer (1.5 M): (100 ml)

Tris-base	18.16 g

Add ~75 ml and adjust pH to 8.8 with HCl. Add water to 100 ml.

8X Stacking buffer (1 M): (100 ml)

Tris-base	12.12 g

Add ~75 ml and adjust pH to 6.8 with HCl. Add water to 100 ml.

10% (w/v) SDS: (100 ml)

Solid SDS	10 g

Add water to 100 ml.

10% APS (prepare freshly): (1 ml)

Solid APS	100 mg

Add water to 1 ml.

TEMED: Use according to Table next page (Store @ 4^{O}C)

10X Electrode buffer: (1 L)

Reagent	Amount	[1x solution]
Tris-base	30.3 g	25 mM
Glycine	144.2 g	192 mM
SDS	10.0 g	0.1% (w/v)

Add ddH_2O to a final volume of 1 L (pH is ~8.3, do not adjust).
To make 1 L of 1X working solution, dissolve 100 ml of 10X stock solution in 900 ml H_2O. Mix well.

Solutions for Tris/Glycine SDS-Polyacrylamide Gel Electrophoresis

6%	5 ml	10 ml	15 ml	20 ml	25 ml	30 ml	40 ml	50 ml
H_2O	2.7	5.3	8.0	10.6	13.3	15.9	21.1	26.5
30% Acrylamide mix[a]	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS[b]	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% APS[c]	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED[d]	0.004	0.008	0.012	0.016	0.02	0.024	0.032	0.04
8%	5 ml	10 ml	15 ml	20 ml	25 ml	30 ml	40 ml	50 ml
H_2O	2.3	4.6	7.0	9.3	11.6	13.9	18.6	23.2
30% Acrylamide mix[a]	1.3	2.7	4.0	5.3	6.7	8.0	10.7	13.4
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS[b]	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% APS[c]	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED[d]	0.003	0.006	0.009	0.012	0.015	0.018	0.024	0.03
10%	5 ml	10 ml	15 ml	20 ml	25 ml	30 ml	40 ml	50 ml
H_2O	2.0	4.0	5.9	7.9	9.9	11.9	15.8	20
30% Acrylamide mix[a]	1.7	3.3	5	6.7	8.3	10.0	13.3	16.6
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS[b]	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% APS[c]	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED[d]	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
12%	5 ml	10 ml	15 ml	20 ml	25 ml	30 ml	40 ml	50 ml
H_2O	1.7	3.3	5.0	6.6	8.3	9.9	13.2	16.4
30% Acrylamide mix[a]	2.0	4.0	6.0	8.0	10.0	12.0	14.0	20.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS[b]	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% APS[c]	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED[d]	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
15%	5 ml	10 ml	15 ml	20 ml	25 ml	30 ml	40 ml	50 ml
H_2O	1.2	2.3	3.5	4.6	5.7	6.9	9.2	11.4
30% Acrylamide mix[a]	2.5	5.0	7.5	10.0	12.5	25.0	20.0	25.0
1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS[b]	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
10% APS[c]	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED[d]	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

STACKING	1 ml	2 ml	3 ml	4 ml	5 ml	6 ml	8 ml	10 ml
H_2O	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
30% Acrylamide mix[a]	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.0 M Tris (pH 6.8)	0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
10% SDS[b]	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
10% APS[c]	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED[d]	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

[a]: Commonly 29.2% acrylamide and 0.8% N,N'-methylene-bis-acrylamide
[b]: Sodium dodecyl sulfate
[c]: Ammonium persulfate
[d]: N,N,N',N'-Tetramethyllethylenediamine

SDS-PAGE MW Standards*		
	Pre-stained	*Unstained*
Phosphorylase b	103,000 Daltons	97,400 Daltons
Serum albumin	77,000	66,200
Ovalbumin	50,000	45,000
Carbonic	34,300	31,000
Trypsin inhibitor	28,800	21,500
Lysozyme	20,700	14,400

*BIORAD: Prestained (Cat. # 161-0305), Unstained (Cat. # 161-0304)

Part F: Protein statistics generated by computer algorithms

There are many tools to analyze proteins from their primary structure. Basic statistics can predict certain characteristics of the protein, such as, solubility, isoelectric point, hydrophobicity, molecular weight and more. More complicated algorithms can be used to predict secondary structure, possible antigenic sites as well as compare the similarity among proteins from different organisms or different forms of the same protein. These predictions made via computer algorithm can help to anticipate how a protein will behave in an experiment. The analyses will ultimately allow you to reduce the trial and error that was common in the early days of research.

Exercise 2a: Using PepTools as an aid for protein analysis.

1. For the following protein exercises, you will want to use the following protocol:

    - Open PepTool
    - Go to the Search Menu (pull down menu)
    - Choose how you want to search for the sequence. If searching by keywords, type "keyword" and click Ok.
    - Choose your sequence. The sequence that is selected is shown in lower box of the window.
    - Go to the Transfer Menu
    - Choose Sequence Editor. Click "new".
    - Choose Protein Statistics from the analyze menu
    - You should see all of the statistics for your protein as you scroll down
    - You may want to print your calculations for later analyses

2. Search for the hCAII protein in the PepTool program.

3. Transfer the sequence to the sequence editor and save the protein sequence.

4. Run the Protein Statistics analysis.

    - What is the molecular weight of the protein?
    - Find 3 statistics that indicate the solubility of the protein. Is this protein soluble?
    - If you were given the molecular weight marker below (size standard), draw where you would expect your band for hCAII to be?

        _____ 60,000D
        _____ 40,000D
        _____ 10,000D
        _____ 500D
        _____ 300D
        _____ 100D

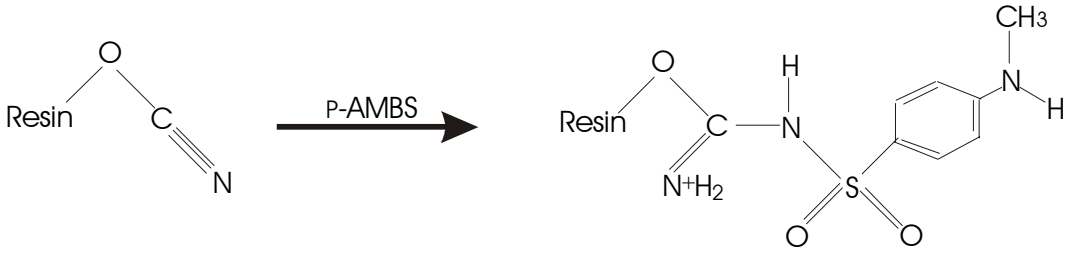
Exercise 2b: Electrophoretic Analysis of Protein Fractions

1. Are there any observable differences in the band pattern between the 'non-induced crude total cell extract' and the 'induced crude total extract'?
2. Is hCAII found mainly in the soluble (supernatant) or insoluble (pellet) cellular fraction?
3. Why is it desirable to express proteins in the soluble fraction? (give at least two reasonsa0.
4. What can you do to improve the proportion of the soluble vs. insoluble recombinant proteins?
5. What would have your gel looked like if you had used a15% Tris-gly SDS-PAGE, while keeping all other electrophoretic paramets the same?
6. Why did you heat the samples in LSB before loading them into the gel?
7. Why did you soak the gel in glycerol before drying?

Experiment 4: Affinity Purification of Recombinant Carbonic Anhydrase

Background

Experiments 4-7 are directed toward assessing the activity of the hCAII recovered from the lysate. Experiment 4 demonstrates purifying proteins from a complex mixture such as a lysate (from Experiment 2), using immobilized affinity ligands. This method exploits specific affinity interactions, often eliminating the need for more laborious steps such as auxiliary ammonium sulfate precipitations, ion exchange, and gel filtration. Typically (though not always), affinity ligands are covalently bound to an activatived agarose gel matrix, or resin, which is packed in a plastic column. Cell lysate is either applied onto the prepacked column or mixed with the resin prior to packing. Proteins from the lysate that have an affinity for the ligand bind to the resin; others remain in solution or wash through. Further washes remove nonspecifically-bound proteins. The specifically-bound protein may be desorbed from the resin by adding a compound that displaces it from the matrix.
In this protocol, hCAII is purified exploiting its affinity to p-aminomethylbenzene sulfonamide (pAMBS). The resin consists of a CNBr activated agarose matrix to which the primary amino group of pAMBS has been attached.



hCAII binds specifically to this resin given a fairly high salt environment. The lysate from Experiment 2 is mixed with the resin at pH 8.7, then packed into the column and washed. A KSCN solution is then used to desorb the purified protein from the resin.

Procedure

Washing of the p-AMBS resin and mixing with cell extract

1. Locate the 15 ml conical tube containing 1.0 ml of Sigma p-AMBS-agarose resin (50% v/v, i.e., resin bed volume is 0.5 ml). Allow resin to settle or centrifuge at 3000 rpm for 30 sec and then use a Pasteur pipet to remove the aqueous layer into the waste container.
2. Add 10 ml cold Wash Buffer "0" (recipe below) to the 15 ml conical tube containing resin. Invert the tube 10 times and centrifuge at 3000 rpm for 30 sec. Carefully remove the wash solution with a Pasteur pipet.
3. Repeat step 2, two more times for a total of three washes.
4. Transfer the bacterial extract from Experiment 2, step 11 to the 15 ml tube of washed p-AMBS resin.
5. Incubate the sample (resin plus extract) for 1-16 hours at 4°C with mixing. For convenience, your laboratory instructor will take your sample and place it in an electric rotator in a cold room.

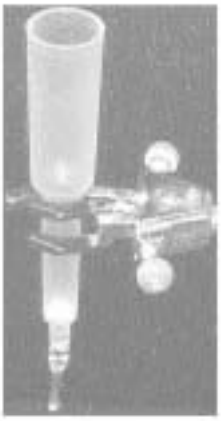
Column preparation, wash and hCAII elution

1. Retrieve the bacterial extract with resin and load into a plastic chromatography column. Make sure that the resin is in suspension before pouring into the column. The column contains a support that allows the

aqueous solution through, but retains the resin. Allow the extract to drain and discard the flow-through into biohazardous waste. A rate of about 1 drop/2 sec is appropriate.

Note: Flow rate control is not necessary using the columns provided in this experiment. When needed, the flow rate can be regulated by using an adjustable clamp or by adjusting a stopcock attached to the column outlet.

Save the flow-through when testing an affinity column procedure for the first time. You may need to optimize the conditions of enzyme binding to the resin.



2. Wash the p-AMBS column with 12 ml of Wash Buffer 1 (recipe below). Discard the column effluent into the biohazardous waste bag. Make sure the flow rate is approximately 1 drop/2 sec (less is Ok) during the washing of the CA-affinity column.
3. Wash the column with 16 bed volumes (8 ml) of Wash Buffer 2 (recipe below). Discard the column effluent as in step 8 above. At this point, all nonspecifically bound protein should have eluted off of the p-AMBS agarose resin. (Near the end of the wash, adjust the flowrate to 1 drop/3 seconds if necessary).
4. Elute the carbonic anhydrase with 1.5 ml of Elution Buffer. Add each 0.5 ml at a time and collect these three fractions in a single microcentrifuge tube. Make sure the collection tube is appropriately labeled (e.g., pure, hCAII/Group No.).
5. Return the columns to lab instructors.
6. Save at 4°C for Experiment 5.

Notes:

-To regenerate the affinity resin for future use, wash the column with 12 bed volumes of Elution Buffer (6 ml). Complete the column regeneration by washing the resin with 12 bed volumes of Storage Buffer and store at 4°C.

-In a typical experiment, many more fractions are applied to the column and collected separately. The purpose is to identify which fraction(s) contain the enzyme (i.e., CAII). Pooling fractions that do not contain enzyme will result in dilution of the enzyme, potential contamination with other proteins non-specifically bound to the resin, and excess salts.

Exercise 3: Affinity Purification

1. Why is KSCN able to elute off CAII from the affinity column?
2. How can you avoid collecting the eluted/pure enzyme in an excessively large volume of elution buffer?
3. Why don't you want too much elution buffer in your purified enzyme solution?
4. How can you tell which fraction(s) from a desalting column contain CAII?
5. Based on the physical chemical characteristics of the enzyme, suggest at least one alternaive method to accomplish some level of purification of CAII.

Solutions

Wash Buffer 0:
(0.1 M Tris-SO_4/0.2 M K_2SO_4/0.5 mM EDTA/pH 7.5)

Reagent	Amount
dH_2O	800.0 ml
Trizma	12.1 g
K_2SO_4	34.8 g
0.5 M EDTA	1.0 ml

Adjust pH to 7.5 with H_2SO_4
Add dH_2O to 1.0 L
Store at 4°C.

Wash Buffer 1:
(0.1 M Tris-SO_4/0.2 M K_2SO_4/0.5 mM EDTA/pH 9.0)

Reagent	Amount
dH_2O	800.0 ml
Trizma	12.1 g
K_2SO_4	34.8 g
0.5 M EDTA	1.0 ml

Adjust pH to 7.5 with H_2SO_4
Add dH_2O to 1.0 L
Store at 4°C.

Wash Buffer 2:
(0.1 M Tris-SO_4/0.2 M K_2SO_4/pH 7.0)

Reagent	Amount
dH_2O	800.0 ml
Trizma	12.1 g
K_2SO_4	34.8 g

Adjust pH to 7.0 with H_2SO_4
Add dH_2O to 1.0 L
Store at 4°C.

Elution Buffer :
(0.1 M Tris-SO_4/0.4 M KSCN/0.5 mM EDTA/pH 6.8)

Reagent	Amount
dH_2O	800.0 ml
Trizma	12.1 g
KSCN	38.8 g
0.5 M EDTA	1.0 ml

Adjust pH to 6.8 with H_2SO_4
Add dH_2O to 1.0 L
Store at 4°C.

Storage Buffer :
(0.1 M Tris-SO_4/0.2 M K_2SO_4/0.5 mM EDTA/1 mM NaN_3/pH 7.5)

Reagent	Amount
dH_2O	800.0 ml
Trizma	12.1 g
K_2SO_4	34.8 g
0.5 M EDTA	1.0 ml

Adjust pH to 7.5 with H_2SO_4
Add dH_2O to 1.0 L
Store at 4°C.

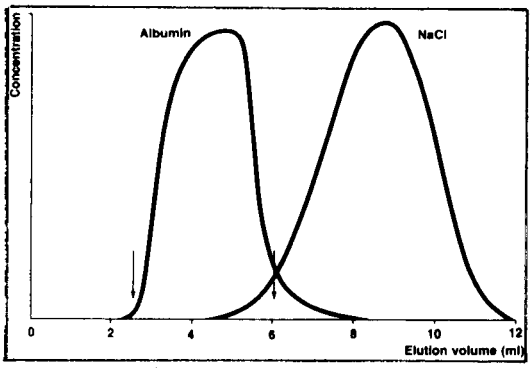
p-AMBS agarose resin:
(Sigma A-0796)
Store at 4°C in storage buffer.
Wash before use thoroughly with Wash Buffer 0.

Experiment 5: Desalting Protein Solutions by Gel Exclusion Chromatography and Concentrating sample by ultrafiltration

The relatively high ionic strength buffer (pH 6.8) used for the elution of hCAII from the affinity column in Experiment 4 is not compatible for measurement of biochemical activity. Salts must be removed from the extract and replaced by a buffer solution for which enzymatic activity is optimum.

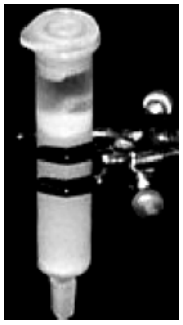
Part A: Desalting of purified hCAII

Desalting of proteins can be attained by dialysis, but this method is time-consuming. A faster and more efficient way to eliminate salts is gel exclusion chromatography. In this method, the protein solution is applied to a small column that has been equilibrated with the desired final buffer. The end result is that small molecules (i.e., salts) in the original solution are largely eliminated (retained in the column), and proteins will now be dissolved in the buffer used to equilibrate the column (buffer exchange). Large molecules such as proteins are excluded from the resin and eluted in the void volume, whereas small molecules such as salts are retained and eluted in later fractions. Proteins recovered from the gel-exclusion column will now be dissolved in the buffer used to equilibrate the column. Therefore, the high salt solution in which the protein was originally dissolved is effectively exchanged for the column equilibration buffer.
The diagram shown below illustrates the pattern of elution from a gel exclusion column when an albumin/NaCl solution was applied.



Removal of NaCl from albumin solution. A column PD-10 was equilibrated with distilled water. The sample contained human serum albumin (25 mg) dissolved in NaCl solution (0.5 M, 2.5 ml). Yield of albumin in 3.5 ml after sample application, (between arrows) 95.3 %; salt content, 2.0 % of total salt originally present.

A PD-10 gel exclusion column (Pharmacia Biotech) already equilibrated with 25 ml of lysis buffer will be provided by the laboratory instructors.



Procedure

1. Mount a PD-10 (Pharmacia) gel exclusion column on a ring stand. Uncap the column and allow the equilibration buffer (same as lysis buffer) to drain completely into a waste container (until no more buffer drips from the column's outlet).
2. Use the combined fractions 1, 2, and 3 from the CA-affinity column (1.5 ml total, Experiment 4, labeled 'pure CAII'). Layer the pooled CA fractions onto the column.
3. Allow the protein solution to enter the column gel completely and discard effluent.
4. Add another 1 ml of lysis buffer and allow to enter the gel completely (do not collect effluent yet).
5. Add an additional 1.6 ml of lysis buffer and collect effluent immediately in two microcentrifuge tubes. These two fractions contain the desalted enzyme. Save on ice for procedure in Exp. 6.
6. Close the desalting column outlet with plastic cap provided and add 2 ml of lysis buffer. The lab staff will collect the columns and regenerate them for reuse in future courses.

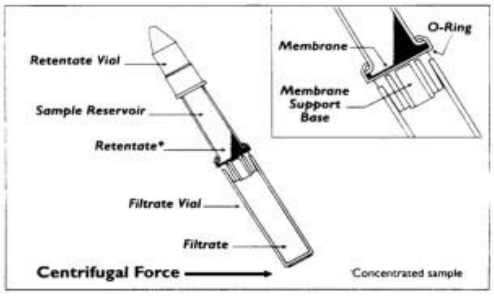
> For immediate reuse of gel exclusion columns, simply add 25 ml of lysis buffer and discard effluent. Column is now ready for protein desalting. For long-term storage, cap column and add a small volume of lysis buffer/0.01% sodium azide to avoid drying and bacterial growth. Rinse column with 25 ml buffer before use.

Part B: Concentration by ultra-filtration

The purified, desalted hCAII is concentrated using a Centricon-10 filtration unit (Amicon®, Inc., Beverly, MA). The filter in this unit has a molecular mass cutoff of 10 kilodaltons; that is, molecules with molecular mass less than 10 kd will pass through along with the solvent. As the protein solution is centrifuged in the filter, small molecules (water, salts, etc) pass through but proteins are retained in the upper compartment of the filtration unit. The net effect is to concentrate the protein in a smaller buffer volume while the buffer concentration remains unchanged.

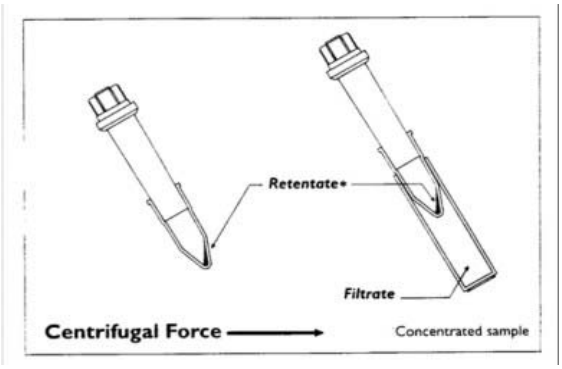
Procedure

1. Add protein solution free of particles such as from a desalting column (A variety of unit sizes are available depending on the volume to be concentrated) to the upper compartment (the one with the membrane) of the Centricon-10 filtration unit.



2. Assemble this compartment onto the lower receptacle of the Centricon-10 filtration unit.
3. Concentrate sample by centrifuging using a non-fixed-angle rotor (such as the Sorvall RT6000B) at 3000 rpm, 4°C. A 1.5 ml sample will take about 60 min to concentrate
4. Label a fresh 1.5 ml microcentrifuge tube to be used as storage for concentrated protein.

5. Disconnect and set aside the lower receptacle of Centricon-10 unit (containing filtrate). Invert upper receptacle (containing concentrated protein), so that conical cap is in the bottom. Centrifuge in this orientation at 3000 rpm for 30 sec, 4°C.



6. The conical cap can serve as a storage tube (properly labeled and capped). If preferred, concentrated protein can be transferred into labeled microcentrifuge tube from step 4. You should have between 50-100 μl of concentrated protein solutions.

Exercise 4: Gel Exclusion chromatography of Proteins

1. Why is it important to desalt purified enzymatic solutions? Give two reasons.

2. The desalting column you used in Experiment 5 had a void volume of 2.5 ml. Explain why did you do step 4 in the procedure described in Part A. (*Hint: void volume is defined as the fraction of the bed volume not occupied by the swollen resin. That is, the volume available for free sample or buffer*).

Experiment 6: Protein Concentration Determination: Dye-Binding Assay (Bio-Rad®)

In this experiment, hCAII concentrations are determined for both the crude supernatant from Experiment 2 and the desalted, concentrated, and purified solution from Experiment 5.
Many methods for estimating protein concentration are available. The appropriate method depends on five major criteria: the amount of protein available for the assay, the concentration of the protein, the specificity of the assay, the presence of chemicals which may interfere with the assay, and the ease of performing the assay.

The more common spectrophotometric methods for determining protein concentration include Biuret, Lowry, Bicinchoninic acid, and Dye-binding. Our protocol employs the Dye-binding method (Bradford, 1976), which uses an acidic solution of Coomassie Brilliant Blue G-250. The dye binds primarily to the basic and aromatic amino acid residues, generating a color change of the solution whose intensity varies in proportion to the concentration of proteins. The absorbance maximum of the dye shifts from 465 nm to 595 nm when it binds to the protein. The dye-binding assay has a linear range from 0.5 to 15 μg protein.

If the absorption coefficient of the protein of interest is not known, a standard curve is constructed using an easily available protein (e.g. BSA, IgG). An absorbance vs. concentration function is calculated for the standard and used to determine the concentration of the protein of interest. This is the method employed in our protocol, using a commercially available bovine CAII as a standard.

If the absorption coefficent for the protein of interest is known, Beer's law may be used to calculate its concentration directly.

Beer's Law: **A = acL**

where: A = absorption at 595 nm
a = absorption coefficient
c = concentration of protein solution
L = length of the light path in cm (usually cuvettes of 1 cm width are used)

Reference

Bradford, M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-252.

Part A: Lab Procedure

A variation of the Dye-binding method will be used to measure the protein concentrations of the "Sup Crude Concentrate" and the concentrated/purified solution of Carbonic anhydrase (Experiment 5B, step 6).
For this assay, we will use a flat-bottom 96-well microtiter plate and a microplate reader such as the one used for ELISA, equipped with a 595 nm optic filter. Large numbers of samples can be assayed in this manner while using a single standard curve per plate.

The laboratory staff will prepare one set of standard solutions per plate according to the following table using a 1.0 mg/ml solution of bovine carbonic anhydrase (bCAII, obtained from SIGMA). Each point of the protein standards will be read twice in 200 µl microtiter plate wells.

Sample Nº	bCAII (µl)	Water (µl)	Dye (µl)	bCAII added/ sample (µg)	A 595 nm		
					1	2	Av
1	0	400	100	0.0			
2	5	395	100	5.0			
3	10	390	100	10.0			
4	15	385	100	15.0			
5	20	380	100	20.0			

1. You will need to prepare two sets of samples, one for the Sup Crude solution (#2.4=Sup. Exp. 6) and another one for the purified CAII solution (Experiment 5, step 5) according to the following tables:

   a) Sup Crude hCAII:

Sample Nº	Sup Crude (µl)	Water (µl)	Dye (µl)	A 595 nm		
				1	2	Av
1	0	400	100			
2	5	395	100			
3	10	390	100			
4	15	385	100			

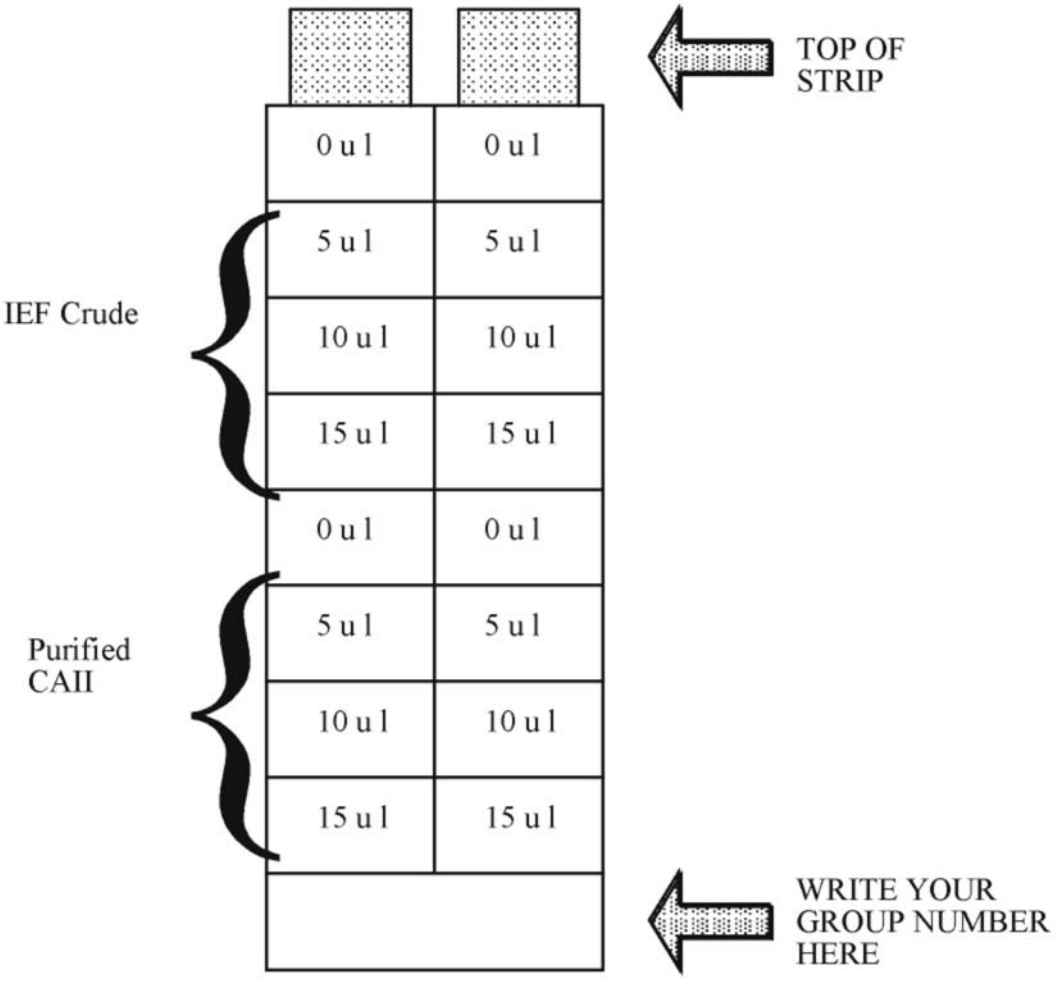
   b) Purified (desalted) carbonic anhydrase (hCAII):

SampleNº	Pure CAII (µl)	Water (µl)	Dye (µl)	A 595 nm		
				1	2	Av
1	0	400	100			
2	5	395	100			
3	10	390	100			
4	15	385	100			

2. Pipet two-200 µl aliquots from each one of the tubes in tables 1a and 1b into parallel wells of the strip of a microtiter plate so that duplicate values can be averaged. See diagram below. Once you have completed pipetting your samples, your lab instructor will reassamble the microtiter plates using everyone's strips. You will also be assigned a known position in the plate.

EXPERIMENT 6: Diagram of microtiter plate strip for protein concentration determination

The volume of the total solution delivered to each well is 200 u l; the listed number refers to the volume of protein.



3. The samples from the entire class will be read simultaneously at 595 nm in a microtiter plate reader.
4. Record in the tables above the A_{595} for each sample and average the duplicates.
5. Construct a standard curve for the bCAII (standard) data and calculate the hCAII protein concentration (crude and pure) as indicated in the "Excel Spreadsheet" exercise below.

Part B: Using Excel to calculate Protein Concentration

Simple calculations of large data sets are tedious and mistakes are easily made. To reduce the headaches associated with large data sets, using simple spreadsheet programs such as Excel will reduce the tedium and the mistakes. Many spreadsheets will also create graphs for your data. Use of spreadsheets for simple statistical calculations from well-known formulae is a relatively easy and extremely useful tool in the lab.

Exercise 5: Introduction to spreadsheet formulae and graphing

Mathematical activators in Excel: + for addition, - for subtraction, * for multiplication and / for division

A. Calculating and Correcting Absorption Averages from Control

a) Enter Volumes: Standard into column A4:8, Crude into A12:15, and Pure into A20:23.
b) Now enter Absorption readings 1&2 in columns B and C, respectively. Absorption readings should line up with their volume.
c) **Standard Absorption Averages:** In column D, calculate the average of the two absorption readings for each volume. To enter first average, click on cell D4 and enter =(B4+C4)/2 and press *Enter*. To calculate the averages of all the other spectrophotometric readings, highlight cell D4, click *Edit* on bar at top of screen, click *Copy*, then click D5 cell and drag down till all cells through D8 are highlighted, again click *Edit* and now *Paste*. This will bring the formula down for each cell corresponding for each volume's absorption reading average.
d) Column E is assigned for calculating the average of 0 μL standard absorption reading subtracted from the average of D5:D8 (normalized average absorption). Enter into cell E4, =D4-D4 and press *Enter*. *Copy* and *Paste* this formula down (D5:D8) to calculate. (The symbol $, is an excel symbol which will make every cell be subtracted by D4) These data for Absorption of Standards will be used to graph a standard curve so to calculate concentrations for our unknown crude and pure volumes.

B. Graphing the Standard curve

a) Generate an Absorption (x-axis) versus Amount in μg (y-axis) graph from your standard calculations. Label cells H1 and I1, x-axis and y-axis respectively. Enter into cells H2:H6 your normalized average absorption readings (E4:E8.) Also enter into cells I2:I6 standard μg amounts (A4:A8.) These will be used to produce a best fit graph of your data.

b) Calculate the Slope and Intercept for the standard curve: Label cell J1 Slope. Enter into cell K1; =SLOPE(I2:I6,H2:H6) and press *Enter*. This formula will calculate the slope for your standard curve data. Label cell J2 y-intercept. Enter into cell K2: =INTERCEPT(I2:I6, H2:H6) and press *Enter*. This will give you the y-intercept value. Both our slope and y-intercept values will be used to calculate the concentrations for our unknown crude and pure data.

c) To view a standard curve, click on the *Chart Wizard* function (bar graph icon), on bar at top of screen, and create (click on) a *XY-scatter* plot for your data. Press *Next*, (a new screen should appear) press *Series*. On *X Value* click the icon to the right of the box, highlight cells H2:H6 and press the icon again. For the y-axis, click *Y Value*, highlight cells I2:I6 and press icon again. Then press *Finish*. A graph should appear. Right click on one of the points, click *Add Trendline, Options, display equations on chart, and Ok*. This will add a best-fit line with your straight line equation (slope and y-intercept) labeled on the graph. Right click inside the graph box, but outside the gray box, and press *Chart Options*, and *Title* to name axes and header.

d) Use Ac) and Ad) to calculate average and normalized averages for crude and pure for columns D and E. (Column E for both crude and pure will not be used to make a graph to find slope or y-intercept, as it was done in standard curve calculations.

C. Calculating the Protein Concentration of Unknowns (crude and pure)

a) To find concentration of our unknowns you solve for 'y'. To do this, enter into cell F13; =(K1*E13+K2)/5. This is our y=mx+b formula, where y=amount of protein in the unknown and x=volume of protein sample for that particular absorption reading (corrected for non-enzymatic hydrolysis). *It is wrong to copy and paste this formula down the cells because absorption was measured for varying volumnes of sample. You will need to enter the formulae individually.* Example (F14); =(K1*E14+K2)/10, etc. This formula should be used for cells F13:F15 (Crude concentrations) and F21:F23 (Pure Concentrations.)

b) Cells F16 and F24 will be your average of concentrations for crude and pure, respectively. Enter into cell F16; =AVERAGE(F13:F15) and press *Enter*. Enter into cell F24: =AVERAGE (F21:F23) and press *Enter*. This will give you the average concentration in mg/mL or ug/uL. These concentrations will be used on the following sheet to calculate your specific activities.

Experiment 7: Spectrophotometric Assay of Carbonic Anhydrase Activity

In the living mammal, CO_2 is produced by metabolic activity in the tissues. CO_2 spontaneously reacts with water to form carbonic acid in the red blood cells. In the lungs, the reverse reaction, the conversion of carbonic acid into CO_2 and water, is catalyzed by carbonic anhydrase.

$$H_2CO_3 \xrightarrow{\text{CAII}} CO_2 + H_2O$$

Although enyzmes are known for their specificity, they often catalyze chemically similar reactions using alternate substrates. If the alternate substrate yields a product that can be recognized and measured in a time-dependent fashion, the enzyme's activity may be assessed *in vitro*. Useful physical properties of the enzyme reaction product include absorbance, fluorescence, light diffraction, conductivity, and others.

Carbonic anhydrase can catalyze the hydrolysis of the ester bond of *p*-Nitrophenylacetate (PNPA). One of the two main products of this reaction is *p*-Nitrophenolate, which is intensely yellow with an absorption maximum at 400 nm in the visible spectrum.

$$\text{p-Nitrophenylacetate} \xrightarrow{\text{CAII}} \text{p-Nitrophenolate (plus p-Nitrophenol)} + \text{Acetate}$$

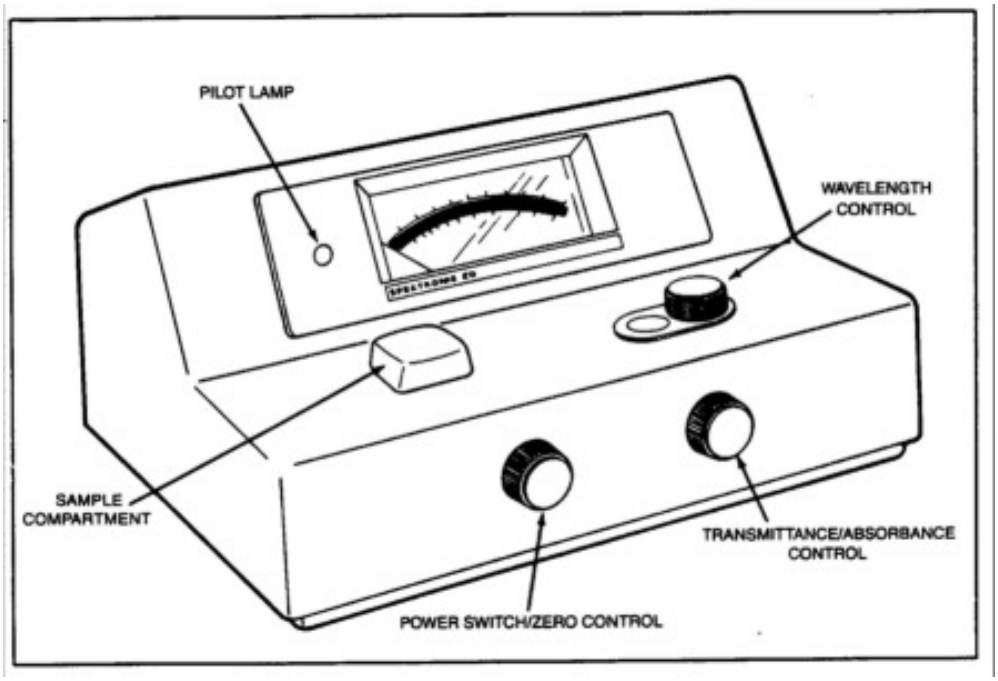
PNPA is unstable in the presence of light, so its rate of non-enzymatic breakdown may be compared to the rate in the presence of the enzymatic extract. For ideal results, the non-enzymatic production should be minimized, using buffers such as malonic acid, pH 8.5. Also, conditions should be such that the enzymatic product increases linearly both with time and with increasing enzyme concentration.

In the case of highly active enzyme preparations, the enzymatic extract should be assayed for several dilutions to make sure that the results are within the linear range of the assay method.

Part A: Laboratory Procedure

Carbonic anhydrase (CAII) activity will be measured in both the crude sample, labeled sample #2.4 (Experiment 2, "Sup Exp. 6") and the purified enzyme solution, labeled "Purified CAII" (Experiment 5, step 5).

> Note: Make certain you have become familiar with the operation of the spectrophotometer before performing procedure beyond step 8. Do not add PNPA to each sample until you are ready to begin monitoring the absorbance.



1. Transfer 27 µl of each enzyme fraction into clean microcentrifuge tubes.
2. Set the spectrophotometer (Spectronic 20 D) to monitor absorbance at 400 nm by using the wavelength control knob (large knob on the top right).
3. Number seven disposable cuvettes for activity measurement of the crude (1cr-4cr) and the purified CAII (5pu-7pu). See Table below.
4. Add water and malonic acid to all cuvettes as indicated in the table below. This can be done ahead of time.
5. Calibrate spectrophotometer as follows:

   -Set the mode to TRANSMITTANCE using the MODE select control button until the "transmittance" LED on the right of the display is lit.

   -With sample compartment empty and cover closed, adjust Zero Control (front, left knob) so that the meter reads zero.

   -Set the mode to ABSORBANCE by pressing the "MODE" select button.
6. Read samples one at a time. Start with control sample by adding PNPA (recipe below) just before monitoring absorbance. Mix well by pipetting gently in and out (no bubbles!). Place cuvette in the cuvette holder (black, plastic adaptor). Insert the control sample into the sample compartment and adjust absorbance to 0.0 by turning knob on bottom right.
7. Record the absorbance readings for the control at time zero, and at every 15 sec thereafter up to 3 min. These are the changes in absorbance due to non-enzymatic hydrolysis of PNPA.
8. For samples 2 through 7, add either diluted-crude sup or diluted-purified CAII first (from step 1 above, also refer to table below), and then add PNPA just before begining to monitor the absorbance. Mix well and immediately place sample in the cuvette holder. **Do not adjust the absorbance to "zero" again in the spectrophotometer before measuring these samples, since what is important is the change of this parameter over time (not its absolute value).**
9. Again, record absorbance from zero to 3 min every 15 sec for each sample.

Exercise 6a: Calculations of enzyme specific activity

MANUALLY, calculate the specific activities and the fold-purification as described below. ALSO, USE EXCEL as described in Part B of this experiment to perform the same calculations. Include both set of calculations in your lab manual.

1. Calculate the change in absorption (ΔA) for every time point of each sample. For example: To calculate the ΔA at 3 min, subtract (A_{400} control at 3 min. minus A_{400} control at zero min.) from (A_{400} sample at 3 min minus A_{400} sample at zero min.)

Sample #	Water (µl)	Malonic acid (µl)	Sup Crude sup. (µl)	Purified CAII	PNPA (µl)
1cr (control)	566	100	-	-	333
2cr } crude	564	100	2	-	333
3cr } crude	562	100	4	-	333
4cr } crude	558	100	8	-	333
5pu } pure	564	100	-	2	333
6pu } pure	562	100	-	4	333
7pu } pure	558	100	-	8	333

2. Later, calculate the specific activity of both the crude and the purified CAII. Then, calculate the number of fold-purification by the affinity column procedure. These calculations can be done as follows:

3 The specific activity is expressed in mol product per amount of enzyme, per time of the assay (we will use nmol/ mg enzyme x min). At the pH (8.5) of the assay and the wave length of the measurement (400 nm), *p*-Nitrophenol concentration is negligible. The concentration of *p*-Nitrophenolate (the predominant product) can be calculated from Beer's law:

$\Delta A = a_m c L$

ΔA is the change in absorption for every time point of the assay (step 9 above),

$\mathbf{a_m}$ is the molar absorptivity of *p*-Nitrophenolate ($18.1 \times 10^3\ M^{-1}\ cm^{-1}$)

c is the concentration of *p*-Nitrophenolate (mol/lit)

L is the light path of the cuvette (1 cm).

13 Multiply the concentration of the product times the reaction volume (in lit) to obtain mol of product. Next, multiply times 10^9 to obtain nmol product. Divide by mg enzyme used in the assay. Divide by the time of the assay (seconds) in which the change in absorbance was observed. This is the specific activity of CAII in nmol/ mg/ sec.

14 Determine the ratio of the specific activity of the purified extract over the specific activity of the crude supernatant. This is the number of fold-purification of the affinity column procedure.

Solutions

Malonic acid buffer:
0.1 M malonic acid in H_2O
Adjust pH to 8.5 using 2 M NaOH

p-Nitrophenylacetate (PNPA):
(make fresh just before using and wrap container with aluminum foil)
dissolve 54.3 mg PNPA in 3 ml acetone
bring volume to 100 ml with H_2O (after PNPA has dissolved)

ABS 400 nm

	(seconds)												
	0	15	30	45	60	75	90	105	120	135	150	165	180
1													
2													
3													
4													
5													
6													
7													

1: control 2,3,4: Sup, crude, supernatant 5,6,7: purified CAII

Exercise 6b: Using Excel to calculate specific activity of CAII from Spectrophotometric data

A. Entering Spectrophotometric Data and correcting for Non-enzymatic Hydrolysis

> Mathematical activators in Excel: + for addition, - for subtraction, * for multiplication and / for division

a) Open new spread sheet and label columns, in appropriate cells, as indicated on the previous page.

b) Enter your absorption readings for crude and pure from 0-180sec in cells C3:O9

c) Calculate the 'normalized' absorption change (ΔA) data for crude and pure samples. This is done by obtaining the chance in absorbance for every time point and then subtracting from it the corresponding change in absorbance due to non-enzymatic hydrolysis of the substrate. For example: at time 30sec for your control, enter into the cell E12: =(E3-C3)-(E3-C3) and press *Enter*. This should equal 0, as we would expect for a normalized control. To find change in absorption for 4uL crude at 60sec enter into cell G14; =(G5-C5)-(G3-C3) and press *Enter*. Find the change in absorption for all crude, pure, and control absorption readings from time 0-180 sec, by entering the appropriate formula into cells C12:O18.

B. Calculating the Molar Concentration and amount (in nmol) of Product Formation

1) Solve for C (mol/L) in cells D22:O27, using the equation $\Delta A = a_m cL$, where a_m=18.1x103 M^{-1} cm^{-1} and L=1cm. To solve for C (in mol/lit) we should enter the formula; =*ΔA*/18100 or =*ΔA*/18.1E3. Type into 1st cell D22; =D13/18100 and press *Enter*. This formula can then be copied and pasted on all cells D22:O27.

2) Calculate amount of product formation in nmol by multiplying (mol/L) x (volume of the assay, $1x10^{-3}$ lit) x ($1x10^{9}$ nmol/mol). This operation is equivalent to entering into all cells D31:O36; =*Cvalue**1000000.

C. Calculating the Amounts of Proteins Used in each Sample in the Assay

From the protein concentration assay (Exp.6), you can determine the amount of protein used in each sample in your activity assay. To determine the actual mg of protein used in each assay sample it is necessary to use the following equation, = 0.001mg/*uL* * #*uL protein used in each assay. This is based on the fact that 1 μg=0.001 mg. In our case, the calculations will be, 2 or 4, or 8 * 0.001mg/uL.* For 2 μL crude (cells D40-O40) enter; =*ug/uLconcentration found for crude**2*0.001 and press *Enter*. Using this example, calculate all the amounts of protein used in your activity assay for each crude and pure sample.

D. Calculation of Specific Activity (in nmol/mg/sec) and Fold Purification

a) Use the arbitrary units of ng/mg/sec to estimate the amount of product formed per amount of protein per time. Divide nmol of product calculated in B (above) by mg protein from C times each second interval. For example: enter in cell D49; =D31/(D40*15) and press *Enter*.

b) Finally to find the Fold Purification, first find the average of crude and pure specific activity values by entering average into cells D56 and D57, respectively. Fold Purification is the average specific activity of pure divided by the average specific activity of crude. In cell D59 enter this equation to find the fold purification of your specific activity.

E. Graphing ΔA Over Time

Validity of a specific assay depends on the linearity of the product detection response over time for a specific amount of enzyme. This will be assessed by plotting each set of change in absorption readings over the time series. Make one graph for pure and one for crude.

1. Using the *Line* graph in *Chart Wizard*, plot time on the X-axis and ΔA (one set for each enzyme amount or volume, i.e., 0, 2, 4 and 8 μl) in the Y-axis.
2. Click *Series*, press *Add* to enter an Y-axis data set.
   - To enter an Y-axis data set; click the icon next to *Values*, highlight data set, and click icon again.
   - Press *Add* again to enter a different uL data set.
   - Continue until all 4 data sets have been entered and click *Finish*.
3. Label x and y-axes.

F. Graphing ΔA Versus Amount of Enzyme

Again, validity of our specific activity calculations depends on the linearity of product formation versus amount of enzyme for a specific assay time. This can be assessed for any time point. Make a graph using ΔA at 180 sec (i.e., 3 min) as an end-point assay, as enzyme amount is varied (0, 2, 4 or 8 μl). Plot data for both crude and pure enzyme preparations.

1. In cells S1:4 enter your μL amounts, this is the X-axis for both graphs.
2. U1:4 enter crude ΔA at 180sec, your y-axis for graph 1.
3. W1:4 enter pure ΔA at 180sec, your y-axis for graph 2.
4. Again make two graphs, one for pure and one for crude, using a *Line* graph. Enter X-axis and Y-axis in the usual manner, and press *Finish*.

Exercise 6c: Enzymatic Activity Questions

1. Is the specific activity of your crude extract lower than the specific activity of your purified extract? Calculate how many folds have you purified hCAII from the crude extract.

2. Was your enzyme assay linear over time? What are possible explanations for a time versus product non-linear graph?

3. Was your enzyme assay linear versus the amount of enzyme? What are possible explanations for a non-linear enzyme amount versus product graph?

4. Calculate how many molecules of PNPA are hydrolyzed by one mg of recombinant hCAII in one second.

Section 2: Protein Chemistry: Protein Characterization

Experiments 8 - 11

Experiment 8: SDS-PAGE of Affinity Purified CAII for Immunological Analysis

Experiments 8, 9 are directed toward the further characterization of Carbonic anhydrase using immunoblotting. You will first cast your own gel. A portion of the resulting gel from Experiment 8 will be used for Western transfer (Experiment 9).

The first step in a Western transfer (immunoblotting) experiment is SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). This technique allows quick analysis of based on their differences in molecular mass. SDS (Sodium Dodecyl Sulfate or Sodium Lauryl Sulfate) binds along polypeptide chains through the hydrophobic tail at a ratio of about 1.4 g SDS per 1 g protein. It generally denatures the proteins' three-dimensional conformation and renders them biologically inactive (with some exceptions, e.g. proteases). Because the negatively-charged heads of the SDS molecules are hydrophilic, facing the solvent (water), proteins in the solution are effectively solubilized. The mass of the negatively-charged SDS-protein complex is proportional to the molecular mass of the protein, so all the proteins present, regardless of their charge distribution, can be separated by electrophoresis on a polyacrylamide gel.

The widely-used Laemmli gel method uses tris/glycine as the electrode buffer (you will be using this system). This method was modified by Schägger and von Jagow (1987) to use tricine in the place of glycine. Such modification substantially improves the resolution of proteins in the low molecular mass range (2-20 kd).

SDS gel electrophoresis resolving power is greatly due to the use of a stacking gel, which concentrates protein samples into small zones prior to their entry into the separating gel. In the stacking gel, as the negatively-charged complexes move toward the positive electrode, the Cl^- ions move fastest and the tricine ions move slowest. The protein-SDS complexes, moving at an intermediate speed, are concentrated into a small zone between the two.
In the separating gel, two phenomena occur. First, glycine is rendered more negative in the altered pH, and it speeds ahead of the proteins. Second, the proteins begin their progress at essentially the same point, and therefore their ultimate separate bands' positions accurately reflect their molecular masses.

References

Bollag, D. M. and S. J. Edelstein. 1991. Protein Methods. Wiley-Liss, New York.

Bradford, M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-252.

Matsudaira, P. T. 1989. A Practical Guide to Protein and Peptide Purification for Microsequencing. Academic Press, San Diego.

Schägger, H. and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 10 kDA. *Anal. Biochem.* **166**: 368-379.

Walker, J. M. 1984. Methods in Molecular Biology Volume 1: Proteins. Humana Press, New Jersey.

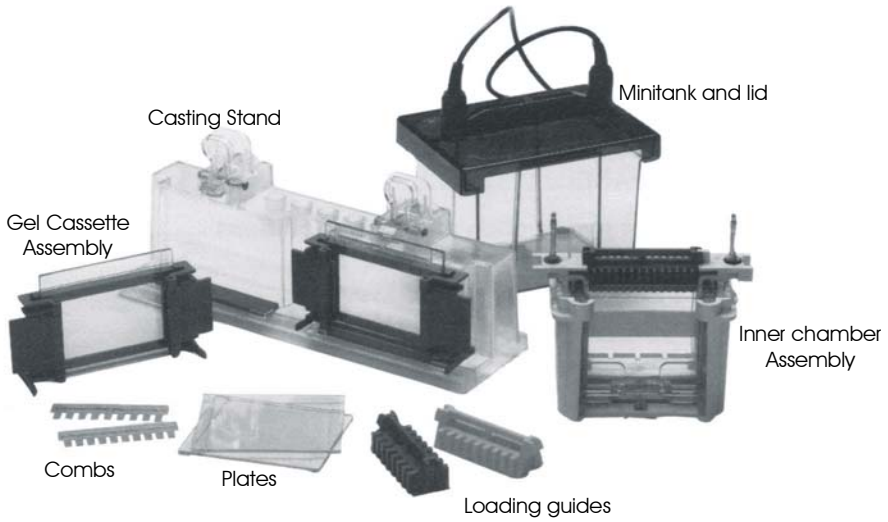
Wray, W., T. Boulikas, V. P. Wray, and R. Hancock. 1981. Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* **118**: 197-203.

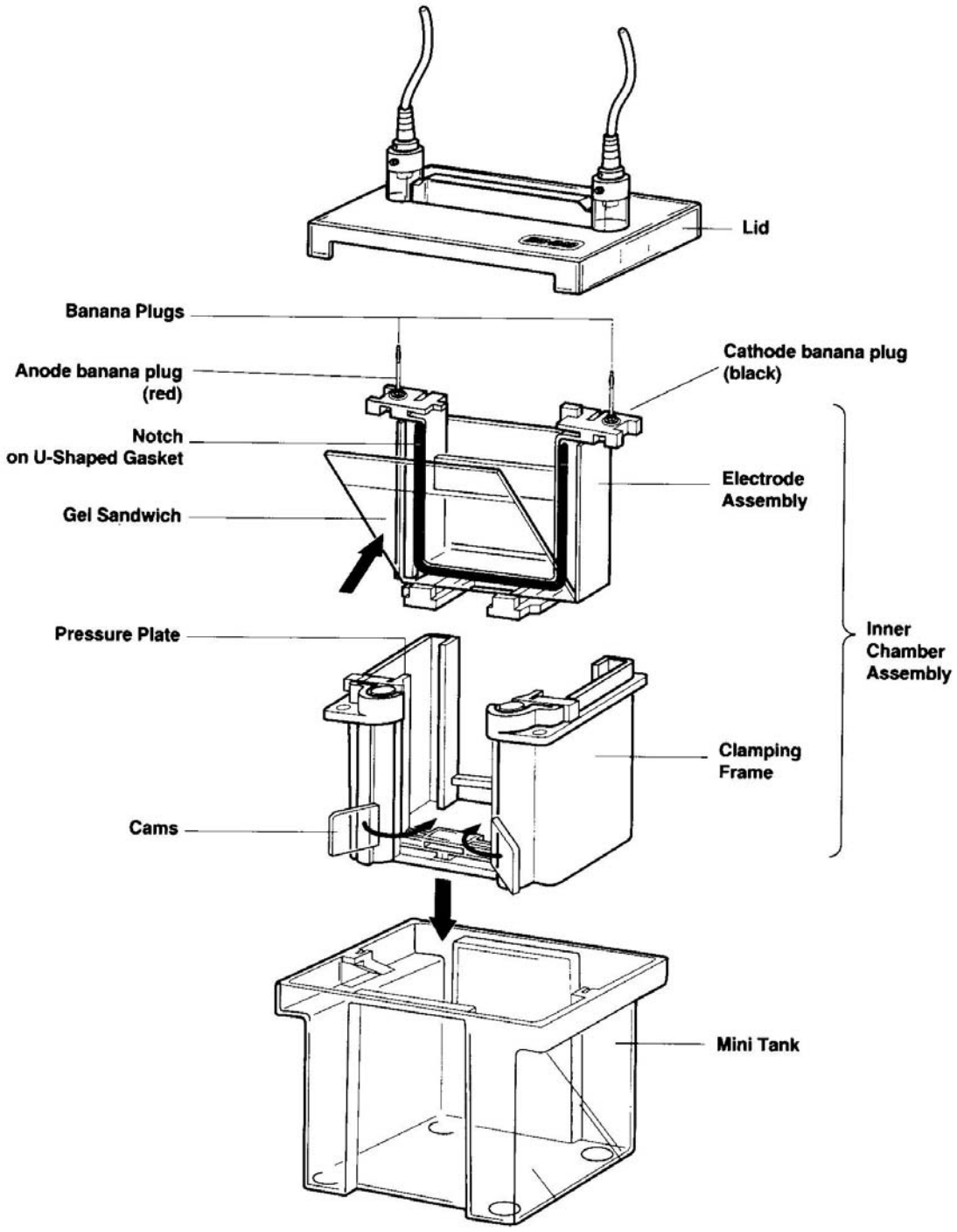
Apparatus and Setup

The gel apparatus you will be using is available commercially from BioRad (Mini Protean 3). It consists of two buffer reservoirs separated by a gel sandwiched between two glass plates. The sample wells are formed by a template "comb" placed within the stacking gel. Gels are run with the negative electrode on the top (or inner chamber) and the positive electrode on the bottom (or outside chamber).

First, the separating gel is poured between the two glass plates and allowed to polymerize. The stacking gel is poured on top of the polymerized separating gel. For the Tris-tricine system, the top and the bottom electrode buffers are different in composition. See appendix 1.

The Mini-PROTEAN 3 system includes a casting stand and glass plates with permanently bonded gel spacers that simplify hand casting. The cell can run one or two gels, and the mini tank is compatible with other BioRad electrode modules for western blotting and two-dimensional electrophoresis.




Lid
Banana Plugs
Anode banana plug (red)
Cathode banana plug (black)
Notch on U-Shaped Gasket
Electrode Assembly
Gel Sandwich
Pressure Plate
Inner Chamber Assembly
Clamping Frame
Cams
Mini Tank

Note: **Wear gloves! acrylamide is a neurotoxin!** Work carefully on top of absorbing paper and inside the area of the tray provided. Mix gel solutions (minus TEMED and APS) and filter through Millipore filter (0.22 μm). These solutions can be kept up to 10 days in the refrigerator. Add TEMED and APS to polymerize gels.

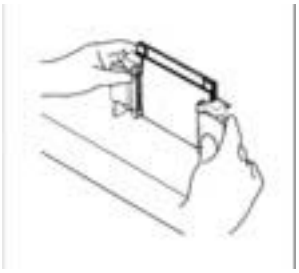
Part A: Casting the gel

Gel Cassette Preparation

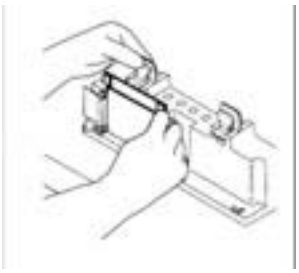
1. Plates should be very clean (laboratory soap and water is adequate). Place a short plate on top of the Spacer plate of the desired thickness (0.75 mm). The orientation of the Spacer plate should be such that the labeling is 'up'.

2. Slide the two glass plates into the Casting frame, keeping the short plate facing the front of the frame (this is the side with the pressure cams. Cams should be in the open position (facing forward).

3. Insure both plates are flush on a level surface. Leaking will develop if plates are misaligned or oriented incorrectly.
4. When the glass plates are in place, engage the pressure cams to secure the glass cassette sandwich in the Casting frame



5. Place the Casting frame into one of the positions in the Casting stand by positioning the Casting frame onto the casting gasket while engaging the spring loaded lever of the Casting stand onto the Spacer plate



6. Fill the space in between the glass plates with water to check for leaks. If no leakage is observed, tilt the unit to discard water and proceed with casting of the separating gel without disassembling the unit. If any leakage is detected, reassemble the unit and test as before.

Casting the Separating Gel (Discontinuous Polyacrylamide Gels)

1. Place a comb completely into the assembled gel cassette. Mark the glass plate 1 cm below the comb teeth. This is the level to which the resolving (separating) gel is poured. Remove the comb.
2. Wearing gloves, obtain 6 ml of the 10% separating gel solution (provided in a falcon tube ready to mix, already degased). Add 3 ul of TEMED and 30 ul of 10% (w/v) APS and mix well by gentle swirling.
3. Pipet separating gel solution (use a one-ml pipet) into the space in between the glass plates.
4. Quickly but gently, overlay the un-polymerized gel solution with 0.5 ml of isobutanol (pre-saturated with water). This keeps the separating/stacking gel interface smooth and flat.
5. Allow gel to polymerize for 30 minutes.

Notes:

When the gell has polymerized, a distinct interface will appear between the separating gel and the isobutanol layer. The gel mold can be tilted slightly to verify complete polymerization.

At this point the gel can be stored at room temperature overnight. However, drying should be avoided by adding 3-5 ml of 1:4 dilution of 1.5 M Tris HCl, pH 8.8 buffer (or the appropriate electrophoresis buffer if a different system is being used).

Casting the Stacking Gel

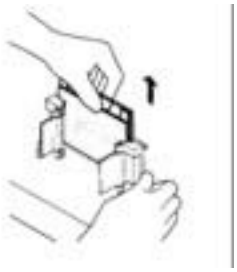
1. Pour off the butanol or the Tris-HCl layer covering the separating gel onto a Kimwipe. Rinse the top edge of the gel with distilled water. Use a piece of Whatman paper to absorb remaining traces of water.
2. Wearing gloves, obtain 2.5 ml of degased stacking gel solution (provided in a falcon tube, ready to use). Add 3.0 µl TEMED and 30 µl 10% (w/v) APS. Gently swirl to mix the components. Pipet stacking gel mix on top of the separating gel until solution reaches top of front plate.
3. Carefully insert comb into gel sandwich until base of teeth line up with the short plate. Be sure no bubbles are trapped below the teeth. Tilting the comb at a slight angle helps to prevent air bubbles.
2. Allow the stacking gel to polymerize for about 30 min.

Module Assembly

Note: You will be sharing an apparuts with the group next to you.

1. Carefully remove comb making sure not to tear the thin "fingers" of gel that divide the individual wells. Rinse wells with water or electrode buffer.

2. Remove the Casting frame from the Casting stand. Rotate the cams inward to release the Gel Cassette



3. Place the Gel Cassettes into the slots at the bottom of each side of the Electrode Assembly. Be sure the Short plate of the Gel Cassette faces inward toward the notches of the U-shaped gaskets.

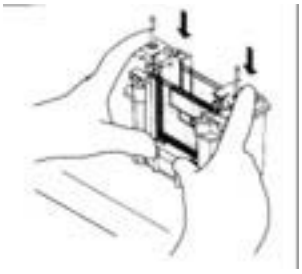
Notes:

The group with which you will be sharing the gel apparatus either will have done this step or will do it next, using the other side of the Electrode Assembly. When running a single gel, the side of the cooling core not being used must be sealed with a clamp assembly containing only the short glass plate.

If only one gel is to be run, use the mini cell buffer dam to create the upper (inner) chamber.

4. Lift the Gel Cassettes into place against the green gaskets and slide into the Clamping frame.

5. **Press down** on the Electrode Assembly while closing the two cam levers of the Clamping Frame to from the Inner the Chamber and insure proper seal of short plate to notch of the gasket.



6. Lower the Inner Chamber Assembly into the Mini Tank. Fill the inner chamber with about 130 ml of electrode buffer. Check for leaks and reassemble if necessary. Do not overfill the Inner Chamber

Assembly as this will cause siphoning of buffer which can result in buffer loss and interruption of electrophoresis.

Note: In Tris tricine gels the inner (top) chamber uses cathode buffer, whereas the lower chamberr uses anode buffer.

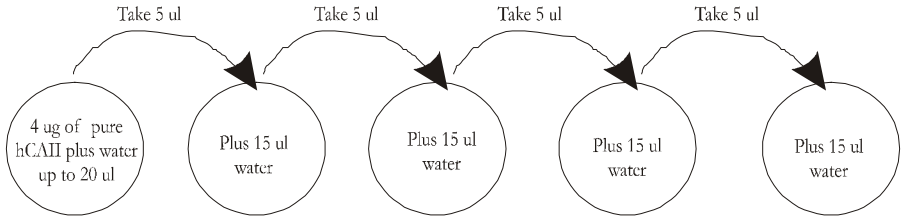
7. Pour 200 ml electrode buffer in the mini tank (outer chamber).
8. Flush the sample wells with electrode buffer using a 1 ml Pipetman to remove un-polymerized acrylamide and any other contaminants.
9. The gel is now ready for sample loading.

Note: Separating gel can be poured one day in advance and stored at room temperature. The stacking gel should be poured immediately before needed.

Part B: Sample preparation

For this experiment, we will use a total of about 125 pmol of purified protein. For CAII (30 kd) that is equal to about 4 μg. You will also need several other samples as indicated in the following procedure and summarized in the table below.

1. Calculate the volume needed for 4 μg of your desalted/purified CAII (Exp. 5). Add this to a tube. The calculation is based on the protein concentration you determined in Experiment 6 (μg/μl).
2. Perform a serial dilution (four-fold each) of this sample as indicated below.



3. Transfer 10 μl from each one of these dilutions into new centrifuge tubes. Mix each separate dilution with 10 μl of 2X Laemmli Sample Buffer (LSB).
4. Prepare an "induced , crude supernatant" sample by mixing 5 μl of fraction #2.2 (Exp. 2) with 5 μl of 2X LSB.
5. The Laboratory staff will provide you with a tube containing 10 μl of BioRad Low Range MW markers ready to be loaded.

SDS-PAGE MW Standards*

	Pre-stained	*Unstained*
Phosphorylase b	103,000 Daltons	97,400 Daltons
Serum albumin	77,000	66,200
Ovalbumin	50,000	45,000
Carbonic	34,300	31,000
Trypsin inhibitor	28,800	21,500
Lysozyme	20,700	14,400

*BIORAD: Prestained (Cat. # 161-0305), Unstained (Cat. # 161-0304)

6. You will also be provided with 10 µl of Sigma carbonic anhydrase standard (final concentration of 0.1 µg/µl) and 10 µl of a "non-induced, crude total extract" samples ready to be heated.

7. Heat all samples (except BioRad MW Standard) to 85°C for 5 min then load as indicated in the chart below.

Lane #	Type of sample	Vol (µl)
1	Non-induced, crude total extract (provided w/o LSB)	10
2	Induced, crude supernatant (Exp. 2)	10
3	Sigma CA standard (0.1 µg/µl) Provided ready to heat!	10
4	Affinity purified/desalted CAII (pCAII) Dilution #1	20
5	pCAII Dilution #2	20
6	pCAII Dilution #3	20
7	pCAII Dilution #4	20
8	pCAII Dilution #5	20
9	Low Range BioRad MW markers (Provided ready to load!)	10
10		-

Part C: Running the gel

1. Attach electrodes and run gel at 125 V until blue dye is 1 cm from bottom of gel.

2. When electrophoresis is done, disassemble the gel using one of the gel spacers to pry apart the glass plates. (**Use gloves**!).

3. Transfer proteins from the gel to a nitrocellulose membrane as described in Experiment 9.

Experiment 9: Characterization of Affinity Purified Protein: Electrophoretic Western Transfer

This experiment utilizes the gel from Experiment 8. SDS-PAGE electrophoresis separates proteins by molecular mass, which is often not conclusive as a criterion to establish the identity or presence of a given protein. The mixture might contain many proteins of similar molecular mass, with the protein of interest being a minor component. The mixture's actual protein composition may be characterized more specifically by probing with an antibody in solution (Enzyme Linked Immuno Assay, ELISA), or, as in this experiment, by transferring proteins isolated by SDS-PAGE to a membrane. Once on the membrane, the proteins may be stained (only if using PVDF or nylon) and the desired band used for micro-sequencing (Edman chemistry). Alternatively, the proteins on the membrane can be probed with antibodies (immunoblotting), as described here.

The term "Western blot" is commonly used for both the transfer from gel to membrane and the immunoblotting, because the procedures were originally developed in combination with one another. However, it must be kept in mind that proteins may be transferred onto a membrane for purposes other than antibody probing.

Three kinds of membranes are most commonly used for transfer: nitrocellulose, nylon and **p**oly**v**inylidene**dif**luoride (PVDF). Although any may be used for western blots, only PVDF is used for Edman Chemistry sequencing of proteins. Nitrocellulose is employed in the protocol described here.

Transfer of proteins to the membrane is accomplished by electrophoresis, with the two most common formats being:

- Semi-dry blotting, in which the gel and the immobilizing matrix are sandwiched between buffer-wetted filter papers, through which a current is applied for 10-30 min.
- Wet (tank) blotting, in which the gel-membrane sandwich is submerged in transfer buffer in a tank. Depending on the applied voltage, this system may require from as little as 1 hour to overnight.

Optimal conditions for the most efficient transfer must be determined empirically since all proteins have different chemical and physical properties. The following guidelines are useful starting points.

Large proteins >80 kd. Use 7.5% acrylamide gels and omit methanol from the transfer buffers. You may also have to extend the blotting time by 2 to 3 times that necessary for smaller proteins.

Average proteins 16 kd to 80 kd. Use 10 to 12% acrylamide gels. Optimal blotting conditions depend on the properties of the protein: if the protein is acidic, it will blot best in the neutral pH range; if it is basic, it will blot better at a higher pH.

Small proteins and peptides <10 kd. Use 15% acrylamide gels. Blotting conditions are best at neutral pH, since individual peptides may have very different properties from the intact protein.

Part A: Assembly of the protein transfer unit

Note: Use the gel from Experiment 8.

1. Identify all the items in the Western Tranfer kit on your bench. You should have one Nitrocellulose membrane (in between two pieces of blue or white wax paper), two pieces of 3MM filter paper the size of gel, 2 fiber sponges, a sandwich container, and the cassette holder.

> Wear gloves! Handle the Nitrocellulose membrane with forceps or gloved hands only! Always pick-up the membrane by the same corner.

2. Equilibrate the Nitrocellulose membrane with Tris-glycine transfer buffer (1 minute).

Tris-glycine: (25 mM Tris, pH 8.3; 192 mM Glycine, 20% (v/v) methanol)

Alternative Transfer Buffer: (10 mM MES, pH 6, 20% (v/v) methanol)

Reagent	Amount
MES, pH to 6	1.95 g
Methanol	200 ml
0.5 M EDTA	1.0 ml
0.5 M EGTA	1.0 ml

> Note: Do not equilibrate gel in transfer buffer. The small amount of SDS remaining in the gel actually helps the protein transfer.

3. Add about 300 ml of transfer buffer to sandwich container. Submerge cassette holder in the buffer with the black side down (this side denotes the negative electrode

4. Open up cassete holder (like you would open a book… while submerged) and assemble the sandwich for transfer by placing items starting from the bottom in the following order: fiber sponge, one piece of 3MM paper, gel (from Exp. 8), Nitrocellulose membrane, second piece of 3MM paper, and second fiber sponge. See also the diagram on the next page. Check after every addition that there are no air bubbles caught between the layers. Close the cassette and keep it submerged until ready for moving onto the transfer chamber.

5. Pour 700 ml of transfer buffer into the transfer chamber, then place the ice block into the unit.

6. Retrieve the transfer cassete from sandwich container and slide it into the slot in the gel tank. Make sure to line up black side of the cassette with black electrode. If cassette has been set up properly the nitrocellulose membrane should be located on the positive side relative to the gel. This is because proteins move toward the positive electrod when current is applied.

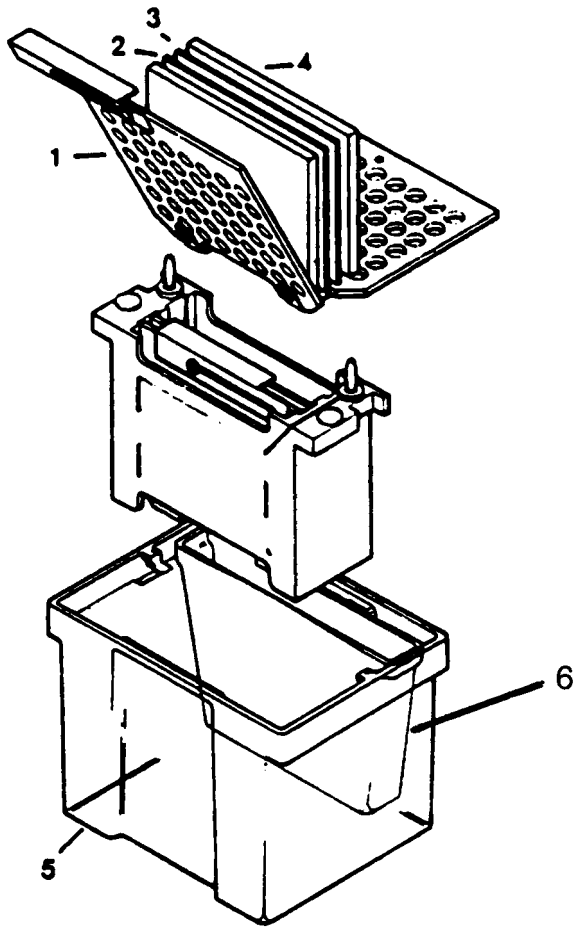
7. Place lid on transfer unit. Transfer proteins at 90 V for 1 hr.

> Protein transfer conditions:
>
> Large gel.....overnight at 20 V at 4°C
>
> Small gel.....small soluble proteins.....1 hr/ 90 V, room temperature
>
> large proteins.....2.5 hr/ 90 V, room temperature

insoluble proteins*.....2.5 hr/ 90 V, room temperature * add 0.01% SDS to transfer buffer and leave out methanol.

8. Turn off transfer unit, and wear gloves to take the transfer cassette out. Disassemble using forceps to put the membrane into a sandwich container and go to Part C, step 1.

Assembly of the gel holder cassette for Western transfer
(Wear gloves for this procedure to avoid contaminating membrane)



Locking the gel cassette clamping system. The cassette (1) holds the gel (2) and membrane (3) while fiber pads and filter paper (4) on both sides provide contact within the gel sandwich. An ice block (6) and the gel cassette are inserted in the buffer tank (5). If preferred, transfer can be done in a cold room to avoid heat build up (in this case an ice block is unnecessary).

Part B: Staining of PVDF Membrane with Coomassie Blue

WARNING!: YOU WILL NOT BE DOING PVDF MEMBRANE STAINING IN THE LAB

Note: This staining procedure works only for PVDF membranes, not for Nitrocellulose or Nylon.

1. Handle membrane with forceps.
2. Wash membrane with distilled water 5 minutes in a petri dish.
3. Stain for 20-30 sec with 15 ml of a solution of 0.05% Coomassie blue made in 40% methanol, 5% acetic acid.

Note: this staining solution has the same composition as the staining solution used for gels, **however do not** use the same solution that you use for staining gels. The gel staining solution will be contaminated with glycine that diffused from the gel. Glycine will then impregnate the membrane, thus yielding the protein spots in the membrane unsuitable for sequencing.

4. De-stain with 20 ml in a solution of 40% methanol, 5% acetic acid for 1-5 min until bleeding stops (change de-stain twice). Dispose the used de-stain solution into the waste container provided.
5. Rinse membrane in distilled water, 3-5 min with at least 3 changes.
6. Air dry, place between two 3MM papers, wrap in foil and store at -20°C.

Choice of membranes:

-For N-terminal sequencing use PVDF type membranes: Immobilon PSQ, Immobilon CD (for problem proteins), Problot, Bio-Rad®, etc.

-For chemical cleavage of a protein use: Immobilon P, Bio-Rad®, Immobilon CD.

-For enzymatic digestions: Nitrocellulose.

-For Western Blots use PVDF or nitrocellulose

Part C: Immunoblotting (Western Blot)

Immunoblotting is a powerful protein characterization tool that makes use of antibodies to detect a protein (antigen) immobilized on a matrix. It is usually preceded by membrane transfer because this makes proteins more accessible, smaller amounts of protein are needed for detection, and because membranes are easier to handle than gels.

Either a monoclonal or polyclonal antibody capable of recognizing the protein of interest must be available. If a protein has already been characterized, it can in turn be used to determine the specificity of sera that contain antibodies of unknown characteristics. It is important to be aware of the fact that some antibodies that recognize an epitope in solution may not be able to recognize the same epitope once blotted onto a membrane. These and other issues will be discussed in workshop lectures.

Antibody probes may be either (1) directly labeled or (2) themselves probed with a secondary antibody that is linked to a marker protein (= labeling agent). The marker uses a substrate that is converted to a colorimetric or chemiluminescent product. Our protocol follows the earlier method.

Detecting the protein (antigen) requires three major steps.

1. The membrane is incubated with the primary antibody for several hours.
2. A secondary antibody, which recognizes an epitope on the first antibody, is incubated with the membrane. This secondary antibody is conjugated to an agent (label) that helps in detection (e.g. alkaline phosphatase).
3. A substrate for the labeling agent is added (BCIP/NBT). The bands with a positive signal are then visualized on the membrane by a color reaction or on an X-ray film (chemiluminescent method).

Limits of detection:	1 pico gram (chemiluminescence)
	10 pico gram (colorimetric detection)

A commercially available secondary antibody used (step 2) is derived by raising goat antibodies against rabbit immunoglobulins for rabbit-generated primary antibodies. The secondary antibody (goat) is conjugated to a labeling agent, such as the enzyme horseradish peroxidase or alkaline phosphatase.

Procedure

1. Incubate membrane in 20 ml TTBS (50 ml tube from the end of Part A) for 10 minutes at room temperature with shaking. There is a rotatory mixer available in the lab for this purpose.
2. Pour off TTBS into waste container and block membrane to reduce non-specific binding by incubating the strip in 15 ml blocking solution. Incubate at room temperature for a minimum of one hour or until needed.
3. Decant the blocking solution back into the drain.
4. Apply primary antibody (sheep anti-hCAII) to membrane. Use a 10000-fold dilution in 15 ml blocking solution. Incubate 1 hr at room temperature with shaking (overnight shaking at 4°C is best).
5. Discard primary antibody solution into the sink. Wash the membrane strip with 15 ml TTBS, 2 times for a total of 30 min.
6. Apply secondary antibody (goat anti-sheep conjugated to ALP) to membrane. Use a 2500-fold dilution in 15 ml blocking solution. Incubate 1 hr at room temperature with shaking.
7. Discard secondary antibody solution into the sink and wash the membrane strip as in step 5.
8. Discard last wash and drain off the excess buffer. Transfer the membrane to a plastic petri dish.
9. Prepare color development mixture by dissolving in 10 ml of ddH_2O a pill made of alkaline phosphatase buffer (AP), BCIP and NBT. Add color development mixture to dish with membrane.

10. Gently swirl until bands become visible (5 to 45 min).

11. Stop development by discarding mixture and rinsing with water. The membrane can be allowed to air dry, then stored at -20°C in between two pieces of Whatman paper and wrapped in aluminum foil until ready for photography.

> Note: Antibody dilutions must be empirically determined for each system.
>
> For chemiluminescent methods such as ECL (Amersham™), the following dilutions are a good starting point:
>
> Primary antibody: 50 to 100 thousand-fold dilution in blocking solution.
>
> Secondary antibody: 50 to a 100 thousand-fold dilution in blocking solution.

Solutions

Tris buffered saline (TBS): (2 L)

Reagent	Amount
Tris (10 mM)	3.12 g
NaCl (150 mM)	17.53 g

Adjust pH to 7.5 and bring volume to 2 L.

Tris buffered saline + Tween (TTBS, 0.1% Tween-20):

Add 1 ml of Tween-20 to 1 L of TBS.
Blocking solution (Blotto): 5% (w/v) dry milk
Add 5 g dry milk to 100 ml TTBS.

Color development mixture: For 200 ml (for 20 membranes; 10 ml each)

Reagent	Amount
0.2 M Tris, pH 8.0	200 ml
2 M $MgCl_2$	400 µl
BCIP in 2 ml DMF	10 mg
NBT	20 mg

BCIP: 5-bromo-4-chloro-3 indolyl-phosphate
DMF: N,N-dimethylformamide
NBT: Nitro blue tetrazolium dye

Algorithms for the Identification of Potential Antigenic Sites

There are many different ways to identify antigenic sites. Initially areas of hydrophilicity, determined by inverting measures of hydrophobicity, were used (Hopp and Woods, 1981). After more study, further analyses were used in combination to better identify antigenic sites. Jameson and Wolfe (1988) created a computer algorithm to generate a value known as the antigenic index. The antigenic index combines hydrophilicity, flexibility, surface probability and turn propensity, based on primary amino acid sequence, to determine areas of antigenic activity. Thus, to determine antigenic sites, it is better to combine all possible statistics available to help identify areas in a protein that might be antigenic.

References

1. Hopp and Woods, Prediction of protein antigenic determinants from amino acid sequences., *Proc. Nat. Aca. Sci.* USA. **78**(6):3824-8, 1981, June
2. Jameson and Wolfe, The antigenic index: a novel algorithm for predicting antigenic determinants., *Computer Applications in the Biosciences.* **4**(1):181-6, 1988, March

Exercise 7: Using PepTools to Identify Potential Antigenic Sites

The following instructions will be useful for the exercises below:

a) Graphing Flexibility
   - Go to the Graph menu and choose the flexibility option
   - This will graph possible antigenic sites (at the peaks)

b) Graphing hydrophobicity
   - Go to the Graph menu and choose the hydrophobicity option
   - This will graph possible antigenic sites (at the troughs)

c) Finding Motifs
   - Go to the Analyze menu and choose Find Motifs
   - A window will pop up. Click the button that says Antigenic Sites
   - The program will search all possible amino acid sequences that are antigenic with your sequence of interest

1. Open the hCAII protein in the PepTools program.

2. Graph the flexibility of the hCAII protein. Determine possible antigenic sites and print the graph.

3. Graph the hydrophobicity of hCAII. Determine possible antigenic sites and print the graph.

4. Compare the two graphs. Look for areas where there is a peak in the flexibility graph and a trough in the hydrophobicity graph (hydrophilicity). Record below (and save) where these possible antigenic sites are.

5. Yet another way to identify antigenic sites, is to identify regions in the protein sequence that elicit an immune response (either by B or T cells). These sequences are known as Epitopes. Search for motifs. Save the results.

6. Compare your results from #4 and #5. Is there any overlap? Are you more certain that your antigenic sites are correct? Where should your antibodies (in experiment 9) bind?

YOU WILL ONLY RESPONSIBLE FOR PART D IN THIS EXPERIMENT. ONE OF THE LAB INSTRUCTORS WILL BE DEMONSTRATING THE IEF GEL PROCEDURE.

Experiment 10: Isoelectric Focusing of Human CAII Crude Extracts (Demonstration)

The SDS-PAGE procedure used in Experiments 3 and 8 separates proteins on the basis of their molecular mass. However, proteins may also be separated along a continuous pH gradient, using the technique of isoelectric focusing (IEF). IEF separates proteins according their isoelectric point –the pH site in a gradient at which the net charge on the protein is zero. The isoelectric point (pI) of a protein is informative about its amino acid composition and it can be an important criterion in deciding which type of chromatographic procedure to use downstream in the purification process.

In IEF, a pH gradient is generated and maintained by passing an electric current through an agarose or polyacrylamide gel in a solution of amphoteric compounds called carrier ampholytes. An ampholyte moves on this gel until its net charge is zero, where it will stop. The stopping position is its *isoelectric point* (pI). The carrier ampholytes have closely spaced pIs in a pH range as broad as 7 pH units (pH 3-10). They are relatively small and stack more quickly than the proteins; in doing so, they establish the pH gradient, increasing from anode to cathode. The proteins (which are also amphoteric compounds) then move along the pH gradient. The points where they equilibrate, or stop, define their pI values. Since IEF is an equilibrium technique, its results do not depend on the mode or site of sample application. Proteins whose pI differ by only 0.02 pH units can be separated into sharp bands.

Several commercial suppliers (e.g., Amersham-Pharmacia biotech) offer IEF polyacrylamide gel strips with preformed pH gradients in which the ampholytes have been covalently linked to the matrix. When all of the amphoteric compounds are at their isoelectric points, the current across the entire IEF gel should be close to zero. Some residual current may be present due to diffusion of compounds away from their pI. IEF is usually run at high voltages so that the near-zero current can be recognized and used as a signal that the procedure is finished.

This Experiment determines the pI of hCAII in the crude cell extract produced in Experiment 5. The protocol used employs pre-poured IEF agarose gels (FMC®) with a flat bed gel system (Bio-Rad®). Cold water is circulated through the system to dissipate heat generated during the focusing process. Between 10-20 μg of hCAII extract is loaded in a 3 μl sample, adjacent to the pI standards. This portion of the gel is fixed, rinsed to eliminate interference of ampholytes with staining, and Coomassie Blue stained. Another sample is added to a separate lane and transferred to a PVDF membrane ("press blotting"), to be probed with anti-hCAII antibodies.

References

Hames, B. D., and D. Rickwood (eds.). 1990. Gel Electrophoresis of Proteins: A Practical Approach. IRL Press, Oxford, New York.

Part A: Flat-bed agarose isoelectric focusing (IEF)

IMPORTANT! IMPORTANT! IMPORTANT! IMPORTANT!

You will be sharing one agarose gel with the group next to you. There are three IEF apparatus. Two gels will be run per apparatus, so you need to coordinate with three other groups for the successful completion of this experiment.

USE GLOVES AT ALL TIMES!

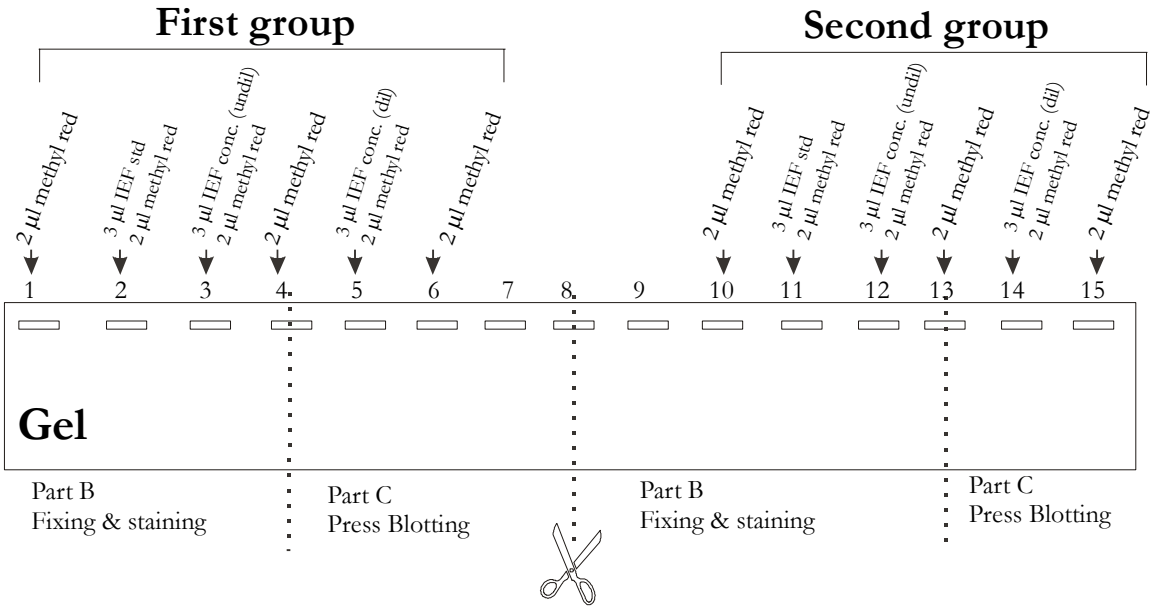
Steps 1 to 7 have been performed by the lab staff. You will begin with step 8.

1. Inspect the IEF apparatus to which you have been assigned. The cover contain the electrodes. Inside the apparatus there is a flat gridded surface on which your gel will be placed. Cold water is circulating through the system to ensure efficient cooling while samples are being focused at a high voltage.
2. Unpack your gel carefully by taking the cover off and then pushing the gel through the round opening at the bottom of the gel container. The gel is attached to a plastic membrane, be careful not to touch the agarose side of the gel.
3. Spray about 1.5 ml of water on the gridded surface in the IEF apparatus and place the agarose gel on it (plastic side down) so that a thin film of water provides direct contact with the cold surface. Avoid trapping air bubbles between the gridded surface of the IEF apparatus and the plastic gel membrane.
4. Blot off excess moisture from the surface of the agarose gel by placing a large piece of adsorbant paper on top of it. Leave the paper on the gel until it looks wet. Repeat this step once using a fresh piece of adsorbant paper. Excess water around the gel should also be adsorbed.
5. Soak one of the electrode strips in 0.5 M acetic acid (anode, positive) and the other one in 1.0 M NaOH (cathode, negative) for at least 2 min.
6. While waiting, gently place the upper lid on the IEF apparatus without closing it. This will mark the sites on the gel where the electrode strips should be placed. Blot the excess acetic acid and sodium hydroxide from the electrode strips by pressing the strips against a few Kimwipes twice. Put the anode strip on the upper end of the gel (away from you) and the cathode strip on the lower end, centering strips over the marks made on gel.
7. Place the sample loading template (green plastic) shiny side down on the gel at about half a centimeter below the anode (upper) strip. Press down gently so that the wells will not leak upon sample application.
8. Dilute 10 μl of desalted, purified CAII (Exp. 5) with 30 μl water. Undiluted sample is loaded in well No. 3. Load your samples in the following order (see below):

   I. Load 2 μl of Methyl Red marker into all of the wells that you will be using.

   II. Load the standard and the diluted CAII samples (see figure below).

   If you are the second group sharing the same gel, load in the same order as above starting with well 10 through 15 (see figure below).



9. Put the lid on the IEF apparatus and close by pressing down to make sure that the thin electrodes are aligned with electrode strips.

10. Connect electric "male" plugs into the power supply (red to red, black to black) and start isoelectric focusing at 1 W for 10 min (set voltage to 1500 maximum and current to 20 mA maximum).

11. Increase power to 25 W for 30 min (The current should approach zero when protein samples have reached their isoelectric point). For 3 gels: triple mA.

12. Just prior to the completion of the IEF, add fixing solution (40 ml) (recipe below) to the large petri dish provided.

13. Disconnect the IEF apparatus and <u>using gloves</u> and a pair of scissors cut through the center of gel without touching the agarose side (top). Identify your half of the gel and cut again through lane 4 (first group) or lane 13 (second group).

<u>Part B</u>: Preparation and staining the IEF gel

Use gloves!!!

1. Quickly, place the **left half** of the gel containing the IEF standards and one of the hCAII samples in the petri dish with 40 ml of IEF-fixing solution.

> **Important:** Immediately put the petri dish containing the IEF gel on the orbital shaker then go to Part C. Return to complete the remainder of Part B after you have begun Part C, step 6

2. Take gel out the fixing solution and lay it on the lid of the petri dish lid.

3. Wet a piece of blotting paper (FMC®) with dH_2O and place it on the gel.

4. Stack eight precut paper towels on top of the filter paper.

5. Place the empty bottom of the petri dish on top of the paper towels. Add a 500 ml bottle containing 300 ml water as a weight on top of the blot.

6. Wait 15 min and then remove the bottle and paper towels.

7. Wet the blotting paper (FMC®) on top of the gel, using a dH_2O spray bottle (if necessary), then remove the blotting paper.
8. Place the gel in a petri dish containing 40 ml of dH_2O and place on the shaker for 10 min.
9. Take the gel out of the water and allow the gel to air dry overnight (this can also be done quickly with a hair dryer). **Return to the Press Blot procedure, Part C, step 7.**

Next day

11. Stain the IEF gel by soaking the gel in 15 ml Coomassie blue IEF staining solution for 5 min.
12. Destain with 20 ml IEF gel destaining solution until the background is clear. Allow the gel to air dry.

Part C: "Press Blotting" of IEF samples onto PVDF membrane.

Use gloves!!!

1. Place the rest of the IEF gel (right side of the cut) plastic side down on the top side of a large petri dish lid.
2. Put a piece of PVDF membrane in a petri dish and wet thoroughly with methanol. Discard the methanol and soak the membrane in 25 ml of "press blot" solution for two minutes. Carefully, place membrane on top of the gel removing any air bubbles caught between the gel and the membrane (transfer begins as soon as the membrane comes into contact with the gel).
3. Wet two pieces of Whatman filter paper in the same "press blot" solution as used in step 2. Place the two filter papers on top of the PVDF membrane.
4. Stack two blotting pads (dry) on top of the filter papers.
5. Discard the press blot solution and place the empty petri dish on top of the two blotting pads. Place a bottle with 100 ml water on top of the plate.
6. Allow the blot-transfer to proceed for 15 min. **In the meantime, return to step 3 of Part B.**
7. Dismantle press blot set up and place the PVDF membrane in the large petri dish you just used (rinsed). Add 30 ml TTBS to this dish and wash for 5 min on the orbital shaker.
8. Remove the membrane and put in the same 50 ml conical tube containing the membrane and blocking solution from Experiment 9. Contact between the membranes can be avoided by separating them with small piece of a nylon mesh.

Solutions and reagents for flat bed agarose IEF gels

Anode buffer (anolyte):
100 ml 0.5 M acetic acid.

Cathode buffer (catholyte):
100 ml 1.0 N NaOH.

IsoGel agarose IEF plates, pH range 3-10:
These plates contain 2.52% ampholytes, 1% IsoGel agarose and 10% d-sorbitol (obtained from FMC®).
IEF standards (pI markers), pH 3-10 range: (Bio-Rad® or FMC®)
Follow manufacturer's instructions to reconstitute and dilute before use.

Methyl Red: (Sigma®)
1.5-2.0 mg/ml in water
As a pH 3.8 marker

Fixing solution: (500 ml)

Reagent	Amount
Methanol	280 ml
Trichloroacetic acid	30 g
Sulfosalicylic acid	18 g

Add ddH_2O to a final volume of 500 ml.

Press blot solution: (0.5 M NaCl/0.2 M Tris, pH 7.5) (2 L)

Reagent	Amount
Tris-HCl	50 g
Trizma base	0.94 g
NaCl	58.44 g

Adjust pH to 7.5 and add ddH_2O to a final volume of 2 L.

Coomassie blue gel stain:(1 L)

Reagent	Amount
Methanol	50 ml
Glacial acetic acid	100 ml
H_2O	850 ml

Tris buffered saline (TBS):(2 L)

Reagent	Amount
Tris	3.12 g
NaCl	17.53 g

Adjust pH to 7.5 and bring volume to 2 L.

Tris buffered saline + Tween (TTBS):
Add 1 ml of Tween-20 to 1 L of TBS.

Exercise 8: Using PepTools to estimate isoelectric points

Isoelectic points have been experimentally determined for some time. There are a number of computer programs that attempt to calculate isoelectric point from the primary sequence of a protein. For this exercise you will determine isoelectric point via a computer algorithm, experimentally determine the isoelectric point, and then compare the two.

1. Open the hCAII protein in the PepTools program.

2. Go to the Analyze menu and choose the option Protein Statistics. Scroll down until you determine the isoelectric point.

3. Do your experimental and calculated values for the isoelectric point match? Why do you think that this is the case? What could you do to determine if the values are close enough to consider non-significant? Which value would you trust more? Why?

Experiment 11: Two-Dimensional Gel Electrophoresis of Crude Human CAII (hCAII) Extract

Introduction

Experiments 3 and 10 illustrate the analysis of proteins according to molecular mass (SDS-PAGE) and isoelectric point respectively. Two-dimensional gel electrophoresis combines these two techniques to provide a powerful mean for the separation of complex protein mixtures. Many variations for this procedure have been devised; the most common format applies the techniques at right angles to one another, such that the resulting gel is something like an XY plot of isoelectric point by molecular mass.

The IEF portion is run first, establishing the first dimension of the gel. It can be done in agarose flat bed gels very quickly (45-60 min) because the highly porous agarose permits the proteins to migrate rapidly to their pI. Gel strips may be cut and sealed on top of a Laemmli gel for electrophoresis in the second dimension. However, an agarose gel is prone to more protein diffusion, protein band smearing, and therefore diminished resolution. Diffusion is minimized by using a polyacrylamide matrix. This results in very tight protein spots (high resolution). In a typical experiment, IEF separation (first dimension) is performed in polyacrylamide tube gels using ampholytes to form the pH gradient during electrophoresis. Despite its improvement over agarose gels, this method can still exhibit performance problems due to gradient drift and batch variability. More recently, an immobilized pH gradient gel (IPG) system has been introduced that eliminates these problems. IPG strips are made with buffering acrylamide derivatives that contain either a free carboxylic acid or a tertiary amino group which are co-polymerized with acrylamide and Bis. The pH gradient is pre-cast into the gel and cannot shift during electrophoresis.

In this experiment, you will be using the PROTEAN® IEF Cell (BIORAD) with ReadyStrip™ IPG Strips to analyze the crude bacterial lysate from the hCAII expression experiment (sample #2.1 from Experiments 1 and 2). Sample will be 'loaded' to the IPG gel strip by adding it as part of a rehydration buffer prior to electrophoresis. Samples can also be loaded in a more conventional manner by using the wells on the channel of focusing trays. However, strip rehydration with a solution containing the protein sample is the preferred method because it allows for larger sample loads and minimizes potential protein solubility problems. To maximize sample solubility, urea and detergent (Urea, CHAPS, Triton X-100 or NP-40) are included in the rehydration buffer and gel solutions. Thiourea is used when maximum solubilization of membrane proteins is needed. It is important to remember that all of these compounds denature the proteins to some extent, so the resulting isoelectric points may be different from those of the native proteins. Separation in the second dimension is done using an SDS polyacrylamide gel. Proteins that have similar pI are now separated based on their molecular mass. Staining of the gel slab should reveal hundreds or even thousands of distinct protein spots that can be further characterized.

Reference

Hames, B. D., and D. Rickwood (eds.). 1990. Gel Electrophoresis of Proteins: A Practical Approach. IRL Press, Oxford, New York.

Reagents

IPG Strips (ReadyStrip™), 0.5 mm thick, 7 cm, 4%T/3%C (125-250 µl rehydration volume)

Rehydration buffer (with protein sample)

Reagent	Concentration Range	Chosen Final Concentration	Amount/10 ml for 1.25X [Final]
Urea[a]	(8-9.8 M): 4.8-5.8 g/10ml	8 M	10 M: 6 g
CHAPS[a]	(1-4%): 100-400 mg/10ml	3.2% (w/v)	4%: 400 mg
DTT[a,b]	(15-100 mM): 23-150 mg/10ml	40 mM	50 mM: 75 mg
Bio-Lytes[a,c]	0-0.2%	0.16 %	0.2%: 20 µl
Orange G[d]	0.001%	0.001% (w/v)	0.00125%: 0.125 mg

[a] The amounts of Urea, CHAPS, DTT and Bio-Lytes depend on the sample solubility. The optimal rehydration buffer composition is determined empirically.
[b, b] DTT can be replaced by Tributylphosphine as an alternative reducing agent..
[c] The pH gradients of Bio-Lytes should be that of the IPG Strip.
[d] Orange G can be replaced by Bromophenol Blue.

Equilibration Buffer I (prepared from stocks immediately before use)

Reagent	Concentration
Urea (6 M)	6 M
SDD (2%)	0.2 g/10ml
Tris-HCl (0.375M), pH 8.8	2.5 ml 1.5 M Tris/10ml
Glycerol (20%)	2 ml/10ml
DTT (130 mM)	200 mg/10ml

This buffer is used to saturate the ReadyStrip IPG Strips with SDS and reducing reagent prior to the second dimension electrophoresis.

Equilibration Buffer II (prepared from stocks immediately before use)

Reagent	Concentration
Urea (6 M)	6 M
SDD (2%)	0.2 g/10ml
Tris-HCl (0.375M), pH 8.8	2.5 ml 1.5 M Tris/10ml
Glycerol (20%)	2 ml/10ml
Iodoacetamide (135 mM)	250 mg/10ml

This buffer is used after Equilibration Buffer I. Iodoacetamide prevents protein re-oxidation during electrophoresis and alkylates residual DTT to minimize vertical streaking in the second dimension.

Electrode buffer for Tris/Glycine gels (Tris/Glycine/SDS Running Buffer):
See Appendix 1.

Gel fixing solution for 2D gels:
50% methanol
5% acetic acid
45% H_2O

Part A: Preparation of Sample and IPG Strip Rehydration

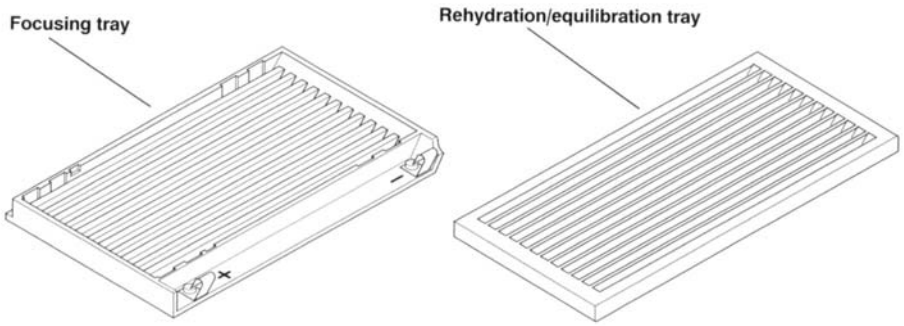
Notes: -**Always wear gloves** when handling IPG strips to prevent contamination.
-Dry IPG strips can be rehydrated with or without the sample in the rehydration solution. In this experiment, you will be rehydrating the IPG strips in the presence of the sample. This is the simplest method to use and it allows loading of larger quantities of proteins (up to 1 mg) while preventing sample precipitation)

1. Prepare sample in rehydration buffer by mixing 30 µl (~50 µg protein) of sample #2.2 ('Induced, crude sup' cell extract of hCAII from Experiment 2, step 4) with 120 µl of a 1.25X rehydration buffer. This buffer will be provided by lab instructors ready to use. Make sure urea is completely dissolved, but do not apply heat since this will cause protein carbamylation.

2. Select an IPG strip with the pH range required for optimum separation of the protein(s) of interest. You will use IPG strips of a pH range of 5-8.

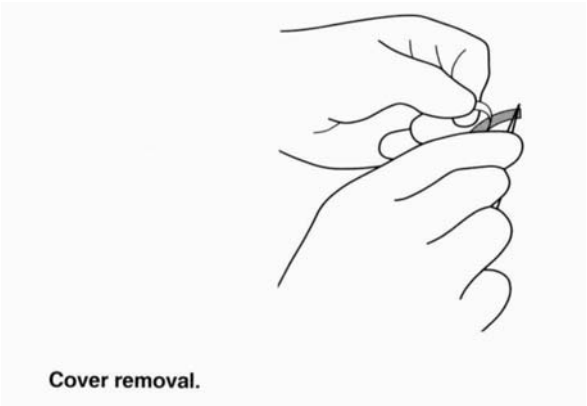
   A broad range (pH 3-10) is a good choice when the pI of a protein is unknown. However, narrower pH ranges are desirable when the expected pI of a protein is known. This will give a better resolution of the potentially numerous protein spots in the gel matrix.

3. Add the entire rehydration solution (containing the sample, 150 µl) to one of the channels of the rehydration tray of the Protean IEF system. Lab instructors will assign your group a specific well.



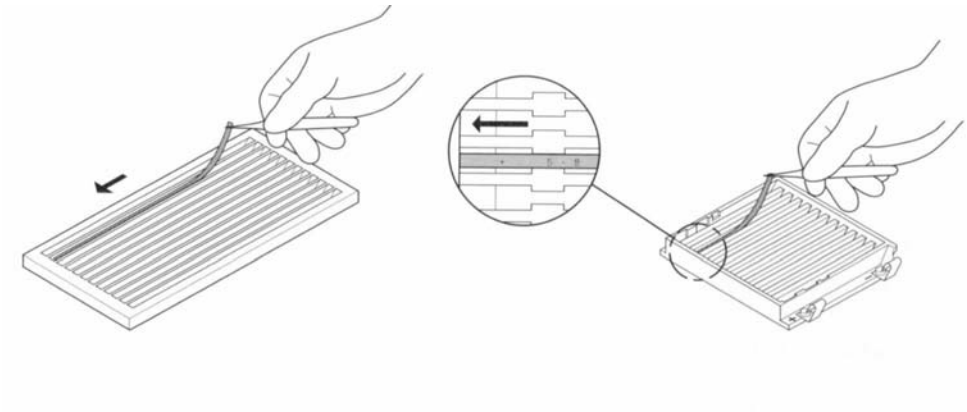
Rehydration/equilibration tray & focusing trays.

4. Open the IPG strip package and carefully remove the protective cover from the IPG strip. The side to which the gel is attached is slightly longer at the end opposite of the end where the pH range of the strip is written. Hold the strip firmly with one hand and bend it from the longer end using forceps to separate the cover from the dry gel. Peel off the cover and discard it.



Cover removal.

5. Place the IPG strip gel side down into a rehydration tray channel. Wet the strip by sliding it through the rehydration solution as you place it into the channel of the tray. Minimize solution on top of the strip and insure that each strip is completely wetted to prevent uneven rehydration.



Rehydration/equilibration tray **b. Focusing tray.**

IPG Strip Placement in Tray

6. Apply mineral oil to each channel containing an IPG strip, sufficient to cover strip and fill chamber half full. Make sure the entire strip is covered.

7. Place the cover lid on the tray.

8. Rehydrate under 'passive' conditions (i.e., diffusion) at 20°C (RT) for 12-16 hours. Lab instructors will guide you in using the instrument, which will be shared by everyone in the class.

   Rehydration can also be done actively at 20°C for 10-12 hours.. Active rehydration decreases the required rehydration time and improves protein entry into the gel strip.

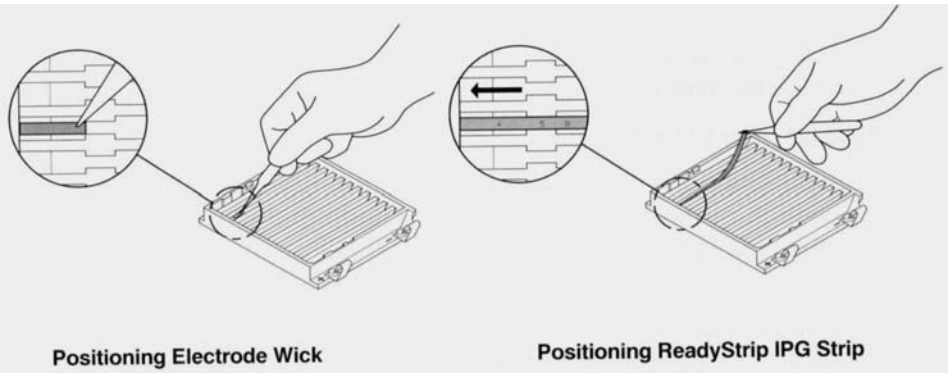
Part B: Isoelectrofocusing conditions

Notes: -Focusing conditions will vary with sample composition, complexity and IPG pH range.
-The current should not exceed 50 μA/strip.

1. Using forceps, place dry wicks directly over electrode wires in IEF focusing tray (See picture below).

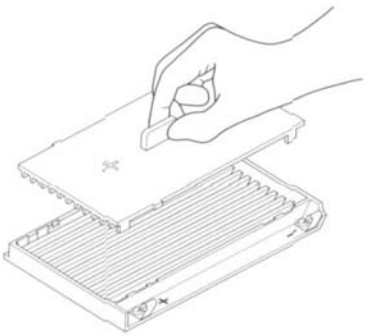
2. Saturate the two wicks with 8 μl water.

3. Use forceps to retrieve ReadyStrip from the rehydration tray. Blot excess oil on a Kimwipe. Place the ReadyStrip gel side down in the IEF focusing tray such that the acidic (marked +) end is at the anode (red/+) electrode of the IEF. Make sure that the gels make contact with the electrode wicks.



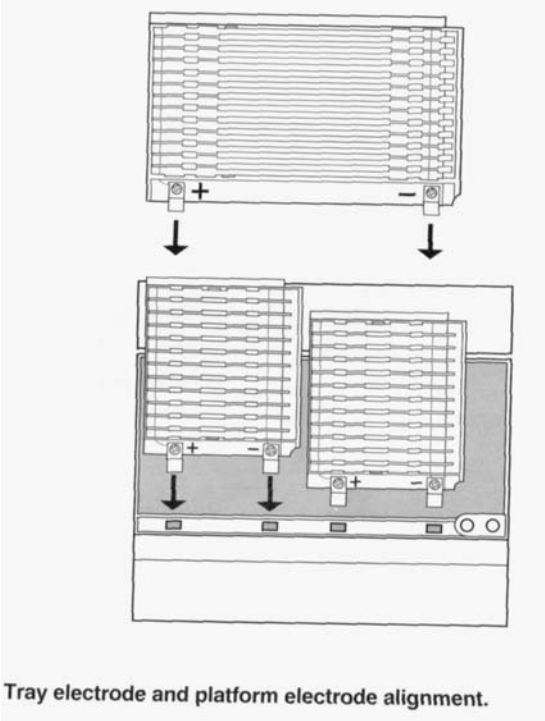
4. Overlay with mineral oil.

5. Place the lid on the focusing tray so that the lid pressure tabs press on the IPG strips directly over the electrodes to insure good contact between the strips and the electrodes.



Interlocking lid and tray

6. Place the tray on the Peltier platform. Be sure the electrodes of the focusing tray contact the color-coded electrodes of the PROTEAN IEF cell (see figure below)



Tray electrode and platform electrode alignment.

7. Lab instructors will program the Bio-Rad PROTEAN IEF cell using the following conditions (7 cm strips):
   Conditioning Step: start voltage of 250 V, 1 hour, 19°C.
   1st Voltage Ramping: start voltage of 250 V, ending voltage of 3,500 V, 3 hours, 19°C.
   Initial Focusing: voltage of 3,500 V, 3 hours (i.e., 10,500 VH), 19°C.
   2nd Voltage Ramping: start voltage of 3500 V, ending voltage of 5,000 V, 2 hours, 19°C.
   Final Focusing: voltage of 5,000 V, 15 hours (i.e., 75,000 VH), 19°C.

Notes: -The time required for voltage ramp will vary with strip length, pH range, nature of the sample, and sample buffer due to the 50 μA current limit. If the final voltage is not achieved at the end of the set ramping time, the ramping time should be increased.
-The total volt hours for the final focusing step will vary with the sample composition and strip length. The range for a 7 cm strip is 8,000-35,000 VH.
-IPG strips may be wrapped in plastic or paraffin wrap and stored at -20°C for at least 3 months or at -80°C indefinitely prior to running the second dimension.

Part C: Equilibration for Second Dimension (SDS-PAGE)

1. Place ReadyStrips in rehydration trays containing 2.5 ml of SDS-PAGE Equilibration buffer I (with DTT).
2. Gently shake on gyratory table for 15 minutes and discard buffer.
3. Add 2.5 ml of SDS-PAGE Equilibration buffer II (with iodoacetamide).
4. Shake for 15 minutes and discard buffer.

Part D: Second Dimension SDS-PAGE

1. Place ReadyStrip onto the larger well of a 1.0 mm thick SDS-PAGE gel (provided)
2. Keep track of the orientation of the strip.
3. Cut ends of the ReadyStrip at the site where they were in contact with the electrodes.
4. Place it on the large well of a SDS-PAGE gel (provided) in such a way that the anode (+) end of the strip is to your left.
5. Place a small (0.5 cm) piece of ReadyStrip (provide by instructors) soaked in MW standard (10 µl/0.5 cm, Bio-Rad Cat. #161-0305) on the anode side (your left) of the first dimension ReadyStrip.
6. Seal in place with 1% (w/v) molten LowMelt agarose prepared in SDS-PAGE running buffer.
7. Snap the gel set up onto the inner core as in Experiment 3. Remember you'll be sharing the gel apparatus with another group. Also, note that the acidic end of the ReadyStrip gel will be flipped to the opposite side as you view it for loading additional samples.

Part E: Running and staining the second dimension gel

1. Run gel at 50 V until the marker dye migrates through the stacker gel, then increase to 125 V for approximately one hour. Refer to the experimental procedures described in Experiment 3, part C for additional details on how to run the gel.
2. Disassemble the SDS-PAGE gel apparatus and fix the gel overnight in 30 ml of 2-D gel fixing solution. Use a large **labeled** petri dish for your gel/fixing solution. This step removes ampholytes that would interfere with the Coomassie blue staining.

Next day

3. Remove the gel from the fixing solution and stain as described in Experiment 3, part D.
4. Dry the gel.

Experiment 12: Mass Spectrometry Analysis of hCAII

Background

Analysis of biological molecules by mass spectrometry provides high accuracy in mass determination on relatively small amounts of sample. The analysis usually takes but a few minutes and sample preparation is relatively simple. The technique can be used successfully to analyze peptides and large proteins, alone or in a mixture. Protein samples are most commonly analyzed by either matrix-assisted laser-desorption ionization time-of-flight (MALDI TOF) or electrospray-ionization (ESI) mass spectrometry.

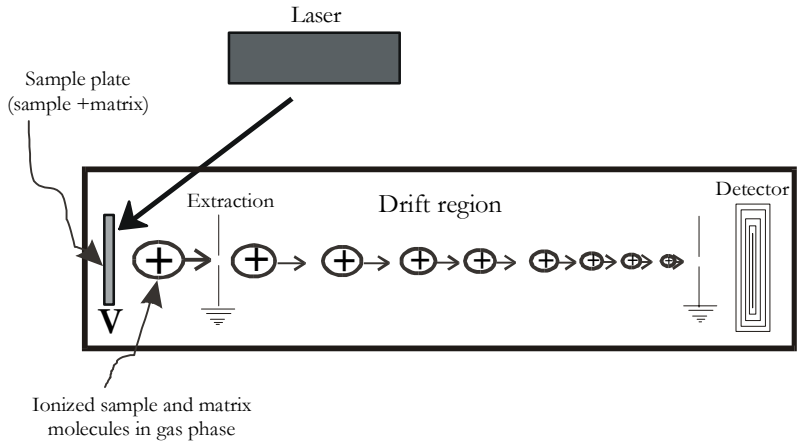
MALDI TOF mass spectrometers can accurately weigh proteins and peptides to within +/- 0.01% of the theoretical mass. This compares to about a 5% mass accuracy provided by SDS gel electrophoresis. With the mass accuracy provided by this instrument, it is possible to determine post-translational modifications, exact proteolytic clip sites, exact lengths of proteins, identification of proteins and much more.

Now that the Genome Project is almost complete, scientists have turned their attention to identifying the function of all the proteins — Proteomics. Usually, proteins of interest are separated by 2D gel electrophoresis, spots are cut out from the gel, digested with trypsin and analyzed by mass spectrometry (e.g. using a MALDI TOF mass spectrometer). The resulting fragments are compared with available databases, also cut with trypsin, to see which proteins best fit the parameters of the proteins of interest. If a good match is found, the protein is identified.

MALDI TOF mass spectrometry relies on a matrix (a small organic molecule such as α-cyano-4-hydroxycinnamic acid) that absorbs the UV radiation from a laser directed onto the sample. The matrix is present in vast excess of the sample. The matrix absorbs the energy from the laser causing the sample and the matrix to become ionized and volatilized. Ions are then accelerated down the flight tube for mass analysis. The laser flash produces neutrals and both positive and negative ions of both the matrix and the sample. Sample ions are ionized by gas phase transfer of protons from the matrix to form $(M+H)^+$.

System components

Matrix-Assisted Laser-Desorption/Ionization
Time of Flight Mass Spectrometer



Ions enter the flight tube with the lighter ions traveling faster than the heavier ions to the detector. The ions separate according to their mass-to-charge ratio. The time-of-flight is proportional to the square root of the mass divided by the number of charges on the ion.

$$(M/z)^{1/2} = At+B$$

t = drift time
z = number of charges on ion
M = mass
A = slope of the line which is a constant relating to the length of the flight tube and kinetic energy
B = intercept

For ions under 10,000 mass units, the best resolution is obtained with the reflector. The reflector is a set of ion mirrors present at the end of the flight tube that extends the flight path length and increases the resolution of the samples by correcting for the kinetic energy spread that occurs for ions of the same mass/charge ratio.

The mass spectrometer can be fine tuned by changing the acceleration voltage, the laser energy, the variable voltage grid and the beam guide wire voltage. These parameters need to be adjusted for each sample to get the best results. This experiment is designed to illustrate the advantages of mass spectrometry for protein identification. The instrument you will be using is a MALDI TOF mass spectrometer (Voyager DE PRO) from PerSeptive Biosystems.

Special micropipet tips (Zip tips) filled with a small amount of reversed phase packing material (C_{18}) can be used to clean up a sample prior to analysis by mass spectrometry. The Zip tips will bind the protein samples and not salts or other contaminants. Once the contaminants are washed out, the protein samples can be eluted with an organic solvent and analyzed directly by mass spectrometry.

Materials

- Gel spot or solution — containing at least 25 pmol of a pure protein (~0.75 μg of a 30 kDa protein).
- 25 mM ammonium bicarbonate, pH 8.0.
- 50% acetonitrile (ACN) in 25 mM ammonium bicarbonate
- 10 mM Tris, pH 8.1.
- 5 μg/ml Promega trypsin in 25 mM ammonium bicarbonate, pH 8.0
- 50% ACN/5% TFA (trifluoroacetic acid)
- 50% ACN/0.1% TFA
- Zip tips (C_{18}) Millipore
- α-cyano matrix (α-cyano-4-hydroxycinnamic acid) — 10 mg
- Scalpel (with a stainless steel blade)
- Siliconized 0.5 ml microfuge tubes and Kontes pellet pestle (Fisher)
- Gel loading pipet tips
- Mass calibration mix 2 in α-cyano matrix — from PerSeptive

Angiotensin I	1296.68 $(M+1)^{+1}$-	Monoisotopic Mass
Neurotensin	1672.92 $(M+1)^{+1}$-	Monoisotopic Mass

Procedure

Part A. Preparation of Sample

Reduction and Alkylation of Glycoprotein

Secondary structure of proteins (i.e., disulfide bonds) reduces their susceptibility to proteolytic digestion. Unaccessible hydrolytic sites may also result in incomplete digestions that complicate analysis of the data. These potential problems are circumvented by reduction and alkylation of the disulfide bonds. One common procedure involves the use β-mercaptoethanol/4-vinylpyridine (reduction/pyridilation). We will be using a dithiothreitol (DTT), followed by carbamidomethylation with iodoacetamide.

In many cases, no reduction/alkylation is needed for protein identification by Mass Spectrometry. This is due to the fact that only a subset of all possible tryptic peptides from a protein can be sufficient for identification, provided there are good matches in the databases.

1. You will be provided with an "unknown" protein solution (1 μg in 5 μl total). The sample is dissolved in Digestion Buffer. Your task in this experiment will be to determine the identity of the protein, using MALDI-TOF. In a separate tube, add 10 μl of Digestion Buffer. This tube will be used as a Control.

   Digestion Buffer: 25 mM NH_4HCO_3, pH 7.8.

2. Add 15 μl of reducing solution directly to the control and unknown protein solutions.

   Reducing solution: 20 mM dithiothreitol, 8 M Urea and 0.2 M Tris HCl, pH 8.0.

3. Incubate for 5 min at 60°C (preferably under nitrogen).

4. Alkylate (carboxymethylate) the resulting cysteine residues by adding 2.5 μl of 0.5 M iodoacetamide (approx. 100 mM final concentration). Incubate for 30 min at room temperature (preferably under N_2). Do this with the control sample also.

5. Desalt by using gel filtration spin columns as indicated in the box below (do this with the control also).

- Resuspend the sephadex matrix in the mini column by inverting the column vigorously several times, making sure that there is no resin in the column cap.
- Remove the top cap from the column.
- Snap off the bottom tip.
- Place the tube in a clean microcentrifuge tube and and spin the setup at 3,000 rpm (~1,000xg) for 1 minute, room temperature.
- Discard the collection tube with the eluted buffer.
- Equilibrate the spin column with 300 μl Digestion Buffer (i.e., add solution to the center of the column, place in a new centrifuge tube and spin again at 3,000 rpm for 2 minute).
- Discard the collection tube with the eluted Digestion Buffer.
- Without delay (to avoid drying of resin), place the column in a new centrifuge tube, and apply sample (~22 μl to the center of the minicolumn (avoid loading the sample on the side of the column).
- Centrifuge the minicolumn/tube setup at 3,000 rpm for 4 minutes at room temperature.
- Save the eluate in the microcentrifuge tube. It should contain the desalted protein.
- Discard the minicolumn in the biohazardous container.

Trypsin Digestion

The choice of the fragmentation enzyme can be very important. If too many peptides are generated, site information may be lost. But if too few peptides are produced, they may be more difficult to isolate and analyze. Ref. David H. Hawke et al. in Techniques in Protein Chemistry III, Academic Press, Inc. 1992. Ed. By Ruth Hogue Angeletti. "*A*

Unified Approach to Glycoprotein Primary Structure Analysis" pp 3315-326. In this experiment we will use trypsin, a proteolytic enzyme that cleaves with a degree of specificity peptide bonds at the carboxylic side of the basic amino acids Lys and Arg.

1. Add 6 µl of 10 ng/µl trypsin (PROMEGA) solution to both the unknown and control samples. This represents a ~1:70 trypsin to glycoprotein mass ratio.

   Trypsin is made in 25 mM ammonium bicarbonate, pH 8.0. Larger ratios of trypsin to protein may also work but this must be tested.

2. Incubate at 37 °C for 60 minutes (incubation can also be done overnight. If doing so, leave in a cell culture incubator or oven in order to avoid volume changes in the sample due to evaporation and condensation of buffer).

Note: Endopeptidase (Asp N) digestion can be performed in a similar manner as described above. However, the recommended E/S mass ratio is 1:100). Incubate at 37°C for 12 hours. Terminate the reaction by adding (trifluoroacetic acid) 5 µl of 10% TFA to bring solution to pH 2.

Note: Trypsin digestion of a protein for Mass Spectrometric analysis can be done directly from a polyacrylamide gel slice. This option may be useful if the protein of interest has not been purified to homogeneity. A procedure for trypsin digestion from a gel slice is provided here but will not be done in the class.

- Excise hCAII spot from Coomassie stained polyacrylamide gel (left side half from Experiment 8) and place in 1.5 ml siliconized eppendorf tube or a tube that has been washed twice with 60% acetonitrile (ACN)/0.1% trifluoacetic acid (TFA). This band should contain approximately 4 µg of pure h CAII. Do this step also with a blank gel slice.
- Add 250 µl of 1:1 solution of ACN: water, and use a small plastic pestle (blue) to macerate gel slice by pressing and rotating. Incubate 3 min and centrifuge at high speed. Remove wash carefully (gel loading tips help).
- Add 250 µl of 1:1 solution of ACN: 50 mM Ammonium bicarbonate to the gel slice to remove Coomassie stain. Incubate 30 minutes at room temperature. Centrifuge and remove wash as before.
- Perform an additional wash with 250 µl of a 1:1 solution of ACN:10 mM Ammonium bicarbonate. Incubate 30 minutes. Centrifuge and remove wash as before.
- Dry destained gel pieces for 20 min in a Speed-Vac.
- Rehydrate the gel particles (assuming these are ~15 mm^3) in 10 mM ammonium bicarbonate buffer containing 7 ng/µl trypsin. Add in 5-10 µl aliquots so that all is absorbed by gel. The trypsin to protein mass ratio should be ~1:20.
- After the trypsin solution is fully absorbed, add an additional 20 µl of 10 mM ammonium bicarbonate buffer (without enzyme), so that the gel pieces remain immersed throughout the digestion.
- Allow digestion to proceed overnight at 37°C.
- Centrifuge for 2 minutes at 14,000 rpm (USE ADAPTORS IN THE CENTRIFUGE) and then use a gel-loading pipet to remove the supernatant into a fresh siliconized 0.5 ml tube. This solution should contain some of the digested protein.
- If higher yield is desired, extract peptides from gel slice by vortexing (on a mechanical agitator for about 30 min) with 50 µl 50% acetonitrile/ 0.5% TFA. Again centrifuge and use a gel-loading pipet to remove the supernatant into the same tube from step 9. The supernatant contains a large proportion of the extracted peptides. If recovery is of no concern go to step 12.
- For an even higher recovery, dry gel pices again in Speed-Vac for 15 minutes. Rehydrate in 25 µl 0.5% TFA and vortex continuously for 5-10 minutes (if available a sonicator can also be used in this step). Add an equal volume of 100% acetonitrile and place on mechanical agitator for 30 minutes. Centrifuge again and save the supernatant in the siliconized tube from step 9. At this point you should have about 100 µl.

- Speed vac samples down to about 10 µl. Use 40 µl water to dilute the acetonitrile and TFA to a level that will allow peptides to bind to the C_{18} tips.

Part B. Preparation of Matrix

Preparation of the α-cyano matrix (10 mg/ml in 50% ACN/50% 0.1 TFA) will have already been done by the laboratory instructors.

Note: The matrix should be re-crystallized before use when bought from Sigma. This can be done as follows:

- Dissolve 100 mg per ml in warm ethanol.
- Filter and then add two volumes of water and let stand in the refrigerator for at least two hours to precipitate the matrix.
- Dry the resulting fluffy yellow powder.
- Stored at -20° C (good for up to three months.
- To use the matrix, simply dissolve in 50% ACN:50% 0.1 TFA.

The matrix we are providing you has already been recrystallized, dried and redissolved in ACN/TFA.

Part C. Preparation of Zip tips

Note: Before beginning washing the Zip tips, locate your samples (part A, step 5) and have them ready for cleaning using the Zip tips.

1. Wash two Zip tips once with 10 ul of acetonitrile (ACN), once with 10 µl of 50% ACN/50% of 0.1% TFA, and twice with 10 µl 0.1% TFA. This is done simple by placing the Zip tip at the end of a P-20 micropipetter that has been set at 10 µl. The wash solution is drawn into the Zip tip slowly and then pipetted out into a waste container. **It is important to keep the tips wet until the sample is applied. Therefore tips should be washed one at a time (or one by each member of the group).**

2. Use one Zip tip per sample (i.e., "Trypsin digestion only" and "UNK digestion"). Immediately after the last wash (step 1) and still using the micropipet set at 10 µl, draw slowly a 10 µl portion of the aqueous sample. Place the tip into a separate siliconized tube and pipet this portion of the sample up and down the Zip tip five times and discard fluid. The peptides should be bound to the hydrophobic particles in the Zip tip. **Be careful to not get solution into the barrel of the micropipet.**

3. Return to the sample with the same Zip tip and draw an additional 10 µl sample. Pipet up and down as before to allow peptides to bind to the hydrophobic particles. Repeat this operation if necessary until all the sample is done.

4. Wash Zip tip at least three times with 10 µl 0.1% TFA. This is done by drawing slowly 10 µl of the TFA solution into the tip and discarding wash each time. Make sure you have a separate TFA wash tube per sample to avoid sample cross-contamination

5. To elute sample from the Zip tip, draw slowly 2 µl of a solution containing **matrix** (part B) in 50% ACN/50% of 0.1% TFA. Again, make sure that you have a separate tube with matrix per sample to avoid cross-contamination. Apply 1 µl of the sample directly onto the Mass spectrometry plate as indicated in Part D. Alternatively, deliver the sample into a clean 0.5 ml siliconized tube that has been appropriately labeled.

Notes:

Peptides that are very hydrophobic may remain stuck to the zip tip under the elution conditions described here. In this case, you can elute with a higher organic solvent.

Samples that are applied to the Mass Spec plate in the absence of the matrix will not desorb (fly) efficiently.

Part D. MALDI TOF Analysis

Apply 1 μl of each of the eluted samples directly onto the positions assigned to your group on the Mass Spectrometer sample plate. Load also a standard calibration mix sample (provided already to load). See below.

Position A: 1 μl standard calibration mix on a spot
Position B: 1 μl of "Trypsin only" digest
Position C: 1 μl of "UNK" digest

2. After samples are dry — apply sample plate to mass spectrometer

3. Mass Spec analysis of standards in reflector mode

Conditions: Acceleration voltage: 20 KV
Grid voltage: 75%
Guide wire: 0.01
Pulse delay: 75 ns

- With joy stick pick a good spot to analyze
- Increase laser power to 2000 — looking to see if there are peaks on the oscilloscope
- Save a good mass spectrum on the computer
- Determine the mass resolution
- Calibrate the instrument

4. Mass Spec Analysis of tryptic digest

Conditions: same as above

- With joy stick pick a good spot to analyze
- Increase laser power to 2000 — looking on oscilloscope
- Save mass spectrum to computer
- Determine resolution of a peak
- Assign mass values to each of the peaks
- Print out the spectrum

Part E. Interpretation of Results

Using the mass values obtained for the tryptic digest, input into MS-FIT program on computer to determine the identity of the protein.

Internet address for program

http://prospector.ucsf.edu

Explanation of terms

Database -- Use the Swiss Prot data base, it provides the most information with the protein hits. Use the following data in the web page.

Species = select all
By stating the species, you increase the speed of the search.

Mass Range — Include the approximate mass of the protein

Enzyme — We are using Trypsin.

Missed Cleavages — Typically this is set to 0 or 1, you can increase this number if you see a lot of larger fragments.

Cysteine Modifications — None

Modifications — None
If you suspected a post-translational modification, you could list that here.

Peptide Masses — List the masses of the peptides from you spectrum to 4 decimal places if possible. Use the higher signal intensity peaks first.

Mass Accuracy — set this to 50 ppm
If no hits are found on the first pass, this parameter can be increased.

Results Size Limitation — The number of hits listed in the results can be limited by using this parameter.
Min. Number of peptides — This sets the number of peptides that need to be matched for a positive identification. Set this number to 5 for the first pass through. If no hits are found, then this number can be decrease

How to search for your protein

- Go to http://prospector.ucsf.edu
- Choose MS-FIT from Prospector Tools
- Change the following parameters
- **Database** choose Swiss Prot
- Cystines modified by choose unmodified
- **Molecular Weight of Protein** input the approximate weight of your protein
- **Mass Tolerance** change to 100 ppm
- Click Start Search

If you have NO results

- Check to make sure your parameters were chosen as above
- Make sure your Mass Spec was calibrated properly
- Your mass tolerance may be too low…increase it
- Decrease the Min. # peptides required to match
- Include more of your peaks
- If you don't have complete digestion, increase your **Max. # of missed cleavages**

If you have TOO MANY results

- Check to make sure your parameters were chosen as above
- Make sure your Mass Spec was calibrated properly
- Your mass tolerance may be too high…decrease it
- You may be matching modified fragments in the database…ignore them
- You may have 'bad' matches…see below

How to determine a GOOD MATCH

- Look at your MOWSE score, the higher the score the better (1e+004 is a really good score)
- Large number of matching fragments (though 2 or more may be enough)
- The amount of sequence matched (the matched peptides cover x% of the protein)

Exercise 9: Mass Spectrometry of Proteins

1. What is the identity of your protein?

2. Did your digestion go to completion? How do you know?

3. How sure are you that you have a good match? How did you determine this match?

Section 3: Immunostaining and Microscopy

Experiment 13

Experiment 13: Immunocytochemical Staining of Cells

Introduction

Immunofluorescence-based microscopic localization techniques have become some of the most powerful and widely used methods in biomedical research. Like recombinant DNA methods and molecular genetic methods, immunofluorescence methods can be used to address many different kinds of biological problems and consist of a broad array of applications that can be customized to suit the special needs of particular experiments.

Antibodies Are Of Primary Importance

Immunofluorescence-based microscopic localization is what its name implies. To determine the position of a protein within a cell, an **immune** reagent (antibody) is used to specifically bind a protein, and the **location** of the bound antibody is reported by an attached **fluorochrome** that can be observed in the light **microscope**. This approach is analogous to the use of histological stains, which typically are small organic compounds that bind cell components and allow them to be visualized by light transmitted through the specimen in the microscope. Because of this similarity, the term "staining" is often used (i.e., "immunofluorescent staining"). However, it is important to bear in mind that there are important distinctions between the use of an antibody and the use of a histological stain. The single most important difference is that, in almost all cases, it is relatively easy to prepare a highly specific antibody that is directed against a particular protein antigen.

Usually, antibodies are directed against polypeptide epitopes, but antibodies can also be directed against a particular post-translational modification (e.g., a phosphotyrosine residue). Because specific antibodies can be prepared against a particular protein, it is possible to determine with precision the distribution of this protein in the cell. In contrast, the typical histological stain adheres to many proteins, as well as to lipids and nucleic acids, and does not yield specific information on a given protein. As a result of the ability to specifically localize a protein within the cell, immunofluorescence localization is especially valuable in characterizing a protein of unknown function; knowledge of a protein's site of action within a cell is an important clue to its function (e.g., a protein associated with the cytoskeleton may function in cell locomotion and/or intracellular transport processes).

Basic Principles

Conceptually, the most straightforward immunofluorescence (IF) approach involves the use of an antibody that is covalently conjugated to a fluorochrome, which is also known as a fluorophore. The antibody-fluorochrome conjugate is then incubated with a fixed and permeabilized cell sample on a slide. The major factor that affects the strength of the IF signal is the availability of antibody binding sites (epitopes). Easily detectable antigens in the cell present a large number of antibody binding sites and/or are present at relatively high local concentrations. The slide is viewed in a microscope equipped for "epi-fluorescence" observation. "Epi" refers to the fact that the specimen is illuminated from above through the objective lens (lens directly above slide). Excited fluorochromes emit light which is focused and collected by the (same) objective lens and transmitted through a filter set that allows only emitted light to reach the eyepieces and camera. This will be illustrated in lecture.

Secondary Antibodies Take An Indirect Approach

The major limitation to the approach mentioned above is that it requires that each antibody be conjugated to a fluorochrome. This requires covalent modification of the antibody molecule, which has certain experimental drawbacks. To overcome this limitation, it is more common to use a secondary antibody conjugated to a fluorochrome. This has two important advantages: (1) one secondary antibody can be used with many primary antibodies; and (2) multiple secondary antibody molecules can bind one primary antibody molecule and in so doing amplify the IF signal. Because the primary antibody is not directly labeled with fluorochrome, but is detected indirectly through its association with the secondary antibody, this approach is often referred to as "indirect" immunofluorescence localization. Secondary antibody-fluorochrome conjugates, usually in affinity purified form, are available from many commercial vendors. The two most common secondary antibody types are directed against rabbit or mouse primary antibodies, because most investigators prepare antibodies in either rabbit or mouse. However, secondary antibodies are available against primary antibodies from human, rat,

guinea pig, goat, horse, donkey, and other sources. Usually antibodies are affinity purified against immunoglobulins they are directed against (e.g., mouse IgG), and are often "cross-absorbed" to eliminate cross-reactivity with (primary) antibodies of related species (e.g., a secondary antibody against mouse IgG may be cross-absorbed against rabbit IgG). In many respects, the use of primary and secondary antibodies for IF localization is similar to the use of primary and secondary antibodies for western blotting.

Adding Colors To Experiments

Fluorochromes have been designed to emit light of different colors. Typically, a given fluorochrome will be excited optimally by a narrow range of wavelengths of light and emit over a narrow range of longer wavelengths. As a practical matter, emissions in the visible spectrum are most useful, but certain fluorochromes are excited by UV light and emit in the blue end of the visible spectrum. Because fluorochromes emit light of different colors they can be used simultaneously. Color filters in the light path of the microscope allow light of different colors to be viewed separately. Images from different color emissions can be combined to yield a composite image. False color can be introduced with computer graphics software to show amount of overlap or exclusion. For example, the area of overlap between a green image and a red image may be shown as yellow (a false color). It is now a routine matter to use 2-3 different antibodies and 2-3 fluorochromes that emit light of different colors to localize 2-3 proteins simultaneously in an experiment.

Co-Localization

To establish where a protein is in the cell it is useful to "colocalize" it with another protein of known localization. This yields a higher degree of certainty than comparison of a protein localization pattern with a cell's morphological features. The reason for this is simple: cells are remarkably complex structurally, and many structures (e.g., the cytoskeleton) and subcompartments are difficult to visualize and identify based on morphology alone. For example, if a protein of unknown localization colocalizes with the cytoskeletal protein tubulin, then this strongly suggests a role for the unknown protein in microtubule structure or function. The use of an anti-tubulin antibody distinguishes microtubules from other cytoskeletal structures such as microfilaments (actin), or cytokeratins, both of which can exhibit a filamentous morphology.

Colors To Dye For

Antibody-based detection may also be used in combination with a fluorescent dye that binds to nucleic acids or lipids (e.g.; Hoescht or DAPI dyes which label chromatin; or $DiOC_6$, which labels the endoplasmic reticulum membrane bilayer). The use of water-soluble fluorescent dyes offers certain advantages: (1) ease of use; (2) labeling of larger intracellular structures, such as organelles; and (3) detection of non-protein macromolecules.

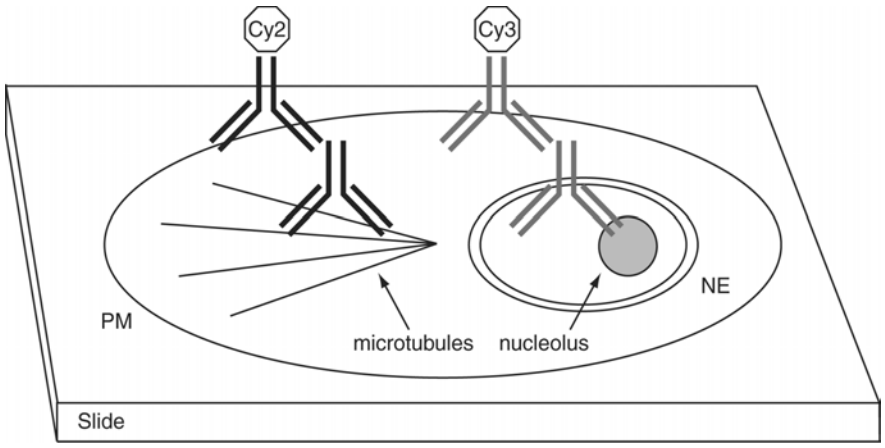


Diagram of immunofluorescence localization of tubulin (microtubules) and fibrillarin (nucleolus).

Extending Limits Of Detection

IF localization methods have become highly sophisticated over the last several years. The two most important advances during this time concern improvements in signal detection, and have involved (1) refinements in generation of signal by fluorochromes and (2) the acquisition of faint signals by the light microscope. Fluorochromes have been designed to generate bright, long-lived signals with relatively discrete absorption and emission spectral characteristics. The efficiency with which a fluorochrome converts incident excitation light into emitted light plays a crucial role in detecting rare or poorly reacting antigens. The current generation of fluorochromes are not as subject to fading as earlier generation fluorochromes (e.g., fluorescein, which remains in wide use). Resistant to fading enables emitted light from a faint source to be collected over a long exposure time—such as a number of minutes—in order to create an image. In addition to this, images are captured in digital format using cooled-charge coupled device (CCD) cameras, which use silicon chip technology to convert incident photons into electrical impulses. CCD cameras permit 10- to 100-fold increases in sensitivity compared to film emulsions. The cost of cooled CCD cameras was prohibitive only a few years ago (~$25,000), but are now available for less than half of this amount. In addition to the CCD camera, enhanced video systems can achieve high sensitivities of signal detection. One of the benefits of video imaging is that faint signals, such as those generated by GFP-protein fusions, can be collected in real time, which allows the dynamic movements of a protein within a cell to be directly observed.

The Sharper Image

Two refinements in acquisition of signal by the light microscope have had a dramatic impact on image quality, or resolution. These are the use of: (1) **confocal** optics; and (2) specialized computational techniques that use "**deconvolution**" algorithms. Both result in elimination of out-of-focal-plane light that blurs the image. In confocal microscopy, out-of-focal-plane light is excluded from the collected image. In "deconvolutional" imaging, three dimensional data are collected in the form of a set of optical sections across the Z-axis. Mathematical approaches are used to identify light from one optical section that is out-of-focus in adjacent optical sections. Thus, the deconvolution approach removes, rather than excludes, out-of-focal-plane light. These two methods are typically used with a cooled-CCD camera. The collection of digital image data that are largely free from out-of-focal-plane information has made possible quantitative analysis of image data.

Some Immunohistochemistry

Immunofluorescence methods emerged during the 1970's from approaches used for immunohistochemistry, which is also known as immunocytochemistry. Immunohistochemistry usually employs a secondary antibody coupled to an enzyme, such as horse radish peroxidase (HRP), to generate an insoluble colored precipitate in a cell or tissue sample in order to localize the site of antibody binding. A common alternative is to use an antibody bound to a gold colloid particle, which can be visualized directly or after silver enhancement. This approach is extremely valuable in staining thin sections that have been prepared for light microscopy, and is in wide use in pathology and toxicology laboratories. Applications in pathology include the determination of such variables as the type of infectious organism present, identification of a neoplastic cell progenitor, or the presence of an inflammatory response. Immunocytochemical approaches are also used with ultrathin sections prepared for electron microscopy.

Experimental Design

Basic Steps

All cell staining techniques make use of labeled antibodies to detect antigens in cells or tissues through four common steps:

1. cell or tissue preparation
2. fixation (and permeabilization if detecting internal molecules)
3. antibody binding and
4. detection

In the first step, the material to be stained is attached to a solid support to allow easy handling in the subsequent procedures. Cells that are naturally adherent can be grown directly on microscope slides, cover slips, or optically suitable plastic. Suspension cells can be centrifuged onto glass slides, bound to slides using chemical linkers, or in some cases handled in suspension. The next step in cell staining is usually fixing followed by

permeabilization to ensure free access of the antibody. This step may be omitted when examining cell-surface antigens. In the third step, cell preparations are incubated with antibodies. Unbound or non-specifically bound antibodies are then washed extensively. If antibodies are labeled, they can now be directly detected. However, most commonly, the antigen-specific antibody (primary antibody) is unlabeled and its binding to antigen is detected by means of a labeled secondary antibody.

For immunostaining of tissue sections, the cells can be preserved in one of three methods: 1) by *in situ* perfusion of the animal, 2) by freezing of dissected tissue sample, or 3) by treatment of tissue with a fixative and embedding in paraffin. Next, sections are cut from the block of tissue with a microtome, and antibodies against specific cell antigens are applied to the tissue section. Unbound antibodies are removed by washing,

Constraints

The major factor that affects the strength of the signal in cell staining is the **local antigen concentration**. Easily detectable antigens present a large number of identical antibody binding sites in a small local environment. Another factor that influences antigen detection is **the type of fixative** and the **characteristics of the antibodies** being used. Many epitopes are masked or altered by certain fixatives so as to make them unrecognizable by antibodies which might recognize them under a different set of conditions. The ideal fixation would immobilize the antigens, while retaining authentic cellular and subcellular architecture and permitting unhindered access of antibodies to all cellular and subcellular compartments. Lastly, the resolution and sensitivity of cell staining largely depends on the **antibody detection method**. For example, enzyme-labeled antibodies are very sensitive but offer lower resolution. On the other hand, flurochrome-labeled antibodies offer higher resolution than light microscope-based procedures. However, fluorescent methods in general are not as sensitive as enzyme-labeled methods.

Choice of Antibodies

Antibody preparations that give satisfactory results for techniques such as immunoprecipitation or immunoblotting may show spurious cross-reactions in cell staining. Surprisingly, antibodies of relatively low affinity often can work well in cell-staining reactions, possibly due to bivalent binding of antibodies to immobilized antigens in high, local concentrations. Three types of antibody preparations can be used for cell staining: polyclonal, monoclonal and pooled monoclonal antibodies. Polyclonal sera normally contains both specific and non-specific antibodies. As a result, high background signal is sometimes observed even when using unimmunized serum. Therefore, these antibodies can be most troublesome for cell staining. However, in some instances it may be advantageous to use polyclonal sera for cell staining because they contain antibodies specific for a broad range of antigen epitopes, including denaturation-resistant epitopes. In fact, polyclonals may be the antibodies of choice for paraffin sections of tissues fixed with paraformaldehyde or with other strong fixative. Also, if the experiment involves colocalization of antigens, the primary antibodies must be of different origin. This means that one of the primary antibodies will be polyclonal since routine monoclonal technology has been developed in mice.

Monoclonal antibodies often work exceptionally well in cell staining tecniques, with minimum background over a broad range of antibody concentrations. However, some monoclonal antibodies may fail to give satisfactory results when they recognize an epitope that is destroyed by cell fixing agents such as paraformaldehyde. This problem may be circumvented by developing monoclonal antibodies from mice that have been immunized with paraformaldehyde-fixed antigens. Furthermore, it is advisable that histochemically-useful monoclonal antibodies be screened using tissue sections.

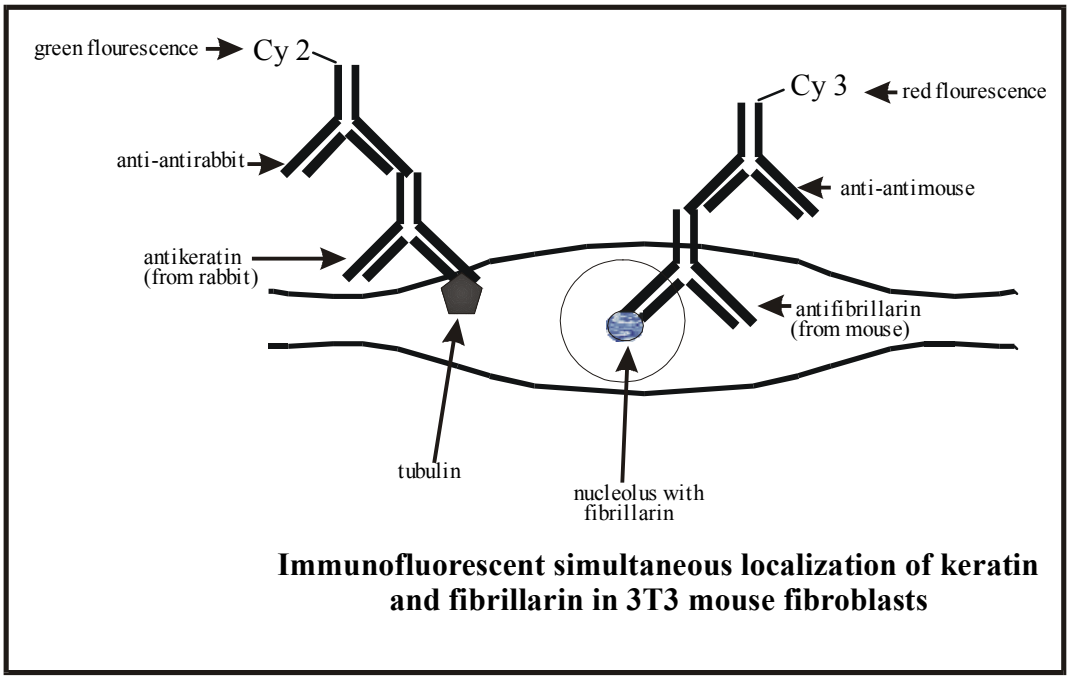
Ocassionally, monoclonal antibodies will cross-react presumably due to the presence of common epitopes on two cellular components. For this reason, the location of an antigen should be confirmed, whenever possible, by using a panel of monoclonal antibodies directed to discrete epitopes on the same antigen.

References

- Antibodies. A Laboratory Manual. Ed Harlow and David Lane. Cold Spring Harbor Laboratory, 1988, New York.
- Antibody Applications. Essential Techniques. P.J. Delves. Ed. By D. Rickwood. John Wiley and Sons, New York.
- Immunochemical Protocols. *Methods in Enzymology*, Vol. 10. Edited by Margaret M. Manson. Humana Press, Totowa, New Jersey; 1992.

Brief description of the Experiment

Previous to today's laboratory, 3T3 NIH Swiss mouse fibroblasts and MDCK (canine kidney) cells were cultured as a monolayer to approximately 50% confluency in 8-chamber multiwell slides. The culture medium was DMEM (high glucose) + 10% fetal calf serum+ penicillin and streptomycin. 3T3 cells are adherent (i.e. they attach to the slide) and acquire an elongated, "spread out" shape as they attach. MDCK cells also adhere to wells on the slide but they remain fairly circular in close contact with each other forming cell islets of varied size depending on the degree of confluency. Adherence is a convenient feature of certain cell types in culture for it allows washing without having to be concerned with dislodging and losing cells during immunostaining procedures. Attachment of cells that normally grow in suspension can be promoted by treatment of slides (or plates) with certain reagents such as polylysine or collagen.



Immunofluorescent simultaneous localization of keratin and fibrillarin in 3T3 mouse fibroblasts

In this experiment, you will be performing simultaneous detection of several pairs of antigens in either 3T3 or MDCK cells (see Experimental plan below). The cellular antigens chosen for this experiment are: tubulin, keratin, fibrillarin (a nucleolar protein) and desmoplakin (a desmosomal protein). Codetection (colocalization) of the two antigens will be possible by the use of a rabbit **polyclonal** antibody against one of the antigens and a **monoclonal** antibody against the other. Following the addition of these "primary antibodies" and appropriate washing, cell samples will be treated with two secondary antibodies conjugated to two different fluorophores. One of the secondary antibodies is directed against rabbit polyclonal antibodies (anti-rabbit) and is conjugated to the dye Cy2 (green fluorescence). The other secondary antibody reacts with the mouse monoclonal antibody (anti-mouse) and is conjugated to the dye Cy3 (red fluorescence).

Preparations Prior to the Experimental Procedure

Many preparations and choices have to be made prior to an *immunolocalization* experiment. Please refer to the "Introduction" section of this experiment (above) for a discussion on these issues. Below, there is a check list of materials and reagents necessary for the experiment described here. Some of these items will already be on your lab station, others will be provided to you by the lab instructors as you need them.

Materials and reagents

1. Absorbent wipes.
2. Latex gloves
3. Coverslips.
4. 8-chamber glass slides with cover (LAB-TEK, Nalge Nunc Intl., Cat. # 177402)
5. Whatman paper
6. 37°C incubator.
7. Cell culture media (GIBCO-BRL, see below)
8. Hemocytometer
9. Micropipettes (P-20, P-200 and P-1000)
10. Fluorescence microscope (equipped with UV, Cy2 and Cy3 filters)
11. Nuclear fast red counterstain (0.1% dye in 5% aluminum sulfate solution).
12. Aqueous mounting medium (e.g. Crystal mount mounting medium, Fisher BM-M02)
13. Doubly distilled water.
14. Phosphate buffered saline (PBS).
15. PBS/$MgCl_2$ (PBS+1 mM $MgCl_2$)
16. PBS/NH_4Cl (PBS+50 mM NH_4Cl)
17. PBS/BSA (PBS+1%Bovine Serum Albumin)
18. PBS/Tx (PBS+1% Triton X-100)
19. *p*-Formaldehyde 30% (*p*FA) stock. **Prepared freshly** as follows:
    a. Add 2.0 g *p*FA and 5 ml ddH_2O to Pyrex tube (16X150 mm).
    b. Heat slowly with burner to boiling and vortex intermittently.
    c. Add 0.1 ml 1M NaOH while vortexing to dissolve *p*FA.
    d. Spin in IEC or equivalent centrifuge for 5 minutes at 2500 rpm at RT. Alternatively, filter through a 0.45 μm sterile filter using a syringe.
    e. Store 30% pFA stock for no longer than one day at room temperature.
20. Fixative: **Prepared freshly** by diluting 30% pFA 1:10 into PBS+1 mM $MgCl_2$ at room temperature. Check pH of PBS+3% pFA. Adjust to pH 7.2 with 0.1 N HCl. Chill on ice before use.
21. Hoechst dye solution (0.2 mg/ml in dd-water, store in dark tubes in the dark).
22. Mountant medium for Immunofluorescence: pipet into a 15 ml round bottom tube the following reagents:
    - 4.5 ml Glycerol
    - 0.5 ml 100 mM Tris.HCl, pH 8.5 (at 25°C)
    - 5 mg *p*-phenylene diamine
    - Mix at RT for 30 min by rotating end over end.
    - Aliquot as 50 μl in 0.5 ml tubes.
    - Store at -70°C.
23. Antibodies. These were obtained from the following sources:

• Antifibrillarin (D77)	-provided by Dr. John Aris	-
• Antikeratin (rabbit polyclonal)	-ICN biomedicals, Inc. -Diagnostic BioSystems	Cat. #10550 Cat # PDR 035-15
• Antikeratin (mouse monoclonal)	-SIGMA	Cat. #C-9687
• Antitubulin (rabitt polyclonal)	-ICN biomedicals, Inc. -Accurate Biochemicals	Cat. #650952 #BYA10811
• Antidesmoplakin	-Maine Biotech. Services, Inc.	Cat. #MAB666
• Antiactin (rabbit polyclonal)	-ICN biomedicals, Inc.	Cat. #650962
• AntiCY3 (antimouse)	-Jackson Immuno Research	Cat. #115-225-146
• AntiCY2 (antirabbit)	-Jackson Immuno Research	Cat. #111-225-144
• Anti-mouse IgM Cy3 conj μ chain specific (min X Hu, Bov, Hrs Sr Prot) ML*		JacksonIR #115-165-075
• Anti-mouse IgG Cy2 conj (subclass 1+2a+2b+3) Fc (γ) frag spec (min X Hu, Bov, Rb Sr Prot)		JacksonIR #115-225-164

NOTE:
GROUPS 1-4 WILL PERFORM EXPERIMENTAL PROCEDURE A, ALL OTHER GROUPS, PLEASE GO TO PROCEDURE B (AHEAD) RESULTS FROM BOTH PROCEDURES WILL BE SHARED BY THE WHOLE CLASS AT THE END.

Experimental Procedure A: Immunolocalization (fluorescence)

Precautions:

-Use gloves during entire procedure.

-Samples should be handled as if capable of transmitting infection.

-Dispose of reagent waste into biohazardous bags located at your bench.

-Never pipette reagents by mouth and avoid their contact with skin and mucous membranes.

-Observe experimental conditions carefully (temperature, incubation times, buffers, etc.).

-Store reagents under proper conditions (as indicated in experimental procedure or as specified by information provided by reagent's vendor).

-Do not allow the cells or tissue to become dry at any time during the detection procedure, as this will increase background staining.

I. Cell culturing

This part of the experiment will have already been done by the lab instructors. A brief description of the culturing steps is provided below for your information. You should begin with Part II "***p*FA fixation**".

1. Culture cells in optimum medium (sterile conditions in a culture hood). **MDCK and 3T3 cells**: 89% DMEM (high glucose)+ 10% fetal calf serum+ 1%(antibiotics+antimycotics).
2. At the point of passing cells, remove medium from flask and add 5 ml fresh trypsin solution (0.25% trypsin, 0.03% EDTA). Incubate for 5 minutes at 37 ^{o}C (visible clumps of cells should be floating in the bottom of the flask. MDCK cells take longer than others to become detached from the bottom of the flask by trypsinization). Disperse cells with pipette, transfer into a 15 ml conical tube and centrifuge for 3-5 minutes at 2,000 rpm (SORVAL centrifuge). Remove supernatant and add fresh medium in an amount equivalent to half the original volume in the flask. Centrifuge again to get rid of all vestiges of trypsin, redissolve as before and count cells in a hemocytometer. Dilute to $0.5x10^5$ cells/ml for passing or for growing on slides.
3. Transfer 400 µl of cells ($1.7x10^4$ cells/ml for 3T3 and $2.5x10^4$ for MDCK) into each of 8 chambers of the slides (Lab-Tek #177402). Incubate at 37 ^{o}C in a 5% CO_2 environment. Cells should adhere to the slides within 16 hours and be approximately 40-60% confluent before use. It is best if cells are allowed to grow on slides for two overnights before using them. The slides can then be used for Immunlocalization.

Note: Cells can also be grown in other types of slides such as HTC super cured glass slides (Cell-Line Associates). The 14 mm well in these slides hold approximately 150 ml of cell solution ($2.5x10^4$ cells/ml). Hydrophobically coated slides are not necessary for self adherent cells such as the lines used here. Transfer slides into a humid chamber or place them in a Petri dish containing four (1x2.5 inch) pieces of wet Whatman paper. Incubate at 37 ^{o}C in a 5% CO_2 environment.

II. *para*-Formaldehyde (*p*-FA) Fixation

Note: The upper row (4 wells) of the slide contain 3T3 and MDCK cells according to the Experimental Plan below. Only groups 1-4 should carry out the immunofluorescent procedure.

1. Lab assistants will retrieve slides containing the cells from the CO_2 incubator just before ready for washing and fixing. Do not keep cells outside the incubator for extended periods of time as the pH of the medium will increase and cause cells to detach from the slide and eventually die.
2. Using a 1 ml pipetor, remove medium from each well of the slide and add 500 µl of PBS to wash cells briefly. Avoid dislodging cells by pointing pipetor to the side of the wells. Do not allow cells to dry during all steps in this procedure.
3. Discard PBS from previous wash and fix cells with 250 µl ice-cold 3% *p*FA made in PBS+1 mM $MgCl_2$ (See Preparation section above). Incubate for 15 minutes at room temperature.

Note: If necessary, fixed cells can be stored for a few days in 0.1% pFA + 1 mM $MgCl_2$ at 4 °C.

III. Permeabilization

1. Wash cells for 5 minutes each time at room temperature as follows:
   - Discard cell *p*FA with pipet (biohazardous waste). Place tip in a corner of the well to draw fluid.
   - Add 500 µl PBS by pointing the pipet tip to a corner of the well to avoid dislodging cells.
   - Remove PBS and add 500 PBS/50 mM NH_4Cl (made by adding 5µl of 5M NH_4Cl into 495 µl PBS).
   - Remove the previous solution added and add again 500 µl PBS.
   - Remove PBS.

   Note: NH_4Cl blocks unreacted fixative (*p*FA) which could cause Ab crosslinking.
2. Permeabilize fixed cells (5 minutes) by adding 500 µl of PBS+1.0% Triton X-100 (PBS-Tx).

IV. Blocking and Primary Antibody Addition

1. Remove PBS-Tx and add 500 µl of PBS+1.0% BSA (PBS-BSA). BSA blocks non-specific binding of antibodies to cellular antigens. Incubate at RT for 5 minutes.
   It is best if cells are blocked with NGS, since the secondary antibodies were raised in goat.
2. While blocking, prepare **primary antibodies** dilutions (1° Abs) in PBS-BSA as indicated in the table below. Each tube already contains the correct amount of 1° Ab. To complete preparation, add the indicated PBS/BSA volume to each tube.

Specificity	Source	µl 1° Ab	µl PBS/BSA	Dilution
Anti-fibrillarin (D77 mAb)	Mouse mAb, ascites fluid	1	99	1/100
Anti-keratin (poly)	Rabbit polyclonal	100	-	prediluted
Anti-tubulin (mAb)	Mouse mAb	1	199	1/200
Anti-actin (poly)	Rabbit polyclonal	2	198	1/100
Anti-desmoplakin (mAb)	Mouse mAb	2	98	1/50
Anti-SR (Mab)	IgM monoclonal Ab	100	-	prediluted

3. Remove PBS-BSA and add 100 µl of the appropriate 1° Ab solution to each chamber according to the **Experimental plan** below. Incubate for one hour at room temperature.

Experimental Plan
(8-chamber slide)

Cells: **3T3 (A)** 1° Abs=mAb α–tubulin + poly α–actin 2° Abs=α–mouse-Cy3 + α–rabb-Cy2	Cells: **3T3 (B)** 1° Abs=IgG mAb α–fibrillarin + IgM mAb α–SR 2° Abs=α–mouse IgG-Cy2 + α–mouse IgM-TR	Cells: **MDCK (C)** 1° Abs= mAb α–tubulin + poly α–actin 2° Abs=α–mouse-Cy3 +α–rabb-Cy2	Cells: **MDCK (D)** 1° Abs=mAb α–desmoplakin + poly α–keratin 2° Abs=α–mouse-Cy3 +α–rabb-Cy2	Name Group
-NONE	-NONE	-NONE	-NONE	

V. Secondary Antibody Addition

1. Discard 1° Abs solution and wash cells three times (5 min each) with 500 µl PBS-BSA.

2. While washing cells, lab assistants will prepare 0.4 ml of secondary antibodies dilutions (2° Abs=fluorochrome) plus Hoechst in PBS-BSA as indicated in the table below. The correct amount of 2° Abs has already been added to the tubes. To complete preparation, add Hoechst dye and PBS/BSA.

Specificity/fluorochrome (2° Abs)	Source/Stock	µl 2° Ab	Hoechst Dye (0.2 mg/ml)	µl PBS/ BSA	Working dilution
Anti-mouse Cy3 conjugate (Red, Rhodamine-like)	Goat	3	3	294	1/100
Anti-rabbit Cy2 conjugate (Green, Fluorescein-like)	Goat	3	3	294	1/100
Whole Anti-mouse IgM Cy3 conjugate µ chain specific (min X Hu, Bov, Hrs Sr Prot) ML*	Goat	2	1	97	1/50
Whole Anti-mouse IgG Cy2 conjugate (subclass 1+2a+2b+3) Fc (gamma) frag spec (min X Hu, Bov, Rb Sr Prot)	Goat	1	1	98	1/100

*Notes: this antibody can be replaced by Jackson ImmunoResearch, Cat# 115-075-020, Lot 41861 (used at ~1:250 fold dilution); Mu chain specific. Anti-mouse IgM Texas Red (TR)- conjugated. Cy3 (A=550, E=570); Cy2 (A=492, E=510). Some cells (e.g., periferal lymphocytes) have Fc receptors. They will, therefore, bind whole IgG. In this case it would be recommended the use of F(ab). Secondary antibodies used on cells that have been cultured in Bovine serum albumen-containing medium, must be preadsorbed with BSA, since cells would have these molecules attached to their surface. If the secondary is raised in goat, then, cells must be blocked with NGS before the addition of the primary antibody.

3. Add 100 µl of **each** of the 2° Ab solutions to all wells in the slide (See Experimental Plan, above). Incubate in the **dark** for 1 hour at room temperature, (e.g. under a piece of aluminum foil).

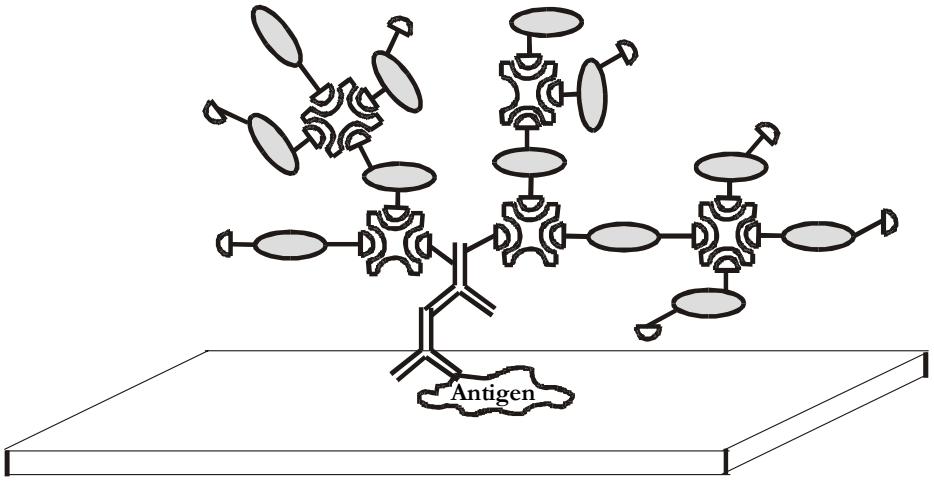
VI. Mounting and Visualization

1. Wash cells three times (5 min each) with 500 µl PBS-BSA. Keep slides in the dark during wash steps.
2. Remove all the PBS-BSA wash buffer. Carefully remove plastic chamber divider. Lab instructor will demonstrate appropriate procedure.
3. Mount in *p*-phenylene diamine/glycerol mountant. Use 4.0 µl per well. Carefully place a single large cover slip over wells. Use 6 very small drops of nail polish at edge of cover slip to seal it to slide. Store slides at -20 °C until viewed.
4. View with Zeiss Axiophot fluorescence microscope. The lab instructors will coordinate appointments for viewing.

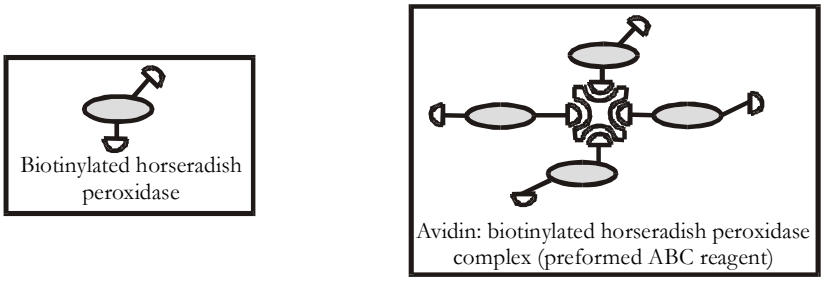
Experimental Procedure B: Immunohistochemical Staining for Colorimetric Detection

The method described here utilizes reagents from the VECTASTAIN® Universal Elite ABC kit (Cat. No. PK-6200) available from Vector Laboratories (www.vectorlabs.com). It relies on the use of antigen-specific, unlabeled primary antibodies, followed by a biotinylated secondary antibody, and then by a preformed **A**vidin and **B**iotinylated horse-radish peroxidase macromolecular **C**omplex. This has been termed the ABC technique (Hsu SM, Raine L., Fanger H: *Am. J. Clin. Pathol.* **75,** 734-738, 1981; and Hsu SM, RaineL, Fanger H*: J. Histochem. Cytochem.* **29**, 577-580, 1981). More information is available at www.vectorslabs.com

Avidin is a glycoprotein (68,000 Daltons) with an extremely high affinity (10^{15} M^{-1}) for biotin (a small MW vitamin). The essentially irreversible binding of avidin to biotin, coupled to the fact that avidin has four binding sites for the vitamin has lead to the effective use of the Biotin/Avidin system in antigen detection schemes that rival fluorescence in sensitivity. The VECTASTAIN® Universal Elite ABC kit exploits the additional fact that most proteins (including antibodies and enzymes) can be conjugated with several molecules of biotin. Although the structure of the Avidin: biotinylated horseradish peroxidase complex is still not defined, evidence suggests that it consists of many biotinylated horseradish peroxidase molecules crosslinked by avidin into a three dimensional array. The ABC unit of the complex apparently retains at least one open binding site that allows it to "dock" on the biotinylated secondary antibody (see diagram).
Because many substrates (of different colors) are available, the VECTASTAIN ABC system allows for multiple antigen labeling in the same tissue. This is done using the detection procedure in a sequential manner.



Schematic of the potential structure of the Avidin: biotinylated horseradish peroxidase complex formed using the *elite* ABC kit for detection



I. Reagent preparation and precautions

(Already done by lab assistants)

VECTASTAIN® *Elite* ABC Universal kit is purchased from Vector Labs, Cat. # PK-6200

Blocking Solution (Normal Serum):		**Biotinylated Universal Secondary Antibody**	
PBS	5.0 ml	PBS	5.0 ml
Stock Normal Serum (provided in the kit)	100 µl	Stock Normal serum	100 µl
		Biotinylated 2° Ab stock	100 µl
Mix well before use.		Mix well before use.	

Wash Solution (PBS-1% BSA)		**ABC Reagent**	
PBS	5.0 ml	PBS	5.0 ml
BSA	50 mg	Reagent A	100 µl
		Reagent B	100 µl
Mix well before use.		Mix well and incubate for 30 min at RT before use.	

AEC Substrate (Vector Labs Cat. # SK-4200)		**DAB Substrate (**Vector Labs Cat. # SK-4100**)**	
Distilled water	5.0 ml	PBS	5.0 ml
Buffer stock solution provided with kit	100 µl (mix well)	Buffer stock solution provided with kit	100 µl (mix well)
AEC stock solution provided with kit	150 µl (mix well)	DAB stock solution provided with kit	200 µl (mix well)
Hydrogen Peroxide solution from kit	100 µl (mix well)	Hydrogen Peroxide solution from kit	100 µl (mix well)
		Nickel solution from kit	100 µl (mix well)

Precautions:

-Use gloves during entire procedure.

-Samples should be handled as if capable of transmitting infection.

-Dispose of reagent waste into biohazardous bags located at your bench.

-Never pipette reagents by mouth and avoid their contact with skin and mucous membranes.

-Observe experimental conditions carefully (temperature, incubation times, buffers, etc.).

-Store reagents under proper conditions (as indicated in experimental procedure or as specified by information provided by reagent's vendor).

-Do not allow the cells or tissue to become dry at any time during the detection procedure, as this will increase background staining.

II. Cell culturing

This part of the experiment will have already been done by the lab instructors under sterile conditions in a culture hood. A brief description of the culturing steps is provided below for your information. You should begin with Part II "***p*FA fixation**".

1. Culture cells in optimum medium. **MDCK and 3T3 cells**: 89% DMEM (high glucose)+ 10% fetal calf serum+ 1%(antibiotics+antimycotics).

2. At the point of passing cells, remove medium from flask and add 5 ml fresh trypsin solution (0.25% trypsin, 0.03% EDTA). Incubate for 5 minutes at 37 °C (visible clumps of cells should be floating in the bottom of the flask. MDCK cells take longer than others to become detached from the bottom of the flask by trypsinization). Disperse cells with pipette, transfer into a 15 ml conical tube and centrifuge for 3-5 minutes at 2,000 rpm (SORVAL centrifuge). Remove supernatant and add fresh medium in an amount equivalent to half the original volume in the flask. Centrifuge again to get rid of of all vestiges of trypsin, redissolve as before and count cells in a hemocytometer. Dilute to $0.5x10^5$ cells/ml for passing or for growing on slides.

3. Transfer 400 µl of cells ($1.7x10^4$ cells/ml for 3T3 and $2.5x10^4$ cells/ml for MDCK) into each of 8 chambers of the slides (Lab-Tek #177402). Incubate at 37 °C in a 5% CO_2 environment. Cells should adhere to the slides within 16 hours and be approximately 40-60% confluent. The slides can now be used for Immunlocalization.

Note: Cells can also be grown in other types of slides such as HTC super cured glass slides (Cell-Line Associates). The 14 mm well in these slides hold approximately 150 ml of cell solution ($2.5x10^4$ cells/ml). Hydrophobically coated slides are not necessary for self adherent cells such as the lines used here. Transfer slides into a humid chamber or place them in a Petri dish containing four (1x2.5 inch) pieces of wet Whatman paper. Incubate at 37 °C in a 5% CO_2 environment.

III. *para*-Formaldehyde (*p*-FA) Fixation

Note: The two rows (4 wells each) of the slide contain 3T3 and MDCK cells in the same arrangement. Please see Experimental Plan below.

1. Lab assistants will retrieve slides containing the cells from the CO_2 incubator just before ready for washing and fixing. Do not keep cells outside the incubator for extended periods of time as the pH of the medium will increase and cause cells to detach from the slide and eventually die.
2. Using a 1 ml pipetor, remove medium from each well of the slide and add 500 µl of PBS to wash cells briefly. Avoid dislodging cells by pointing pipetor to the side of the wells. Do not allow cells to dry during all steps in this procedure.
3. Discard PBS from previous wash and fix cells with 250 µl ice-cold 3% *p*FA made in PBS+1 mM $MgCl_2$ (See Preparation section above). Incubate for 15 minutes at room temperature.

   Note: If necessary, fixed cells can be stored for a few days in 0.1% *p*FA + 1 mM $MgCl_2$ at 4 °C.

IV. Permeabilization

1. Wash cells for 5 minutes each time at room temperature as follows:
   - Discard cell *p*FA with pipet (biohazardous waste). Place tip in a corner of the well to draw fluid.
   - Add 500 µl PBS by pointing the pipet tip to a corner of the well to avoid dislodging cells.
   - Remove PBS and add 500 PBS/50 mM NH_4Cl (made by adding 5µl of 5M NH_4Cl into 495 µl PBS).
   - Remove the previous solution added and add again 500 µl PBS.
   - Remove PBS.

   Note: NH_4Cl blocks unreacted fixative (*p*FA) which could cause Ab crosslinking.
2. Permeabilize fixed cells for 5 minutes by adding 500 µl of PBS+1.0% Triton X-100 (PBS-Tx).

V. Blocking and Primary Antibody Addition

1. Remove PBS-Tx and add 200 µl of Blocking Solution (Normal serum in PBS). Normal serum proteins block non-specific binding of antibodies to cellular antigens. Incubate at RT for 20 minutes.

2. While blocking, prepare **primary antibodies** dilutions (1° Abs) in PBS-BSA as indicated in the table below. Each tube already contains the correct amount of 1° Ab. To complete preparation, add the indicated PBS/BSA volume to each tube.

Specificity	Source of 1° Antibody	µl 1° Ab	µl PBS/BSA	Dilution
Anti-fibrillarin (D77 mAb)	Mouse mAb, ascites fluid	2	198	1/100
Anti-tubulin (mAb)	Mouse mAb	1	399	1/400
Anti-desmoplakin (mAb)	Mouse mAb	4	196	1/50

3. Remove Blocking Solution and add 100 µl of the appropriate 1° Ab solution to each chamber according to the **Experimental plan** below. Incubate for one hour at room temperature.

Experimental Plan
(8-chamber slide)

Cells: **3T3 (A)**	Cells: **3T3 (B)**	Cells: **MDCK (C)**	Cells: **MDCK (D)**	
1° Ab= mAb α–tubulin 2° Ab= Universal, biotinylated **DAB**	1°Ab=IgG mAb α–fibrillarin 2° Ab= Universal, biotinylated **DAB**	1° Ab= mAb α–tubulin 2° Ab= Universal, biotinylated **DAB**	1°Ab=mAb α–desmoplakin 2° Ab= Universal, biotinylated **DAB**	Name Group
1° Ab= PBS 2° Ab= Universal, biotinylated **DAB**	1°Ab= IgG mAb α–fibrillarin 2° Ab= Universal, biotinylated **AEC**	1° Ab= PBS 2° Ab= Universal, biotinylated **DAB**	1°Ab= mAb α–desmoplakin 2° Ab= Universal, biotinylated **AEC**	

VI. Secondary Antibody Addition and peroxidase treatment

1. Discard 1° Abs solution and wash cells three times (2 min each) with 500 µl PBS-BSA.

2. While washing cells, lab assistants will prepare secondary antibody solution in PBS-Normal Serum as indicated in the table below.

Specificity (2° Ab)	Stock Normal Serum	µl 2° Ab	µl PBS	Working dilution
Universal polyclonal: raised in horse against mouse and rabbit	20	20	1000	1/52

3. Add 120 µl of **each** of the 2° Ab solutions to all wells in the slide (See Experimental Plan, above). Incubate for 30 minutes at room temperature.

4. Remove 2° Ab solution and add 200 µl of 3% hydrogen peroxide (v/v in water) and incubate for 3-5 minutes. Peroxide treatment quenches endogenous peroxidase activity that would result in increased background. This step may not be necessary depending on the cell type or tissue.

VII. ABC Reagent Addition

1. Wash cells three times (2 min each) with 500 µl PBS-BSA.
2. Add 150 µl of ABC reagent (prepared as described above) to each well. Incubate a room temperature for 30 minutes.
3. Remove ABC reagent and wash cells three times (2 minutes each) with 500 µl PBS-BSA.

VIII. Substrate Addition

1. Remove previous PBS-BSA from the wells that will receive AEC as a substrate ONLY (see Experimental Plan above). Add 200 µl of AEC to the wells according to the Experimental Plan.
2. Incubate a room temperature for 30 minutes. Roughly 18 minutes into the AEC incubation time, remove the last PBS-BSA from the wells that will be developed with DAB. Add DAB at the appropriate time and continue to incubate all well (both sets of substrates) for 10 minutes.
3. Remove DAB substrate from the appropriate wells and wash cells three times (2 minutes each) with 500 µl water.
4. Remove AEC substrate next and wash cells twice (2 minutes each) with 500 µl PBS-BSA, and once with 500 µl of water.

IX. Samples Mounting and Viewing

1. Working carefully, snap off the plastic top, chamber divider from slide as demonstrated by lab instructors. Check to see that all glue residues are removed from the sites of contact between the glass slide and the plastic chamber divider. This can be done using a scalpel.
2. Mount in glycerol mountant. Use 5.0 µl per well. Carefully place a single large cover slip over wells. Use 6 very small drops of nail polish at edge of cover slip to seal it to slide. Store slides at -20 ^{o}C until viewed.
3. View samples under a light microscope. AEC stains brown, while DAB-Nickel stains grey-black.

Notes on cell Immunolocalization

1. In the fluorescent microscope, red filter is set for Rhodamine (or Cy3), green filter is set for Fluorescein (or Cy2) and blue filter is set for Hoechst.

2. Trypsinization (i.e., trypsin treatment) is required to detach adherent cells from the surface of culture flasks. After splitting, cells require approximately one day to recover from trypsinization.

3. Sometimes even cells that are normally adherent will fail to attach to glass slides. Attachment can be promoted by increasing the FBS concentration to 15%-20% in the culture medium and by washing the cells in culture medium at least once after trypsinization. Also, plastic slides can be used since cells normally adhere better to plastic. However care must be taken to ensure that the plastic material is suitable for microscopic viewing.

4. In the case of non-adherent cells, these can be cytocentrifuged or grown on slides that have been treated with polylysine, glycogen or any of several propriatary reagents that are commercially available. In extreme cases, cells can be crosslinked to silanized glass with reagents such as glutaraldehyde. However, this treatment may be deleterious to antigens of interest on the cells or tissue.

5. Antigens can be detected colorimetrically by using a secondary Ab that has been conjugated to an enzyme such as horse radish peroxidase or alkaline phosphatase. Endogenous activity enzymatic activities may have to be blocked when using these detection methods. Methanol inhibits peroxidase but may also cause antigenic denaturation. Colorimetric detection offer less localization resolution than fluorescent methods.

6. If using an alkaline phosphatase (AP) conjugated Ab as the secondary Ab, it might be necessary to inhibit the endogenous AP of the cells or tissue prior to the addition of the secondary Ab/Detection steps. This can be done by incubating the samples in PBS/levamisole (1 mM).

7. Freshly made *p*-Formaldehyde is preferred as a fixative over formalin for antigen immunolocalization experiments. This is because formalin solutions contain up to 15 % methanol as a stabilizing agent. Methanol causes antigenic denaturation, especially when used on cryostat sections or tissue.

Section 4: Gene Cloning Techniques

Experiments 14 - 16

Experiment 14: Isolation, Quantitation and Agarose Gel Fractionation of RNA from Mouse Liver

Part A: RNA isolation

The preparation of pure and undegraded RNA is the critical first step for a wide variety of molecular biology procedures (e.g., Northern transfer, Ribonuclease Protection Assay, cDNA synthesis, *in vitro* translation and mRNA Differential Display). However, the ubiquitous presence of RNases (both within the cells and in the environment), represent a major obstacle in RNA isolation. Fortunately, very effective RNase inhibitors cocktails are available. One such commonly used cocktail is composed of 4 M guanidinium isothiocyanate, N-lauryl sarcosine and ß-mercaptoethanol. These reagents rapidly disintegrate cellular structures, dissociate nucleoprotein complexes and cause the rapid denaturation of proteins. RNase activity is inhibited almost immediately. The intact RNA is further purified by a rapid, one-step acidic-phenol/chloroform extraction (Chomczynski and Sacchi, 1987). Under these conditions, RNA selectively partitions into the aqueous phase, free from DNA and protein. The RNA is then recovered by isopropanol precipitation.

References

Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**: 156-159.

Promega Technical Bulletin #87.

Note: RNases are difficult to inactivate. Autoclaving is not effective at destroying RNase activity. The key to isolation of good quality of RNA is to minimize RNase activity. Other than endogenous RNases in tissues or cells reagents, glassware, hands, saliva etc. can be the source of RNase contamination. Care must be taken to avoid such contamination by practicing the following rules:

1. Most common sources of RNase contamination are the user's hands, bacteria and molds that may be present in airborne particles. Proper sterile technique should be observed when handling reagents. Do not breath or cough into tubes and wear surgical gloves at all times.
2. Whenever possible, sterile disposable plasticware should be used for handling RNA. It is best to use filter pipette tips for pippetting.
3. Non-disposable labware should be treated to make them RNase-free. Glassware should be treated with 0.05% (v/v) diethyl pyrocarbonate (DEPC) and baked at 220°C overnight. Plasticware should be rinsed with 0.1 N NaOH/1 mM EDTA followed by RNase-free water, and then autoclaved for 30 min. Alternatively, commercially available products, such as RNase Zap™ (Ambion®, Inc.) work well for cleaning glassware, plasticware and electrophoresis equipment.
4. All solutions should be treated with 0.05% DEPC overnight at room temperature and then autoclaved for 30 min to remove any trace of DEPC. Tris buffer cannot be DEPC-treated because DEPC reacts with amino groups of the buffer.

Procedure

Note: The procedure described here is a variation of the Perfect RNA, Eukaryotic RNA isolation kit by Eppendorf (Cat # 0032 006.108). Some of the reagents used in this procedure are from the Eppendorf kit. **Steps 1, 2, 3 and 4** have already been performed by the laboratory instructors prior to today's laboratory. **IT IS OF UTMOST IMPORTANCE THAT THESE STEPS ARE PERFORMED AS QUICKLY AS POSSIBLE TO MINIMIZE RNA DEGRADATION.** You should begin with step 3. Use **GLOVES** and "aerosol resistant" pipet tips throughout the RNA isolation procedure.

Tissue handling and homogenization (done in advance by lab instructors)

1. Quickly, remove liver from mouse using sterile techniques.
2. Place the tissue in a culture dish or a clean weighing dish. Rinse briefly and cut tissue in small 50 mg fragments. Transfer liver fragments directly into an RNase-free, 2 ml tubes (wide bottom).

> Note: The amount of tissue must not be more 50 mg. Using larger amounts of tissue with the same amounts of other reagents described in this procedure will result in RNA of significantly lower quality.

3. Flash-freeze the tube with tissue in liquid nitrogen or an ethanol/CO_2 bath. Store tissue at -80°C if it won't be processed immediately, or proceed to step 4.

> Note: The RNA isolation procedure described here works equally well for 10^7 cells from suspension cultures. In such case, cells are lysed by the addition of the denaturing solution and vortexed until the viscosity disappears.

4. Retrieve tubes containing a liver fragment and add 350 µl of Lysis buffer (from the Eppendorf kit) plus 2.625 µl of Proteinase K Mix (20 mg/ml in water). Vortex thoroughly.
5. Homogenize the tissue using a nuclease-free, hand-held homogenizer. The probe tip is introduced into the tube and activated for 15 sec. Make sure that the tissue is caught by the rotating probe.

> Note: Alternatively, tissue can be homogenized by grinding to a fine powder in liquid nitrogen using a mortar and a pestle. Likewise, denaturation solution (10 ml/g tissue) can be added to freshly minced tissue and homogenized using a Polytron, a Dounce, or a conical glass homogenizer. Other laboratories utilize Mini-Bead Beaters (1.5 g Zirconium beads per ml of denaturation solution) to process hard-to-disrupt tissues, such as tumor tissues.

RNA Binding to Matrix

1. Centrifuge the homogenate in a microcentrifuge for 5 minutes at 16,000xg.
2. Place the supernatant in a fresh tube being careful not to transfer any of the solid material.
3. Add 350 ul of 70% Ethanol to the tube and mix thoroughly with very **gentle** inversion. Do not mix too vigorously or this will decrease the yield by 2 to 3 fold. (A fine precipitate may be visible at this point, this is normal and will not affect the preparation).
4. Add 200 ul of Perfect RNA Binding Matrix to the above lysate ethanol mixture. Make sure that the binding resin has been fully resuspended before addition to the mixture. Invert **gently** to mix.
5. Pipet the lysate/Binding Matrix mixture onto a RNA Mini spin column evenly.
6. Centrifuge for 60 seconds at 16,000xg. Discard the filtrate from the 1.5 ml collection tube and return the spin column to the same microcentrifuge tube.

Washing and Elution of RNA

1. Wash the bound RNA with 700 ul Wash Solution I. Centrifuge for 60 seconds at 16,000xg.
2. Discard the filtrate and return the spin column to the same collection tube. Spin again at 16,000xg to remove any residual wash solution.
3. Place the spin column into a new 1.5 ml collection tube.
4. Wash RNA by adding 500 ul of Wash Solution II. Centrifuge for 60 seconds at 16,000xg. Discard filtrate and return the spin column to the same collection tube.
5. Wash the RNA a second time with another 500 ul of Wash Solution II. Centrifuge for 60 seconds at 16,000xg. Discard filtrate and return the spin column to the same collection tube.

6. Spin again at 16,000xg to remove any residual wash solution.
7. Transfer the spin column to a new 1.5 ml collection tube (RNase–free), and add 50 ul of DEPC treated water.
8. Vortex the spin column assembly for 1 second to thoroughly moisten the binding matrix.
9. Incubate the spin column assembly in a 50°C heat block for 5 minutes.
10. Vortex the spin column assembly for 5 seconds and immediately centrifuge for 2 minutes at 16,000xg.
11. Discard the spin column, store the RNA at –80°C.

DNase digestion

1. Digest residual DNA by adding **6 µl** of 10X DNase buffer and **1 µl** (2 units) of DNase I (RNase-free)
2. Mix gently and incubate at 37°C for 20 min. DNase digestion is necessary when the RNA preparation is intended as a template for reverse transcription-PCR.
3. Resuspend the DNase Inactivation Reagent (from the Eppendorf kit) by flicking or vortexing the tube. Add 6 ul to the above mixture.
4. Incubate for 2 minutes at room temperature. Flick tube one time during the incubation to re-disperse the Inactivation Reagent.
5. Centrifuge for 60 seconds at 16,000 x g. Remove the supernatant to a new tube. Some carry over of the Inactivation Reagent is not a problem and will not affect further reactions.
6. Store at -80°C.

For long-term storage, resuspend the RNA in DEPC-treated water, add sodium acetate (2 M, pH 4.0) to 0.25 M and 2.5 volumes of ethanol. Store at -80°C.

Spectrophotometric analysis

1. To determine the concentration and purity of the RNA solution, transfer 2 µl of your RNA solution into an RNase-free tube containing 198 µl of DEPC-water. The lab instructors will measure the A_{260}/A_{280}. Pure RNA will give a ratio of approximately 2.0.

   1.0 A_{260}= 40 µg/ml RNA

   dilution factor in spectrophotometric cuvette= 100

   RNA solution conc. (µg/ml)= (A_{260})(100)(40µg/ml)

2. Check the integrity of the RNA by fractionation on a formaldehyde agarose gel as described in Part B.

Solutions

I. Denaturing solution (4 M guanidinium thiocyanate, not used in this procedure, but it is similar to the Lysis solution):

- 25 g Guanidinium thiocyanate
- 0.25 g Sodium lauryl sarcosine
- 1.25 ml 1M Sodium citrate, pH 7.0
- 0.35 ml ß-mercaptoethanol
- Bring the volume to 50 ml with water, stir until dissolved (may be heated to 65°C) and filter through 0.45 micron membrane.

II. 10X DNase buffer (5.5 µl) (not used here, but of similar composition)

DNase digestion mix

Reagent	Amount
1 M Tris (pH 7.5)	2.5 µl
0.2 M $MgCl_2$	2.5 µl
RNasin (RNase inhibitor)	0.5 µl

Part B: Formaldehyde agarose gels

Once cellular RNA has been isolated, its quality and yield can be quickly assessed by size fractionation on a denaturing formaldehyde agarose gel and ethidium bromide staining.

Good quality RNA in a gel should have the following features:

- Two prominent bands corresponding to the 28S and 18S ribosomal RNAs with the first band being about twice the intensity of the second.
- A light smear between the two ribosomal bands that represents most mRNAs.

Undesirable RNA degradation is evident when a smear, sometimes more intense than the ribosomal band, can be seen at the low molecular weight region of the gel.

Procedure (Wear gloves at all times)

1. Clean the gel apparatus thoroughly to inactivate RNases as follows: Add a small amount (~5 ml) of RNase ZAP™ (Ambion®, Inc., Austin, TX) solution into the bottom of the gel tray. Use four Kimwipes to gently scrub all surfaces that might come into contact with the RNA sample (e.g. comb, casting tray, wedges). Treat for 5 min and then discard the RNase ZAP™ solution. Rinse apparatus three times with 40 ml of DEPC-treated water.

2. Prepare a 1% formaldehyde agarose gel in an RNase-free, 250 ml Erlenmeyer flask (provided) by mixing the components listed below (Perform this task in collaboration with the group next to you. Therefore, follow the "2 gels" column):

Reagent	**1 gel**	**2 gels**
DEPC-treated H_2O	26.9 ml	53.8 ml
High melt agarose	0.31 g	0.62 g
10X MOPS-AE buffer*	3.1 ml	6.2 ml

* See MOPS-AE preparation below.

3. Heat this solution in a microwave oven (medium power) to dissolve the agarose. Do not allow to boil. Mix well and repeat heating to make sure agarose is completely dissolved.

4. Allow the molten agarose solution to cool to about 65°C (about 5 min), and then add 1.75 ml of 37% formaldehyde (3.5 ml for two gels). Work with the formaldehyde under a chemical fume hood. This makes a 0.66 M formaldehyde, agarose gel.

> Warning: formaldehyde is a suspected liver carcinogen. Handle appropriately under a chemical hood.

5. Mix the agarose-formaldehyde solution well and pour into the clean gel apparatus. You should proceed with step 7 while the gel solidifies.

6. After the gel has solidified, add enough RNase-free, 1X MOPS-AE buffer to cover the gel. Remove the comb by shaking it and pulling it upwards.

7. Prepare RNA samples (from isolated RNA in Part A) by mixing the reagents listed in the Table below. In addition to two different amounts of your own RNA sample, you will be provided with 2 µl of RNA prepared by the lab instructors.

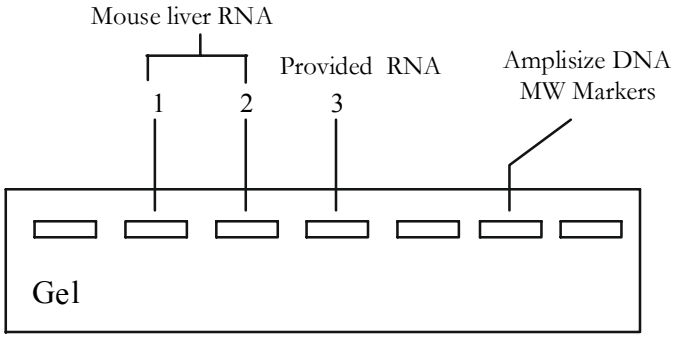
RNA Sample preparation for loading on the formaldehyde gel

	Sample 1**(µl)**	Sample 2 **(µl)**	Sample 3 (provided) **(µl)**
RNA	1	3	2
*RNA loading dye	24	22	23

*Denaturing RNA loading dye: 95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 0.5 mM EDTA, 0.025% SDS.

8. Incubate at 60-65°C for 15 min to denature the RNA.

9. Mix and load the entire tube volume into the appropriate wells.



10. Electrophorese at 70 V for 1.5 hr.

11. At the end of the electrophoresis, stain the gel for 15 minutes in a clean tray (weighing boat) with 15 ml of ethidium bromide solution (1 µg/ml).

12. Destain with 30 ml of ddH_20 for 10 minutes and photograph.

Size of AmpliSize™ DNA size marker:
2,000
1,500
1,000
700
500
400
300
200
100
50

Solutions

Non-denaturing RNA-loading dye: (6X)
50% glycerol
1 mM EDTA, pH 8.0/0.25% bromophenol blue/0.25% xylene cyanol
Make in DEPC H_2O, then autoclave

Phosphate Buffered Saline, pH 7.2-7.4 (1 liter):
8 g NaCl
0.2 g KCl
1.44 g Na_2HPO_4
0.24 g KH_2PO_4

10X MOPS-AE (100 ml):
0.2 M MOPS, pH 7.0 (20 ml of a 1.0 M solution pH 7.0 made in 0.05% DEPC-H_2O and autoclaved).
50 mM Na^+ Acetate (2.5 ml of 2 M NaOAc, made in 0.05% DEPC-H_2O and autoclaved).
10 mM EDTA, pH 8.0 (2.0 ml of 0.5 M EDTA, pH 8.0, made in 0.05% DEPC-H_2O and autoclaved).
(75.5 ml H_2O)

Experiment 15: cDNA Synthesis and PCR Amplification (RT-PCR)

Introduction

Conventional RT-PCR is performed in two separate steps. First, mRNA species are reversely transcribed into cDNA through the enzymatic activity of one of many possible reverse transcriptases (e.g., MMLV, AMV or RAV-2 transcriptase) in the presence of dNTPs and an oligo dT primer. Second, the cDNA products are amplified using gene specific primers and *Taq* polymerase. There are multiple commercial providers of kits pre-optimized for RT-PCR (e.g., Invitrogen, Roche, Amersham-Pharmacia, GIBCO BRL, Hybaid, Perkin Elmer, etc.) All of these companies are reputable reagent sources, but in practice, not all reagents perform successfully in every experiment, and users have to take into consideration many factors before deciding what source to use. For example, some mRNA may have a greater degree of secondary structure and it might be desirable to perform the reverse transcription at an elevated temperature. It is therefore relevant to know what type of reverse transcriptase is the one included in a given kit, since RAV-2 and AMV are more temperature-stable than MMLV.

Another important issue is the extent of RNA sample handling. Ideally, this should be minimized. To this end, both the reverse transcription and the PCR reactions can be performed in the same tube and using the same buffer. In fact, a thermostable polymerase (i.e., *Tth*) has both enzymatic activities at 68-75°C. However, it is not always necessary to use *Tth* for single tube RT-PCR reactions. In this experiment you will employ a combination of RAV-2 (Amersham-Pharmacia) and High Fidelity Taq (Roche) to perform single-tube RT-PCR as a step in the process to clone murine CAII. Success in the synthesis of first-strand cDNA also depends on the integrity and purity of the RNA. The presence of chromosomal DNA in the RNA preparation will result in unwanted products during PCR of the cDNA products.

The highest yield synthesis of full-length, first-strand cDNA can be obtained from poly(A) RNA (mRNA). However mRNA quality can difficult to verify. Therefore, total RNA is commonly used with adequate results.

> Note: Continue to observe the same guidelines for RNA isolation (i.e., RNase-free handling) during the cDNA synthesis step.

Description of the polymerase chain reaction

The polymerase chain reaction (PCR) permits us to make multiple copies ("amplify") of a target DNA region. It is perhaps the most used tool in modern molecular biology and is being applied in a multitude of research areas in a wide variety of creative ways.

The PCR is a temperature-driven process and it operates as follows:

- The double-stranded template DNA is heated (92-95 °C) until it denatures (unzips) exposing the nitrogenous bases (**denaturation**).
- Oligonucleotide primers are allowed to bind by hydrogen bonding to the regions flanking the target DNA segment contained in the template. This is done by cooling down the reaction to 35-70°C (**annealing**).
- The DNA target is synthesized at the optimal operating temperature of a thermostable DNA polymerase (72 °C). This enzyme utilizes the 3' ends of the primer as the synthesis initiation sites (primer **extension**).

The temperature cycle repeats and the target gene segment is exponentially amplified because the newly synthesized DNA products become themselves available for primer binding.

In order to perform PCR it is necessary to have prior knowledge of at least the nucleic acid sequence flanking of the DNA segment to be amplified. A typical PCR reaction includes the following components:

1. **Template**: a DNA solution that contains the target sequence. This could be relatively simple molecules such as plasmids or complex molecules as genomic preparations.
2. Two different single-stranded oligonucleotide **primers** (15 to 45 bases long). One of the primers is complementary to the 5' end of the sense strand of the target sequence (also called 5' primer or upper primer). The other primer corresponds to the 3' end of the sense strand of the target sequence (also referred to as 3' primer or lower primer).
3. DNA precursor molecules (**dNTPs** or deoxyribonucleotide triphosphates: dATP, dGTP, dCTP, and dTTP).
4. **Buffer** solution, at a pH and ionic strength optimal for the DNA polymerase activity
5. **$MgCl_2$**, at a concentration optimal for each particular PCR reaction.
6. Thermostable **DNA polymerase**.
7. Water baths set at appropriate temperatures or a computer-controlled **thermocycler**.

Suggested Readings

- Erlich, H., and N. Arnheim. 1992. Genetic analysis using the polymerase chain reaction. *Ann. Rev. Genet.* **26**: 479-506.
- Erlich, H., and N. Arnheim. 1992. Polymerase chain reaction strategy. *Ann. Rev. Biochem.* **61**: 131-156.
- Porter-Jordan, K., E.I. Rosenberg, J.F. Keiser, J.D. Gross, A.M. Ross, S. Nasim, and C.T. Garrett. 1990. Nested polymerase chain reaction assay for the detection of cytomegalovirus overcomes false positives caused by contamination with fragmented DNA. *J. Med. Virol.* **30**: 85-91.
- Vaishnav, Y.N., and F. Wong-Staal. 1991. The biochemistry of AIDS. *Ann. Rev. Biochem.* **60**: 577-630.
- White, T.J., R. Madej, and D.H. Persing. 1992. The polymerase chain reaction: clinical applications. *Adv. Clin. Chem.* **29**: 161-196.
- PCR Troubleshooting: http://info.med.yaly.edu/genetics/ward/tavi/Trblesht.html

---

Part A: Experimental Plan

Always **wear gloves** and maintain all reagent solutions on ice, unless indicated otherwise.

In this experiment, you are given the option of using one out of five possible sets of gene-specific primers to perform RT-PCR on CAII mRNA from the murine RNA you isolated. Use BioTools and the reported cDNA sequence for CAII to assess which one of the primer sets would be best suited for your experiment.

Forward Primers		Reverse Primers	
F1	5'- CGT GAC CAT GTC CCA CCA CT - 3'	R1	5'GCCCTTCGGACCCCTGCT 3'
F2	5'GGCACAAGGACTTCCCCATT 3'	R2	5'GCCGTGGCAGAGAAAAGATG 3'
F3	5'TGGGGATACAGCAAGCACAAC 3'	R3	5'ATGCGAGTCGGGATCCAAAT 3'
F4	5'TGGGGATACAGCAAGCACAACG 3'	R4	5'CCTGGGTCTTGCTTTTCACATT 3'
F5	5'CTGCTCTGCCCCAATCACC 3'	R5	5'- AAT CAC CCA GCC GTA ACT GC - 3'

Because the quality of the results from every PCR reaction depends on a multitude of interdependent factors, you will have to vary some of these parameters to optimize your RT-PCR results.

Due to constraints in time, reagents and instrument availability, each group in the class will be asked to vary only one parameter. The conditions for the non-varying parameters should be those indicated in the "Reference RT-PCR Conditions" listed below. Also, you must include among your samples two different controls. a) For the CAII(+) control, the template will be the plasmid pCR 2.1/CAII(+), used in combination with primers provided by lab instructors for the amplification a 869 bp CAII cDNA fragment. b) For the ß-actin control, 3 µg of your murine RNA will be used a the template, in combination with two ß-actin primers (provided) to amplify a 303 bp cDNA fragment of this house keeping gene. The latter of these controls serves as an indication of the suitability of the RNA preparation for RT-PCR.

General Procedure

YOU NEED TO PREPARE TWO SETS OF REACTIONS: ONE FOR THE REFERENCE SAMPLES (2X) AND ONE FOR THE VARIABLE OPTIMIZATION EXPERIMENT (5X)

1. Start by adding to each sample all reagents indicated in two Tables below under a) "Procedure for the Reference Samples" and b) the worksheet for the "Variable Optimization Samples), **except those reagents included in the "Master Mix" described in step 2**.

2. All samples (7 total) contain the same amounts of 10X Buffer, Rnasin, DTT, HF TAQ and RAV-2 RT. Therefore, these reagents can be prepared as a "Master Mix" to be added to all samples. Prepare the following "Master Mix" for 9 samples to account for pipetting errors. Don't forget to mix well.

Reagent	Amount per sample (µl)	Amount per 9 samples (µl)
10X Buffer	5	45
RNasin (RNase inhibitor)	1	9
DTT (500 mM)	0.5	4.5
HF TAQ (Roche, 0.35 U/ul)	4	36
RAV-2 RT (Amersham, 2.5 U/ul)	1	9
TOTAL VOLUME/SAMPLE	11.5	-

3. Add 11.5 µl of the "Master Mix" to each set of samples and mix reagents well by pipetting in and out 3 times.

4. Pulse spin the samples to collect reagents in the bottom of the PCR tubes (use adaptors in the centrifuge!).

5. Place your samples on ice until everyone in the class is done, then bring samples to the thermocycler.

6. Place tubes in the thermocycler that has been programmed using the following parameters:

42 degrees, 1 hour	(Reverse transcription step)
94 degrees, 30 seconds/ 55, 1 minute/ 72, 5 minutes	(35 cycles)
72 degrees 5 minutes	Final amplification
4 degree hold	

Procedure for the "Reference RT-PCR conditions" (control samples)

1. Label two control PCR tubes and label them clearly to distinguish them from the tubes from other groups. Include your group number. For example: Group No. 1 will label its tubes 1-CA(+) and 1-ßAc. PCR tubes must be certified RNase-free. These are the smallest tubes on your bench (0.2 ml) and must be labeled on the side (**NOT ON THE TOP**).

2. Add into the ßAc control tube 6 µl of your murine RNA containing 3 µg of material. This could be done by first preparing a 0.5 ug/µl RNA solution. The other control PCR tube (CA+) gets 2 µl (1 ng) of a plasmid into which CAII cDNA has been cloned.

Note: As little as 1 ng of material can be used for the first-strand synthesis of a moderately abundant message when using mRNA as the starting material.

3. Close the caps and heat the tubes at 70°C heating block for 5 minutes, then place the tube on ice (3 minutes). This treatment removes some secondary structure from the RNA.

4. Pulse-spin the tube (5 sec) in the microcentrifuge (use tube adaptors!) to collect the reaction mixture.

5. Set up RT-PCR reactions by adding to the tubes containing the denatured RNA, all other reagents according to the "Reference RT-PCR Conditions" table below, **except those reagents that are included in the "Master Mix" described above**.

6. Continue with the setting-up of samples for the "Procedure for Variable Optimization of RT-PCR..." (see below), before addition of Master Mix as described above under "General Procedure"

Note: If working with low-abundant messages or starting from less than 100 ng of mRNA, repeat the cDNA synthesis by adding more Reverse Transcriptase and incubating again at 42°C for 60 min.

ß-Ac Upper primer (ß-UP): 5' - GAA ACT ACC TTC AAC TCC ATC ATG- 3'
ß-Ac Lower primer (ß-LP): 5' - CTA GAA GCA TTT GCG GTG GAC - 3'

REFERENCE RT-PCR CONDITIONS

Reagent	**CA(+) Control**	**β-Actin Control**	**Final conc.**
Water	27 ul	23 ul	-
10X Buffer	Master Mix	Master Mix	1X
RNasin	Master Mix	Master Mix	1 µl/50 µl rxn
25 mM $MgCl_2$	5 ul	5 ul	2.5 mM $MgCl_2$
dNTP's (5 mM each)	2 ul	2 ul	0.2 mM each
CA Primer 1 (10 uM)	1.25 ul	-	0.25 uM
CA Primer 2 (10 uM)	1.25 ul	-	0.25 uM
ß-Ac Upper primer (10 uM)	-	1.25 ul	0.25 uM
ß-Ac Lower primer (10 uM)	-	1.25 ul	0.25 uM
DTT (500 mM)	Master Mix	Master Mix	5 mM
HF TAQ (Roche, 0.35 U/ul)	Master Mix	Master Mix	0.028 U/ul
RAV-2 RT (Amersham, 2.5 U/ul)	Master Mix	Master Mix	0.05 U/ul
Total RNA (0.5 ug/ul)	-	6 ul	~3 ug
1 ng pCR 2.1/CAII plasmid (1:1000)	2 ul	-	~1 ng
Total reaction	**50 ul**	**50 ul**	**-**

-Hi Fidelity (HF) Taq is normally provided at 3.5 U/ul. We have diluted 1:10 in 1X PCR buffer for ease of pipetting.
-RAV-2 RT is normally provided at 25 U/ul. We have diluted it to 2.5 U/ul in 1x RT buffer for ease of pipetting
-10X Buffer :
500 mM KCl
100 mM Tris pH 8.5
0.1% (w/v) Gelatin
1% Triton X-100

Procedure for Variable Optimization of RT-PCR of CAII

OPTIMIZATION PLAN

Group Number	Parameter to optimize	Range	No. of points/range
1, 2, 3,4,5,	$MgCl_2$ conc.	1.0-3.5 mM	5
6,7,8,9,10	Primers conc.	0.2-1.5 μM	5
11, 12,13,14,15	dNTPs conc.	0.1-0.5 mM	5
16, 17, 18, 19, 20	RNA template	1-10 μg	5
21, 22, 23, 24, 25	Annealing temperature	50-60°C	5

1. Make sure you identify correctly the variable you are assigned to optimize. At the end of the experiment, every group will share its results with the rest of the class.
2. Label 5 PCR tubes on their side including your group number. For example: Group 1 will have the following tube labels: I-1, I-2, I-3, I-4, I-5, where "I" is the roman number numeral one.
3. Add reagents following the worksheet shown below. This will help you avoid potential confusion. Reagents showing numbers are those already included in the Master Mix above (under "General Procedure"). Non-varying reagents should be added as in the Reference RT-PCR Table.
4. Proceed with step 2 above under "General Procedure".

SUGGESTED WORKSHEET FOR THE RT-PCR OPTIMIZATION EXPERIMENT

Reagent	Sample Number and Vol. (μl)						Check box
	1	2	3	4	5		
Water							
10X Buffer	5	5	5	5	5		
RNasin	1	1	1	1	1		
25 mM $MgCl_2$							
dNTP's (5 mM each)							
Forward Primer of your choice (10 uM)							
Reverse Primer of your choice (10 uM)							
DTT (500 mM)	0.5	0.5	0.5	0.5	0.5		
HF TAQ (Roche, 0.35 U/ul)	4	4	4	4	4		
RAV-2 RT (Amersham, 2.5 U/ul)	1	1	1	1	1		
Total RNA (0.5 ug/ul)							
Total reaction volume (μl)	50	50	50	50	50		
Variable value							

Part B: Computer-aided Primer Design

There is a multitude of undesired consequences that can result from poor primer design, whether one is performing PCR or sequencing a template. Fortunately, there are many different computer programs and some Internet sites, which will help you to design better primers than those designed by hand. These programs will check your sequence for false priming sites, for temperature incompatibilities, for dimer formation and for hairpin loops among other problems. These are problems, which are, at best, difficult to detect when designing primers by hand. The programs that help you to design primers will also give you a more accurate estimate of your melting and annealing temperatures that those computed by hand. Designing your primers on one of these computer programs is an ideal way to save time and money, because your primers are more likely to work the first time and you save time in the design step itself.

Exercise 10: Using BioTools to design PCR primers

1) Find and save the genomic cDNA sequence of mouse carbonic anhydrase II (MusCAIIm, Accession # K00811).

2) Using BioTools and the following instructions design primers:

1. Go to the Analyze menu
2. Choose Find PCR Primers
3. To design primers by hand:
    - Click and hold down the mouse button on the strand where you want to create a primer
    - Drag the mouse as you hold down the button to make the primer the length that you desire
    - If you want to design a forward (a.k.a. sense/upper) primer, click and drag on the upper strand
    - If you want to design a reverse (a.k.a. antisense/lower) primer, click and drag on the lower strand
4. To have the computer find appropriate primers
    - Once you are in the primer window (after step 2)
    - Go to the Analyze menu
    - Choose Find Primers
    - Enter parameters for your primers
        i. Length of primer +/- number of bases
        ii. Annealing temperature +/- ^{o}C
        iii. The range in the sequence where the primers will be located
        iv. Minimum product length
    - Click OK
    - The primers that the computer locates will be listed in two columns (Forward and Reverse Primers)
    - The primers are scored based on how closely they match your parameters and how 'good' the primers are (primer score is=90-hairpin penalty-est. free energy of the 1st (5') 5 bases in the primer+est. free energy in the last (3') 5 bases in the primer)
    - If there is an * next to a primer there is a problem with that primer (e.g. Hairpin loop, inappropriate internal stability, false priming etc)
    - To choose a primer from the list simply click on that primer and it will be displayed in the sequence window
5. To check your primers for problems
    - Once you have your primers selected there are two buttons (one for each primer) to the far right middle of the window the 'Show Problem' buttons
    - If the Show Problem button is visible click on it and a window will pop up that indicates the problem with the primer
    - If the Show Problem button is shaded you will not be able to click on it…this means that there are no structural problems with that particular primer
6. To save your primers
    - You can save your primers to open up in GeneTools by choosing Save or Save As from the File menu
    - To save the primers as a text file suitable for printing choose Save Primer As from the File menu
    - You can also save the entire product of your PCR reaction with the 2 primers you have created by going to the Transfer menu and choosing Sequence Editor. You will be able to save this file as a sequence at that point and to be able to use it for future projects.

3) Design primers that will amplify the protein coding region of MusCAIIm. Save the primers in the three ways mentioned in instructions **6a-c** above.

4) Design an experiment (with your group) to test which of the following components is the most important to the success of a PCR reaction.

- Primer design
- Primer concentration
- Mg+ concentration
- Annealing temperature
- dNTP concentration
- Buffer concentration
- Reaction volume
- Template (DNA) concentration
- Template (DNA) quality
- Amount of Taq

Experiment 16: Purification, Quantitation and Cloning of PCR Products

Part A: Preparative agarose gel electrophoresis of PCR products

Although PCR amplification of a target DNA sequence can be done very specifically, non-specific amplification products are sometimes observed. This problem is due to less than optimal primer design and/or reaction conditions, and/or the complexity (size) of the template DNA. Even in the case where only the expected PCR product is observed, there might be non-specific products that just are not visible by the chosen gel staining method. In most cases, direct cloning of PCR products will increase the complexity of the screening for the desired ligation product. It is therefore advisable that PCR fragments intended for cloning be first isolated and purified before ligating them to a vector. In Part A of this experiment, you will use agarose gel electrophoresis as a first step in the purification of the PCR product we intend to clone (i.e., CAII cDNA).

A.1 Casting the agarose gel

1. Place a well-forming comb in the agarose apparatus.
2. Prepare a 1.0 % agarose solution in 1X TAE

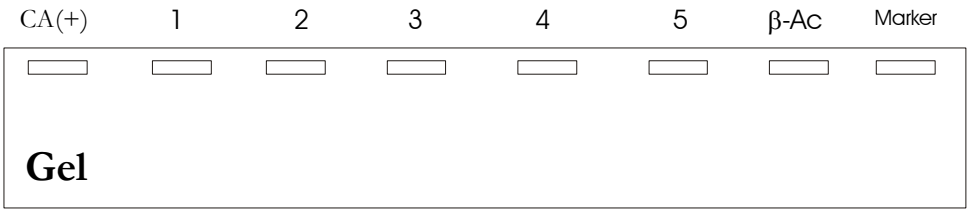
> The laboratory staff has prepared the agarose solution. The molten agarose can be found in the 60°C water bath at the side of the classroom.

50X TAE (one liter):
242 g Tris base
57.1 ml glacial acetic acid
100 ml 0.5M EDTA (pH 8.0)

3. Obtain the agarose solution from 65°C water bath and pour the melted agarose into the tray. Make sure that no bubbles are trapped underneath the comb.
4. After the gel is completely set, unseal the ends of the casting tray, add 1X TAE to just cover the surface of the gel and remove the comb carefully.

A.2 Electrophoresis procedure

1. Remove 25 µl from PCR reactions CAII and CA(+) (Exp. 15, Part B) and transfer to separate 1.5 ml microcentrifuge tubes. Add 5 µl of loading dye (6X) to each tube. Close tube cap, mix by tapping, and spin briefly. Save the remainder of the PCR products (~25 µl) on ice (Lab instructors will collect them for future use).
2. Prepare sample from the ß-Actin control by mixing 25 µl of the PCR product (Exp. 14, Part B) and 5 µl of loading dye (6X).
3. Using the micropipet, slowly load the entire volume (30 µl) from the tubes prepared in step 1 into separate preparative wells of the agarose gel (see diagram below). Load entire 30 µl of the sample prepared in step 2 into one of the analytical wells.
4. Also, load to the right of the ß-Actin sample, 12 µl of AmpliSize™ DNA size marker (provided by the instructors, Bio-Rad® Laboratories, Inc., Hercules, CA) leaving one well vacant between the sample and the molecular size marker.



5. Close the lid of the gel tank and connect electrical leads appropriately (i.e., red to red and black to black).
6. On the power supply set the voltage to 0. Turn the unit on and then increase the voltage until the voltmeter indicates 80 V. The power supplies we are using can run two gel electrophoresis units at once. The voltage supplied to the left and right sides of the unit is adjusted separately and the voltmeter must be switched to the correct side when the voltage is being adjusted or monitored. Bubbles should be visible at the electrodes and the bromophenol blue tracking dye should enter the gel.
7. Run the gel until the fastest moving dye front (bromophenol blue) has migrated 3/4 of the way down the gel.
8. Turn off the power supply and remove leads.
9. Carefully remove the gel and stain in the staining dish with ethidium bromide solution (1 μg/ml) for 10 min.

Caution: Ethidium bromide is a known carcinogen. Wear protective gloves when handling ethidium or ethidium-stained gels.

10. Drain off the ethidium bromide solution into a collection vessel for reuse.
11. Rinse gel and tray with tap water.
12. Visualize the DNA bands by placing on a UV transilluminator and photograph. Remember that UV light damages DNA. Therefore the long wave UV light setting must be used for visualization and exposure time must be minimized.
13. Cut the desired DNA band from the lane containing the mouse CAII cDNA. The expected size is approximately 869 bp. The band should be excised as close as possible to the fluorescent DNA band in order to minimize the amount of agarose carried into the DNA recovery step (Exp. 17, Part B). The "volume" of the gel slice should be approximately 200 μl.
14. Place the gel slice in a single 1.5 ml microcentrifuge tube and proceed to Exp. 16, Part B.

Part B: Recovery of the PCR product from the agarose gel

Following electrophoretic separation and visualization of PCR products, the desired DNA band is excised from the gel with either a clean microscope slide cover slip or a scalpel. The next step involves the recovery of the DNA fragment from the agarose gel slice. You will be using the method (QIAGEN Inc.; Chatsworth, CA) for this purpose.

The QIAEX II® procedure is rapid and does not require the use of phenol or ethanol precipitation. The gel slice containing DNA is first dissolved by the addition of a chaotropic salt and heating to 50°C. Next, the DNA is adsorbed to a proprietary DNA-binding resin under conditions of high ionic strength and low pH (< pH 7.5). Salts are removed by rinsing the resin with an ethanolic solution. The DNA remains bound to the resin during the ethanolic washes, but it is desorbed from the resin by the addition of water or Tris-EDTA buffer, pH 8.5.

Removal of DNA from agarose gel slice using the QIAGEN II system

Note: Many other kits and reagents are available commercially for DNA removal from ge slices. Examples of these sources are: Bio 101, Inc. (GeneClean kit), PROMEGA, BIORAD and Amicon, Inc. All of these reagents work reasonably well. However, in our experience we obtain better yields using the Qiagen kit as described here. This method is suitable for the extraction of 40 bp to 50 kb DNA fragments from 0.3-2% standard or low-melting-point agarose gels in TAE or TBE buffers. Procedure can be performed in one1.5 ml tube for gel slices weighing up to 250 mg.

1. Estimate the volume of the gel slice containing DNA (Part A.2) by weighing the gel piece and assuming that 1 mg = 1 µl. Alternatively, add a small known volume of Buffer QX1 and look at the graduation on the microcentrifuge tube to estimate the total volume of gel plus buffer. Subtract added QX1 Buffer added from total volume to obtain approximate gel slice volume.
2. Add 3 volumes of **Buffer QX1** to 1 volume of the gel slice, plus 10 µl of **QIAEX II resin (make sure that QUIAEX II resin is resuspended well before transferring).**
3. Incubate at **50°C** for 10 min to dissolve the gel slice. Vortex the tube every minute during the incubation. When the gel has dissolved, check the solution is clear or yellow. This indicates that the pH of the solution is <7.5.
4. If the pH is >7.5 (the color will be orange or purple), add 10 µl of 3 M sodium acetate, pH 5.0, mix well, and recheck that the pH is <7.5. Incubate for an additional 5 minutes.

   QX1 buffer contains a pH indicator dye that is yelow below neutral pH. Efficient binding of DNA to the resin only occurs at pH<7.5.
5. Centrifuge the sample for 30 sec and carefully discard the supernatant with a pipet. The QIAEX II resin will appear as a small opaque pellet.
6. Add 500 µl of **Buffer QX1** and resuspend the QIAEX pellet by vortexing. Centrifuge the sample for 30 sec and discard supernatant with a pipet.
7. Centrifuge the sample again for 10 sec and **remove all traces** of supernatant with a pipet.
8. Add 500 µl of **Buffer PE** and resuspend the QIAEX pellet by vortexing. Centrifuge the sample for 30 sec and discard supernatant.
9. Wash the QAIEX pellet again with 500 µl of **Buffer PE.** Resuspend the QIAEX pellet by vortexing. Centrifuge the sample for 30 sec and discard supernatant.
10. Centrifuge again for 10 sec and **remove all traces** of supernatant with a pipet. The PE washes removes all residual salts.
11. **Air-dry** the QIAEX pellet for 10-30 minutes or until the pellet becomes white (not grey). Do not over-dry as this may result in decreased elution efficiency.
12. To elute DNA from QIAEX II resin, add 30 µl of **10 mM Tris-HCl, pH 8.5** and resuspend the pellet by vortexing. Incubate at room temperature for 5 minutes.
13. Centrifuge for 30 sec and carefully pipet the DNA-containing supernatant into a clean tube.

Several points about the treatment of the PCR products should be noted:

Direct purification and cloning of the PCR products works only if the desired PCR product is the predominant (or only) product of the PCR reaction. This is not usually the case, especially when the target DNA for the amplification reaction is a complex genomic DNA. Usually, the desired PCR product must be purified by agarose gel electrophoresis before it can be used for cloning. There are a variety of techniques available for the extraction of DNA fragments from gel slices, these techniques include enzymatic digestion with agarase, low melt agarose, phenol, freeze-thaw, electroelution, and DNA binding resins such as the one we used here.

References

Innis, M. A., D.H. Gelfand, J. J. Sninsky, T. J. White (eds.) 1990. PCR Protocols. Academic Press, San Diego.

Mullis, K. B., and F. A. Faloona. 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Meth. Enzymol.* **155**: 335-350.

Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988.

Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**: 487-491.

Part C: Analytical agarose gel electrophoresis of purified PCR products

C.1 Casting the agarose gel

1. Seal edges of the gel-casting tray and position the comb close to the negative (black or cathode) electrode.
2. Prepare a 1.0 % agarose solution in 1X TBE.

> The laboratory staff has prepared the agarose solution. The molten agarose can be found in the 65°C water bath at the side of the room.

5X TBE:
54 g Tris base
27.5 g boric acid
20 ml 0.5 M EDTA (pH 8.0) per liter. (45 mM Tris-borate, 1 mM EDTA)

3. Retrieve melted agarose solution from the 65°C water bath and pour into the gel-casting tray. Make sure that no bubbles are trapped underneath the comb.
4. After the gel is completely set, unseal the ends of the casting tray, add 0.5X TBE to just cover the surface of the gel and remove the comb carefully.

C.2 DNA Electrophoresis

In this experiment you will be analyzing an aliquot (5 µl) of your purified PCR products (from Part B). We will use this gel electrophoresis step to estimate the relative concentrations of purified PCR product by comparing with a known marker DNA standard. Thirty nanograms of the purified PCR product will be used in the ligation reaction described in Part D of this experiment.

1. Pipet 5 µl from the purified PCR product (Part B) into a clean microcentrifuge tube. Add 5 µl of water and 2 µl of loading dye (6X) to the tube. Close tube cap, mix by tapping, and spin briefly.
2. Using the micropipet, slowly load the entire 12 µl into one well of the 1.0% agarose gel.
3. In an adjacent well, load 12 µl of the marker DNA standard (1 µg of *Hae* III ΦX174 + in 9 µl dH_2O + 2 µl loading dye) on the agarose gel.
4. On the power supply set the voltage to 0. Turn the unit on and then increase the voltage until the voltmeter indicates 85 V. The power supplies we are using can run two gel electrophoresis units at once. The voltage supplied to the left and right sides of the unit is adjusted separately and the voltmeter must be switched to the correct side when the voltage is being adjusted or monitored. Bubbles should be visible at the electrodes and the bromophenol blue tracking dye should enter the gel.
5. Run the gel until the fastest moving dye front (bromophenol blue) has migrated 3/4 of the way down the gel.
6. Turn off the power supply and remove leads.
7. Carefully remove the gel and stain in the staining dish with ethidium bromide solution (1 µg/ml) for 10 min.

> **Caution:** Ethidium bromide is a known carcinogen. Wear protective gloves when handling ethidium or ethidium-stained gels.

8. Drain off the ethidium bromide solution into a collection vessel for reuse.
9. Rinse gel and tray with tap water.
9. Visualize the DNA bands by placing on a UV transilluminator and photograph.

ΦX174 *Hae* III fragment (bp)	% Total Mass	Proportion of 1.0 μg (ng)	Bio-Rad Mass Ruler fragment (bp)	Mass of band (ng)
1353	25.12	251	1000	100
1078	20.01	200	700	70
872	16.19	162	500	50
603	11.20	112	200	20
310	5.76	58	100	10
281	5.22	52		
271	5.03	50		
234	4.34	43		
194	3.60	36		
118	2.19	22		
72	1.34	13		
5386	100.00	1000		

Size of AmpliSize™ DNA size marker:
2,000
1,500
1,000
700
500
400
300
200
100
50

Part D: TOPO TA Cloning

Background

(Adapted from Invitrogen TOPO™ TA Cloning Instruction Manual)

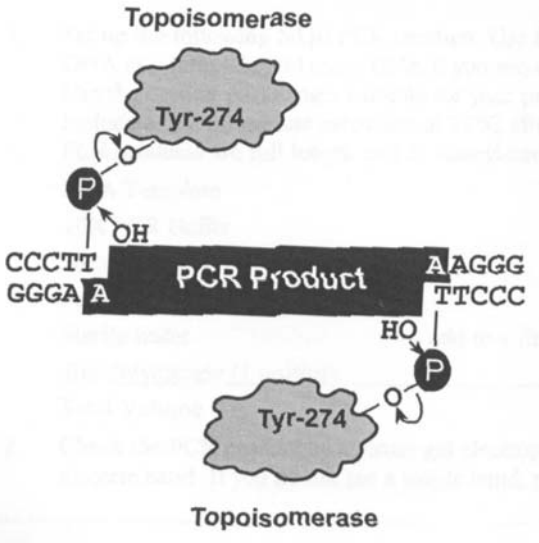
Like no other technique in modern molecular biology, PCR can be applied to a large number of research problems. For example, PCR can be designed so as to facilitate DNA cloning.

TOPO™ TA Cloning (Invitrogen Corp.) provides a highly efficient, 5 minute, one-step cloning strategy for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector. No ligase, post-PCR procedures, or PCR primers containing specific sequences are required.

The plasmid vector (pCRII-TOPO or pCR2.1-TOPO) is supplied linearized with:

- Single 3' thymidine (T) overhangs for TA Cloning
- Topoisomerase covalently bound to the vector (referred to "activated" vector)

Taq polymerase has a nontemplate-dependent terminal transferase activity which adds a single deoxyadenosine (A) to the 3'-ends of PCR products. The linearized vector supplied in the TOPO™ TA Cloning kit has single, ovehanging 3' deoxythymidine (T) residues. This allows inserts to ligate efficiently with the vector.



TOPO™ Cloning exploits the ligation activity of topoisomerase by providing an "activated", linearized TA vector using proprietary technology (Shuman, 1994). Ligation of the vector with a PCR product containing 3' A overhangs is very efficient and occurs spontaneously within 5 minutes at room temperature. The TOPO™ Cloning reaction can be transformed into chemically competent cells or electroporated directly into electrocompetent cells. Transformation has to be done immediately following the ligation reaction. This is because topoisomerase contains a nuclease activity which appears to relinearize the ligation product. The efficiency of transformation decreases approximately 50% if transformation is performed 60 minutes after ligation.

References

Shuman, S. (1994). Novel Approach to Molecular Cloning and Polynucleotide Synthesis Using Vaccinia DNA Topoisomerase. *J. Biol. Chem.* 269: 32678-32684.

Sambrook, J., D.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A laboratory manual. 2nd Edition. Cold Spring Harbor Press, New York. (particularly pp. 9.16-9.23).

Ligation

1. Thaw on ice one vial of competent cells for each transformation. A tube already containing 1 ul of PCR-TOPO vector is provided. Add the remaining reagents to this tube to set-up reaction.

2. Set up the TOPO cloning reaction as follows:

Fresh QIAEX II PCR Product	2.0 µl
Water	1.0 µl
Salt Solution (1.2 M NaCl, 60 mM $MgCl_2$)	1.0 µl
pCR2.1-TOPO vector	1.0 µl
Total	**5.0 µl**

Note: if the yield of the PCR product clean up step was low, use up to 4 µl of the PCR product and decrease the amount of water accordingly to maintain a total of 10 µl final reaction volume.

3. Mix gently and incubate for 5 minutes at room temperature. For best results do not leave for more than 5 minutes or the transformation and cloning efficiencies will decrease.

4. Briefly centrifuge and place the tube on ice. Immediately proceed to the transformation reaction. Ligation products can be stored at –20°C for up to 24 hours. However, the transformation efficiency will be diminished.

Transformation

In this experiment, you will introduce the DNA products of your ligation reaction (plasmid plus insert) into *E. coli* cells of the TOP10 strain (transformation). When any DNA is added to competent *E. coli* cells in a high ionic strength buffer containing a mixture of divalent cations (e.g., Mg^{++}, Ca^{++}), the DNA forms a DNAase resistant complex that adheres to the cell surface. This complex is taken up by the cell during a brief heat pulse at 42°C, probably through zones of adhesion on the cell surface.

After growth in rich medium (e.g., SOC broth) for the recovery of cells and the phenotypic expression of the antibiotic resistance gene (*amp*^{r}), cells that acquired the plasmid (transformants) can be isolated by plating on a selective medium such as LB/KAN. This step eliminates the cells which do not acquire the plasmid DNA during the transformation step. This is important because less than 1% of the bacterial cells will take up the plasmid DNA. The kanamycin resistance phenotype ensures that only the cells that have acquired the pCR2.1-TOPO vector are selected for growth, but does not ensure that the plasmid has the desired insert DNA. A simple *in vivo* genetic complementation test will be used to identify kanamycin resistant transformants that are also likely to have acquired the PCR DNA inserted into the pCR2.1-TOPO vector.

> **Note:** Prior to today's lab, *E. coli* cells were made "competent" (i.e. capable of plasmid DNA uptake) by the Hanahan procedure (see reference and appendix). In this procedure, permeabilization of bacterial cell membrane is accomplished. by treatment with a mixture of divalent cations. Cells can also be made competent with a greater efficiency by exposing cells to an electric pulse in a low salt solution in a procedure referred to as electroporation.

1. Add 1.0 µl of the TOPO cloning reaction into a tube containing 25 µl of competent cells (TOP10 one-shot cells, Invitrogen). Mix gently.
2. Incubate on ice for 30 minutes.
3. Heat shock the cells for 30 seconds at 42°C in the heater block.
4. Immediately transfer the tubes to ice and incubate for two minutes.
5. Add 250 µl of room temperature SOC medium.
6. Cap the tube tightly and incubate at 37°C for 1 hour.
7. At the end of incubation, place the tube on ice if not ready with the plate.
8. While the transformed cells are incubating, prepare one LB-kanamycin (50-100 µg/ml) agar plate for the blue-white genetic complementation test (see the box below for an explanation of this test). Add 80 µl of X-gal (20 mg/ml in dimethylformamide) to the center of the plate and spread as follows:
    - Dip the spreading rod in 70% ethanol to sterilize the rod and then ignite the excess ethanol by passing the rod **briefly** through a flame.
    - Touch the agar surface with the rod to be sure that the rod is cool.
    - Spread X-gal and IPTG by moving spreading rod in a circular motion.
    - Resterilize the spreading rod before beginning the next operation.

> X-gal (5-Bromo-4-chloro-3-indolyl-ß-D-galactopyranose) is a synthetic, chromogenic substrate for ß-galactosidase. ß-galactosidase is encoded by the *E. coli lac* Z gene. *E. coli* colonies that express a functional *lac*Z gene appear blue when grown on media supplemented with X-gal. If the *lac*Z gene is effectively disrupted by the insertion of a piece of DNA, no expression of ß-galactosidase is possible. Colonies on the same media that do not express the *lac* Z

gene appear white. X-gal should be stored at -20°C until just prior to use. X-gal is light sensitive so the plates should be covered with paper towels or aluminum foil.

IPTG (Isopropylthio-ß-galactoside) is a synthetic inducer of the *lac* operon by binding to the *lac*I repressor protein. IPTG is not required in the media in this experiment because *E. coli* TOP10 cells do not express the *lac* I repressor protein.

9. Centrifuge cells for 15 seconds at 14,000 rpm. Pipet 150 ul of the supernatant and discard into biohazardous bag.

10. Resuspend cells with pipette and plate entire solution (~100) µl onto a Luria Broth-kanamycin plate that has X-gal.

11. Incubate overnight at 37°C with the plate lid on the bottom. This keeps droplets from falling on the agar, thus possibly cross-contaminating clones.

Part E: Mini-preparation of Plasmid DNA

Your LB/Kan plates should contain two types of *E. coli* colonies, white ones and blue ones. The white colonies are most likely the result of cells transformed with a recombinant plasmid in which the pCR2.1-TOPO vector has acquired the PCR DNA insert.

You will subculture two of the white colonies from our LB/Kan plate in liquid media. After overnight incubation to allow the bacterial cells to multiply, you will extract the plasmid DNA from each of the bacterial cultures and analyze it by restriction enzyme digestion.

The procedure described here for the small-scale purification of plasmid DNA utilizes the Perfectprep® Plasmid Mini Kit marketed by Eppendorf. This system is based upon the methods published by Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981). The plasmid DNA is purified by binding the plasmid DNA to a proprietary silicon-based resin. Briefly, overnight cultures of stationary phase *E. coli* cells are treated with EDTA to chelate divalent cations thereby de-stabilizing the cell membrane. Next, treatment with SDS solubilizes the phospholipid and protein components of the membrane. This causes rupture of the cell membrane. When NaOH is added to the ruptured cell, the hydrogen bonds in the non-supercoiled chromosomal DNA molecule will be broken, the double helix will unwind and the two strands will separate completely. However, the two strands of the supercoiled plasmid DNA remain intertwined.

Treatment with potassium acetate neutralizes the solution and causes the two strands of the plasmid DNA to re-associate. The re-aggregated chromosomal DNA forms a tangled mess with an insoluble precipitate of K-SDS-lipid-protein. This insoluble network can be precipitated by centrifugation, while the plasmid DNA remains in the supernatant. The plasmid DNA is further purified by binding the DNA to a silicon-based resin and eluting the DNA with water or TE buffer. Following the completion of the Perfectprep® protocol, the plasmid DNA is suitable for restriction enzyme digestion or DNA sequencing.

Suggested reading

1. Birnboim, H.C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acid Res.* 7: 1513-1523.
2. Ish-Horowicz, D. and J.F. Burke. 1981. Rapid and efficient cosmid cloning. *Nucleic Acid Res.* 9: 2989-2998.

Part a: Liquid Subculture of Bacterial Transformants

1. Add 3 ml LB-Kan (Luria broth with 50 μg/ml kanamycin) to two Falcon culture tubes.
2. Start a 3 ml culture from one of the white colonies growing on your LB-Kan/X-gal plate as follows:

   a) pick the colony by **lightly** touching it with a sterile plastic loop (it is not necessary to scrape the entire colony from the plate)

   b) uncap falcon tube

   c) submerge the loop into the LB-Kan in the Falcon tube

   d) rapidly move the loop back and forth through the LB-Kan a few times (to dislodge the cells), then discard the loop

   e) recap the tube
3. From another group that has a different dengue serotype, start a 3 ml culture from one of their white colonies using the procedure in step 2. Be sure to innoculate the colony into a separate culture tube.
4. Grow both cultures at 37°C with vigorous shaking (300 rpm) for 20-24 hours.

Part b: Cell Lysis and Plasmid Purification

1. Pre-heat a 1.5 ml microcentrifuge tube with approximately 200 μl dH_2O in a water bath. This water will be used to elute the purified plasmid DNA from the spin column.
2. Transfer 1.5 ml of each bacterial culture to a 1.5 ml microcentrifuge tube. Take care to label each tube to its corresponding culture and by your group #. Pellet the bacteria by centrifuging each 1.5 ml microcentrifuge tube at 14,000 rpm for 30 seconds.
3. Remove the supernatant by decanting or aspirating into the waste collection tube. Do not disturb the bacterial pellet.
4. Add 100 μl of Solution I and completely resuspend the pelleted bacteria by either vortexing the tube or rubbing it along the holes in your rack. The mixture should have a smooth and even consistency with no clumps.
5. Lyse the bacteria by adding 100 μl of Solution II to the resuspension and mix several times by repeated **gentle** inversion each tube. The lysate should be relatively clear with no visible clumps of cell material.
6. Neutralize the bacterial lysate by adding 100 ul of Solution III and immediately mixing thoroughly by repeated inversion of each tube. After mixing, the whitish precipitate should have a curd-like appearance.
7. Pellet out the cellular debris and precipitated proteins by centrifuging the neutralized lysate in a microcentrifuge for 5 minutes at 14,000 rpm.
8. While your tubes are being centrifuged, label two spin columns and two collection tubes with your group # and the identification to its corresponding culture.
9. Carefully transfer all the clear supernatant to a spin column in a collection tube. Take care to not pick up any of the white precipitate that could possibly clog your column. You **do not** have to get all of the supernatant.
10. Vigorously mix the DNA Binding Resin by vortexing for about 15 seconds. Using a wide bore tip, add 450 μl of DNA Binding Matrix to the supernatant in each spin column. Mix by capping the spin column and vortexing the column/collection tube assembly.

11. Purify the bound plasmid DNA by centrifuging the spin column/collection tube assembly for 30 seconds at 14,000 rpm. Decant the filtrate into the waste collection tube and place the spin column back into the collection tube.

12. Add 400 µl of Purification Solution to each spin column. Cap each tube and vortex briefly. Centrifuge the spin column/collection tube for 30 seconds at 14,000 rpm.

13. Decant the filtrate and place each spin column back into their respective collection tubes. Remove the residual Diluted Purification Solution by centrifuging each spin column/collection tube assembly for 30 seconds at 14,000 rpm.

14. Transfer each spin column to a new collection tube (be sure to label the new tubes). Add 50 µl of dH_2O that has been pre-heated to 65°C to each spin column. Cap each tube and briefly vortex each column to mix .

15. Centrifuge each spin column/collection assembly for 60 seconds at 14,000 rpm.

16. Discard the spin columns and store the eluted plasmid DNA at either 4°C or –20°C.

Part F: Determining the cloning orientation of mouse CAII cDNA by restriction enzyme digestion analysis

In this experiment, the recombinant molecule composed of pCR™2.1 plasmid ligated to the mouse CAII cDNA insert (i.e. plasmid miniprep from Part E) will be digested to verify both the presence and ligation orientation of the cDNA insert. Determining the orientation of the insert in the vector can be very important. For instance, one may want to determine if the insert contains an open reading frame (i.e., for the purpose of protein expression). Likewise, insert orientation must be determined if one wishes to use the plasmid construct to perform transcriptional analysis.

First, you will need to complete the Bioinformatics exercise at the end of this chapter in order to obtain a detailed restriction enzyme map analysis of the pCR™2.1/CAII construct.

Restriction enzyme digestion procedure

1. Label two clean, 1.5 ml microcentrifuge tubes, one for each of you mini-prep DNAs, with your group number and the experiment number.

2. For each separate plasmid, add the following reagents in the order indicated below:

Reagent	**Volume (µl)**
H_2O	4
10X Enzyme buffer	1
10X BSA	1
Plasmid DNA 1 or 2 (Exp. 16, Part b)	3
Restriction enzyme	1

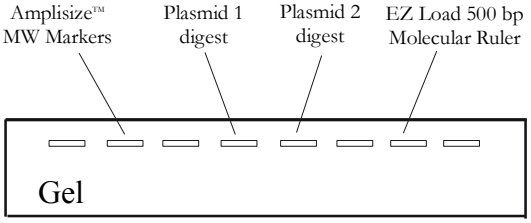
3. Mix and pulse-spin to pool reagents in the bottom of the tube.

4. Incubate at the enzyme optimum temperature for a minimum of 30 minutes. Optimum temperature will depend on the enzyme being used and is usually 37 degrees C. *Bsa I* is @ 50 degrees C.

5. At the end of the incubation, add 2 µl 6X loading dye and save for Exp. 16, Part G.

Part G: Gel electrophoresis

1. Pour a 1.4% agarose solution in 1X TBE gel.
2. After agarose has hardened, remove comb and wedges from the casting tray. Then add 1X TBE buffer until the surface of the gel is covered.
3. Load the entire 12 µl of each restriction digest (Part F, step 5) in two separate wells (See figure below).



4. Also, load 12 µl of the EZ Load 500 bp Molecular Ruler (size markers, provided by instructors) in a well adjacent to the digested plasmid samples.
5. Close the lid of the gel tank.
6. Run the gel until the fastest moving dye front (bromophenol blue) has migrated 3/4 of the way down the gel.
7. Turn off the power supply and remove the power leads.
8. Carefully remove the gel and stain in the staining dish with ethidium bromide solution (1 µg/ml) for 10 min.

Caution: Ethidium bromide is a known carcinogen. Wear protective gloves when handling.

9. Drain off the ethidium bromide solution into a collection vessel for reuse.
10. Rinse gel and tray with tap water.
11. Visualize the DNA bands by placing on a UV transilluminator and photograph.

ΦX174 *Hae* III fragment (bp)	% Total Mass	Proportion in 1000 ng (1 µg)	Bio-Rad Mass Ruler (#170-8207) fragment (bp)	Mass of band (ng)
1353	25.12	251	1000	100
1078	20.01	200	700	70
872	16.19	162	500	50
603	11.20	112	200	20
310	5.76	58	100	10
281	5.22	52		
271	5.03	50		
234	4.34	43		
194	3.60	36		
118	2.19	22		
72	1.34	13		
5386	100.00	1000		

Size of AmpliSize™ DNA size marker (Biorad # 170-8200)	EZ Load 500 bp MW Ruler (Biorad # 170-8354)
2,000	5000 (darkest band)
1,500	4500
1,000	4000
700	3500
500	3000
400	2500

300	2000
200	1500
100	1000
50	500

Exercise 11: Computer-Simulated Cloning, Restriction Enzyme mapping, and Restriction enzyme fragment determination (BioTools)

Use the following sequence of steps (within BioTools) to simulate the cloning of the CAII insert, and to determine a suitable restriction enzyme-based experiment that can be used to screen for the two possible cloning orientations of the insert in the plasmid construct. A detailed procedure for this process is described below under **'In Silico Cloning'**.

- Obtain and save the sequence of the pCR™2.1 vector.
- Clone (in silico) the murine CAII fragment into the vector's T/A cloning site in order to generate the plasmid/insert construct.
- Generate a restriction enzyme map containing restriction enzymes that cut the vector at unique sites and that also cut the insert in an asymmetric fashion.
- Generate a table (such as the one below) with all the restriction fragments for each potentially useful enzyme.

Plasmid	Expected fragment sizes after *enzyme* digestion (in bp)
pCR 2.1/CAII, orientation A	
pCR 2.1/CAII, orientation B	
pCR™2.1	

- Run a simulated gel to determine in which case you can unambiguously distinguish the cloning orientation of the insert in the vector.
- Choose the enzyme that you think is best suited for your experiment. Please choose one from the following list.

ENZYME	CUT SITE
BamHI	G GATCC CCTAG G
Bgl II	GCCNNNN NGGC CGGN NNNNCCG
Bsa I	G GTCTC C CAGAG
Bst XI	CCANNNNN NTGG GGTN NNNNNACC
Dra III	CACNNN GTG GTG NNNCAC
Mbo II	nnnGAAGAnnn n n nnCTTCTnnnn
Pst I	CTGCA G G ACGTC
Rsa I	GT AC CA TG

Note: If the DNA sequence of the construct were unknown, this will have to be obtained by sequencing.

Restriction Enzyme Databases

Most molecular biology software packages come with a database of commonly used restriction enzymes, and for most people, these are enough. However, if you need to find out information on these enzymes, or if you need

to find a rare restriction enzyme to cut a specific site, the 'Rebase' database that has compiled just about every known restriction enzyme is probably the most definitive source of information. However, you should be aware that some of the enzymes contained in this database are not commercially available. Thus, it is important to choose a few enzymes that would work for your project.

Restriction Enzyme Mapping
When a sequence is known, mapping restriction enzyme cut sites, while tedious, is often necessary. Computer programs make this task much quicker and easier. Most basic biotech software and Internet software will map restriction enzymes for a known sequence (see appendix for Internet software).

Clone Insertion
To test the direction of a clone insert, you must look at the pattern of restriction enzyme cut sites. There are computer programs to test for the different patterns of restriction enzymes cut sites in clones with a correctly inserted DNA fragment versus a backward DNA fragment. This also simplifies your work and reduces the possibility for errors.

In Silico Cloning

1. Find the sequence for the pCR™2.1 vector in either the common vectors folder in GeneTools or the Invitrogen website (www.Invitrogen.com).

2. From the mCAII sequence that you saved, use the instructions below:

    a) Highlight the sequence that represents your PCR Product
    b) Copy the sequence by going to the Edit menu and choosing copy
    c) Go to the vector sequence that you opened in the sequence editor
    d) The insert will be placed at position 294 in the pCR-2.1 sequence (i.e., cloning site. Place your cursor there.
    e) Go to the Edit menu and paste the sequence into the vector sequence
    f) Go to the File menu and **Save As mCAII-F**
    g) Go to the Edit menu and choose reverse complement (this will change the orientation of your insert)
    h) Go to the File menu and **Save As mCAII-R**

    *Note after inserting the mCAII sequence in both orientations, go to the Edit menu and choose Circular DNA. This will make the plasmid circular *in silico*, just as ligating mCAII cDNA did *in vitro*.

3. In the lab, to determine the orientation of insert we must cut the vector/insert DNA with restriction enzymes. To get some idea of what enzymes we will use in the lab, we can use the computer to test the enzymes. First we must get some idea of what enzymes cut the vector and what enzymes cut the vector/insert construct.

    a) Use BioTools to obtain a total restriction map of the vector/insert construct (select all enzymes-and select 2 or 3 cut sites).
    b) Identify 10 restriction enzymes that cut the insert and the vector. Which of these enzymes might be most useful in determining the orientation of your insert (mCAII)? *Hint what would happen if you picked an enzyme that cut in the middle of the insert?*
    c) Cut the vector/insert sequence (for each) with *Rsa* I. Where are the cut sites of this enzyme in relation to the insert/vector connection (are they in the middle of the insert or at the ends)?

4. Run the gel simulation by going to the Analyze menu and choosing Gel Simulation for the mCAII-F and mCAII-R sequences.

    a) What enzymes from part 3b would be useful in determining the orientation of the insert? Tabulate the fragments for the informative (useful) enzymes.
    b) What are the fragment sizes that you will expect to see with *Rsa* I? Is this the best enzyme to determine orientation of the insert?

5. Suggest 3 more restriction enzyme analysis strategies (other than using *Rsa* I) that will allow you to determine the cloning orientation of the hCAII insert in the vector.

Section 5: DNA Detection and Analysis

Experiments 17 - 18

Experiment 17: Southern Blot Hybridization

Overview

Localization of particular sequences within genomic DNA is accomplished by transfer techniques originally described by Southern (1975). The first step consists of genomic DNA isolation followed by digestion with a type II restriction enzyme. Type II restriction enzymes recognize a specific DNA base sequence and cut double stranded DNA by hydrolyzing one phosphodiester bond on each DNA strand.

In the second step, the DNA fragments are separated according to their size by horizontal agarose gel electrophoresis, denatured *in situ* and then vertically transferred by capillary action to a nylon or nitrocellulose membrane. The relative positions of DNA fragments are preserved during their transfer to the membrane and specific fragments are subsequently identified by hybridization with a labeled DNA probe.

Part A: Isolation of mammalian genomic DNA

Isolated DNA to be used for Southern blotting should be of high molecular weight and free of exonucleases. Excessively sheared genomic DNA limits the number of molecules that can be digested by sequence-dependent cleavage (restriction enzymes). Random physical shearing will diminish the target signal and increase the background upon detection.
You will be isolating genomic DNA from mouse liver tissue utilizing reagents from the Easy-DNA™ Kit supplied by Invitrogen® (San Diego, CA). Other mammalian DNAs and one avian DNA sample will be provided. A procedure for DNA isolation from blood is also described in Appendix 2. Also, refer to the manual of Sambrook *et al.* (1989)., pp. 9.16-9.23 for additional procedures for high molecular weight DNA isolation from cells in culture and tissue.

Principles of genomic DNA isolation

Cells are lysed by the addition of Solution A and incubated at 65°C. Proteins and lipids are precipitated and extracted with Solution B and chloroform. The solution is then centrifuged resulting in two phases with a solid interface. The DNA is in the upper, clear aqueous phase, the proteins and lipids are in the solid interface, and chloroform makes the lower phase. The recovered DNA is precipitated with ethanol and resuspended in TE buffer. The quality of the DNA obtained by this procedure is suitable for PCR or Southern blot analysis. The DNA solution may be stored at +4°C or -20°C.

A.1 DNA isolation procedure

> Note: The procedure described here uses a hand-held tissue homogenizer. An alternative method which uses a mortar and pestle over liquid nitrogen is offered in Appendix 1.

Homogenization and extraction

1. You will be provided with a 2 ml microcentrifuge tube containing a frozen, ~80-100 mg fragment of mouse liver which has been homogenized in 350 ul of Solution A plus 2.6 ul of Proteinase K (20 mg/ml in water).
2. Incubate the cell suspension at 65°C for 10 minutes in a heating block. Make sure the wells in the heating block have water.
3. While the tissue is incubating, obtain the tube with the phase lock gel and centrifuege at 14,000 rpm for 1 minute.
4. Remove the tube from the 65°C water bath and add 150 µl of Solution B. Again mix gently by manual, rocking motion for approximately 2 minutes until the precipitate moves freely.

5. Add 500 µl of chloroform/isoamyl alcohol (24:1) solution. Mix phases **gently but thoroughly** by repeatedly inverting the tube for a total of 3 min. The viscosity of the solution should decrease and the mixture should become homogenous.
6. Using a wide bore pipet tip (cut tip), transfer the mixture to the tube containing the spun phase lock gel (from step 4 above).
7. Centrifuge at 14000 rpm at room temperature for 5 min in the microcentrifuge. After centrifugation three phases should become obvious. The phase lock gel should be in the middle along with the cell debris and proteins. The upper aqueous phase contains the genomic DNA.
8. Without disrupting the layers, remove the aqueous **(upper)** phase using a wide bore tip (cut tip) and transfer into a new 1.5 ml microcentrifuge tube.

Ethanol precipitation and RNase digestion

1. Add 1 ml of cold (-20°C) 100% ethanol and mix well by repeated inversion of the tube (30 sec). **Leave on ice for 30 min**.

> Note: If a white, particulate (loose) material is observed at the bottom of the tube, this should be removed with a fine-tipped pipetor before centrifugation. Genomic DNA can be observed as a string-like material in the body of the solution, sometimes mixed with the particulate material. If so, transfer the stringlike material (i.e., DNA) to a fresch 100% ethanol tube (1 ml) and centrifuge as in step 2.

2. Pellet the DNA by centrifugation at 14000 rpm for 10 minutes (4°C).
3. Carefully discard the supernatant fluid by pipeting without disturbing the DNA pellet.
4. Add 500 µl of 70% ethanol (**RT**) to wash residual salts out the DNA pellet. Mix by flicking tube gently and inverting a few times.
5. Centrifuge at 14000 rpm for 3 min.

> Note**:** Place the centrifuge tube in the rotor with the hinge that connects the cap to the tube facing the outside of the rotor. This will help you orient the tube so it is easier to locate the small opaque DNA pellet after centrifugation.

6. **Repeat** 70% ethanol wash at **RT** (steps 13 and 14).
7. After centrifugation, remove as much ethanol as possible using a pipet and taking care not to disturb the DNA pellet.
8. Re-spin the tube in the microcentrifuge for 10 sec and use a P200 pipetor to eliminate the last remnants of ethanol.
9. Allow the pellet to air-dry in a 37°C bath for 5 min.
10. Resuspend the DNA pellet in 48 µl of TE buffer (pH 8.0) and 2 µl of a 2 mg/ml RNase solution (80 µg/ml final concentration). Pipet solution in and out 6 times while gently teasing the pellet **with a wide bore pipet tip** to aid in dissolving the pellet.
11. Incubate at 37°C for 30 min. DNA is now ready for further experiments. Store at 4°C.

> Note: TE buffer is a commonly used solvent for storing DNA. Tris maintains the pH at 8.0 and the EDTA chelates free Mg^{++} thus inhibiting the action of DNases.

A.2 Spectrophotometric evaluation of mouse genomic DNA

1. Pipet 195 µl of water into a 1.5 ml microcentrifuge tube. Be sure to label the tube with your Group number and the experiment number.
2. Using a large orifice pipet tip (cut tip), transfer 5 µl of your mouse DNA to the 195 µl of water prepared in previous step. The laboratory staff will collect this diluted DNA sample for spectrophotometric analysis.

   1.0 A_{260}= 50 µg/ml

   Dilution factor in spectrophotometric cuvette= 40 (i.e. 200/5)

   DNA conc. (µg/ml)= $(A_{260})(40)(50\mu g/ml)$

Solutions

RNase: 2 mg/ml in water
Solution A (cell lysis solution): EDTA, dodecyltrimethylammonium bromide
Solution B (DNA precipitating solution): hexadecyltrimethylammonium bromide

Suggested Reading

Sambrook, J., D.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A laboratory manual. 2nd Edition. Cold Spring Harbor Press, New York. (particularly pp. 9.16-9.23).

Part B: Visualization of genomic DNA by agarose gel electrophoresis

Genomic DNA isolated in Part A will now be analyzed by agarose gel electrophoresis. to asses its size, purity, and approximate concentration. This information will supplement the information obtained from spectrophotometric analysis of the DNA preparation. Absorbance readings are useful but they do not discriminate between low and high molecular weight DNA, or between DNA and RNA. Therefore, spectrometry can result in an overestimation of genomic DNA concentration. In contrast, electrophoresis provides an accurate evaluation of the size and purity of the DNA preparation. It can also be used to assess the DNA concentration when a reference DNA is loaded in the gel (e.g., λ DNA).

Agarose gel electrophoresis, first introduced by Daniel Nathans in 1970, is a simple and efficient method for resolving DNA fragments of different sizes (100-50,000 bp, see table below). DNA molecules are negatively charged due to the presence of phosphate groups. Consequently, the DNA molecules migrate towards the anode (positive electrode) in an electric field. The agarose gel matrix acts as a molecular sieve to sort fragments based on size. Electrophoretic mobility of the DNA fragment is inversely proportional to the $\log_{10}$ of the number of base pairs (Helling *et al.*, 1974). Walter Schaffner invented the currently used configuration of horizontal agarose gel electrophoresis. Papers by McDonell *et al.* (1977) and Southern (1977) describe details of this technique.

Percent agarose versus fragment size separation

% agarose	Fragment size to separate
0.3% (w/v) - 0.6% (w/v)	15,000 - 50,000 bp
0.6% (w/v) - 1.0% (w/v)	5,000 - 15,000 bp
1.0% (w/v) - 1.8% (w/v)	100 - 5,000 bp

References

Helling, R.B., H.M. Goodman, and H.W. Boyer. 1974. Analysis of endonuclease R - *Eco*RI fragments of DNA from lambdoid bacteriophages and other viruses by agarose gel electrophoresis. *J. Virol.* **14**: 1235.

McDonell, M.W., M.N. Simon, and F.W. Studier. 1977. Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. *J. Mol. Biol.* **110**: 119-146.

Southern, E. 1977. Gel electrophoresis of restriction fragments. *Meth. Enzymol.* **68**: 152-176.

B.1 Casting the agarose gel

1. Seal edges of the gel-casting tray and position the comb close to the negative (black or cathode) electrode.

Agarose: The melted agarose is located in the 65°C water bath at the end of the room.

2. Pour the melted 0.5% agarose gel (made in 0.5X TBE) into the tray until it reaches the upper edge. Make sure that no bubbles are trapped underneath the comb.

3. After the gel is completely set (approximately 10 min), unseal the ends of the casting tray, add 0.5X TBE buffer to cover the surface of the gel and carefully remove the comb.

0.5X TBE buffer:
50 mM Tris
45 mM Boric acid
1.0 mM Na_2EDTA, pH 8.4

B.2 Electrophoresis of genomic DNA

1. Use a large orifice pipet tip to transfer a 2.5 µl aliquot of your genomic DNA sample the DNA isolation experiment (Exp. 16.A.1) into a 1.5 ml tube. Add 18 µl of distilled water and 5 µl of 6X loading dye to the DNA sample. Mix the DNA, water and loading dye by tapping the tube with your finger or by slowly pipetting mixture in and out.

   6X loading dye:

   0.25% bromophenol blue (BPB)
   0.25% xylene cyanol FF (XC-FF)
   15% Ficoll (type 400; Pharmacia Biotech, Uppsala, Sweden)

> Note: The loading dye serves two functions: first, the Ficoll makes the sample more dense than the electrophoresis buffer and allows the sample to "sink" to the bottom of the well that was created by the comb in the solidified agarose gel; second, the BPB and the XC-FF make the sample visible during the loading operation and, more importantly, allows the progress of the electrophoresis to be monitored by observing the movement of the dye toward the positive electrode.

2. Use the 1X loading dye solution* to prepare three different, further dilutions of the genomic DNA sample (step 1) as follows: Label three tubes: DNA-1, DNA-2, DNA-3. Mix reagents according to the table below.

Tube label	Genomic DNA sample from step 1 (µl)	1X loading dye (µl)
DNA-1	10	5
DNA-2	5	10
DNA-3	2.5	12.5

   *1X loading dye was made by mixing 20 µl of 6X loading dye and 100 µl H_2O.

3. Pulse spin the tube in the microcentrifuge to pool the reagents.

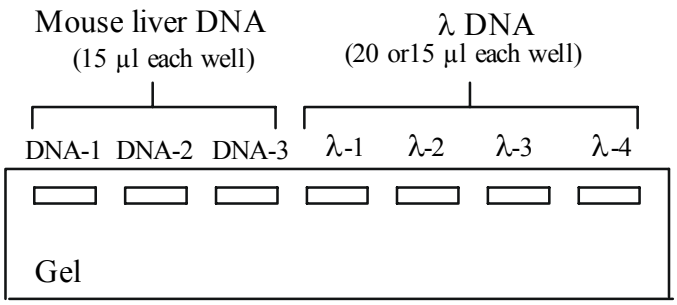
4. You are provided with a tube containing 50 µl of a 0.025 µg/µl bacteriophage λ DNA solution in 1X loading dye.

> The λ genome is ~5 x 10^4 bp which has a similar electrophoretic mobility as the average fragment size of good quality genomic DNA after isolation from tissue.

5. Prepare three different dilutions of the λDNA sample according to the Table below.

Standards	λDNA provided (µl)	1X loading dye (µl)
λ-1	20	0
λ-2	10	5
λ-3	4	11
λ-4	2	13

6. Load the total volume from the three samples of mouse liver DNA (step 2) and λ DNA (step 5) onto the gel as in the diagram (below).



7. Close the lid of the gel tank and connect electrical leads between gel box and power supply (red to red and black to black).

8. On the power supply, set the voltage to 0. Turn the unit on and then increase the voltage until the voltmeter indicates 75 V. The power supplies we are using can run two gel electrophoresis units at once. The voltage supplied to the left and right sides of the unit is adjusted separately and the voltmeter must be switched to the correct side when the voltage is being adjusted or monitored. Bubbles should be visible at the electrodes and the bromophenol blue tracking dye should enter the gel.

9. Run the gel until the fastest moving dye front (bromophenol blue) has migrated 3/4 of the way down the gel (This takes approximately 50 min).

10. Turn off the power supply and remove leads.

11. Carefully remove the gel and place in the staining dish provided at your station.

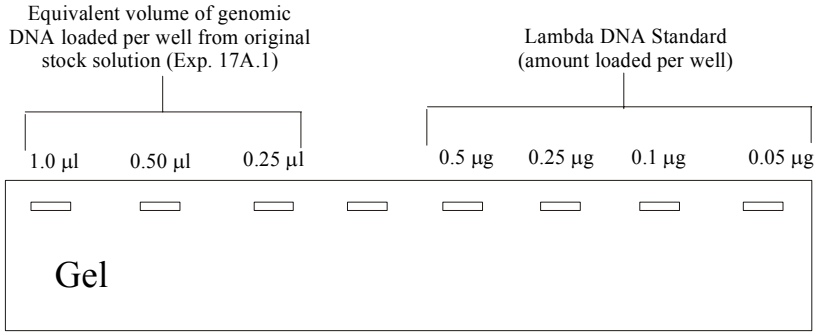
12. Bring the gel in the dish to the staining station. The instructors will stain your gel for you with ethidium bromide solution (1 µg/ml) for 10 min.

> **Caution:** Ethidium bromide is a known carcinogen. Wear protective gloves when handling ethidium or ethidium-stained gels.

13. The instructors will drain the ethidium bromide stain into a collection vessel for reuse and rinse the staining dish with tap water.

14. Visualize the DNA bands by placing on a UV transilluminator and photograph.

15. Estimate the mass of the genomic DNA by comparing the band brightness of the mouse genomic DNA lanes to the band intensity of the λDNA standard. The brightness of the ethidium staining is proportional to the mass of the DNA present in each lane. Choose one of the mouse DNA lanes of intermediate brightness relative to the gradient of staining intensities exhibited by the four concentrations of the λDNA standards. **Similar intensity means similar mass**. The mass of λDNA present in the four lanes is as follows: λ-1 = 0.50 µg, λ-2 = 0.25 µg, λ-3 = 0.1 µg, λ-4 = 0.05 µg. To calculate the approximate concentration of your murine liver DNA sample, divide your estimate of the murine DNA mass by the aliquot volume of the DNA solution in that lane. This is the concentration of your genomic DNA solution in µg/µl).



Part C: Incremental restriction digestion of genomic DNA

Background

Eukaryotic genomes are relatively large. In the human haploid genome there are approximately 3 x 10^9 base pairs of DNA. An appropriate amount of genomic DNA is necessary in order to obtain a detectable hybridization signal. The amount of DNA needed depends on factors such as:

- the proportion of the genome that is complementary to the probe,
- the size and specific activity of the probe,
- and the transfer efficiency of DNA to the membrane.

Under optimal conditions, Southern blotting allows the detection of a 1000 bp sequence that occurs only once in the mammalian genome (i.e. 1 part in 3 million) after an overnight exposure, if 10 µg of genomic DNA is used and hybridized to a probe of several hundred nucleotides in length.

Restriction enzymes cleave double stranded DNA by binding to the molecule, moving along the molecule until an unprotected (unmethylated) cleavage sequence is encountered. Digestion of large masses of complex genomic DNAs are enhanced by long incubations (e.g. 10-16 hr), repeated (incremental) addition of restriction enzyme, and mixing the reaction reagents periodically. The more complex the genome, the more difficult it is to obtain a complete digestion. In this experiment, the genomic DNA will be digested with *Rsa*I by adding fresh enzyme to the reaction mixture in two increments.

Digestion procedure

1. Set up the restriction enzyme digestion of the various genomic DNAs in separate 1.5 ml microcentrifuge tubes as indicated in the table below. The correct amounts of the two "Control" DNAs (human and chicken) has been provided. Add the remaining reagents (buffer, enzyme, water) directly to the tubes containing the control DNA samples.

Important: Be sure to add the enzyme last!

Samples	Tube A	Tube B	Tube C
Your mouse DNA (**2.5 µg**)	X µl		
Human K562 DNA (2.5 µg)		12.5 µl	
Bovine DNA (2.5 µg)			12.5 µl
10X *Rsa* I buffer	2 µl	2 µl	2 µl
Rsa I enzyme	1 µl	1 µl	1 µl
Water	up to 20 µl	4.5 µl	4.5 µl

The final volume should be 20 µl after addition of DNA, buffer, *Rsa* I enzyme and H_2O.

2. Tap each tube gently to mix the contents. Pulse spin in a microcentrifuge to bring the reactants to the bottom of the tube.
3. Incubate overnight at 37°C.
4. The instructors will add an additional 1 µl *Rsa* I (~10 Units) into each tube tomorrow morning before class.
5. Incubate at 37°C an additional 2 hr.

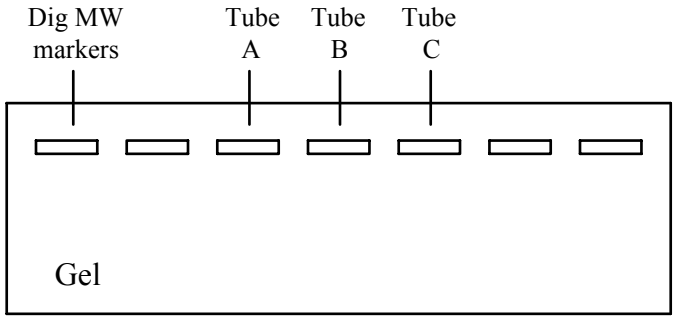
Part D: Agarose gel fractionation of *Rsa* I digested genomic DNA

Each group will run a 0.8% agarose 0.5X TBE buffer gel to fractionate the restriction enzyme-digested genomic DNAs.

1. Cast the 0.8% agarose gel as before (Part B). Cover the surface of the gel with 0.5X TBE buffer and carefully remove the comb.
2. Add 4 µl of 6X loading dye directly to Tubes A-C from Part C. Digoxigenin-labeled MW markers DNA will be provided by the instructors (10 µl, ready to load in 1X dye).

> Note: The DIG-labeled markers consist of 12 digoxigenin-labeled fragments of the following sizes: 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234, 220 and 154 (Boehringer Mannheim, Cat. No. 1218611). For chemiluminescent detection using CDPStar as an ALP substrate, 2 ng (0.2 µl of stock) MW standards is adequate.

3. Mix and pulse-spin in a microcentrifuge.
4. Load 24 µl of the digested genomic DNAs and 10 µl of the DNA markers ladder into the wells of agarose gel in the following order (be sure to skip Lane 2):



5. Electrophorese at 50 V for approximately 2 hours.
6. Stain the gel with ethidium bromide (1 µg/ml) for 10 min (as in Exp. 1, Part B).
7. Visualize the DNA in the gel on the UV transilluminator. Check Lanes 3-5 for complete digestion of genomic DNA. Complete digestion of complex genomic DNA is indicated by an even smear with relative brightness decreasing from the region of high molecular weight DNA to the region of low molecular weight DNA. It is not uncommon for some genomic DNA to remain in the wells. The presence of too much DNA in the wells is an indicator of an incomplete digestion and/or overloading the well with DNA.

Part E: Capillary transfer

The DNA fragments that are created by restriction enzyme digestion are now separated by agarose gel electrophoresis. To prepare for transfer to a nylon membrane, they are subjected to the following treatment while still in the gel.:

Partial acid depurination. Acid depurination chemically cleaves a fraction of the N-glycosidic bonds that link purine nitrogenous bases to carbon number 1' (C1') of deoxyribose residues in DNA.
Alkaline cleavage. The sugar-phosphate backbone is cleaved at many of the depurinated sites by alkaline treatment. This increases the transfer efficiency of large molecular weight DNA fragments from the agarose gel to the nylon membrane since the length of the fragments is reduced.
Alkaline denaturation. The alkaline solution also denatures the DNA (i.e., separates the strands).
Capillary transfer of the now single-stranded DNA is carried out in a high-salt buffer from the gel to a nylon membrane. In order to avoid disloging of the DNA during the hybridization steps, the DNA is crosslinked to the membrane by exposure to UV radiation. The nitrogenous bases hydrogen bonding sites on the DNA are now available for hydrogen bonding events with the single-stranded probe DNA.

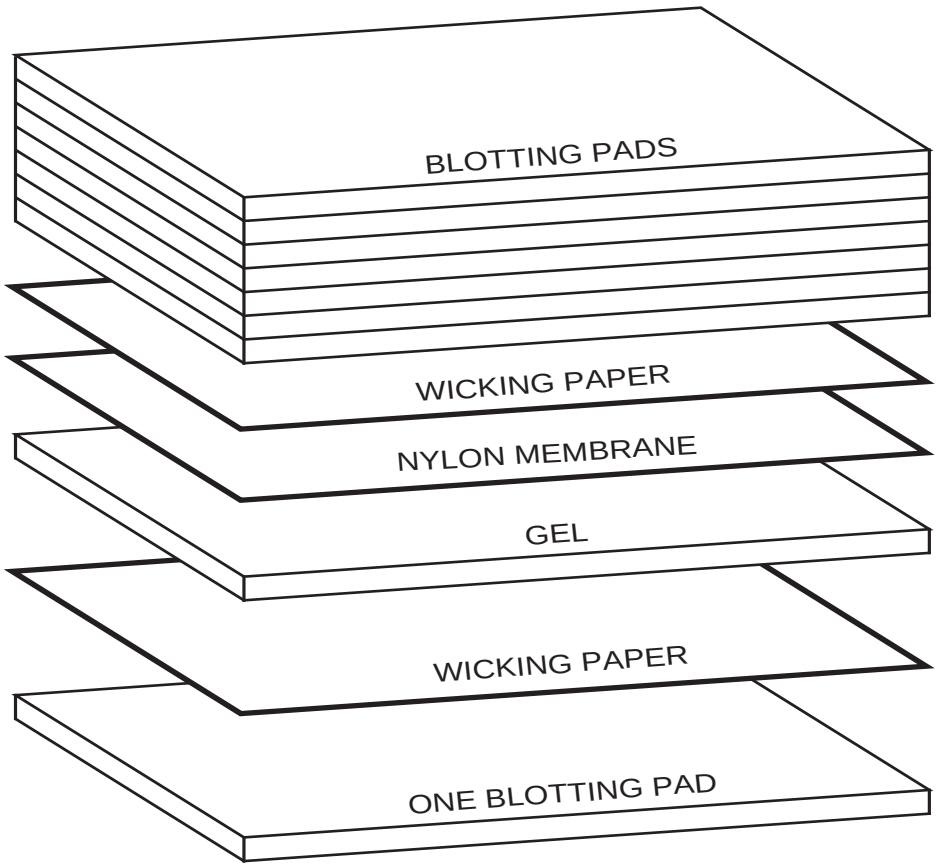
Reference

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**: 503-517.

Procedure for capillary transfer of DNA to a nylon membrane

1. Gently place the gel (Part D, step 7) in a clean plastic sandwich container. Add 100 ml of 0.5 N NaOH/ 1.5 M NaCl to the gel in the plastic container. Gently shake for 10 min at room temperature.
2. Discard the 0.5 N NaOH/ 1.5 M NaCl. Add 100 ml of dH_2O to the gel and gently shake for 30 sec at room temperature.
3. Discard the dH_2O. Add 100 ml of 1 M Tris (pH 7.4), 1.5 M NaCl to the gel and gently shake for 10 min at room temperature.
4. Discard the 1 M Tris (pH 7.4), 1.5 M NaCl. Add 100 ml of fresh 1 M Tris (pH 7.4), 1.5 M NaCl to the gel and gently shake for 10 min at room temperature.
5. While the gel is soaking, place one blotting pad (thickest pad in your blotting kit) in the middle of bottom of the plastic box (see the diagram below). Saturate the blotting pad with 20 ml of 20X SSC. Remove any air bubbles trapped between the pad and the plastic box by gently massaging the pad with a gloved finger. Place a piece of wicking paper (pre-cut, clean Whatman 3MM paper) squarely on the saturated blotting pad. Remove any air bubbles trapped between the blotting pad and the wicking paper as previously described.
6. Carefully place the gel, sample wells up, squarely onto the saturated wicking paper. Remove any air bubbles trapped between the gel and the paper.

> Note: If the DNA fragment to be detected in the Southern blot procedure is >15 Kbp, then it is first necessary to soak the gel in 0.1 N HCl (10 min). This treatment depurinates the DNA fragments at random sites. Upon NaOH treatment, depurinated sites are hydrolyzed, thus rendering smaller DNA fragments that are more efficiently transferred onto a membrane. When incrementally digested DNA is HCl-treated, an additional NaOH wash (10 min) is also included in the procedure.



Guidelines for manipulating the nylon transfer membrane.

a. Wear latex gloves when working with the membrane.

b. Wash off the corn starch on the outside of the gloves.

c. Use forceps to handle the membrane when possible.

d. Always handle the same corner of the membrane when holding the membrane with forceps.

e. Work with the membrane over a clean piece of Whatman 3MM chromatography paper to protect the membrane if it is accidentally dropped on the table.

9. Use a soft ball-point pen or a soft pencil to mark the top-right-hand corner of the side of the nylon membrane that will contain the DNA after transfer. Make sure to include your group number. Wet the membrane by immersing the membrane for 30 sec into dH_2O in a plastic sandwich container.

10. Carefully place the membrane onto the gel such that the side of the membrane marked "DNA" is in direct contact with the gel. Remove any trapped air bubbles between the membrane and the gel.

11. Place a piece of wicking paper squarely on the saturated membrane. Remove any trapped air bubbles between the wicking paper and the membrane.

12. Place 7 blotting pads squarely on the wicking paper. Gently place the inverted lid of the plastic box on the blotting pads. Add a weight of approximately 200 g (60 ml of H_2O in a 100 ml Gibco bottle) in the middle of the lid.

13. Allow capillary transfer of DNA to proceed overnight.

> Capillary transfer of the majority of the DNA fragments in the gel to the membrane is typically completed in 2 hr. An overnight transfer increases transfer efficiency of high molecular weight DNA fragments.

Next day (Resume blot procedure)

14. Unpack the blot; remove the (7) blotting pads and the uppermost sheet of wicking paper to expose the membrane.

15. Add 100 ml of 5X SSC to a clean plastic sandwich container. Carefully remove the membrane with forceps. Be sure that the gel is separated from the membrane. Transfer the membrane to the plastic container and rinse in this solution for 5 min with gentle shaking at room temperature.

16. Check transfer efficiency by re-staining the gel with ethidium bromide (1 μg/ml) for 10 min. The majority of the DNA should not be present although some high molecular weight DNA is usually seen.

17. Carefully remove the membrane and place it DNA side up on a clean large sheet of Whatman 3MM paper. Leave the membrane on the paper until the obvious puddles of 5X SSC are absorbed (approximately 1 min). Do not allow the membrane to dry completely.

18. Place the membrane DNA side up on the sheet of Whatman 3MM filter paper. Place the membrane in the Bio-Rad® UV cross linker DNA side up. Close the door and press the start button. Retrieve your membrane after the cross-linking is complete. Exposure to UV light facilitates the formation of covalent bonds between the DNA and the membrane (UV cross linking).

Part F: Hybridization and detection

Overview

A specific DNA sequence (target) is detected by means of the label on the probe molecule after it has annealed (i.e. hydrogen bonded to complementary nitrogenous bases) to immobilized target DNA on the membrane. The target DNA and labeled DNA probe form a hybrid complex. Hence, this portion of the Southern blot protocol is referred to as hybridization.

Several procedures are used to label DNA probes. These are generally separated into two categories: radioactive and non-radioactive. Radioactively labeled probes are typically synthesized *in vitro*. Usually, one type of radioactive dNTP is included in the *in vitro* synthesis reaction along with unlabeled dATP, dTTP, dGTP, and dCTP. The isotope most frequently used to label probes for Southern blots is phosphorous-32 esterified to the C5' of deoxyribose. The decay of the radioisotope within the probe emits energy (mostly beta particles) which reveals the location of the probe when detected by exposure of the membrane to X-ray film. This allows an investigator to determine the presence and location of a particular DNA fragment that is complementary to the probe.

There are several non-radioactive labeling and detection systems available. The most sensitive non-isotopic detection systems utilize enzyme linked probes (e.g., alkaline phosphatase or horse radish peroxidase) and a chemiluminescent substrate to induce an *in situ* chemiluminescent reaction that is detectable with X-ray film. Colorimetric detection systems are also available, but they lack the sensitivity afforded by chemiluminescent or radioactive methods. This experiment illustrates the detection of a DNA target (murinte CAII) using chemiluminescent detection.

F.1 Prehybridization and hybridization

Before DNA on the membrane is allowed to hybridize to the probe, the membrane must be treated with a "prehybridization solution" in order to block non-specific nucleic acid binding. This ultimately serves to lower background signal. Many different prehybridization solutions have been described in the literature. Optimal prehybridization and hybridization components as well as other conditions have to be determined experimentally. An additional important variable to consider is the hybridization temperature that depends on the length of the probe and on the extent of sequence homology with the target sequence.

Procedure

Hybridization plan: Two different hybridization temperatures and two different probe amounts will be tested by the class. Although the procedure is described for 300 ng of probe and 60°C hybridization temperature, you should perform the experiment at the suggested conditions for your group in the following table.

Group Number	Hybridization Temperature	Probe Amount
1-6	50°C	300 ng
7-12	60°C	300 ng
13-18	50°C	100 ng
19-24	60°C	100 ng

1. Place the UV-crosslinked membrane in a 50 ml conical tube containing 15 ml of prehybridization solution. Cap tube and incubate in a small hybridization oven (middle of your bench) at 60°C for 1 hr.
2. From the DNA probe concentration you obtained in Experiment 19 (page 135), estimate the volume required for a total of 300 ng. Pipette this volume into a 1.5 ml tube containing 1 ml of prehybridization solution. This constitutes the hybridization solution.
3. Cap this tube and place it in a boiling water bath for 5 min to denature the DNA.
4. Chill quickly over ice for at least 5 min.
5. Discard prehybridization solution and quickly pipette entire 1.5 ml of hybridization solution into the 50 ml conical tube containing the membrane.
6. Cap tube and place in a hybridization oven set at 60°C. Tube clamp is slightly twisted to maximize contact of hybridization with the entire membrane. Allow the hybridization to proceed for 1 hr.
7. After hybridization, pour the hybridization solution back into a 15 ml conical tube and return it to the lab instructors.
8. Wash the membrane twice, 5 min each time, with 15 ml Wash A solution at room temperature (over a gyratory platform). Continuos agitation is important to reduce background.
9. Wash the membrane three times, 15 min each time, with 15 ml of Wash B solution at 60°C in hybridization oven.
10. Before discarding the last wash solution, continue with step 1 of Chemiluminescence Detection procedure (below).

Notes on the Hybridization procedure

Step 1: Longer prehybridization times (e.g. overnight) are acceptable. However, the minimum possibe hybridization time is recommended if the probe has been directly labeled with an enzyme such as alkaline phosphatase or horse raddish peroxidase. These labeling enzymes can be deactivated by long incubations at common hybridization temperatures. Several membranes can be processed in the same bag as long as there is sufficient prehybridization solution to cover all the membranes, and the membranes can move freely.

Step 2: When using Dig-labeled probes other than those made from DNA, the following concentrations are recommended: RNA probes, 100 ng/ml; tail-labeled oligonucleotides, 0.1-2 pmol/ml; end-labeled oligonucleotides, 1-10 pmol/ml.

Step 3: Single-stranded RNA and oligonucleotide probes do not require denaturation unless extensive secondary structure is predicted from the sequence.

Step 7: Solutions will be saved at -80°C for reuse. Dig-labeled probe solutions are good for about one year when stored under these conditions.

Steps 8 and 9: The washes remove non-specifically bound probe which will lead to high backgrounds if not removed

Solutions

Prehybridization solution (For one liter):

5X SSC	(250 ml of 20X SSC)
0.1% (w/v) N-lauroylsarcosine	(1 gram)
0.02%(w/v) SDS	(2 ml of 10% w/v SDS)
1% Blocking reagent	(10 grams)

Wash A (2X wash solution)(For one liter):

2X SSC	(100 ml of 20X SSC)
0.1% SDS	(10 ml of 10% SDS)

Wash B (0.5X wash solution)(one liter):

0.5X SSC	(25 ml of 20X SSC)
0.1% SDS	(10 ml of 10% SDS)

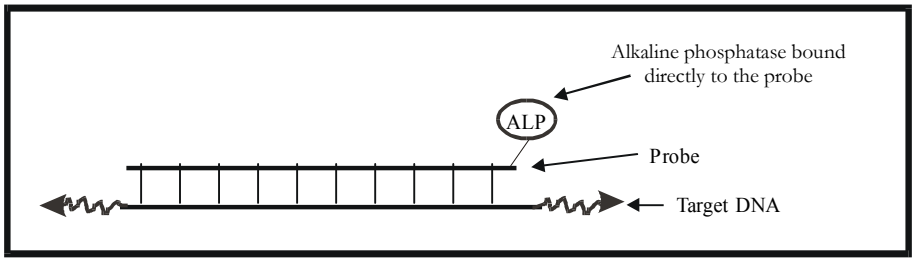
20X SSC:

3 M NaCl
0.3 M sodium citrate
pH 7.0, autoclaved

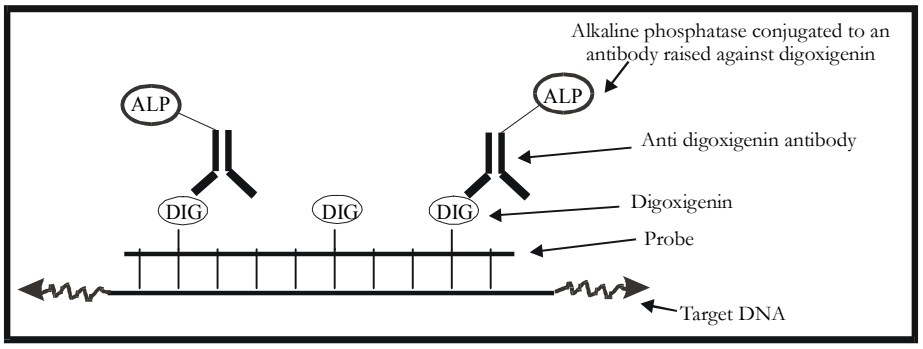
F.2 Detection of dig-labeled probes: Chemiluminescent method

There are several non-radioactive labeling and detection systems available. A brief description of these systems is given below.

1. Direct: A signaling enzyme is covalently (and directly) attached to the nucleic acid probe. The most commonly used enzymes are alkaline phosphatase (ALP) and horse radish peroxidase (HRP). In the direct enzyme labeling format, the colorimetric or chemiluminescent substrate for the enzyme is added after hybridization of probe to the target.



2. Indirect: A hapten is incorporated into the probe molecule by either internal incorporation, end labeling or chemical modification. Examples of haptens are: digoxigenin (steroid hapten), biotin, sulfonated bases, and dinitrophenol. Detection is afforded through recognition of the hapten by a moiety that is itself coupled to a signaling enzyme (e.g. ALP or HRP). Examples of hapten-binding moieties are: Antibodies and streptavidin (or avidin). Indirect methods could potentially yield high background due to non-specific binding of secondary reagent to the membrane. This possibility is minimized by controlling the stringency of the washes after hybridization.



In this experiment you will be using an indirect digoxigenin (DIG)-based system (Boehringer Mannheim). The DIG-labeled probe is detected after hybridization to target nucleic acids, by an enzyme-linked immunoassay employing an antibody-conjugate (anti-digoxygenin alkaline phosphatase conjugate, anti-DIG-ALP).

After the antibody reaction, one of many chemiluminescent substrate is added. Examples of these substrates are: CSPD [disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1.1.]decan}-4-phenylphosphate], Lumigen PPD, Lumi-Phos 530 or CDP-Star. When using a nylon membrane, the chemiluminescent substrates have a detection limit of 0.03 pg of DIG-labeled DNA, comparable to the level of sensitivity obtained with P^{32}-labeled probes. As an alternative to chemiluminescent detection, BCIP and NBT can be used as substrates for alkaline phosphatase. These compounds produce an insoluble blue precipitate, which allows direct visualization of hybrid DNA molecules on the surface of the nylon membrane. However, the sensitivity of BCIP/NBT detection is much lower than for chemiluminescent substrates.

Procedure

Note: In order to minimize background, wear gloves always. Use sterile, blunt-ended forceps to pick up membranes and handle membranes only at the edges. Wash powder off gloves with clean water before handling membranes.

Note: Perform these steps in a clean sandwich container

1. Without allowing the membrane to go dry after the last hybridization wash, discard the wash solution and add 20 ml of Buffer 1. Incubate at room temperature for 1 min.
2. At this point, make sure that the chemiluminescent substrate is allowed to come to room temperature so that it is ready when needed.
3. Discard Buffer 1, then add 20 ml of Blocking solution. Gently agitate the wash solution for 30 min at room temperature using a gyratory shaker.

4. Prepare 15 ml of a 1:10000 dilution of the Anti-DIG antibody:alkaline phosphatase conjugate in Blocking solution (i.e. dissolve 1.5 µl in 15 ml of Blocking solution).
5. Pour off the Blocking solution from step 3 and add the entire Antibody solution prepared in step 4. Incubate at room temperature for 30 min. Continue gentle agitation.
6. Discard the Antibody solution and wash the membrane three times at room temperature, 15 min each, in 25 ml of Buffer 1 (agitate!).
7. Pour off Buffer 1 and add 20 ml of Detection Buffer. Incubate at room temperature for 2 min.
8. Using clean forceps, transfer the membrane into a plastic bag. It is easiest if the bag is sealed on only two edges so that the wet membrane can be conveniently placed.
9. Pipette 0.5 ml (1.0 ml/100 cm^2) of the chemiluminescent substrate (CDP-Star) directly on top of the DNA side of the membrane. Try to cover as much as possible of the membrane surface.
10. Fold the top sheet of the of the plastic bag across the surface of the membrane, allowing the substrate to spread evenly over the entire membrane surface. With a clean Kimwipe, wipe across the top of the bag in order to remove any bubbles and excess substrate.
11. Seal all sides of the plastic bag (development folder) as close as possible to the membrane. Rinse the outside of the bag with soap and water. Dry and bring the bag to the center table so that your membrane can be exposed in an X-ray film cassette.
12. Expose for 20 min and develop in the usual manner for X-ray films.

Solutions

Buffer 1: For 1.2 liter

100 mM maleic acid, pH 7.5	(19.2 g maleic acid, disodium salt, pH 7.5)
150 mM NaCl,	(10.53 g NaCl)
3% (v/v) Tween 20	(36 ml Tween 20)

Maleic acid buffer: For 1 liter

100 mM maleic acid, pH 7.5	(16 g maleic acid, disodium salt, pH 7.5)
150 mM NaCl	(8.8 g NaCl)

Blocking solution:

1% Blocking reagent (same used for nucleic acid hybridization) in Maleic acid buffer.

Detection Buffer: For one liter

100 mM Tris, pH 9.5	(12.1 g Trizma base, pH 9.5)
100 mM NaCl	(5.85 g Na Cl)

Exercise 12: Southern Blot Analysis

1. What was the approximate size of the CAII (exon VII) target detected by your Dig-labeled probe?
2. Which DNA sample(s) had target fragments that were detected by the probe?
3. What were the effects on the blot signal when the hybridization temperature was lowered?
4. How can you decrease background in your blot without decreasing the signal intensity?

Experiment 18: Digoxigenin Labeling of a Mouse CAII Probe

Nucleic acid can be labeled with Digoxigenin (DIG) by means of incorporation of DIG-dUTP in the probe using any of the common labeling procedures (see below).

Common labeling procedures

DNA labeling	RNA labeling	Oligonucleotide labeling
• PCR • Random primed • Nick translation • PCR	• *In vitro* transcription	• 3'-end labeling • tailing • 5'-end labeling

In this experiment you will be using the Polymerase Chain Reaction (PCR) to incorporate DIG-11-dUTP into a CAII cDNA fragment that will be used as a probe. A PCR DIG probe synthesis kit is available from Roche Diagnostics (Cat. # 1 636 090). The amount of DIG-dUTP in the PCR labeling reaction affects both the yield and sensitivity of the probe. Incorporation of the label is controlled by varying the ratio of DIG-dUTP to dTTP in the labeling reaction. Usually, ratios of 1:2 to 1:5 are ideal for producing maximally sensitive probes for Southern blot procedures. However, DIG-dUTP ratios of 1:6 through 1:9 can be used for less demanding applications.

Part A: Preparation of DIG-labeling mixture

Note: This Part will have been done in advance by the lab instructors. Continue with Part B. Reagents were from the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Cat. # 1 636 090).

1. Add the following components to prepare a 1:5.7 "DIG-dNTP mix (10X)":

Reagent	Amount
*DIG-11-dUTP probe synthesis mix (10X)	3.0 µl
**dNTP stock solution (10X)	3.0 µl

* DIG-11-dUTP probe synthesis mix (10X): contains dATP, dCTP, dGTP (2 mM each); 1.3 mM dTTP, 0.7 mM DIG-11-dUTP, alkali-labile; pH 7.0.
**dNTP stock solution (10X): contains dATP, dCTP, dGTP, dTTP (2 mM each), pH 7.0.

2. This constitutes the "DIG-dNTP mix (10X)". Save for use in the PCR labeling reaction (Part B, Table below).

Part B: PCR labeling reaction and probe purification

The mouse cDNA probe will be synthesized and labeled in a PCR reaction using DIG-11-dUTP (see above) and the following two primers:

Upper primer: 5'-CTC ATC CGT TGT GCT TAC TA-3' (position 931 of cDNA sequence KOO811 Accession No.)
Lower primer: 5'-TGA CTG TTT ACC TGT TTT GC-3' (position 1222 of cDNA sequence KOO811 Accession No.)

The expected PCR product is a 292 bp fragment corresponding to a region within the seventh exon of the mCAII mRNA. This is an untranslated region located 3' of the stop codon. The probe was designed so that the probe would hybridize to a contiguous region of the DNA locus comprising the CAII gene.

When compared to MW standards, the DIG-labeled product will appear slightly larger than the Control product (Control is the PCR product with unlabeled dNTPs). Therefore, a simple electrophoretic analysis of the PCR products can be used as an indication a successful DIG-labeling reaction.

The template in the PCR reaction for DIG-labeling of the Southern hybridization probe will be a gel purified 292 bp cDNA fragrment corresponding to the 7th exon of CAII mRNA (obtained from an RT-PCR of mouse total RNA, using the same primers described above for probe labeling).

Procedure

1. Label two separate 0.2 ml PCR tubes on their upper side. Include your group number. For example, Group number 1 will have tubes: 1-C and 1-DIG. The first tube will contain control PCR reagents (i.e. deoxy nucleotides without DIG-11-dUTP).

2. Add reagents according to the Table below (*Taq* polymerase is added last).

Reagent	DIG (µl)	Control	[Final]
PCR buffer (10X)*	4.65	4.65	1X (1.5 mM $MgCl_2$)
DIG-dNTP mix (10X) Part A	2.5	-	200 µM each
dNTP stock solution (10X)	-	2.5	200 µM each
Upper primer	1.25	1.25	1 µM
Lower primer	1.25	1.25	1 µM
292 bp 7th exon CAII fragment	1	1	2 pg/µl
Expand High Fidelity *Taq* pol**	3.75	3.75	0.05 U/µl
Water	10.6	10.6	-
Total volume	25	25	

*10X PCR buffer:
100 mM Tris-HCl, pH 8.3 (at 25°C)
500 mM KCl
0.01 (w/v) gelatin
15 mM $MgCl_2$

** *Taq* DNA polymerase is normally supplied at 3.5 U/µl. We have made a 1:10 dilution of the supplier's stock in 1X PCR buffer for ease of pipetting.

3. Mix samples by lightly flicking them with your fingers and centrifuge them briefly to pool components in the bottom of the tube.

4. Keep your PCR sample tubes on ice until everyone in the class is ready to begin the thermocycling process. Then transfer tubes to the thermocycler and carry out amplification using the following parameters:

94°C 3 min — Initial denaturation

94°C, 45 seconds
55°C, 1.5 minutes — **3 cycles initial amplification**
72°C, 45 seconds

94°C, 45 seconds
45°C, 1.5 minutes — **35 cycles main amplification**
72°C, 45 seconds

72°C 5 min hold at 4°C — Final extension

Probe purification

Note: THE PROBE WILL NOT BE PURIFIED IN THIS CLASS. Proceed to quantification by gel electrophoresis (Part C). Sometimes it is necessary to purify probes from unincorporated label. If so, the procedure described below using QIAGEN II kit components can be used. Eliminating unincorporated (free) DIG-dUTP can often be helpful in reducing background signal. However, the yield of the probe is significantly diminished by purification procedures.

a. Add 3 volumes (75 µl) of **Buffer QX1** to 1 volume of the PCR product solution.

b. Resuspend the QIAEX II by vortexing for 30 sec.

c. Add 10 µl of **QIAEX II resin** to the solution from step "a" and incubate at **RT** for 10 min. Mix the solution every minute to keep QIAEX II in suspension.

d. Centrifuge the sample for 30 sec and carefully discard the supernatant with a pipet. The QIAEX II resin will appear as a small opaque pellet.

e. Add 500 µl of **Buffer QX1** and resuspend the QIAEX pellet by vortexing. Centrifuge the sample for 30 sec and discard supernatant with a pipet.

f. Centrifuge the sample again (10 sec) and **remove all traces** of supernatant with a pipet.

g. Add 500 µl of **Buffer PE** and resuspend the QIAEX pellet by vortexing. Centrifuge the sample for 30 sec and discard supernatant.

i. Wash the QAIEX pellet again with 500 µl of **Buffer PE.** Resuspend the QIAEX pellet by vortexing. Centrifuge the sample for 30 sec and discard supernatant.

j. Centrifuge again for 10 sec and **remove all traces** of supernatant with a pipet. The PE washes removes all residual salts.

k. **Air-dry** the QIAEX pellet for 10-30 minutes or until the pellet becomes white (not grey). Do not over-dry as this may result in decreased elution efficiency.

l. To elute DNA from QIAEX II resin, add 30 µl of **10 mM Tris-HCl, pH 8.5** and resuspend the pellet by vortexing. Incubate at room temperature for 5 minutes.

m. Centrifuge for 30 sec and carefully pipet the DNA-containing supernatant into a clean tube. Repeat elution for anextra 15% recovery.

n) Save this solution for electrophoretic analysis. The probe solution should now be separated from unincorporated dNTPs (DIG-dUTP included), as well as from other PCR reaction components.

Note: DIG-labeled PCR products can also be cleaned by the following LiCl procedure:

a) Add 1 µl of a 20 mg/ml Glycogen solution (made in water) to the 50 µl PCR sample.

b) Precipitate labeled nucleic acids by adding 5 µl of 4 M LiCl and 150 µl of chilled ethanol. Mix well and incubate at -80°C for 30 min.

c) Thaw briefly at room temperature and centrifuge at 13000 rpm for 15 min.

d) Use a pipetor to remove the ethanol/LiCl supernatant and add 100 µl of 70% ethanol to get rid of salts. Centrifuge at 13000 rpm for 5 min.

e) Remove the ethanol with a pipetor and centrifuge again for 30 sec to bring down ethanol droplets from the side of the tube. Remove the last remnants of ethanol.

f) Dry the pellet by leaving tube open for 10 minutes. Redissolve with 50 µl TE/SDS buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA and 0.1% w/v SDS). Heat at 37°C for 10 min if necessary.

Part C: Electrophoretic analysis of DIG-labeled probe

C.1 Casting the agarose gel

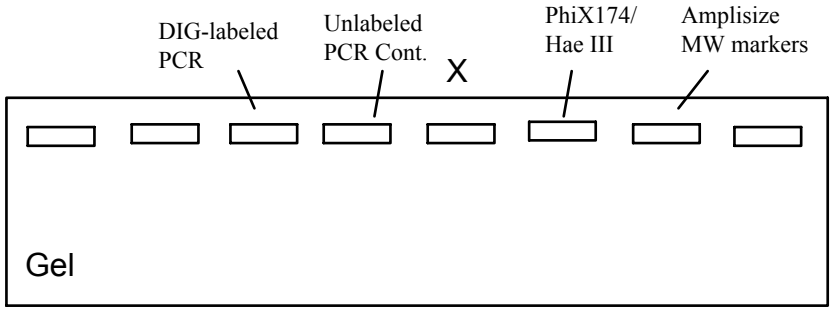
1. Place a well-forming comb in the agarose apparatus.
2. Prepare a 1.5 % agarose solution in 0.5X TBE.

> The laboratory staff has prepared the agarose solution and has been placed in 70°C bath.

3. Obtain the agarose solution from 70°C water bath and pour the melted agarose into the tray. Make sure that no bubbles are trapped underneath the comb.
4. After the gel is completely set, unseal the ends of the casting tray, add 0.5X TBE to just cover the surface of the gel and remove the comb carefully.

C.2 Electrophoresis procedure

1. Remove 5.0 µl from each PCR product (Control and purified DIG-labeled probe) and transfer to two separate 1.5 ml microcentrifuge tube. Add 5.0 µl of water and 2.0 µl of loading dye (6X) to each tube. Close tube cap, mix by tapping, and spin briefly. Save the remainder of the PCR products (~45.0 µl) on ice.
2. Using the micropipet, slowly load the entire volume from each tube (12 µl) prepared in step 1 into separate wells of the agarose gel.
3. Also, load 12 µl of *Amplisize*™ DNA size marker (provided by the instructors, Bio-Rad® Laboratories, Inc., Hercules, CA) and 12 µl (0.5 µg total) of ΦX174/Hae-digested DNA. Leave one well vacant between the DIG-labeled sample and the molecular size markers.



4. Close the lid of the gel tank and connect electrical leads approximately (red to red and black to black).
5. On the power supply set the voltage to 0. Turn the unit on and then increase the voltage until the voltmeter indicates 80 V. The power supplies we are using can run two gel electrophoresis units at once. The voltage supplied to the left and right sides of the unit is adjusted separately and the voltmeter must be switched to the correct side when the voltage is being adjusted or monitored. Bubbles should be visible at the electrodes and the bromophenol blue tracking dye should enter the gel.
6. Run the gel until the fastest moving dye front (bromophenol blue) has migrated 3/4 of the way down the gel.
7. Turn off the power supply and remove leads.
8. Carefully remove the gel and stain in the staining dish with ethidium bromide solution (1 µg/ml) for 10 min.

Caution: Ethidium bromide is a known carcinogen. Wear protective gloves when handling ethidium or ethidium-stained gels.

9. Drain off the ethidium bromide solution into a collection vessel for reuse.

10. Rinse gel and tray with tap water.

11. Visualize the DNA bands by placing on a UV transilluminator and photograph.

Caution: Remember that UV light is harmful to the eye. Wear UV-protective glasses

Size of AmpliSize™ DNA size marker:
2,000
1,500
1,000
700
500
400
300
200
100
50

12. Estimate the amount of PCR DNA product by comparing the intensity of the band in the purified PCR sample to the band intensity of the ΦX174 DNA size marker standards (see the table below for the approximate relative mass for each of the ΦX174 *Hae* III fragments). Make a rough calculation of the concentration of your purified PCR product "stock." To calculate the concentration of your PCR product stock divide your estimate of the mass by the volume of the DNA sample applied to the gel (2.5 µl). These calculations are necessary to determine the exact amount of the probe to be used in the hybridization step of the Southern blot procedure.

For example: Assume that the analysis of the PCR product in the agarose gel indicates an approximate concentration of 10 ng PCR product per µl. Since you will need 300 ng of probe for the hybridization, the volume of clean, DIG-labeled PCR product needed is 30 µl. If your PCR product solution is too concentrated, dilute an aliquot of the sample in sterile water to yield an appropriate concentration.

ΦX174 *Hae* III fragment (bp)	% Total Mass	Proportion of 1.0 µg (ng)	Bio-Rad Mass Ruler fragment (bp)	Mass of band (ng)
1353	25.12	251	1000	100
1078	20.01	200	700	70
872	16.19	162	500	50
603	11.20	112	200	20
310	5.76	58	100	10
281	5.22	52		
271	5.03	50		
234	4.34	43		
194	3.60	36		
118	2.19	22		
72	1.34	13		
5386	100.00	1000		

Experiment 19: Microarrays for Differential Gene Expression Analysis

Introduction

Our generation is witnessing an unprecedented explosion in data production that promises to shed an increased understanding of how biological systems work. A large component of these data is in the form of genomic sequences containing genes, many of which do not have any clear function (50-90%). Such a volume of information represents both an immense challenge and opportunities in research. It does not only give us an indication of the enormous task ahead, but also provides us with the necessary reagents that can be used to unravel the operation of genes in functional networks.

The scientific challenges in genomics are illustrated by the presence of nearly 30,000 distinct mRNA species in a human cell (1), 99% of which are rare (2) (i.e., < 1 copy per 20,000 mRNA molecules). Only about 300 genes can account for half of the mRNA mass in any given cell. Therefore, tools for gene expression analysis should be sensitive enough to detect the rare messages, and should have a large enough dynamic range to also assess the abundant messages.

Several techniques have been developed to assess mRNA expression on a global scale. These include SAGE (3), Differential Display (4), Oligonucleotide arrays (5), and cDNA microarrays (6). The most common use of all these techniques is for the determination of gene expression patterns between two or more identical samples, but that have been subjected to different experimental conditions.

What are microarrays?

Microarrays consist of miniature arrays of gene fragments (spots) attached to glass slides (chips). These biochips are hybridized to fluorescently labeled cDNA samples. Following hybridization, the chips are read with a high-speed fluorescent detector and the intensity of each spot is quantified. The amount and identity of each gene present in the hybridized sample is revealed by the intensity and location of each spot. The data are then analyzed (i.e., mined and modeled) using computational biology tools.

Advances in the field of microfluidics have made it possible to generate chips containing tens of thousands of gene fragments. Therefore, gene profiles from entire genomes are possible from a simple microarray experiment. Such a powerful, data-generating technology will allow researchers to examine biological questions that were not possible before. It allows researchers to observe the effect of perturbations on gene expression at the organismal level.

As an exploratory tool, the use of biochips is not limited to the existence of a hypothesis. However, since the ratio of experimental time versus analysis time for microarrays is enormous, careful experimental design and planning should precede any use of the technology. Adherence to this paradigm implies that the focus, scope, and intent of a study would have been carefully formulated prior to an experiment. This should also make the so-called microarray data glut more manageable and meaningful.

For a thorough and up-to-date discussion on Microarrays, please read 'Microarray Biochip Technology' (7).

Currently, there are two different approaches for the creation of microarrays:

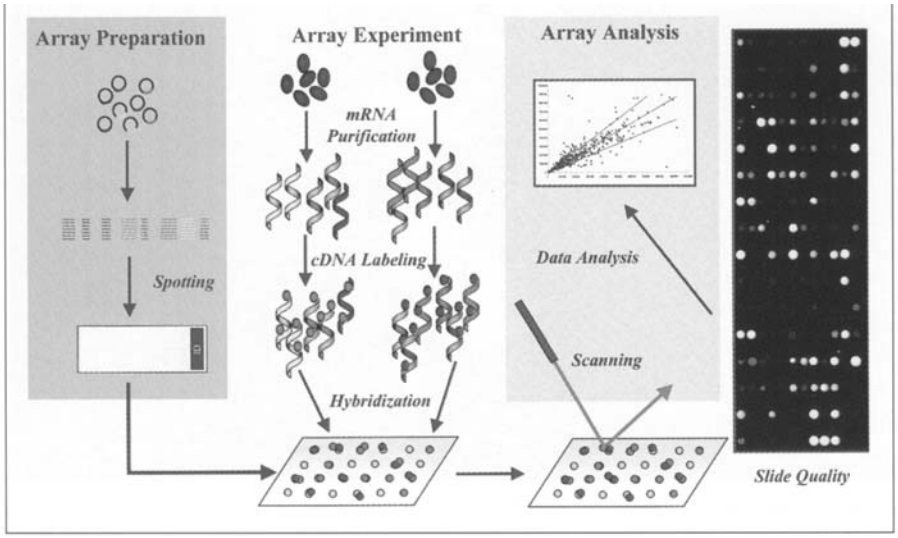
A. Light-directed, in vitro synthesis of oligonucleotide arrays.

This method was pioneered by Fodor et al (8a, b) and commercialized by Affymetrix. It uses precision photolithographic masks to define the positions at which single, specific nucleotides are added to growing single-stranded oligonucleotide chains. High-density arrays of defined oligonucleotides (<25 nt in length) are

synthesized on a solid substrate. Although sophisticated and powerful, this system does not allow custom fabrication of arrays in the user's laboratory (at least in its present form).

B. Spotted microarrays of nucleic acids.

Spotted arrays (6) are created by depositing small volumes of probe solutions onto the array surface using a pen or pin-deposition tool. Microfluidic delivery techniques offer the flexibility of generating microarrays that could consist of genes obtained from commercial sources, cDNA clones, DNA probes or PCR amplification products from personal cDNA libraries, without the need for sequence information. Other types of deposited material suitable for this method include plant, animal, human, fungal, and bacterial cells; viruses, peptides, antibodies, receptors, and other proteins; peptide nucleic acids, genomic DNA, RNA probes, oligonucleotides, chemicals, and any type of small particulate material in suspension. These materials are suitable for deposition on any of several substrates, including chemically treated microscope slides, coverslips, plastics, membranes, gels, etc.



Flow chart of two-color microarray experiments. The major phases of array preparation, differential gene-expression experiment, and analysis are listed, with the topics discussed in this chapter labeled in italics. (See Chapter 4.)

Basic Considerations in Microarray Construction and Experimentation

The complexity of microarray technology represents a warning for what could be a hopelessly unreliable method unless potential sources of variations are properly addressed. Instrumentation, chemistry and software must be successfully integrated to make microarrays useful and practical for scientific discovery. Since the goal of most microarray applications is to be able to detect changes, the variability inherent in the technology must be kept to a minimum.

Herein, we discuss some of the important factors that must be evaluated and optimized upon the undertaking of a microarray experiment. Although the considerations described here are largely based on the spotted arrays method, they are broadly valid for other systems. Available microarray systems should be evaluated having these (and other) issues in mind.

For an in depth discussion on Technology Standards in Microarray fabrication, see references below.

Potential sources of variation

- Instrumentation: sample spotters and fluorescent scanners
- Array surface chemistry
- Probe attachment
- Sample isolation and preparation
- Sample labeling and detection
- Hybridization conditions

1. Instrumentation

a) Spotter. Spotting tool design in a microarray system affects not only spot size and density of the array, but also reproducibility of the results. Samples must be positioned precisely and uniformly deposited on the array surface. Carryover between samples must be minimized. The best results have been obtained with fully automated instruments capable of depositing subnanoliter volumes of samples from standard 384 well microplates onto coated glass slides. The spotting is performed in a humidity controlled enclosure to minimize dust and other environmental influences on the process.

Deposition devices follow one of many designs (e.g., pins, tweezers, and capillary pens), all paying special attention to precise volume delivery and reproducibility. Sample carryover is avoided by a wash and dry procedure after each set of samples is deposited on the slides. Even the best deposition devices available only have a spot to spot reproducibility that varies within a 15% standard deviation.

In the affymetrix system spotting is circumvented by *in situ* synthesis of oligonucleotide probes on the chip (please see Appendix on 'Affymetrix System Overview').

b) Scanner. Fluorescent detection is critical step in microarray experiments. The imaging requirements for microarrays fall in between the size of gel bands or dots on membrane arrays, and objects normally observed by a microscope. The microarray scanning system needs to have a moderately high resolution, while remaining capable of imaging a fairly large area (e.g., 2x6 cm) in a short time. To accommodate differential gene expression experiments using two fluorescent dyes, the system must be able to distinguish at least two fluorescent spectra. In addition, the scanning instrument must have a low fluorescent detection threshold to allow quantification of rare messages, and a large linear dynamic range to measure abundant ones simultaneously. Finally, the entire area of the microarray must be scanned uniformly to ensure exprimental reproducibility.

Current providers of microarray scanners include GSI Lumonics, Amersham Pharmacia Biotech/Molecular Dynamics, Axon, HP, Virtek, and others. Most of these instruments use some form of scanning technology with photo multiplier tube (PMT) detection. Multicolor detection is facilitated by utilizing either gas- or solid -

state lasers and a number of filter sets to provide spectral separation into each of the different channels. In addition, confocal optics and special wide-field objective lenses help collect a high-quality, quantitative microscopic image. The response variation of these machines can be as little as +/-4% over an entire scan field of 1x6 cm. In general, the resolution of the scanning instrument should be such that the pixel size is 1/10 the diameter of each spot ot the chip.

Other detection systems utilize charge-couple devices (CCD) with a greater spatial resolution than laser-based scanners (i.e., pixel size is approximately 20 μm when imaging a 20x20 mm area). However, these systems are best suited for imaging small arrays (< 1.0 cm^2).

For microarray dyes to be able to emit fluorescent photons for detection, first they must be excited. The excitation light must be carefully gauged to avoid drastic reduction (photobleaching) in fluorescent readout of the microarray during successive scans. Photobleaching should be < 1% per imaging cycle and this is accomplished by commercial scanners using 5-35 mW light sources with scanning speeds fo 5-25 lines per second.

Another important issue in fluorescent scanning has to do with differential photostability between dyes. This is particularly problematic in experiments involving multiple fluors. For example, fluorescein thiocyanate (FITC) is more susceptible to photobleaching than the Cy dyes.

One last concern that must be addressed by fluorescent scanners is a phenomenon referred to as cross-talk. This occurs because emission spectra are typically broad, with as much as 10% of the emitted signal observable at wavelengths 100 nm longer than the maximum emission wavelength. As dictated by the Stokes shift, the emission wavelength is always longer than the absorbance wavelength. Therefore, Cy3 (550 nm emission) cross-talk into the Cy5 (650 nm emission) channel is a practical problem, but not viceversa. The least expensive method to minimize cross-talk involves the use of emission filters that reject light outside the desired wavelengths. Optical cross-talk should be kept to a level of < 0.1% per channel, with even lesser levels desired for applications such as gene expression analysis where high sensitivity is essential.

2. Array surface chemistry

Common microscope slides (1x3 in) were used for the early microarray experiments. However, many commercial sources now offer offer ultraflat, low-fluorescent surface microscope slides for microarray work at an affordable price. Slides must be scrupulously clean (hot nitric acid wash) and chemically derivatized (3-aminopropyltrimethoxysilane) to induce DNA binding to its surface. The coating must be uniform (vapor phase coating is preferred) with no bare patches. In addition, the slide mus able to withstand harsh conditions such as boiling, baking, and soaking in warm detergent-containing solutions for long incubation times.

Advantages of microscope slides:

a) compatibility with many fluorescent detection and robotic fabrication systems.
b) large surface area (19 cm^2), which allows arrays of >100,000 elements, using microspotting and ink-jetting technologies.
c) compatibility with many existing microscopes and microscopy tools.
d) ease of surface modification, hybridization and washing.
e) low hybridization volumes (5-20 μl) using coverslips. This also allows the use of high probe concentations and rapid kinetics.
f) low intrinsic fluorescence.
g) low cost.

Disadvantages:

a) open microarray surface is susceptible to contamination with dust, and other airborne particles (clean room is required).
b) susceptible to surface damage (scratching, etc.).

Another widely used format is the proprietary Affimetrix cassette. It consists of a plastic enclosure that seals the glass chip from the environment.

Advantages of enclosure-protected glass chips:

a) the chip is protected from environmental contaminants.
b) protected from user-inflicted damage
c) provides a fixed-reagent volumes to ensure consistency in reactions.
d) greater ease of handling.
e) allows automated loading.

Disadvantages:

a) incompatibility with most scanning systems (except Hewlett Packard).
b) increased cost.

Yet another format that has been developed by Protogene uses a 4x4 inch slide. This symmetrical format allows chips to be spun during manufacturing, thereby allowing reagents to be washed off the surface by centrifugal force during each cycle of phosphoramidite coupling.

3. DNA attachment chemistry

In spotted arrays, cDNA or PCR products can be attached either covalently or non-covalently to silane-coated glass slides. The latter of these methods is more flexible, simpler and less expensive. A commonly used procedure for DNA attachment uses sodium thiocyanate (NaSCN) as follows:

-Dissolve cDNA or PCR fragments in 3-5 M NaSCN

-Perform spotting using a reproducible procedure

-Heat slides at 80^{o}C for 2 hours to dehydrate the spots

-Prior to hybridization, wash in isopropanol for 10 minutes

-Boil in water for 5 minutes

This procedure results in 20-30% DNA retention after a hybridization experiment. The test conditions included DNA concentrations of 100 ng/μl (corresponding to 100 pg/spot) for a 1 nl deposition and fragment size of 250-750 bp.

The Affymetrix system (i.e., oligonucleotide arrays) makes use of photolithographic and solid state methods to synthesize oligonucleotides *in situ* on a previously attached synthetic linker (see Affymetrix System Review chapter).

4. Sample isolation and preparation

The large data flow and present cost of microarray experiments are two important factors that place a premium on high-quality sample preparation. Meticulous and consistent sample preparation is particularly crucial in gene expression experiments where differential degradation of RNA can lead to erroneous conclusions about both relative and absolute mRNA levels in samples under study. Poor sample preparation will have a negative impact on every step of the process, starting with labeling all the way to data output and assembling of databases based on microarray experiments.

When working with RNA, the main consideration is the fast and complete inactivation of ribonucleases. In the case of DNA, one has to be concerned with the isolation efficiency of high-molecular-weight molecules. For comparative studies, it is absolutely important that all biological samples be processed in an identical manner (i.e., reagents from the same lot, same procedural conditions, same experimentalist, etc.). Although many commercial kits or other procedures available in the literature can be used to isolate RNA (or DNA) of acceptable quality, the percentage yield between methods may vary greatly for a large number of mRNA species in a sample.

5. Sample labeling and detection

Important factors for deriving high-quality sample labeling for microarrays experiments include:

Intact and clean RNA, free of polysaccharides, proteins and DNA.
Removal of unincorporated nucleotides and small fragments (< 200 bases) following cDNA synthesis.
Optimum specific activity of dye incorporation into the cDNA as determined by the absorbance at 260 and 550 (Cy3) or 650 (Cy5), using published extinction coefficients. Very high specific activity (>1 dye molecule/20 nucleotides) results in a decreased hybridization signal, due to destabilization of the hybridization complex. On the other hand, very low specific activity (< 1 dye/100 nucleotides) gives unacceptably low hybridization signals.
Careful handling of both the fluorescent nucleotides. This is especially important for Cy5 which is more easily photobleached and more prone to freeze-thaw degradation than Cy3.

Nucleic acids samples intended for microarray analysis can be labeled using one of many direct or indirect methods. To maximize the precision of direct fluorescent labeling procedures involving multiple dyes for comparative analysis, chemically related dyes (absorbing and emitting light at different wavelengths) should be chosen. This is important because fluorescent compounds that differ widely in structure could produce hybridization and detection artifacts that are sequence dependent. Alternatively, this potential problem can be addressed by performing reciprocal labeling in which dyes are "swapped" with respect to the mRNA source in separate experiments. Fluorescent moeities are incorporated directly into the sample by enzymatic action in the presence of either labeled nucleotides or PCR primers.

In indirect fluorescent labeling schemes, nucleic acids are first labeled with a moeity such as biotin. Samples are then treated with a biotin-binding protein derivatized with a fluorescent dye (i.e., streptavidin-phycoerythrin conjugate). This approach has the advantage that it can provide 10-100-fold amplification when using formats such as in the biotin/streptavidin-HRP/cyanine tyramide system. This relatively recent approach works through the catalytic (HRP) deposition of Cyanine-tyramide (Cy3-tyr, or Cy5-tyr) compounds onto the microarray surface. Although indirect labeling methods have the potential to increase the sensitivity in microarray experiments, they are somewhat more difficult to implement and less precise for comparative studies. This is largely due to the fact that the enzyme (HRP) may have different affinities for the Cy3-tyr and Cy5-tyr.

Matching of sample signal in dual-label experiments

This is an important requirement due to the fact that Cy3 has a lower quantum yield (0.15) than Cy5 (0.28). Signal balancing is attained instrumentally as follows: a) the solid-state green laser used to excite Cy3 is more powerful that the HeNe laser used to excite Cy5; b) the PMT used to collect the fluorescent signal is more sensitive in the green (Cy3) part of the spectrum than in the red (Cy5); and c) data collection can be obtained using a higher PMT-voltage setting during scans of the dye with the weaker signal.

6. Hybridization conditions

Accurate and specific quantification of gene induction requires that the arrayed DNA be present in excess relative to the labeled sample. Empirical tests indicate that 20-30 pg of spotted DNA on glass constitutes an excess even for the most abundantly expressed genes when added at a 0.004-0.1 ng/μl concentration. In bacterial gene expression studies, the maximum amount of sample hybridized to DNA spots was 8.5 pg . These observations underscore the importance of determining the amount of spotted DNA actually retained following the hybridization step, and whether the hybridization signal is within the range of linear response for the system being used.
Combining two labeled cDNA populations does not affect the quantitation of expression results, provided the spotted DNA is in excess relative to the sum of both samples.

Although porous membranes have a larger DNA binding capacity than glass slides, only 4% of membrane-bound DNA is available for hybridization. Moreover, the uneven surface of membranes makes them unsuitable for high-density, two-color, confocal fluorescence imaging.

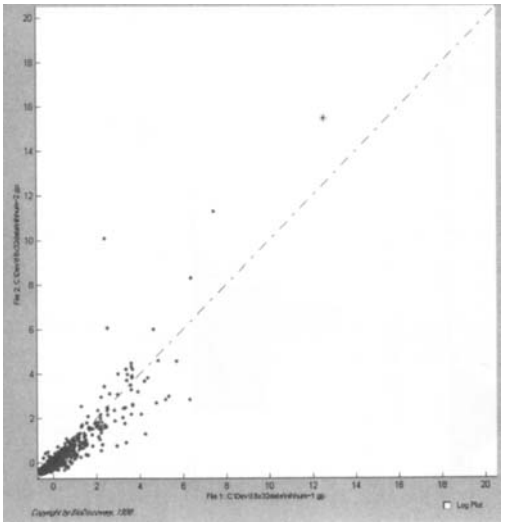
Additional factors to consider during hybridization are:

a) Probe depletion from the hybridization site due to slow sample diffusion. This suggests that in the absence of mixing, it may be important to have replicate spots well separated on a slide, as adjacent replicates may be depleting probe from the same hybridization solution microenvironment.
b) Temperature. All other conditions being equal, typical hybridization temperatures used for membrane hybridization (50-65°C) are too stringent for some high-density glass-based array experiments. The most reproducible results both across the slide and between slides have been obtained using formamide-containing buffer at 42°C.

Data Files and mining

The microarray industry relies heavily on bioinformatics. Interpretation of the vast amounts of microarray-generated data requires powerful graphical and computing modeling tools. The most common graphical output format fluorescence imaging instruments is the 16-bit tiff (.tif) file. This format allows a theoretical dynamic range in excess of four orders of magnitude, a useful feature in microarray work.

There are a number of data quantitation packages that deal adequately with microarray data output (e.g., QuantArray™ from GSI Lumonics, ImaGene™ from BioDiscovery, etc.). Typically, a user-defined gridding pattern is overlaid on the image, and the areas defined by the regular pattern of circles (or squares) are subjected to data extraction. Once values are quantified from the microarray, the data are commonly displayed in a variety of ways, most commonly as a scatter plot. For a two-color experiment, large numbers of data points can be plotted as a function of ratio and signal intensity. Data points with ratios greater than 1.0 appear above the diagonal, while those with ratios less than 1 appear below the diagonal. A simple mouse click on any given data point usually provides the user with information about the gene sequence present at that position on the microarray.



Following quantitation and identification of the genes of interest, the data are ready for mining. This process requires additional software also available from a variety of sources (e.g., Stingray™ from the Molecular Applications Group, GeneSpring™ 2.2 from Silicon Genetics, etc.). A common exercise consists of grouping genes (clustering) according to expresssion profile, thereby identifying functionally related genes such as those in common signaling pathways.

Nomenclature and Glossary

General

There is some degree of confusion regarding the use of certain terms in the microarray field. Duggan et al (9) proposed a nomenclature where the immobilized nucleic acids are called **'probes'** because they are used to interrogate the samples of interest. The labeled, free nucleic acid that becomes hybridized to the probe is called **'target'** or simply the 'sample'. ***This nomenclature is adopted in this manual.***

Linear dynamic range: the range of sample concentrations that has the same detection efficiency.
(The dynamic range of a microarrray scanning system can be defined in at least three different ways:
i) The electronic dynamic range of the scanner; ii) the chemical dynamic range of the fluorescent dyes used, and iii) the biological dynamic range of the system under study. Valid biological data results from experimental conditions where the electronics or fluorescent chemistry is not limiting.

Limit of detection (LOD): The amount of fluorescent dye for which the signal-to-noise ratio (SNR) is 3.

Signal-to-noise ratio (SNR): (Signal minus background)/(standard deviation of background). Under optimum conditions SNR can be approximately 0.2 amol of Cy3 and 0.4 amol of Cy5 per spot (i.e., 3-6 fluorescent molecules/μm^2) at a photo multiplier voltage of 600V.

Oligonucleotide Arrays

Standard tiling: Probe design approach in which for any given nucleotide position, there are two sets of probes complementary to the sense and antisense strands of the reference probe sequence.

Reference probes: Probes that match the target sequence exactly.

Partner probes: Probes that contain a single base mismatch in the center of the reference probe sequence.

Feature: Designated regions of an array containing many (10^6-10^7) copies of one synthetic probe.

References

1. Bishop, J.O., et al. (1974). Three abundant classes in HeLa cell messenger RNA. *Nature* **250**: 199-204.

2. Wan, J.S., et al. (1996). Cloning differentially expressed m RNAs. *Nat. Biotechnol.* **14**:1685-1691.

3. Velculescu. V.E., et al. (1995). Serial Analysis of Gene Expression. *Science* **270**: 484-487.

4. Liang, P. and A.B. Pardee (1992). Differential display of eukaryotic messenger RNA by ;means of the Polymerase Chain Reaction. *Science* **257**: 967-971.

5. Lockhart, D.J., et al. (1996). Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat. Biotechnol.* **14**: 1675-1680.

6. Schena, M., et al. (1995). Quantitative monitoring of gene expression patterns with complementary DNA microarray. *Science* **270**: 467-470.

7. 'Microarray Biochip Technology' (2000). Edited by Mark Schena. Chapters 1- 4. Eaton Publishing. BioTechniques Books Division, 154 East Central Street. Nattick, MA 01760.

8. Fodor , S.A., et al (1991). Light-directed, spatially addressable parallel chemical synthesis. *Science* **251**: 767-773.

9. Fodor , S.A., et al (1993). Multiplexed biochemical assays with biological chips. *Nature* **364**: 555-556.

10. Duggan, D.J., et al. (1999). Expression profiling using cDNA microarrays. *Nat. Genet.* **21**(1 Suppl.): 10-14.

Custom-made, Membrane Gene Arrays: Experimental Procedures

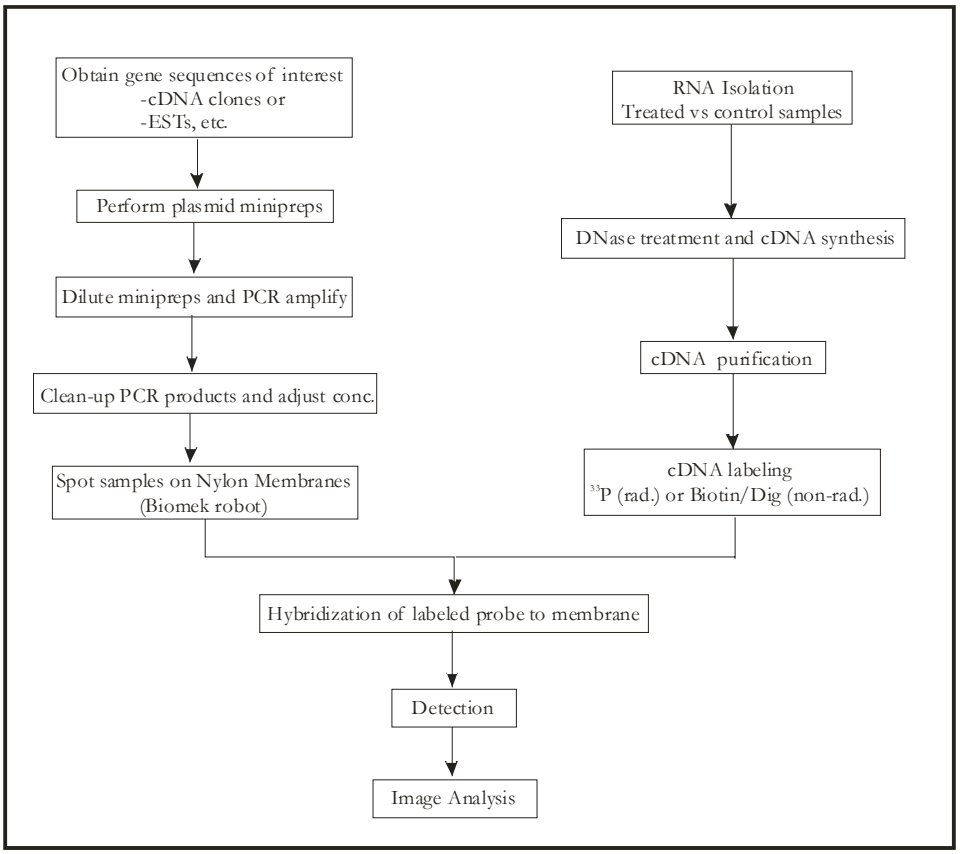
Gene arrays (in the form of glass slides or membranes, containing thousands of pre-spotted gene sequences) are commercially available from an increasing number of providers. Unfortunately, this is the case only for the best known "model" organisms (e.g., humans, mice, arabidopsis, etc.) for which there is a significant amount of sequence data. Researchers working on less popular models are confronted with the need to generate their own material to spot on slides or membranes.

In this experiment you will be spotting PCR products representing clones of a cDNA library constructed from fish RNA. For the purpose of this lab, only 12 clones will be spotted. However, thousands of cDNA species (i.e., mRNAs) can be arrayed on a single slide or membrane to be used in a single experiment. Small volumes of probe solutions (PCR products) are deposited onto the array surface using a pen or pin-deposition tool. Microfluidic delivery techniques offer the flexibility of generating microarrays that could consist of genes obtained from commercial sources, cDNA clones, DNA probes or PCR amplification products from personal cDNA libraries, without the need for sequence information.

Once the membranes/chips have been constructed, labeled mRNAs (or their corresponding cDNAs) from animals that have been exposed to different conditions (e.g., treated with estrogen, *versus* untreated) can be allowed to hybridize to the arrayed material, and evaluated for differences in signal intensity at every spot (i.e., differential expression).

The flowchart below outlines the number of steps needed to generate a custom-made membrane array.

Membrane Array Preparation Scheme for Differentially Gene Expression Analysis



A. Overnight culture of *E. coli* bacterial clones transformed with plasmids containing unique, fish cDNA fragments: Plasmid minipreps

The bacterial clones used here were obtained from Dr. Nancy Denslow's laboratory (Univ. of Florida). cDNA from fish (bass) was cloned into the pGEM-T Easy plasmid (PROMEGA) and transformed into *E. coli*. Twelve different clones (containing unique sequences) will be picked and grown in selective medium in order to obtain plasmids that can be used as templates in PCR reactions.

Part a: Liquid Subculture of Bacterial Transformants

1. Add 3 ml LB-Amp (Luria broth with 50 μg/ml Ampicillin) to two Falcon culture tubes.
2. Start two 3 ml cultures from one of the white colonies growing on each of the LB-Amp plates assigned to your group as follows:

   Groups 1 and 2: plates 1 and 2
   Groups 3 and 4: plates 3 and 4
   Groups 5 and 6: plates 5 and 6
   Groups 7 and 8: plates 7 and 8
   Groups 9 and 10: plates 9 and 10
   Groups 11 and 12: plates 11 and 12

   a) pick the colony by **lightly** touching it with a sterile plastic loop (it is not necessary to scrape the entire colony from the plate)
   b) uncap falcon tube
   c) submerge the loop into the LB-Kan in the Falcon tube
   d) rapidly move the loop back and forth through the LB-Kan a few times (to dislodge the cells), then discard the loop
   e) recap the tube

3. Be sure to innoculate each colony into <u>separate</u> culture tubes (falcon).
4. Grow both cultures at 37°C with vigorous shaking (300 rpm) for 20-24 hours.

Part b: Cell Lysis and Plasmid Purification

Caution: The DNA purification resin contains 7M guanidine HCl. This substance is toxic and irritant. Harmful by inhalation, skin contact or ingestion. Qiagen miniprep kit Catalog #27104.

1. Pre-heat a 1.5 ml microcentrifuge tube with approximately 200 μl dH_2O in a water bath. This water will be used to elute the purified plasmid DNA from the spin column.
2. Transfer 1.5 ml of each bacterial culture to a 1.5 ml microcentrifuge tube. Take care to label each tube to its corresponding culture and by your group #. Pellet the bacteria by centrifuging each 1.5 ml microcentrifuge tube at 14,000 rpm for 30 seconds.
3. Remove the supernatant by decanting or aspirating into the waste collection tube. Do not disturb the bacterial pellet.
4. Add 100 μl of Solution I and completely resuspend the pelleted bacteria by either vortexing the tube or rubbing it along the holes in your rack. The mixture should have a smooth and even consistency with no clumps.

5. Lyse the bacteria by adding 100 µl of Solution II to the resuspension and mix several times by repeated **gentle** inversion each tube. The lysate should be relatively clear with no visible clumps of cell material.
6. Neutralize the bacterial lysate by adding 100 ul of Solution III and immediately mixing thoroughly by repeated inversion of each tube. After mixing, the whitish precipitate should have a curd-like appearance.
7. Pellet out the cellular debris and precipitated proteins by centrifuging the neutralized lysate in a microcentrifuge for 5 minutes at 14,000 rpm.
8. While your tubes are being centrifuged, label a spin column and collection tube with your group # for each one of the cultures.
9. Carefully transfer all the clear supernatant to a spin column in a collection tube. Take care to not pick up any of the white precipitate that could possibly clog your column. You **do not** have to get all of the supernatant.
10. Vigorously mix the DNA Binding Resin by vortexing for about 15 seconds. Using a wide bore tip, add 450 µl of DNA Binding Matrix to the supernatant in each spin column. Mix by capping the spin column and vortexing the column/collection tube assembly.
11. Purify the bound plasmid DNA by centrifuging the spin column/collection tube assembly for 30 seconds at 14,000 rpm. Decant the filtrate into the waste collection tube and place the spin column back into the collection tube.
12. Add 400 µl of Purification Solution to each spin column. Cap each tube and vortex briefly. Centrifuge the spin column/collection tube for 30 seconds at 14,000 rpm.
13. Decant the filtrate and place each spin column back into their respective collection tubes. Remove the residual Diluted Purification Solution by centrifuging each spin column/collection tube assembly for 30 seconds at 14,000 rpm.
14. Transfer each spin column to a new collection tube (be sure to label the new tubes). Add 50 µl of dH_2O that has been pre-heated to 65°C to each spin column. Cap each tube and briefly vortex each column to mix .
15. Centrifuge each spin column/collection assembly for 60 seconds at 14,000 rpm.
16. Discard the spin columns and store the eluted plasmid DNA at either 4°C or –20°C.

Composition of the buffers

Cell Resuspension Solution
50 mM Tris-HCl, pH 7.5
10 mM EDTA
100 µg/ml RNase A

Column Wash Solution
100.0 mM NaCl
10.0 mM Tris-HCl, pH 7.5
2.5 mM EDTA
50% Ethanol

Neutralization Solution
2.55 M KOAc, pH 4.8

Cell Lysis Solution
0.2 M NaOH
1% SDS

B. Plasmid Digestion: Testing for the presence of cDNA inserts

EcoR I restriction enzyme digestion

The plasmid used for cloning of fish cDNA fragments (pGEM-T Easy) contains an *Eco* RI site on both sides of the Multiple Cloning Site. Therefore, digestion with this enzyme should result in the generation of at least two fragments: one corresponding to the nearly complete linearized pGEM-T Easy vector (approximately 2700 bp),

and another fragment corresponding to the cloned insert (between 200-600 bp, assuming there aren't any Eco RI sites present in the insert). Digestion reactions are analyzed by agarose gel electrophoresis (Part C).

1. Label two tubes with the corresponding plasmid clone numbers. Make sure to include your lab group number to avoid any confusion during centrifugation.
2. Mix reagents according to the following table

Reagent	Amount (µl)	
	Tube 1	**Tube 2**
dH_2O	4.0	
10X Buffer H	1.0	
10X BSA	1.0	
*Eco*R I	1.0	
Plasmid 1 or 2	1.0 (plasmid 1)	1.0 (plasmid 2)

3. Incubate at 37°C for 1 hour.

Casting an agarose gel for analysis of plasmid digestion fragments

1. Seal edges of the gel-casting tray and position the comb close to the negative (black or cathode) electrode.
2. Prepare a 1.3 % (w/v) agarose solution in 1X TBE and heat until agarose is completely dissolved.

> The laboratory staff has prepared the agarose solution. The molten agarose can be found in the 65°C water bath at the side of the room.

5X TBE:
54 g Tris base
27.5 g boric acid
20 ml 0.5 M EDTA (pH 8.0) per liter. (45 mM Tris-borate, 1 mM EDTA)

3. Retrieve melted agarose solution from the 65°C water bath and pour into the gel-casting tray. Make sure that no bubbles are trapped underneath the comb. (USE GLOVES. THE AGAROSE SOLUTION CONTAIN ETHIDIUM BROMIDE, A SUSPECTED CARCINOGEN).
4. After the gel is completely set, unseal the ends of the casting tray, add 1X TBE to just cover the surface of the gel and remove the comb carefully.

DNA Electrophoresis

1. Add 2 µl of loading dye (6X) to the entire 10 µl digestion mix from each tube in part B. Close tube cap, mix by tapping, and spin briefly.
2. Using the micropipet, slowly load the entire 12 µl into one well of the 1.3% agarose gel.
3. In an adjacent well, load 12 µl of a DNA mass standard (Amplisize DNA markers, BIORAD) .
4. On the power supply set the voltage to 0. Turn the unit 'ON' and then increase the voltage until the voltmeter indicates 90 V. The power supplies we are using can run two gel electrophoresis units at once. The voltage supplied to the left and right sides of the unit is adjusted separately and the voltmeter must be switched to the correct side when the voltage is being adjusted or monitored. Bubbles should be visible at the electrodes and the bromophenol blue tracking dye should enter the gel.

5. Run the gel until the fastest moving dye front (bromophenol blue) has migrated 3/4 of the way down the gel.
6. Turn off the power supply and remove leads.
7. Carefully remove the gel and drain off the ethidium bromide-contaminated solution into a collection vessel.

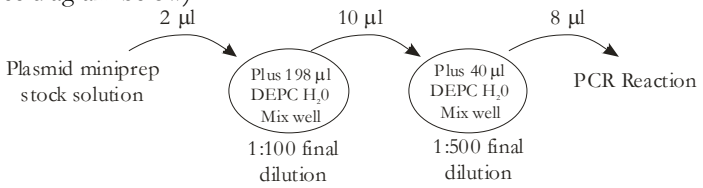
> **Caution:** Ethidium bromide is a known carcinogen. Wear protective gloves when handling ethidium or ethidium-stained gels.

8. Rinse gel and tray with tap water.
9. Visualize the DNA bands by placing on a UV transilluminator and photograph.

Size of AmpliSize™ DNA size marker:	Bio-Rad Mass Ruler fragment	
	bp (band size)	Mass of band (ng)
2,000	1000	100
1,500	700	70
1,000	500	50
700	200	20
500	100	10
400		
300		
200		
100		
50		

C. PCR Amplification of inserts from plasmid minipreps

1. Make a 1:500 dilution in DEPC-water of plasmid minipreps (A and B). If the yield of a miniprep seems low, make a 1:100 dilution (see diagram below).



2. Label four PCR tubes (small tubes) on the sides. One set of two tubes will be duplicates for one of the plasmids. The other two will be used for triplicate runs of the other plasmid. Make sure you include your group number to distinguish your samples from the rest of the of the class.

3. Prepare the PCR Master Mix by adding reagents according to the table below. DO NOT add reagents separately to each tube. Go to step 4 after preparing the Master mix

Reagent	**Master Mix (µl) (5 samples)**	**Amount per tube (µl)**	**Final concentration**
DEPC-H_20	303	60.6	-
10X PCR buffer	50	10	1X
2 mM dNTPs each	40	8	160 µM each

25 mM $MgCl_2$	40	8	2 mM
20 µM M13 forward primer*	10	2	0.4 µM
20 µM M13 reverse primer**	10	2	0.4 µM
3.5 U/µl Taq pol	7	1.4	0.05 U/µl

*Forward primer: 5'-GTT TTC CCA GTC ACG ACG TTG-3'
**Reverse primer: 5'-GCG GAT AAC AAT TTC ACA CAG GA-3'

4. Mix reagents well in the Master Mix. Transfer 92 µl into each of the 4 PCR tubes from step 2. Place samples on ice.

5. Add 8 µl of the 1:500 diluted plasmid A solution into each tube of one of the sets of PCR tubes. Make sure tubes are labeled well. Add 8 µl of the diluted plasmid B to the other set of tubes.

6. Mix well using a pipet and filter tips (change tips in between samples). Keep samples on ice until the rest of the class is ready for thermocycling.

7. Transfer samples to thermocycler and use the following parameter for amplification.

94°C	2 minute	1 cycle
80°C	1 minute	1 cycle
94°C	1 minute	32 cycles
57°C	1 minute	
72°C	2 minutes	
72°C	10 minute	1 cycle
4°C	Hold until ready for analysis	

D. Purification and Concentration of PCR Products

Purification using QIAquick® Spin Columns

The QIAquick system combines the convenience of spin-column technology with the selective binding properties of a uniquely-designed silica-gel membrane. DNA adsorbs to the silica-membrane in the presence of high salt while contaminants pass through the clumn. Impurities are efficiently washed away, and the pure DNA is eluted with Tris buffer or water. Adsorption of DNA to silica depends both the salt concentration and pH of the solution. Typically, 95% of the DNA in a solution will bind at pH ≤7.5. If the pH of the solution is too high, it can be lowered using 3M sodium acetate, pH 5.0. The binding solution in the kit contains an pH indicator that turns the solution yellow when the pH is optimal.

Many applications require PCR product purification after amplification. These include: restriction digestion, labeling, in vitro transcription, hybridization, ligation/transformation, radioactive and fluorescent sequencing, and microinjection.

Purification Procedure (QIAquick PCR Purication kit, QIAGEN # 28104)

1. Combine the two PCR reactions in a single tube.
2. Add 5 volumes of Buffer PB to 1 volume of the PCR sample mix (e.g., add 1000 µl Buffer for 200 µl PCR)
3. Place a QIAquick spin column in a provided 2 ml collection tube.
4. Apply the sample to the column and centrifuge for 30-60 seconds at 6,000 rpm (sample binds).
5. Discard flow-through. Place the column back into the same tube (collection tubes are re-used to reduce waste)

6. To wash, add 0.75 ml Buffer PE to the column and centrifuge for 30-60 sec (6,000 rpm).
7. Discard flow-through and place the column back in the same tube. Centrifuge the column for an additional 1 minute at maximumn speed (Note: residual ethanol from Buffer PE will not be efficiently removed unless the flow-through is discarded before the additional centrifugation).
8. Place QIAquick column in a clean 1.5 ml microcentrifuge tube. Make sure tubes are well labeled.
9. To elute DNA add 50 µl Buffer EB (10 mM Tris.Cl, pH 8.5) or water to the center of the QIAquick membrane. Centrifuge the column for 1 minute (Note: Maximum elution efficiency is pH dependent. Elution can also be accomplished with TE: 10 mM Tris.Cl, 1 mM EDTA, pH 8; however, EDTA may inhibit subsequent enzymatic reactions).

Concentration Procedure

1. Place your tubes containing the purified PCR products in a Centrivap or Speed-vac (centrifugal evaporator). Lab assistants will be available to help you with this step.

2. Concentrate samples down to 15-20 µl.

E. Analytical agarose gel electrophoresis of clean, concentrated PCR products: Estimating the concentration of samples

Casting the agarose gel

1. Seal edges of the gel-casting tray and position the comb close to the negative (black or cathode) electrode.
2. Prepare a 1.3 % agarose solution in 1X TBE.

> The laboratory staff has prepared the agarose solution. The molten agarose can be found in the 65°C water bath at the side of the room. USE GLOVES! The agarose solution contains Ethidium bromide, a suspected carcinogen.

5X TBE:
54 g Tris base
27.5 g boric acid
20 ml 0.5 M EDTA (pH 8.0) per liter. (45 mM Tris-borate, 1 mM EDTA)

3. Use gloves to retrieve melted agarose solution from the 65°C water bath and pour into the gel-casting tray. Make sure that no bubbles are trapped underneath the comb.
4. After the gel is completely set, unseal the ends of the casting tray, add 1X TBE to just cover the surface of the gel and remove the comb carefully.

DNA Electrophoresis

We will use this gel electrophoresis step to estimate the relative concentrations of purified PCR product by comparing with a known marker DNA standard.

1. Make a 1:10 fold dilution of each one of the PCR product solutions (i.e., 1 µl sample, 9 µl water)
2. Pipet 2 µl from the diluted PCR product into a clean microcentrifuge tube. Add 10 µl of water and 2 µl of loading dye (6X) to the tube. Close tubes, mix by tapping, and spin briefly.
3. Using the micropipet, slowly load the entire 12 µl from each sample into separate wells of the 1.3% agarose gel.
4. In an adjacent well, load 12 µl of the marker DNA standard (BioRad Mass Ruler) on the agarose gel.

5. Connect electrophoretic chamber to power supply. Set the power supply to 90 V. Bubbles should be visible at the electrodes and the bromophenol blue tracking dye should enter the gel.
6. Run the gel until the fastest moving dye front (bromophenol blue) has migrated 3/4 of the way down the gel.
7. Turn off the power supply and remove leads.
8. Use glove to carefully drain electrophoresis buffer into collection vessel. Buffer contain traces of ethidium bromide.

> **Caution:** Ethidium bromide is a known carcinogen. Wear protective gloves when handling ethidium or ethidium-stained gels.

9. Rinse gel and tray with tap water.
10. Visualize the DNA bands by placing on a UV transilluminator and photograph.
11. Estimate the amount of PCR products by comparing band intensities to the Standard (Mass Ruler). Lab assistants will guide you through this exercise.
12. Adjust the concentration of PCR products to 160 ng/µl. You will need about 19 µl of PCR products

Bio-Rad Mass Ruler fragment (bp)	Mass of band (ng)
1000	100
700	70
500	50
200	20
100	10

NOTE: PCR product concentration can also be estimated by spectrophotometry (you will not do this!)

1. To determine the concentration of the PCR products by spectrophotometry, for each sample, transfer 1 µl of the concentrated solution into an RNase-free spectrophotometric cuvette containing 99 µl of DEPC-water. The lab instructors will help you measure the A_{260}/A_{280}. Pure PCR products will give a ratio of approximately 2.0.

   1.0 A_{260}= 30 µg/ml PCR products

   dilution factor in spectrophotometric cuvette= 100

   PCR product solution conc. (µg/ml)= (A_{260})(100)(30µg/ml)

2. Adjust the concentration of the PCR products solutions to 200 ng/µl

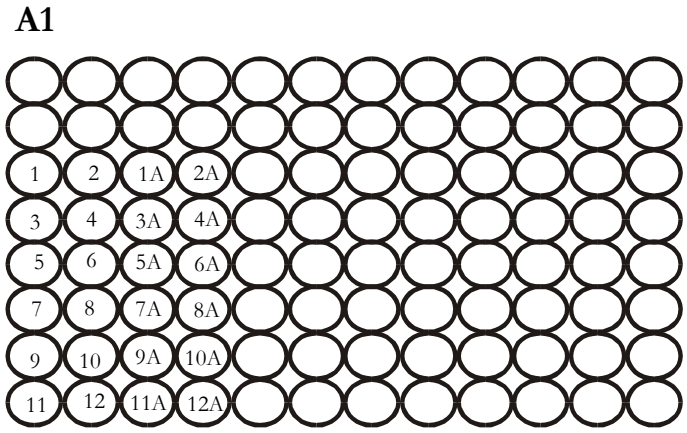
---

F. Spotting the samples

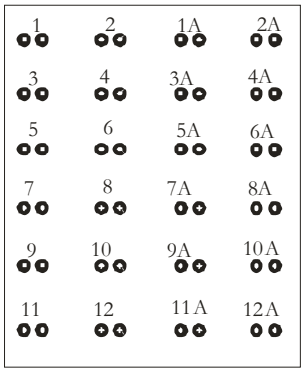
1. Take 18.75 uL of the 160 ng/uL samples and add it to the a new tube.
2. Add 1.85 uL 3 M NaOH to each of the samples.
3. Heat tubes at 65°C for 10 minutes in a water bath and then immediately put tubes on ice.
4. Add 9 uL 20X SSC to each tube (add some bromophenol blue to the 20XSSC stock to give it some color, see lab instructors about how much to add).
5. The final concentration of is now 0.3M NaOH, 6X SSC, and 100 ng/µL cDNA template.
6. Load the samples into a 96 well plate (Fischer, #05-500-68) in the following orientation. Since each of the twelve clones is being processed by two different groups, one of the sets is identified by the clone number plus the letter A. Even number groups, please label your clones with the number plus the letter A.
7. Spin the 96-well plate down in a suitable centrifuge.

8. Spot samples in duplicates onto a piece of neutral nylon membrane (Fischer Sci, #12-565-306). Spotting is done using a Biomek robot (Biomek 2000, Beckman Coulter) equipped with 100 nl pins.
9. After spotting, UV cross-link membranes at 1000X100 μJoules (Stratalinker), and store under vacuum until used for hybridization.

Clone number	Groups to work with clones	Original ID
1	Groups 1&2	clone #12-1
2	Groups 1&2	clone #5-10
3	Groups 3&4	clone #2-2
4	Groups 3&4	clone #6-1
5	Groups 5&6	clone #15-1
6	Groups5&6	clone #135-1
7	Groups 7&8	clone #1-3
8	Groups 7&8	clone #25-1
9	Groups 9&10	clone #5-13
10	Groups 9&10	clone #7-3
11	Groups 11&12	clone #8-2
12	Groups 11&12	clone #9-1



Nylon Membrane
Each sample is spotted twice using a robot



G: RNA Isolation from culture cells or animal tissue (treated vs untreated)

Disruption and Homogenization

For the purpose of this experiment, RNA would have already been isolated from fish liver (bass) by laboratory assistants. This was done in the interest of saving time. However, the procedure is described here for the sake of completeness (see below QIAGEN RNeasy kit). The procedure used was similar to that described in Experiment 14 (See also Par B below).

More recently, a proprietary solution has become available from AMBION (RNA later ™) that allows from convenient storage of tissue samples for up to many days, at room temperature until ready for processing. Such remarkable reagent is of great use in field work where liquid nitrogen and/or homogenization equipment might be of limited availability. Tests have indicated that the integrity of RNA is maintained in the RNA Later reagent.

Note: The procedure described here is a variation of the RNeasy isolation kit by QIAGEN (Cat #s 74103, 74104 or 74106). The DNA removal reagents used in this procedure is from AMBION (Cat # 1906). **IT IS OF UTMOST IMPORTANCE THAT THESE STEPS ARE PERFORMED AS QUICKLY AS POSSIBLE TO MINIMIZE RNA DEGRADATION.** Use **GLOVES** and "aerosol resistant" pipet tips throughout the entire RNA isolation procedure.

QIAGEN RNeasy Kit Principle and Recommendations

The RNeasy procedure combines the selective binding properties of a silica-gel-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows the binding of up to 100 μg of RNA (longer than 200 bases) to the RNeasy silica gel membrane. Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine isothiocyanate (GITC)-containing buffer which immediately inactivates RNases to ensure isolation of intact RNA. Ethanol is added to provide appropriate binding conditions and the sample is then applied to an RNeasy mini spin column where the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in 30 μl, or more of water. Small RNAs (<200 bases), such as 5.8S RNA, 5S RNA, and tRNAs, approximately 160, 120, and 70-90 nucleotides in length, respectively, will not bind quantitatively under the conditions used.

Tissue handling, disruption and homogenization

Samples require different handling depending on the source. Please refer to the literature provided provided with RNA isolation kits (e.g., RNeasy® Mini Handbook) for recommendations on source-specific lysis procedures (e.g., bacteria, yeast, animal cell cultures, animal tissue, plant tissue, or filamentous fungi). In general, the quality of RNA is best from fresh samples. Tissue samples must be removed a promptly, cut into 30-50 mg segments, and disrupted and/or homogenized in denaturing buffer. Fungi and plant tissue are first ground in liquid nitrogen and then lysed under highly denaturing conditions. Alternatively, the tissue can be quickly removed from the animal, cut into 30-50 mg segments and flash-frozen in liquid nitrogen. Flash-frozen samples can be subsequently stored in a -80°C freezer until ready for RNA isolation.

> ***Disruption:*** *Complete disruption of cell walls and plasma membranes of cells and organelles is absolutely required to release all the RNA contained in the sample. Different samples require different methods to achieve complete disruption. Incomplete disruption results in significantly reduced yields.*
>
> ***Homogenization:*** *Homogenization is necessary to reduce the viscosity of the cell lysates produced by disruption. Homogenization shears the high-molecular-weight genomic DNA and other high-molecular-weight cellular components to create a homogenous lysate. Incomplete homogenization results in significantly reduced yields.*

Important Points before Using RNeasy Kits

It is absolutely essential that the recommended amount of starting material is used. The two main factors that determine the amount of starting material are:

- The volume of lysis buffer required for efficient disruption of cells in the sample.

- The RNA binding capacity of the RNeasy mini spin column (100 μg)

RNeasy column specifications

Variable	Optimum quantity
Maximum binding capacity	100 μg RNA
Maximum loading volume	700 μl
RNA size distribution	RNA>200 nucleotides
Minimum amount of starting material	
Animal cells	$1x10^7$
Animal tissue	30 mg
Bacteria	$1x10^9$
Yeast	$5x10^7$
Plant tissue	100 mg
Maximum Yield	10-60 μg (sample-dependent)

A. Culture Cells

1. Loosen cell pellet by flicking the tube and add 350 μl of denaturing solution (Buffer RLT).

   Make sure that β-mercaptoethanol is added to Buffer RLT before use (see QIAGEN kit user's manual).

   This volume of Buffer RLT is good for up to $5x10^6$ cells.

2. Vortex or pipet to mix. No cell clumps should be visible before proceeding to step 3.
3. Homogenize the sample by pipetting lysate directly onto a QIAshredder column sitting in the 2 ml collection tube provided, and centrifuge for 2 min at 14,000 rpm.

B. Animal Tissue (fish liver)

1. Weigh quickly approximately 20 mg of tissue (fresh or frozen at -80°C) and place directly into a microcentrifuge containing 350 μl of denaturing solution (Buffer RLT). Homogenize immediately for about 30 seconds
2. Centrifuge lysate for 3 minutes at 14,000 rpm.
3. Transfer supernatant into a clean microcentrifuge tube.

Binding of RNA to column

1. Add 350 μl of 70% ethanol to the homogenized lysate, and mix well by pipetting. Do not centrifuge.

   If some lysate is lost during homogenization, adjust volume of ethanol accordingly. A precipitate may form after the addition of ethanol. This will not affect the RNeasy procedure.

2. Apply 700 μl of samples, including any precipitate which may have formed, to an RNeasy mini spin column in a 2-ml collection tube, and centrifuge for 15 sec at 12,000 rpm.

   If the volume of the mixture exceeds 700 μl, successively load aliquots onto the RNeasy column and centrifuge as above. Reuse the collection tube but discard flowthrough after each step. Reuse the collection tube in the following step.

Washing: Eliminating nonspecifically bound material

1. Pipet 700 μl wash buffer 1 (Buffer RW1) onto the RNeasy column, and centrifuge for 15 sec at 12,000 rpm.
2. Discard flow-through and collection tube.
3. Transfer RNeasy column into a new 2-ml collection tube. Pipet 500μl of wash buffer 2 (Buffer RPE) onto the RNeasy column, and centrifuge for 15 sec at 12,000 rpm.

   Discard flow-through and reuse the collection tube in the following step.

Ensure that ethanol is added to Buffer RPE before use (see QIAGEN RNeasy user's manual).

4. Pipet another 500 µl of wash buffer 2 (Buffer RPE) onto the RNeasy column. Centrifuge for 2 min at 14,000 rpm to dry the RNeasy membrane.
5. Following the spin, remove the RNeasy column from the collection tube carefully so that the column does not contact the flow-through as this will result in carryover of ethanol.

   It is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent reactions. The following spin ensures that no ethanol is carried over during elution.

6. Place the RNeasy spin column in a new 2-ml collection tube, and discard the old collection tube with the filtrate. Centrifuge at 14,000 rpm for 1 min.

Elution of RNA from the column

1. Transfer the RNeasy column into a new 1.5-ml collection tube, and pipet 30 µl of RNase-free water directly onto the RNeasy membrane. Centrifuge for 1 min at 12,000 rpm to elute.

   A second elution step can be performed using another 30-50 µl RNase-free water

2. Store RNA at –80°C or proceed with DNase treatment.

   For long-term storage, resuspend the RNA in DEPC-treated water, add sodium acetate (2 M, pH 4.0) to 0.25 M and 2.5 volumes of ethanol. Store at -80°C.

DNase Treatment and Removal of Reagents

Description

Adapted from Ambion's User's pamphlet (Cat. # 1906)

The DNA-free™ Kit is designed to remove contaminating DNA from RNA preparation, and to subsequently remove the DNase and divalent cations from the sample. Contaminating DNA is removed to levels below the limit of detection by routine RT-PCR. DNA removal by this method does not require phenol/chloroform extraction, alcohol precipitation, heating, or the addition of EDTA.

The amount of RNA that can be treated in a single DNA-free reaction depends on the amount of DNA contamination present. RNA isolated from spleen, kidney, thymus, for example, typically has significant DNA contamination. The RNA isolation procedure can also affect the amount of DNA present in an RNA preparation. For example, carry-over of the interphase from organic extractions, or overloading the silica matrix in solid-phase RNA purification methods can cause increased DNA contamination.

Procedure

1. Determine the volume of the RNA solution and add 0.1 volume of 10X DNase I Buffer and 1 µl of DNase I (2 units) to the RNA. For example, a 50 µl RNA solution will require 5 ml of 10X Buffer.

   For severely contaminated RNA preparations, dilute the sample to contain ~0.5 mg/ml of nucleic acid before DNase treatment, and use 2-3 µl of DNase and /or extend the incubation time to 1 hour.

2. Mix gently and incubate at 37°C for 20-30 min.
3. Resuspend the DNase Inactivation Reagent by flicking or vortexing the tube (solution should be homogenously white). Before reagent resettles, add 0.1 volume (original sample volume) or 5 µl, whichever is greater, of the slurry to the sample. (If more than 2 µl of DNase was used, add 0.2 volumes of slurry). Mix well.
4. Incubate the tube for 2 min at room temperature.

   Flick the tube at least once during the incubation to redisperse the DNase Inactivation Reagent.

5. Centrifuge the tube at 10,000 xg for 1 min to pellet the DNase Inactivation Reagent.

   Transfer RNA solution into a clean RNase-free tube for long term storage or before use for RT-PCR.

Notes: Make sure that the EDTA concentration in the RNA preparation is <0.1 mM before DNase I treatment. This enzyme requires divalent cations for activity.

Samples with excessive DNA contamination may benefit from a second round of DNase I treament with a preliminary heat denaturation step as follows: Heat RNA for 3 min at 95°C then quench on ice. This will denature any RNA:DNA heteroduplexes, which are poor substrates for DNase I.

RNeasy RNA Cleanup Procedure

Some researchers recommend an additional cleanup step for the RNA sample before using in a microarray experiment. This can be done using the RNeasy reagents as follows (procedure was adapted from user's instructions in the kit):

Notes: Do not exceed the RNA binding capacity (100 μg) of the RNeasy mini spin columns.

Buffer RLT may form a precipitate upon storage. If this happens, warm up to redissolve.

Add 10 μl of β-ME to 1 ml of Buffer RLT before use (stable for 1 month).

Buffer RPE is provided as a concentrate. Before using for the first time, add 4 volumes of ethanol (96-100%) as indicated on the bottle to obtain a working solution.

All centrifugations are done at room temperature.

1. Adjust sample to a volume of 100 μl with RNase-free water, add 350 μl Buffer RLT to the sample, and mix thoroughly.
2. *Note: Ensure that β-ME is added to Buffer RLT before use.*
3. Add 350 μl Buffer RLT to the sample, and mix thoroughly.
4. *Note: Ensure that β-ME is added to Buffer RLT before use.*
5. Add 250μl ethanol (96-100%) to the lysate, and mix well by pipetting. Do not centrifuge.
6. Apply sample (now 700 μl) to an RNeasy mini spin column sitting in a collection tube. Centrifuge for 15 seconds at 12,000 rpm.
7. Discard flow-through and collection tube.
8. Transfer RNeasy column into a new 2-ml collection tube. Pipet 500μl of wash Buffer RPE onto the RNeasy column, and centrifuge for 15 sec at 12,000 rpm to wash.
9. *Ensure that ethanol is added to Buffer RPE before use.*
10. Discard flow-through and reuse the collection tube in the following step.
11. Pipet 500 μl of wash Buffer RPE onto the RNeasy column. Centrifuge for 2 min at 14,000 rpm to dry the RNeasy membrane.
12. Following the spin, remove the RNeasy column from the collection tube carefully so that the column does not contact the flow-through as this will result in carryover of ethanol.
13. *It is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent reactions. The following spin ensures that no ethanol is carried over during elution.*
14. Place the RNeasy spin column in a new 2-ml collection tube, and discard the old collection tube with the filtrate. Centrifuge at 14,000 rpm for 1 min.
15. Transfer the RNeasy column into a new 1.5-ml collection tube, and pipet 30 μl of RNase-free water directly onto the RNeasy membrane. Centrifuge for 1 min at 12,000 rpm to elute.
16. *A second elution step can be performed using another 30-50 μl RNase-free water. This might improve yield.*
17. Dilute 1 μl of the reaction into 99 μl of water and use this for a spec reading using the Biophotometer (Eppendorf).
18. Store the rest at –80°C or proceed with the next step.

Solutions

Denaturing solution (4 M guanidinium thiocyanate, not used in this procedure, but it is similar to the Lysis solution):

- 25 g Guanidinium thiocyanate
- 0.25 g Sodium lauryl sarcosine
- 1.25 ml 1M Sodium citrate, pH 7.0
- 0.35 ml ß-mercaptoethanol

Bring the volume to 50 ml with water, stir until dissolved (may be heated to 65°C) and filter through 0.45 micron membrane.

Non-denaturing RNA-loading dye: (6X)

- 50% glycerol
- 1 mM EDTA, pH 8.0/0.25% bromophenol blue/0.25% xylene cyanol
- Make in DEPC H_2O, then autoclave

Phosphate Buffered Saline (1 liter):

- 8 g NaCl
- 0.2 g KCl
- 1.44 g Na_2HPO_4
- 0.24 g KH_2PO_4
- pH 7.2-7.4

10X MOPS-AE (100 ml):

- 0.2 M MOPS, pH 7.0 (20 ml of a 1.0 M solution pH 7.0 made in 0.05% DEPC-H_2O and autoclaved).
- 50 mM Na^+ Acetate (2.5 ml of 2 M NaOAc, made in 0.05% DEPC-H_2O and autoclaved).
- 10 mM EDTA, pH 8.0 (2.0 ml of 0.5 M EDTA, pH 8.0, made in 0.05% DEPC-H_2O and autoclaved).

Denaturing RNA loading dye (6 ml)

- 2.5 ml formamide(41.7%)
- 0.875 ml of 37% formaldehyde (5.4%)
- 0.5 ml 10X MOPS-AE (0.83X)
- 0.5 glycerol (8.3%)
- 0.025% xylene cyanol
- 0.025% bromophenol blue
- Add DEPC water up to 6 ml

Spectrophotometric analysis

1. To determine the concentration and purity of the RNA solution, transfer 2 µl of your RNA solution into an RNase-free tube containing 98 µl of DEPC-water. The lab instructors will measure the A_{260}/A_{280}. Pure RNA will give a ratio of approximately 2.0.

   1.0 A_{260}= 40 µg/ml RNA

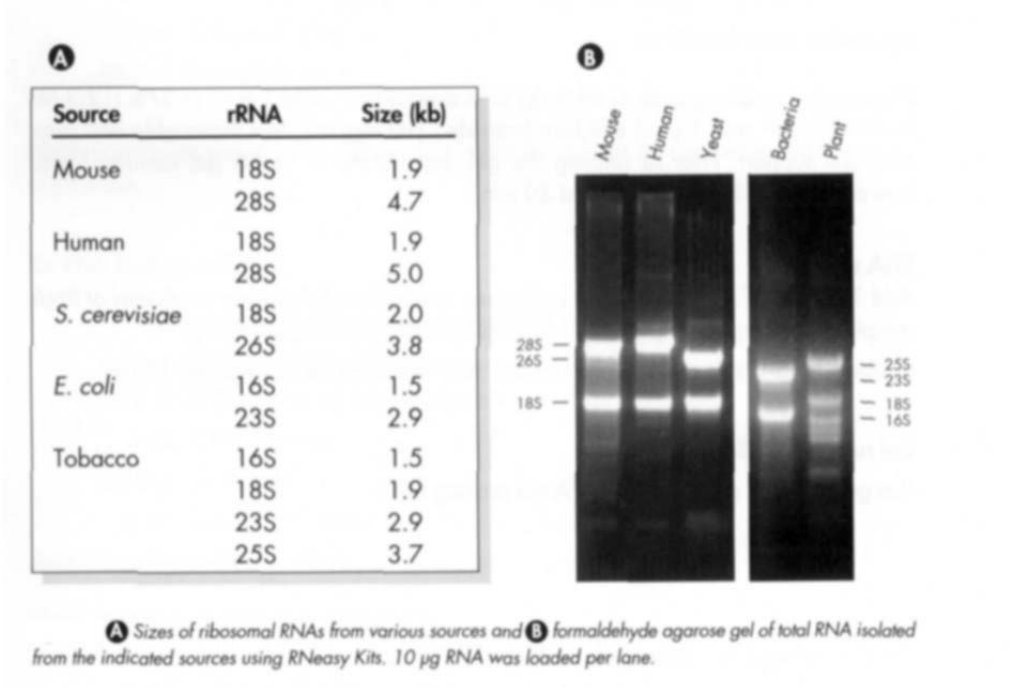
   Dilution factor in spectrophotometric cuvette= 50

   RNA solution conc. (µg/ml)= (A_{260})(100)(40µg/ml)

2. Check the integrity of the RNA by fractionation on a formaldehyde agarose gel as described below.

Formaldehyde agarose gels: Checking for RNA integrity

Good quality RNA in a gel should have the relevant ribosomal species (see figure below) as sharp band. 28S and 23S ribosomal RNA bands should be present at approximately twice the amounts of the 18S and 16S RNA. Possible RNA degradation is indicated by smear of the ribosomal RNA bands towards the lower molecular weight region of the lane.



A

Source	rRNA	Size (kb)
Mouse	18S	1.9
	28S	4.7
Human	18S	1.9
	28S	5.0
S. cerevisiae	18S	2.0
	26S	3.8
E. coli	16S	1.5
	23S	2.9
Tobacco	16S	1.5
	18S	1.9
	23S	2.9
	25S	3.7

A *Sizes of ribosomal RNAs from various sources and* **B** *formaldehyde agarose gel of total RNA isolated from the indicated sources using RNeasy Kits. 10 µg RNA was loaded per lane.*

Procedure (Wear gloves at all times)

1. Clean the gel apparatus thoroughly to inactivate RNases as follows: Add a small amount (~5 ml) of RNase ZAP™ (Ambion®, Inc., Austin, TX) solution into the bottom of the gel tray. Use four Kimwipes to gently scrub all surfaces that might come into contact with the RNA sample (e.g. comb, casting tray, wedges). Treat for 5 min and then discard the RNase ZAP™ solution. Rinse apparatus three times with 40 ml of DEPC-treated water.

2. Prepare a 1% formaldehyde agarose gel in an RNase-free, 250 ml Erlenmeyer flask (provided) by mixing the components listed below (Perform this task in collaboration with the group next to you. Therefore, follow the "2 gels" column):

Reagent	1 gel	2 gels
DEPC-treated H_2O	26.9 ml	53.8 ml
High melt agarose	0.31 g	0.62 g
10X MOPS-AE buffer*	3.1 ml	6.2 ml

* See MOPS-AE preparation below.

3. Heat this solution in a microwave oven (medium power) to dissolve the agarose. Do not allow to boil. Mix well and repeat heating to make sure agarose is completely dissolved.

4. Allow the molten agarose solution to cool to about 65°C (about 5 min), and then add 1.75 ml of 37% formaldehyde (3.5 ml for two gels). Work with the formaldehyde under a chemical fume hood. This makes a 0.66 M formaldehyde, agarose gel.

Warning: formaldehyde is a suspected liver carcinogen. Handle appropriately while working in a chemical fume hood.

5. Mix the agarose-formaldehyde solution well and pour into the clean gel apparatus. You should proceed with step 7 while the gel solidifies.

6. After the gel has solidified, add enough RNase-free, 1X MOPS-AE buffer to cover the gel. Remove the comb by shaking it and pulling it upwards.

7. Prepare RNA samples (from isolated RNA in Part 2d) by mixing the reagents listed in the Table below. In addition to two different amounts of your own RNA sample, you will be provided with 2 µl of RNA prepared by the lab instructors.

RNA Sample preparation for loading on the formaldehyde gel

	Sample 1 (µl)	Sample 2 (µl)	Sample 3 (provided) (µl)
RNA	1	3	2
*RNA loading dye	24	22	23

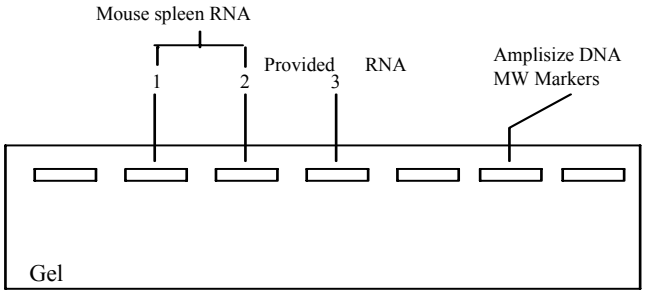
*Denaturing RNA loading dye recipe (see below)

8. Incubate at 60-65°C for 15 min to denature the RNA.

9. Using gloves, add 1 µl of ethidium bromide (200 µg/ml) per reaction.

Caution: ethidium bromide is a suspected carcinogen. Handle appropriately with gloves.

10. Mix and load the entire tube volume into the appropriate wells.



11. Electrophorese at 70 V for 1.5 hr.

12. At the end of the electrophoresis, use gloves to empty running buffer into an ethidium bromide disposal bottle. Rinse gel with water (sink) and bring to gel documentation station for photography.

Size of AmpliSize™ DNA size marker:

2,000
1,500
1,000
700
500
400
300
200
100
50

H. cDNA Synthesis (First-strand only) and Labeling

In this section you will be using reagents from the Strip-EZ RT kit (Ambion, Cat. # 1490) to produce unlabeled cDNA probes starting with RNA from either estrogenized male fish liver, or from its untreated counterpart. These probes will later be labeled nonradioactively with biotin (Section I). For gene arrays, it is recommended that cDNA synthesis is primed with Oligod(T) in order to minimize undesired probe synthesis from ribosomal RNA (since these species do not contain poly(A) tails. However, use of random sequence decamers generate probes of the highest specific activity when labeling is done during cDNA synthesis.

RNA has been isolated by lab instructors prior to the workshop. Based on the spectrophotometric reading of the total RNA, you are given 5 µl of total RNA to use in the 1st strand reaction. This is approximately 2 µg of RNA. **Work in groups of two.**

Procedure

1. Using an RNase-free 1.5 ml centrifuge tube, mix reagents according to the following Table.

STEP 1

Reagent	**Volume (µl)**
RNA (0.4 µg/µl)	5
Random decamers (10X)	2
TOTAL	7

2. Incubate @ 65°C in a water bath for 5 minutes.
3. Spin briefly and place on the bench at room temperature for 3-5 minutes.
4. When incubation is done, to the same tube, add reagents according to the Table below.

STEP 2

Reagent	**Volume (µl)**
Tube from step 1	7
10X RT buffer	2
0.5 mM dATP	1
10X dNTP mix	2
[α-^{33}P]dATP (3000 Ci/mmol, 10 mCi/ml)	2
DEPC-water	4
MMLV reverse transcriptase	2

TOTAL	20

5. Mix well with a pipette (carefully, in and out) and incubate at 37-42 °C, one hour.
6. Remove unincorporated dinucleotides as described in the next session.

I. Removal of Unincorporated Dinucleotides from cDNA

Procedure

This procedure utilizes a nucleotide removal kit provided by QIAGEN (QIAquick spin column, nucleotide removal kit, # 28304).

1. Add 10 volumes of Buffer PN to 1 volume of sample mix (e.g., add 200 µl Buffer for 20 µl sample).
2. Place a QIAquick spin column in a provided 2 ml collection tube.
3. Apply the sample to the center of the column and centrifuge for 60 seconds at 6,000 rpm (**sample binds**).
4. Discard flow-through containing unincorporated material (radioactive waste). Place the column into a new tube.
5. To wash, add 0.5 ml Buffer PE to the column and centrifuge for 60 sec (6,000 rpm).
6. Discard flow-through into radioactive waste and place the column back into a clean tube.
7. Wash again by adding 0.5 ml Buffer PE to the column and centrifuging for 60 sec (6,000 rpm).
8. Discard flowthrough and centrifuge the column for an additional 1 minute at maximumn speed (13,000 rpm) (Note: residual ethanol from Buffer PE may not be efficiently removed unless the flow-through is discarded before the additional centrifugation).
9. Place QIAquick column in a clean 1.5 ml microcentrifuge tube. Make sure tubes are well labeled.
10. To elute DNA add 50 µl of EB buffer to the center of the QIAquick membrane. **Wait** for 1 minute and centrifuge the column for 1 minute at 6,000 rpm (Note: Maximum elution efficiency is pH dependent. Elution can also be accomplished with TE: 10 mM Tris.Cl, 1 mM EDTA, pH 8; however, EDTA may inhibit subsequent enzymatic reactions).
11. Pipet 1 µl of clean probe into a scintillation vial containing scintillation fluid. Read sample in a scintillation counter and estimate specific activity.
12. Store at -20°C until probe is ready to use.

J. Membrane Prehybridization and Hybridization

Suitable recipes for prehybridization and hybridization solutions cna be found in most manuals of basic moleclar biology techniques. In this lab, we will be using the ULTRAarray™ hybridization buffer (Ambion). This proprietary recipe increases sensitivity by 10-100 fold over commonly used hybridization buffers.

Procedure

1. Using gloves transfer the spotted membrane onto a 50 ml conical tube. Your membrane and your conical tube must be labeled in a unique manner (include your group number).

2. Add 5 ml of ULTRAarray™ hybridization buffer and incubate in hybridization oven (rotating) for 1 hour at 64°C.
3. Shortly before the end of prehybridization, calculate the amount of labeled probe that you need for a total of $5X10^6$ dpm (for example, if you had a clean probe solution at 300,000 dpm/µl, you'll need a total of 16.7 µl).
4. Dissolve your probe stock (e.g. 16.7 µl in the example above) in 20 times the volume of 10 mM EDTA, pH 8.0. For example, if you calculated that you'll need 16.7 µl of probe, mix it with 334 µl of 10 mM EDTA.
5. Heat the diluted probe for 5 min at 95°C, followed by rapid cool on ice (2 minutes). Make sure your tube has a lid lock in order to avoid possible spill of radioactive material.
6. Pulse-spin (5 sec) and transfer denatured probe directly onto the prehybridization tube (i.e., 50 ml conical tube containing the membrane in 5 ml prehybridization solution).
7. Hybridize for 12 hours or overnight at 64°C.
8. At the end of hybridization, discard solution into radioactive waste container, and rinse the membrane at room temperature with 15 ml of low stringency solution (1 minute).
9. Wash blot 4 times, 15 minutes each, with 10 ml of low stringency solution (2X SSC, 0.5% SDS), 64°C. Alternatively, membranes can be washed twice, 30 minutes each time.
10. Wash blot 4 times, 15 minutes each, with 10 ml of high stringency solution (0.5X SSC, 0.5% SDS), 64°C. Alternatively, membranes can be washed twice, 30 minutes each time.

K. Detection

1. Following high stringency washes, transfer the membrane onto a piece of filter paper. Blot any residual puddles of stringent solution that might still be on the membrane.
2. Cover the membrane with a piece of saran wrap and bring to the lab instructors who will expose all membranes to an X-ray film overnight.

Appendix

Appendix 1: Solutions for Tris-Tricine SDS-PAGE

Anode buffer: (Bottom) (10X Stock) (For 500 ml)

Reagent	Amount
2 M Tris, pH 8.9	121 g

To make 1X anode running buffer: Mix 40 ml 10X stock buffer with 360 ml H_2O before use.

Cathode buffer: (Top) (10X Stock) (For 500 ml)

Reagent	Amount
1 M Tris, pH 8.25 (do not adjust pH)	60.5 g
1 M Tricine	90 g
1% (w/v) SDS	25 ml of 20%

To make 1X cathode running buffer: Mix 20 ml 10X stock buffer with 180 ml H_2O before use.

Gel buffer : (10X Stock) (For 200 ml)

Reagent	Amount
3 M Tris, pH 8.45	72.6 g
0.3% (w/v) SDS	3 ml of 20%

20% (w/v) SDS:

Reagent	Amount
SDS	20 g/100 ml

30% acrylamide:

Reagent	Amount
Acrylamide	30 g/100 ml

pH > 6.0

40% (w/v) acrylamide:

Reagent	Amount
Acrylamide	40g/100 ml

pH > 6.0

2% (w/v) Bis-acrylamide: (crosslinker)

Reagent	Amount
Bis-acrylamide	2 g/100 ml

pH > 6.0

Laemmli sample buffer: (2X LSB)
(For 10 ml)

Reagent	Amount
62.5 mM Tris, pH 6.8	1.25 ml of 0.5 M
2% (w/v) SDS	1 ml of 20%
720 mM 2-mercaptoethanol	0.5 ml of 14.4 M
Glycerol	2 ml
0.1% (w/v) Bromophenol blue	10 mg

This sample buffer is for SDS-PAGE reducing gels (2-mercaptoethanol is the reducing agent). The glycerol increases the density of the samples so they will sink into the wells when loading.

TEMED (N,N,N',N',-tetramethylethylenediamine): Catalyst of acrylamide/ bis-acrylamide polymerization.

10% (w/v) Ammonium persulfate: Initiator of acrylamide/bis-acrylamide polymerization.

Useful proportions for separating and stacking gel mixtures

Separating Gel Mix	For 10% T*/3% C**		For 15% T/3% C	
	200 ml solution	6 ml solution	120 ml solution	6 ml solution
Gel Buffer	67 ml	2.01 ml	40.0 ml	2 ml
30% or 40% (w/v) Acryl.	67 ml (30%)	2.01 ml (30%)	45 ml (40%)	2.25 ml(45%)
2% (w/v) Bis	31 ml	0.93 ml	27.3 ml	1.37 ml
20% (w/v)SDS	1 ml	30 µl	0.6 ml	30 µl
H_2O	34 ml	1.02 ml	7.1 ml	0.355 ml
TEMED	-	2.5 µl/6 ml gel	-	2.5 µl/6 ml gel
10% (w/v) APS	-	25 µl/6 ml gel	-	25 µl/6 ml gel

*T = Total sum of the weights of the acrylamide monomer and the crosslinker (bis). For example, 10% T gel would contain 10% (w/v) of acrylamide plus bis. As the %T increases, the pore size of the gel decreases.

$$\%T = \frac{\text{Acryl. Weight + Bis weight}}{\text{Volume}} \times 100\%$$

**C = Percent crosslinker of the total sum of monomer and crosslinker (bis). For example, a 10%T/3%C gel would have 10% (w/v) of acrylamide plus bis solution, and the bis would account for 3% of the total weight of the acrylamide plus bis.

$$\%C = \frac{\text{Bis weight}}{\text{Acryl. Weight + Bis weight}} \times 100\%$$

Stacking Gel	3% T/3% C	
	50 ml solution	2.5 ml solution
Gel Buffer	12.4 ml	0.62 ml
30% (w/v) Acrylamide	5 ml	250 µl
2% (w/v) Bis	2.4 ml	120 µl
20% (w/v) SDS	250 µl	12.5 µl
H_2O	30 ml	1.5 ml
TEMED	-	2.5 µl/2.5 ml gel
10% (w/v) APS	-	25 µl/2.5 ml gel

Appendix 2: Silver staining of SDS-PAGE gels

Silver Staining is a very sensitive method for detecting proteins. As little as 2 ng of protein in a single band can be visualized. It is roughly 20 times more sensitive than Coomassie blue stain. This sensitivity is useful for a pilot analysis of the effectiveness of endoprotease digestion. Only a small amount of the protein for digestion is needed (approx. 9 μg of a 30 kd protein, or 50 pmol) for the endoprotease products to be detectable. Once the conditions of the experiments are optimized, the reaction can be scaled up for electrophoretic transfer onto a PVDF membrane followed by Coomassie blue staining and micro sequencing of the internal peptides.

Procedure

1. Prepare solutions A, B and C (see below).
2. Add silver stain solution "C" to gel and incubate on a shaker for 15 min with constant but gentle agitation. Decant solution into the waste container.
3. Rinse gel twice in deionized water, then soak in water for 2 min. with gentle agitation.
4. Prepare solution D.
5. Add solution D (Developer) to gel in container. Shake gently and constantly until bands appear (less than 10 min). Develop to desired intensity. If a pale yellow background appears, reaction should be stopped. Decant solution into the waste container.
6. Stop development by rinsing in 1% acetic acid.
7. Photograph the gel using the light box and Polaroid camera.

- If protein deposits are too dark, de-stain gel with Kodak Rapid Fix. Wash with distilled water 3 times. Stop de-stain with Kodak hypo clearing agent. Then wash in 50% methanol/10% acetic acid.
- If desired, the silver-stained gel can be soaked in 10% ethanol/5% glycerol for 30 min and dried overnight.

Solutions

Solution A:
0.8 g silver nitrate in 4 ml distilled water.

Solution B:
18.8 ml water plus 190 μl of 10 N NaOH (Final conc. 100 mM NaOH).
Then add 2 ml 14.8 M (30%) ammonium hydroxide.

Solution C:
Add entire solution A to Solution B drop-wise with constant vigorous stirring, allowing brown precipitate to clear. Add water to 100 ml. Use solution C within 15 min.

Solution D: (Developer)
Mix 0.5 ml 1% citric acid with 50 μl 38% formaldehyde, add water to 100 ml. Solution must be made fresh.

Appendix 3: Endoproteinase Digestion of Proteins for sequencing of internal fragments

Recent technical advances have made protein sequencing a relatively routine procedure. Sequential digestion of the N-terminal end of polypeptides is done by Edman degradation and the product from each cycle detected by HPLC. This procedure can only be performed a relatively small number of times (30-35 cycles) with a reliable signal to noise ratio for the N-terminal product. In some cases, the N-terminus of a protein may be blocked and resistant to the Edman chemistry. For these reasons, most proteins must first be fragmented into a set of polypeptides of smaller MW suitable for Edman degradation. The sequence of the original protein can be assembled from a series of overlapping sub-fragments. Several endoproteinases have been identified for this purpose. The choice of the endoproteinase and the experimental conditions of the digestion should be such that distinct, low MW bands can be identified by SDS-PAGE (using the Tris/Tricine system).

In this appendix, we describe there are two pilot experiments, one using Endo Lys-C to digest CAII (as an example) and another using Asp N. Once the conditions have been optimized, the experiment can be scaled up. The protein fragments can be fractionated by SDS-PAGE, transferred into a PVDF membrane and stained. Micro sequencing of each protein band can be performed directly on the membrane.

EndolysC procedure

Wear Gloves!!!

1. Using a clean blade cut out a gel slice from an SDS-PAGE gel containing the protein of interest (e.g., hCAII). Use forceps to transfer gel slice to a 1.5 ml microcentrifuge tube (4 μg represents about 118 pmol, although 50 pmol is usually enough. CAII MW = 30 kd).
2. Wash the gel slice 4 times with 1 ml of water (each wash) over 15 min. Discard washes by pipetting into a waste container.
3. Add 1 ml of extraction buffer and incubate at RT for 5 min. Discard the extraction buffer.
4. Add 100 μl of fresh extraction buffer and macerate the gel using the small, plastic pestle provided.
5. Add 100 μl of extraction buffer into a separate microcentrifuge tube as a control.

   Extraction buffer:
   (5 ml solution, already prepared by the laboratory staff)

Reagent	Amount
0.1% SDS	25 μl of 20% stock
50 mM Tris, pH 8.8	160 μl of 1.5 M stock
0.1 mM EDTA	5 μl of 100 mM stock (pH 8.0)
0.2 M ammonium bicarbonate	79 mg

6. For complete digestion, add 0.003 U enzyme per μg protein in the gel slice. Since your gel slice should contain 4 μg protein, 0.012 U of Endo Lys-C is provided in 5 μl of solution. Add 5 μl of the protease to both the protein sample and the control tube.
7. Vortex both samples for 1 min.
8. Incubate both samples at 37°C overnight (about 16 hr).

Next day: For both CAII gel sample and control.

9. Heat the Endo Lys-C digestion tubes at 85°C for 5 min.
10. Allow the digests to cool to room temperature and spin at 5000 rpm for 30 sec. in the microcentrifuge.
11. Transfer the supernatant fluid (not necessary for the control) into a 2 ml- micro filtration unit (spinX, 0.22 μm) and centrifuge at 5000 rpm for 1 min.

12. Transfer 100 µl from the control and 100 µl from the spinX unit into separate microcentrifuge tubes.
13. Acetone precipitate the 100 µl samples by adding 9 volumes ice-cold acetone (900 µl) and let sit either overnight at -20°C or 2 hr in an EtOH-dry ice bath.
14. Centrifuge at 12,000 rpm for 20 min to pellet the precipitate. Carefully remove as much of the acetone supernate as possible with a pipet
15. Air dry the pellets at 37°C for 3 min.
16. Add 15 µl Laemmli sample buffer (LSB) and 15 µl of water to each tube.
17. The entire volume of the sample (30 µl) can be loaded onto a Tris-tricine gel.
18. Silver stain the gel and identify protein fragments that are not located in the control. These are the bands of interest, since endoproteinases will self hydrolyze.

Asp N Procedure

1. Transfer one gel slice into a special 1.5 ml microcentrifuge tube. (Each band contains about 118 pmol, although 50 pmol is usually enough).
2. Wash band 4 times with 1 ml water over 15 min. Discard washes into waste container.
3. Wash once with 1 ml extraction buffer for 5 min.
4. Add 200 µl fresh extraction buffer and macerate the gel using the small, plastic pestle provided. Add 200 µl also to a control tube.

Extraction buffer (previously prepared by laboratory staff):
(5 ml solution)

Reagent	Amount
0.01% SDS	2.5 µl of a 20% stock
50 mM Tris, pH 8.8	160 µl of a 20% stock
0.2 M ammonium bicarbonate	79 mg

5. For complete digestion add 1 µg enz/ 10 µg protein in the slice.
6. Let digestion go overnight at 37°C (about 16 hr).

Next day

7. Add 1 µl of 10% (w/v) SDS and 1 µl of 10 mM EDTA (pH 8.0) so that protein extraction from the gel is done efficiently.
8. Heat sample at 85°C for 5 min.
9. Allow to cool to about room temperature and spin at 5000 rpm for 30 sec.
10. Transfer the supernatant fluid (not necessary for the control) into a 2-ml micro filtration unit (spinX, 0.22 µm) and centrifuge at 5000 rpm for 1 min.
11. Speed vac down sample and control to 100 µl.
12. Acetone precipitate by adding 9 volumes ice-cold acetone (900 µl) and let sit either overnight at -20 or 2 hr in an EtOH-dry ice bath.
13. Centrifuge at 12000 rpm for 20 min to precipitate pellet. Remove as much acetone as possible.
14. Air dry sample at 37°C.
15. Add 15 µl Laemmli sample buffer (LSB) and 15 µl water.
16. Load onto a Tris/Tricine gel and stain with silver nitrate stain.

Appendix 4: Synthesis and Biotin Labeling of a CAII RNA Probe by *in vitro* Transcription

Background

(Parts of this section are adapted from "Molecular Cloning: A laboratory Manual" Sambrook *et al.* pp. 10.27-10.37, (1989) and Ambion's BIOTINscript™ and RPA II Manuals (Ambion, Inc. 1995))

A preliminary requirement for a ribonuclease protection assay (RPA) is the synthesis of the labeled antisense RNA transcript which is used as a hybridization probe for detection of the mRNA of interest. The technical advance that made it possible to synthesize single-stranded RNA probes of high specific activity was the development of plasmid vectors containing polycloning sites placed downstream from powerful transcriptional promoters derived from the *Salmonella typhimurium* bacteriophage SP6 (Green *et al.* 1983) or from *the E. coli* bacteriophages T_7 and T_3 (Studier and Rosenberg, 1981; Davanloo *et al.*, 1984; Tabor and Richardson, 1985). These transcription promoters are recognized specifically by DNA-dependent RNA polymerases encoded by their respective bacteriophages. Each enzyme has a distinct specificity for its cognate promoter and does not use promoters recognized by other polymerases. In addition, these bacteriophage enzymes do not recognize bacterial or plasmid promoters or eukaryotic promoters in cloned DNA sequences. Furthermore, they do not efficiently initiate transcription at nicks in double-stranded DNA templates, will transcribe homopolymeric tracts and are able to transcribe long segments of cloned DNA *in vitro*. Thus when a linearized plasmid is incubated *in vitro* with the appropriate RNA polymerase and the four rNTPs, virtually all RNA synthesis is initiated at the bacteriophage promoter. The transcripts (single-stranded), which terminate at the end of the linear DNA molecule can be used as strand-specific reagents in hybridization reactions.

The use of RNA probes in Ribonuclease Protection Assays is better than using single-stranded DNA probes for several reasons:

- Labeling of RNA is much more efficient than labeling of single-stranded DNA. The yield of RNA can exceed the weight of the template several fold because the templates used to synthesize the RNA can be transcribed many times.
- Bacteriophage DNA-dependent RNA polymerases function efficiently *in vitro* in the presence of low concentrations of rNTPs (1-20 μM), so that full length probes of high specific activity can be synthesized inexpensively.
- RNA probes generally yield stronger signals in hybridization reactions than do DNA probes of equal specific activity. This is due to the innately higher stability of hybrids involving RNA (Casey and Davidson, 1977).
- RNA probes do not have to be denatured because they are already single-stranded.

RNA probes can be labeled radioactively or nonradioactively. The latter of these methods has become increasingly popular with the advent of chemiluminescent detection methods. Besides avoiding the obvious inconvenience of handling and disposing radionuclides, non radioactive probes can be stored for over a year without any loss in specific activity. Any of several commercially available *in vitro* transcription kits can be used in conjunction with a modified nucleotide to obtain a nonisotopically labeled probe. Commercial sources of modified nucleotides include: Biotin-14-CTP (GIBCO-BRL), Biotin-16-UTP (Boehringer Mannheim Corp. or Enzo Diagnostics), Digoxigenin-11-UTP Boehringer Mannheim Corp.) and Fluorescein (Boehringer Mannheim Corp.).

Biotin can be incorporated into the probe by chemical crosslinking (e.g. Ambion's BrightStar™ Psoralen-Biotin™ Nonisotopic Labeling Kit) or by including a biotinylated nucleotide in the transcription reaction mixture. Once labeled, nonisotopically modified probes can be used in ribonuclease protection assays (RPA), Northern, Southern and dot blots. Synthesis of nonisotopically labeled probes is quite robust since there is no limiting nucleotide, and 4 μg of transcript can be synthesized from each reaction. This is sufficient probe for hundreds of assays (Northern, Southern and dot blots require about 10 ng probe, RPA require 0.2-0.8 pg probe).
The presence of the modified nucleotide may affect the efficiency of the labeling reaction, as well as the effectiveness of the probe in subsequent applications. The type, size and location of the modification on the

nucleotide will determine how well it is incorporated into the transcript by the RNA polymerase. Moreover, the modified nucleotide within the probe might also interfere with hybridization of the probe to the target sequence through steric hindrance. In the case of RPA, nuclease digestion becomes an additional concern.

In this experiment, Biotin-16-UTP will be used to label an RNA probe for murine CAII (mCAII). A linearized plasmid containing a 554 bp fragment of mCAII is used as a template in the *in vitro* transcription reaction. In addition, a ß-actin probe will be synthesized and used as an internal control. Such a control is used in cases when it is desirable to determine relative amounts of a certain message in RNA isolated from several tissues.

References

Casey, J. and Davidson, N. (1977) Rates of formation and thermal stabilities of RNA:DNA and DNA:DNA duplexes at high concentrations of formamide. *Nucl. Acids Res.* **4**: 1539-1552.

Davanloo, P., A.H. Rosenberg, J.J. Dunn, and F.W. Studier (1984) Cloning and expression of the for bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci.* **81**:2035

Green, M.R., T. Maniatis, and D.A. Melton. (1983) Human ß-globin pre-mRNA synthesized *in vitro* is accurately spliced in *Xenopus* oocyte nuclei. *Cell* **32**: 681.

Studier, F.W., and A.H. Rosenberg. (1981) Genetic and physical mapping of the late region of bacteriophage T7 DNA by use of cloned fragments of T7 DNA. *J. Mol. Biol.* **153**: 503

Part A: Synthesis of DNA template

Note: This Part would have been done in advance by the Lab instructors.

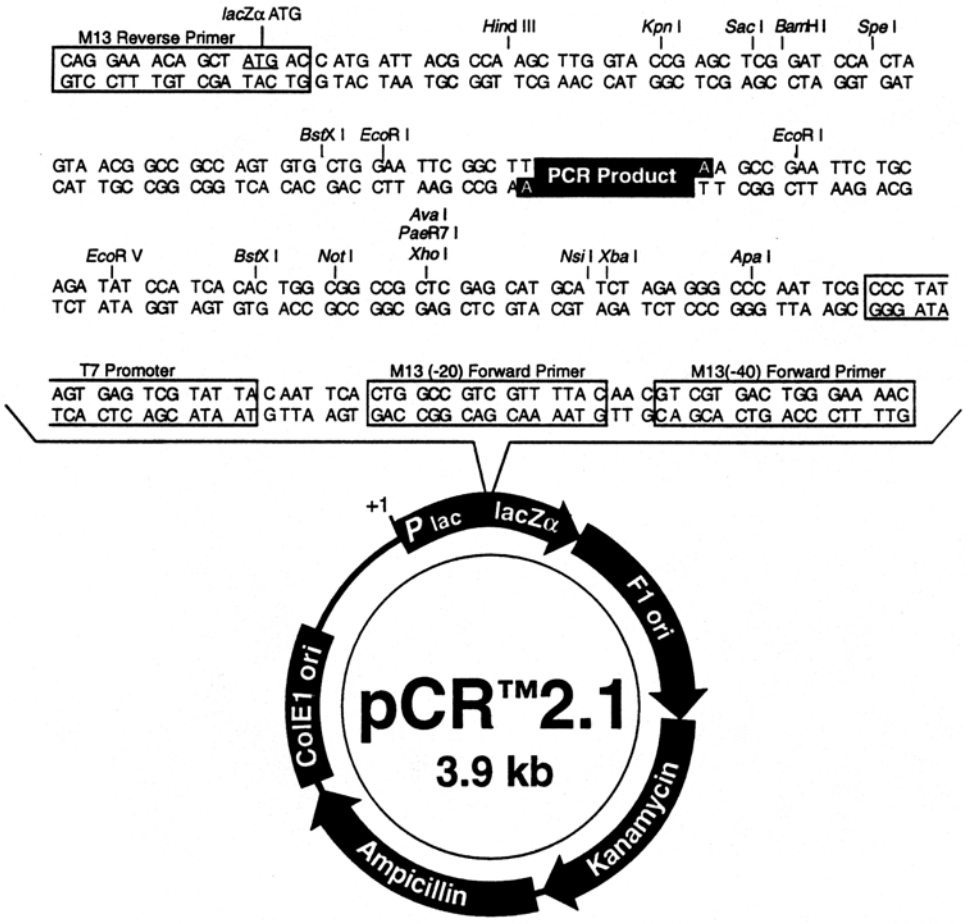
Background

(Parts of this section are adapted from: Ambion's BIOTINscript™ Manual; Ambion, Inc. 1995).
During *in vitro* transcription reactions, RNA molecules are synthesized by run-off transcription from a DNA template. There are three varieties of transcription templates that can be used. The most common is in the form of a linearized plasmid. The fundamental requirement is that the desired transcript is cloned downstream from a strong bacteriophage promoter (e.g. SP6, T_7 and T_3). The second type of transcription templates is PCR products in which a bacteriophage polymerase promoter has been incorporated into the 5'-end of one of the PCR primers. A third alternative is to use synthetic oligonucleotides (short probes). For this approach, two oligonucleotides need to be synthesized - the bottom strand includes sequence for a phage promoter (-17 through +6) and the coding sequence to generate the desired transcript; the top strand only needs to have the complementary promoter sequence from minus 17 through -1.

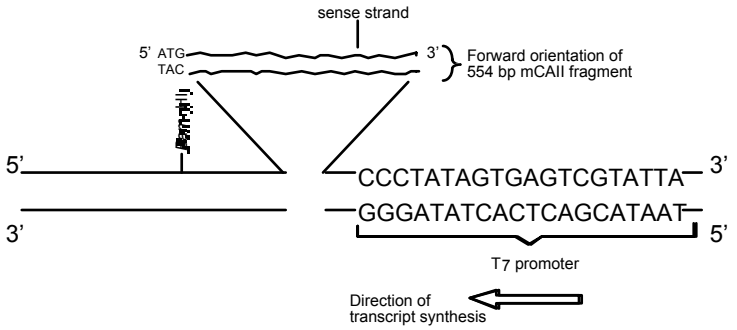
In this experiment, a plasmid (pCR™2.1) containing a 534 bp fragment of the CAII cDNA (between positions 142 and 676 of the CAII cDNA sequence, Accession Number K00811) was linearized by restriction digestion using *Bam*HI or *Eco*RI, enzymes that generate a 5' overhang**.** Enzymes that generate blunt ends can be also utilized for linearization, but enzymes that create protruding 3' termini (e.g. *Kpn* I or *Pst* I) are not used because such termini may serve as promoter-independent transcription sites for the RNA polymerase. These illegitimate transcription products may co-migrate with the probe during gel purification and give rise to background bands by hybridizing to the probe. A map of the pCR 2.1 plasmid is given below (Invitrogen®'s Original TA Cloning® Kit Manual).

Note that the 554 bp fragment is cloned at the site in between the two *Eco* R I sites in the pCR 2.1 vector (i.e. "PCR product" site) and that the T_7 promoter is located to the right of the "PCR product" cloning site. This means that the 554 bp fragment must be cloned in the forward orientation, that is, the orientation in which the ATG-containing strand (sense strand) of the CAII cDNA can be read in the usual manner (5'--3'). Choosing the appropriate orientation of the PCR insert in the plasmid construct is essential, so that the run-off transcript synthesized by T_7 polymerase corresponds to the antisense strand (complement of the mRNA). Recall that the

cDNA sense strand corresponds to the mRNA sequence. The orientation of the 554 bp CAII fragment was determined by restriction enzyme analysis using *Pst* I. Upon digestion with this enzyme the "forward" orientation construct yields three fragments of sizes: 2959, 1167 and 334 bp. In constrast, the "reverse" orientation construct yields fragments fo 3047, 1167 and 246 bp.



This plasmid has a cloned copy of the bacteriophage T_7 promoter adjacent to the polycloning site. Therefore, a clone with the CAII cDNA fragment in the appropriate orientation was selected in order to transcribe the antisense strand. However, many of the available plasmids contain two different bacteriophage promoters (T_7 and SP6) so that any of the DNA strands can be transcribed by simply choosing the appropriate RNA polymerase. In other words, the antisense strand can be transcribed from a plasmid construct containing the insert in any of the two possible cloning orientations, provided the appropriate RNA polymerase is used.



RNA probe synthesis by *in vitro* transcription

Part B: Synthesis of RNA probe by *in vitro* transcription

Note: This part has been done in advance by Lab instructors. You will be using a CAII and a ß-actin probes in Experiment 21. The conditions and components of the reaction for the synthesis and labeling of the CAII probe are included here for reference. The probe generated from the template in this procedure contains complementary sequences to the target mRNA as well as plasmid sequences that are not expected to be complementary to the target. This is a convenient feature of the probe since the size of the free probe can be easily differentiated in denaturing gels from the protected fragment in the RPA experiment.

B.1 Transcription Reaction Procedure

1. Working at room temperature, add the reagents listed in the table below paying attention to the suggested order (Note: spermidine present in the transcription buffer can cause precipitation of template DNA at lower temperature. The phage RNA polymerase should be maintained on ice at all times).

Reagent	mCAII sample	ß-actin control
-Nuclease-free water	10 µl	10 µl
-10X Transcription buffer*	2 µl	2 µl
-10 mM ATP	1 µl	1 µl
-10 mM CTP	1 µl	1 µl
-10 mM GTP	1 µl	1 µl
-10 mM UTP	0.6 µl	0.6 µl
-10 mM Biotin-16-UTP	0.4 µl	0.4 µl
-linearized pCR2.1/mCAII template** (0.5 µg/µl)	2 µl	none
-linearized pTRIPLEscript plasmid*** (0.5 µg/µl)	none	2 µl
-T7 RNA polymerase + RNase inhibitor (5 U/µl each	2 µl	2 µl

*10X Transcription Buffer:
400 mM Tris.Cl (pH 7.5 at 37°C)
60 mM $MgCl_2$
20 mM Spermidine
50 mM NaCl
1 mg/ml bovine serum albumin
100 mM DTT
** contains a 554 bp fragment of murine CAII gene in the antisense orientation under the transcriptional control of T_7 promoter.

*** contains a 250 bp fragment of mouse ß-actin gene in the antisense orientation under the transcriptional control of T_7, T_3 or SP6 promoter.

2. Incubate the reaction mixture at 37°C for 2 hr.

> Note: In some cases the proportion of full-length transcripts may be maximized by carrying out the transcription reaction at room temperature 15°C or at a temperature as low as 4°C.

DNase Digestion

1. Heat the transcription reaction to 95 °C for 5 minutes.
2. Chill on ice.
3. Add 1 µl of DNase I.
4. Incubate at 37 °C for 15 minutes.

B.2 Gel purification of the CAII RNA probe (polyacrylamide/ Urea gel)

Introduction:

Gel purification of probe is not necessary for Northern blots. In such case, precipitation with Lithium chloride or ammonium acetate/ethanol is adequate for removal of unincorporated nucleotides. Spin column filtration is an alternative procedure with equivalent results to the probe precipitation procedures. However, for Ribonuclease Protection Assays (RPA) is important to purify the probe by gel electrophoresis in order to make sure that only full-length probe is used in the hybridization step. In Northern blots, since hybridization is done by applying the probe solution to the RNA target on a membrane, even truncated probe will result in a sharp-band signal, provided the target RNA was intact. In contrast, in the RPA the hybridization step is done in solution, and is followed by nuclease digestion of the single-stranded portion of the target that did not hybridize to the probe. Consequently, truncated probe molecules will result in increased (sometimes excessive) background signal below the expected molecular mass of the probe.

The polyacrylamide gel procedure described below works well for purification of biotinylated mCAII RNA probe to be used in the RPA. All surfaces of the gel apparatus that come into contact with the gel should be soaked overnight in 0.1 N NaOH or cleaned thoroughly with RNase ZAP (i.e. comb, gel casting tray, damns and buffer chamber). 5X and 1X TBE must be prepared with DEPC-water and filtered through a 0.45 µm sterile filter.

Procedure:

1. Prepare a 5% Acrylamide/8M Urea denaturing polyacrylamide gel by mixing the following reagents (15 ml):

Reagent	*Amount*
-High quality urea	7.2 g
-5X TBE	3.0 ml
-40% acrylamide (19:1 acrylamide:bisacryl.)	1.875 ml
-DEPC water	up to 15 ml (mix well at RT)
-10% ammonium persulfate in water (fresh)	120 µl
-TEMED	16 µl

2. Pour into minigel system and allow to polymerize for 30 min.
3. After gel has polymerized, add an equal volume of Gel Loading Buffer (GLB) to the ß-actin control probe and the mCAII probe. (GLB: 95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 0.025% SDS, 0.5 mM EDTA).
4. Heat probe samples at 95 °C for 5 minutes to denature any RNA probe secondary structure and then place on ice to prevent renaturation (Gel migration of undenatured RNA is aberrant).

5. Flush urea from wells with tank buffer (1X TBE) using a syringe/needle or a P-1000 pipetor.
6. Load the entire probe samples onto separate wells on the gel.
7. Electrophorese at 200 volts (minigel) for 30 minutes until the blue dye front (fastest moving) reaches the bottom of the gel.
8. After electrophoresis, remove one of the glass plates and cover the gel with plastic wrap. Place the gel, gel side down, on a flat surface and carefully remove the gel from the second glass plate. Now cover other side of the gel with plastic wrap.
9. In a darkened room, place the plastic-wrapped gel on top of the white, opaque side of a fluor-coated TLC plate and visualize the bands by shining a hand-held UV light source (254 nm) on the surface of the gel (directly from above). The RNA trancripts appear as purple "shadow" bands.
10. Excise tightly the band representing the full-length transcript using an RNase-free razor (i.e. cleaned with RNase ZAP). The mCAII probe should be about 554 bases and should appear larger than the ß-actin control RNA probe (250 bp).

> Note: RNA transcripts may also be visualized by staining the gel with ethidium bromide or acridine orange. However, the stain should be removed before hybridization as it may compromise hybridization eficiency.

Probe Elution, Quantitation and Storage

1. Using a RNase-free blade, cut gel fragment into small pieces and transfer the pieces to a nuclease-free tube containing 350 µl of elution buffer (elution buffer: 0.5 M NH_4OAc/ 1 mM EDTA/ 0.2% SDS). EDTA and SDS are included in the elution buffer to inactivate low levels of nuclease.
2. Incubate the tube overnight (12-16 hours) at 37 ^oC.
3. Next day, pipet 2.5 µl of eluted probe into a tube containing 197.5 µl water. Mix well and transfer into a spectrophotometric cuvette.
4. Measure the Absorbance at 260 nm.
5. Calculate the yield of probe synthesis by assuming 1 A_{260}= 40 µg/ml RNA
6. Aliquot probe in 5 µl portions in nuclease-free tubes and store at -80 ^oC. Stability of the probe is approximately one year under these conditions. Avoid repeated freezing and thawing of probe solutions.

Appendix 5: Ribonuclease Protection Assay

Background (Adapted from: Ambion's RAPII Manual, Ambion, Inc. 1995)

The Ribonuclease Protection Assay (RPA) is an extremely sensitive procedure for the detection and quantitation of RNA species (usually mRNA) in a complex sample of total cellular RNA. For the RPA, a labeled RNA probe is synthesized that is complementary to a portion of the target RNA to be analyzed. This is done by cloning the probe fragment into one of the common transcription vectors under control of a bacteriophage promoter (either the T_3, T_7, or SP6 promoter) and using the corresponding T_3, T_7, or SP6 RNA polymerase to generate an RNA transcript of high specific activity. The labeled probe is then mixed with the sample RNA and incubated under conditions that favor hybridization of the complementary transcripts. After hybridization, the mixture is treated with ribonuclease to degrade single-stranded, unhybridized probe. Labeled probe that hybridized to complementary RNA in the sample mixture will be protected from ribonuclease digestion, and can be separated on a polyacrylamide gel and visualized by autoradiography. When the probe is present in molar excess over the target mRNA species in the hybridization reaction, the intensity of the protected fragment will be directly proportional to the amount of complementary mRNA in the sample mixture.

Compared to hybridization protocols that rely on RNA bound to a solid support (i.e. Northern blots), low abundance mRNAs are detected more readily and quantified more accurately by RPA because hybridization is allowed to occur in solution (Lee and Costlow, 1987). Since the probes used in RPA are generally significantly shorter than the mRNA species being detected, the target RNA preparation need not be completely intact (breaks in mRNA that occur outside the region that hybridizes to the probe will have no effect on the RPA, but will result in loss of signal on Northern blots). Due to the high resolution of the acrylamide gel system used to analyze the protected fragments, the RPA is well-suited for mapping positions of internal and external junctions in mRNA, for example transcription initiation and termination sites and intron/exon boundaries (Kekule *et al.*, 1990, Melton *et al.*, 1984, and Calzone *et al.*, 1987). By adjusting the ribonuclease concentration in the digestion reaction, the assay can be used to detect small differences between the probe and target mRNA. Ribonuclease protection can therefore be used to distinguish between different mRNAs coded for by genes of multigene families which cross-hybridize or show a single band on Northern blots.

References

1. Calzone, F.J., R.S. Britten, and E.H. Davidson. 1987. Mapping of gene transcripts by nuclease protection assays and cDNA primer extension. *Meth. Ezymol.* **152**: 611-632.
2. Kekule, A.S., U. Lauer, M. Meyer, W.H. Caselmann, P.M. Hofschneider, and R. Koshy. 1990. The pre S2/S region of integrated hepatitis B virus DNA encodes a transcriptional transactivator. *Nature* **343**: 457-461.
3. Lee, J.J., and N.A. Costlow. 1987. A molecular titration assay to measure transcript prevalence levels. *Meth. Enzymol.* **152**: 633-648.
4. Melton, D.A., P.A. Krieg, M.R. Rebagliati, T. Maniatis, K. Zinn, and M.R. Green. 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucl. Acids Res.* **12**: 7035-7056.

Part A: Hybridization and ribonuclease digestion

Procedure

> Note: You will be given biotin-labeled CAII and ß-actin probes (400 pg/µl solution). Using 2 µl of this probe solution (i.e. 800 pg) is sufficient to obtains a molar excess of probe over target, for even moderately abundant mRNAs such as ß-actin in 20 µg sample of total RNA.

1. Adjust the concentration of the RNA sample you isolated from mouse spleen (Exp. 15) to 0.5 µg/µl using DEPC-treated water.

2. Prepare RNA samples as indicated in the following table:

Nº	Mouse spleen RNA (µl)	CAII probe (µl)	ß-actin probe (µl)	DEPC-Water (µl)
1	2	2	2	4
2	6	2	2	-
3	2	-	2	6

3. Add 1.1 µl of 5 M NH_4OAc and 27.5 µl EtOH (2.5 volumes) to each of the 3 tubes prepared in step 2.
4. Mix well and incubate at -20°C for 15 min to precipitate the RNA and probe.
5. Centrifuge at 12000 rpm for 15 min at 4°C.
6. Carefully remove the ethanol supernatant using a pipetor. Leave behind about 5 µl and centrifuge again at 12000 rpm for 3 min. Carefully, remove the last traces of supernatant.
7. Dry the samples for 5 min at room temperature with the tube lid open.
8. Dissolve the pellets in 20 µl of hybridization buffer (see below). Vortex for 5 sec and centrifuge for 5 sec. Place lid locks on the tubes.
9. Incubate tubes at 90°C (± 5°C) for 4 min to denature RNA and increase solubilization. Vortex 5 sec and centrifuge 5 sec.
10. Incubate at 45°C overnight (12 hr) in a hybridization oven to allow hybridization of the probe to the target RNA.

> Note: If the probe and sample RNA are in sufficiently small volume (<15 µl), steps 1-10 can be replaced by the following streamlined procedure:
>
> a) Complete steps 1-3 as above.
>
> b) Add 20 µl of hybridization buffer to each tube. Vortex for 5 sec and centrifuge for 5 sec.
>
> c) Incubate tubes at 90°C (± 5°C for 4 min) to denature RNA and increase solubilization. Vortex for 5 sec and centrifuge for 5 sec.
>
> d) Incubate overnight (12 hr) at 45°C to allow hybridization of probe to the target.

Next day

11. Add 200 µl of digestion buffer **with** RNase to each of the RNA sample tubes.

> Lab instructors prepared RNase-containing digestion buffer by diluting 11 µl of RNase mixture (RNase **A** + RNase **T1** concentrated mixture in glycerol) in 1100 µl of RNase digestion buffer (Ambion, proprietary information)

12. Vortex the sample tubes for 5 sec, centrifuge for 5 sec, and incubate at 37°C for 30 min to digest unprotected (unhybridized) single-stranded RNA.
13. Add 300 µl of precipitation solution and 100 µl of EtOH to each tube.
14. Incubate at - 20°C for 15 min to precipitate the protected RNA.
15. Pellet the precipitated products from the RNase digestion by centrifugation at 12000 rpm for 15 min at 4°C.
16. Remove all supernatant carefully as in step 5.

17. Resuspend the pellets in 10 µl of nondenaturing gel loading dye. Samples are now ready for polyacrylamide electrophoresis.

Solutions

Hybridization Buffer:
80% deionized formamide
100 mM sodium citrate, pH 6.4
300 mM sodium acetate, pH 6.4
1 mM EDTA

Concentrated RNase solution (450 µl):
250 units/ml RNase A (Ambion)
10,000 units/ml RNase T1 (cloned, Ambion)

Solution F: (Probe diluting solution)
0.5 M ammonium acetate 1 mM EDTA 0.2% (w/v) SDS

Nondenaturing gel-loading dye (6X):
50% glycerol
1 mM EDTA, pH 8.0/0.25% bromophenol blue, 0.25% xylene cyanol
or
48% sucrose
0.155% bromophenol blue

Part B: Polyacrylamide gel analysis of protected RNA fragments

The products of ribonuclease protection assays (RPAs) can be separated by either denaturing or nondenaturing polyacrylamide gels. Native (nondenaturing) gels produce sharper bands, less background, and give more reliable and sensitive RPAs. This is because RNA:RNA duplexes are more resistant to RNase cleavage than the single-stranded species. However, some researchers prefer to use denaturing (containing urea) gels because unlike nondenaturing gels, they allow the use of single-stranded molecular size standards.

The formulation for native gels is identical to that of denaturing gels except that the urea is omitted. Denaturing gels are made in 0.5X TBE, 8.0 M urea and polyacrylamide at the percentage appropriate for the size of the RNA being analyzed. The following guidelines are suggested for choosing an appropriate acrylamide percentage:

< 30 base	20% →
30-60 bases	15% →
61-150 bases	10% →
151-500 bases	5% →
> 500 bases	4% →

Components for 4-20% polyacrylamide/8 M urea gels (denaturing)*:
For a 15 ml solution (enough for 13 cm x 15 cm x 0.75 cm thick gel)

% Acrylamide	DEPC H_2O (ml)	40%acrylamide (acryl:bisacry 19:1) (ml)	10X TBE (ml)	High qual. Urea (g)	50% APS (µl)	TEMED (µl)
4%	9.25	1.5	0.75	7.2	22.5	15
5%	8.85	1.9	0.75	7.2	22.5	15
10%	7.00	3.75	0.75	7.2	22.5	15
15%	5.15	5.6	0.75	7.2	22.5	15
20%	3.25	7.5	0.75	7.2	22.5	15

*For non-denaturing gels, leave urea out and adjust final volume to 15 ml.

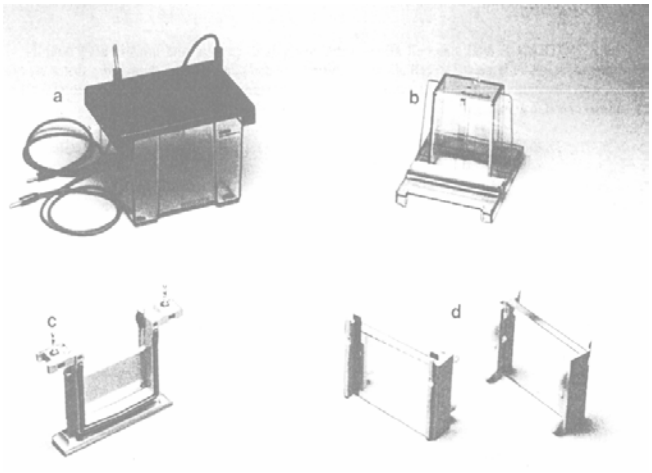
Solutions

10X TBE: (Do not mix with DEPC)
109 g Tris base (0.9 M [Final])
55 g boric acid (0.9 M [Final])
40 ml 0.5 M EDTA, pH 8.0 (20 mM [Final])
Add DEPC - treated H_2O to 1 liter

Apparatus and Set-up

The apparatus used here is available commercially from BioRad®. It consists of two buffer reservoirs separated by a gel sandwiched between 2 glass plates. The sample wells are made by a template "comb" placed within the stacker gel. The gel is poured between the two glass plates and allowed to polymerize. Refer to the figure below for a description of the major parts of the apparatus.

Description of major parts of the SDS-PAGE apparatus:

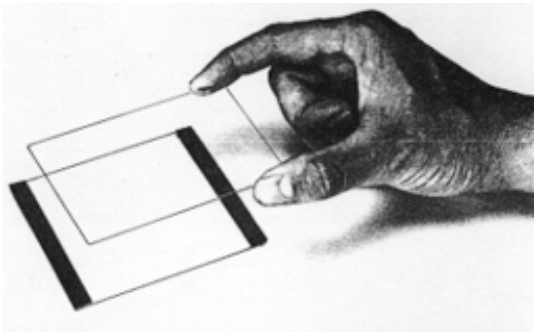


Mini-PROTEAN II slab cell (BioRad): Lower buffer chamber and lid (a), casting stand (b), inner core (c), sandwich clamp assemblies (d).

B.1 Casting the gel

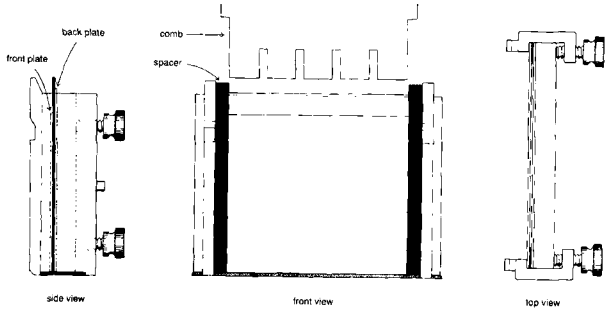
Use gloves while performing this procedure!

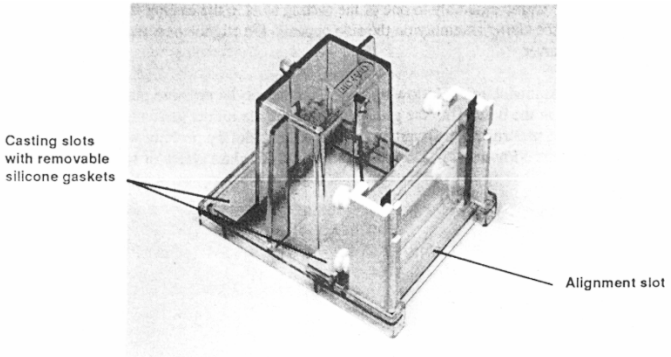
1. Clean all inner surfaces of polyacrylamide gel apparatus with RNase ZAP™ and place on a clean surface (these parts include buffer chamber, clamps, inner core plates, glass plates, spacers, alignment card and comb). Lay the longer plate down first, then place two spacers of equal thickness (0.75 mm) along the edges of the plate (use an alignment card to keep spacers properly positioned). Next, place the shorter plate on top of the spacers.



2. Loosen the screws on one of the clamp assemblies and stand it so that the screws are facing away from you. Firmly grasp the glass plate sandwich (or pre-poured gel) and gently slide it into the clamp assembly along the front face of the acrylic plate so that the longer glass plate faces away from you.

3. Place the clamp assembly/glass plate sandwich into the alignment slot of the casting stand. Make sure that the bottom of both gel plates, spacers and clamp assembly are perfectly flush against the flat surface. A slight misalignment will result in a leak.

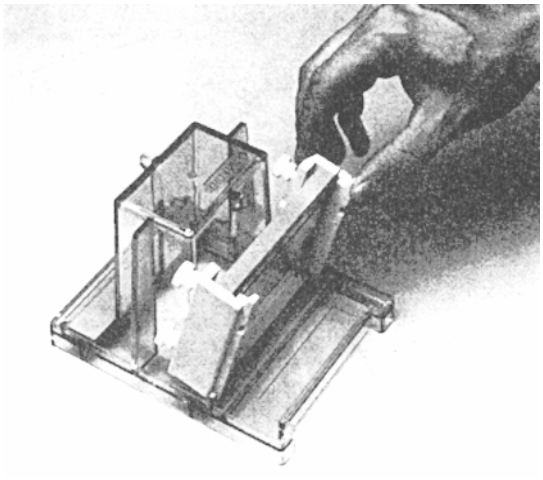


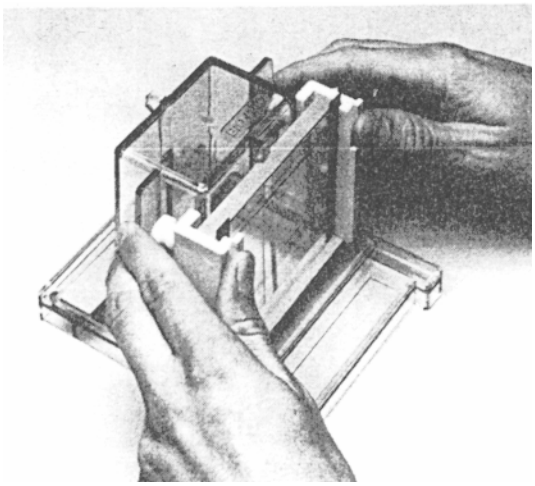


4. Tighten all four screws on the clamp assembly in a criss-cross pattern. Do not over tighten. Recheck the alignment of the spacers/glass plates and clamps.

5. For a good seal, it is helpful to place a piece of parafilm on top of the rubber piece of the casting slot in the casting stand. Transfer the clamp assembly to one of the casting slots in the casting stand. Do this by butting the acrylic plate against the wall of the casting slot at the bottom, so the glass plates rest on the

rubber gasket. Snap the acrylic plate underneath the overhang of the casting slot by pushing the white portions of the clamps.





6. Test for leaking by adding about 5 ml DEPC-water between the glass plates. Reassemble if any leaking is detected. Pour off any water trapped between plates and dry with a piece of filter paper.

7. Wear gloves. Follow the recipe below to prepare 7.5 ml of a 5% native gel solution in a 50 ml conical centrifuge tube. (Note: The lab instructors will have already mixed TBE, acrylamide and water. You only need to add APS and TEMED to the filtered and de-gassed solutions).

Reagent	Amount
10X TBE	0.375 ml
30% acrylamide (acrylamide: bis acrylamide, 19:1)	1.25 ml

Add DEPC-water to 7.5 ml and mix by swirling.
Add 8 µl TEMED and 60 µl of 10% w/v APS.

Note: **Acrylamide is a neurotoxin!** Work carefully on top of adsorbing paper and inside the area of the tray provided. **Wear gloves!**. Mix gel solutions (minus TEMED and APS) and filter through Millipore filter (0.22µm). These solutions can be kept for 10 days in the refrigerator. Add TEMED and APS to polymerize gels.

8. Swirl gently to mix solutions and pour solution into gel space (in between glass plates) using a syringe equipped with a needle or a 1 ml pipetor. Fill the plate compartment up to the top of the shorter glass plate.

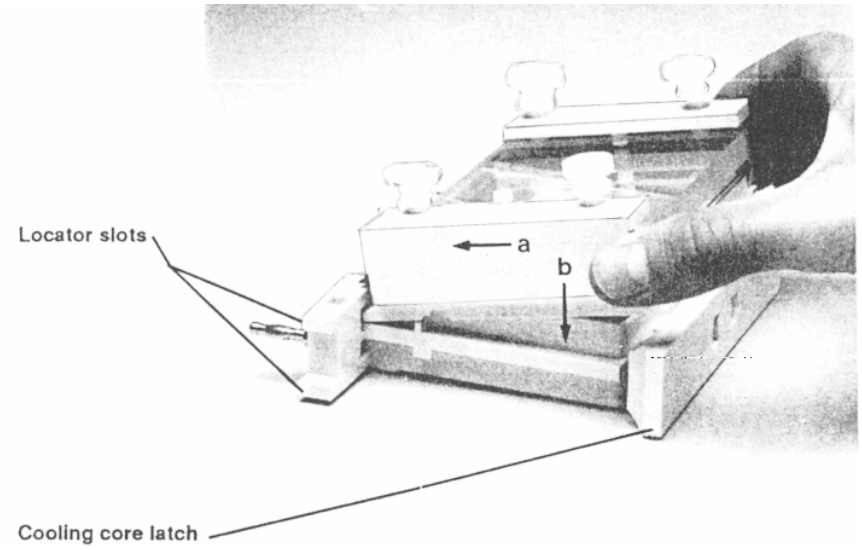
9. Carefully insert the comb between the plates until base of teeth lines up with the top edge of the short glass plate. Be sure no bubbles are trapped below the teeth. Tilting the comb at a slight angle helps to prevent air bubbles. Allow the gel to polymerize for 30 min.

10. Carefully remove comb making sure not to tear the thin "fingers" of gel that divide the individual wells.

11. Remove the clamp assembly/gel sandwich from the casting stand (or from the alignment slot if you used a pre-poured gel)

12. Lay the inner cooling core down flat on the lab bench. Carefully, slide the clamp assembly wedges underneath the locator slots on one side of the inner cooling core until the inner glass plate of the gel sandwich butts up against the notch in the U-shaped gasket. While pushing the clamp assembly up toward the top of the locator slots, snap the clamp assembly fully onto the cooling core by pressing at the bottom of the clamp assembly until the cooling core latch engages each side of the clamp assembly (see diagram below).

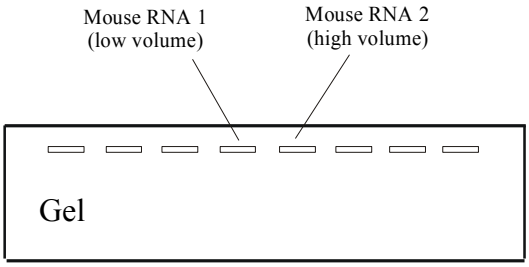
The group with which you will be sharing the gel apparatus either will have done this step or will do it next, using the other side of the cooling core. When running a single gel, the side of the cooling core not being used must be sealed with a clamp assembly containing only the short glass plate.



13. Stand the inner chamber assembly (top chamber) upright and add 0.5X TBE buffer to the reservoir until wells are filled (you need about 130 ml). Allow to stand on a clean paper towel to check for leaks. Reassemble if necessary.
14. Pour 350 ml of 0.5X TBE buffer in the gel tank (outer chamber) and place inner chamber unit into it. Remove air bubbles trapped along the bottom of the gel. This can be done by squirting anode buffer with a syringe equipped with a bent needle.
15. Flush the sample wells with 0.5X TBE buffer using a 1 ml pipettor to remove un-polymerized acrylamide and any other contaminants.
16. The gel is now ready for sample loading.

B.2 Sample preparation

1. Retrieve your samples from the RPA experiment.
2. Pulse spin samples in microcentrifuge for 5 sec.
3. Introduce the samples into the wells as indicated in the figure (next page) using a sample loading tip. Be careful not to poke the gel well. Layer the sample on the bottom of each well and raise tip as the dye level rises. Avoid introducing air bubbles as this may allow some of sample to be carried to an adjacent well.



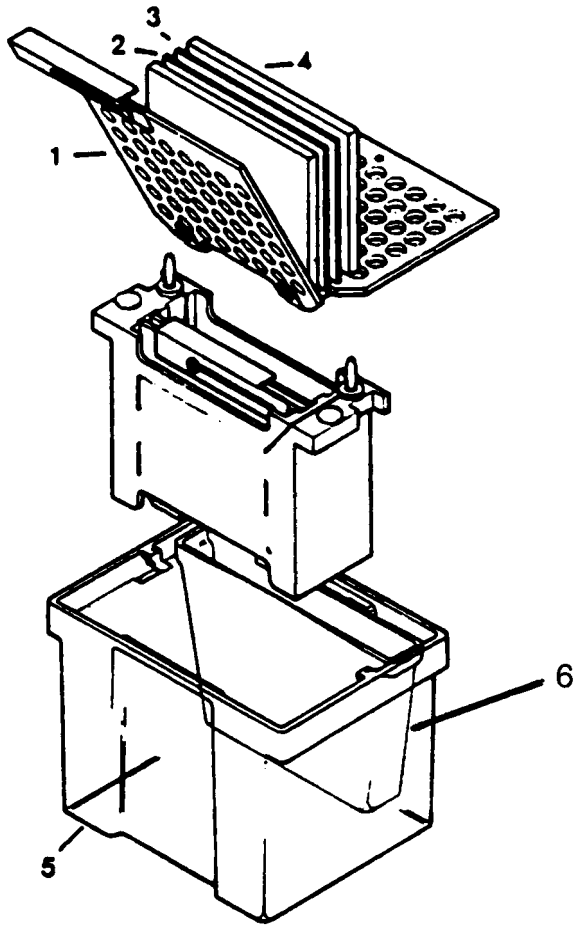
B.3 Gel electrophoresis

1. Attach the power leads to the proper electrodes. (-) black to top and (+) red to bottom.
2. Turn on the power supply and adjust the output to 60 V.
3. Electrophorese for about one hour or until the green dye reaches the bottom of the gel.
4. While the gel is running, prepare for electroblotting as described below.
5. Turn off the power supply and disconnect the electrode plugs.
6. Use gloves to remove gel plates from assembly.
7. Carefully remove a spacer and use it to pry apart the gel plates. The gel will stick to one of the plates.
8. Use gloves. Remove gel carefully and assemble for RNA transfer onto a nylon membrane as described below.

Part C: Electroblotting from Polyacrylamide

Electroblotting of Protected RNA Fragment from Polyacrylamide Gels onto a Nylon Membrane will be done using a BioRad blotting system and the same gel box used for running polyacrylamide gels. Refer to the figure below for a description of the components of this system. Many other transfer systems are commercially available, and they all perform well.
Assembly of the electrotransfer unit

Note: All parts of the protein transfer unit are soaked overnight in 0.1 N NaOH to inactivate RNases.



Locking the gel cassette clamping system. The cassette (1) holds the gel (2) and membrane (3) while fiber pads and filter paper (4) on both sides provide contact within the gel sandwich. An ice block (6) and the gel cassette are inserted in the buffer tank (5). If preferred, transfer can be done in a cold room to avoid heat build up (in this case an ice block is unnecessary).

Wear gloves at all times!

1. Using a pencil, label the RNA side of the nylon membrane the size of the gel. Always pick-up the membrane by the same corner.
2. Place cassette holder on a clean plastic box containing 0.5X TBE. Assemble the sandwich for transfer as indicated in the figure. Start with **dark side of the cassette holder down**. Place in sequential order, a fiber sponge, one piece of 3MM paper, gel, nylon membrane (RNA side facing the gel), second piece of 3MM paper, second sponge. Check after every addition that there are no air bubbles caught between the layers.
3. Close the cassette and lock it using the plastic sliding clamp. Slide t cassette holder into one of the two slots of the transferring unit (unit with electrodes), making sure black side of the cassette holder is on the same side of the black side of the transferring unit. If done correctly, the membrane should be located in the positive electrode side relative to the gel.
4. Pour 700 ml of transfer buffer (0.5X TBE) into the transfer chamber, then place the ice block into the unit.
5. Place lid on transfer unit. Transfer protected RNA fragments at 200 milliamperes for 1 hr.

Wear gloves !!!

6. Turn off transfer unit, take the transfer cassette out and disassemble.
7. Remove the membrane from the transferring assembly and place it in the plastic box containing 40 ml 5X SSC. Shake gently at RT for 2 min to remove any pieces of polyacrylamide that may have attached to the gel.
8. Put the membrane (RNA side up) on a large, clean piece of Whatman paper and wait for two minutes until the last puddles of buffer on the membrane have dissappeared.
9. While the membrane is still damp, cross-link RNA in the UV crosslinker chamber. Exposure: 120 millijoules. Crosslinking can also be accomplished by baking at 80 ^{o}C for 15 min.

Equipment and solutions

1. Equipment (Exp. 21, part C)

   -One nylon membrane (gel size)
   -Two 3MM filter papers (gel size)
   -Two fiber sponges
   -Cassette holder
   -Transferring unit

2. Reagents for the preparation one liter of 5X TBE

Reagent	Amount
0.445 M Tris	54.5 g Tris base
0.445 M Boric Acid	27.5 g boric acid
10 mM EDTA	20 ml 0.5 M EDTA, pH 8
DEPC-water	Up to 1 liter

3. To prepare transfer buffer (0.5X TBE), Mix one part of 5X TBE with nine parts of water.

<u>Part D:</u> Detection of Biotin labeled RNA

The detection of the protected RNA fragment will be done using the *BrightStar™ BioDetect™* solutions marketted by Ambion, Inc. The key reagents in this kit are a **Streptavidin-Alkaline Phosphatase conjugate** and an Alkaline Phosphatase **chemiluminescent substrate**. All solutions used for hybridization, blocking and washing of the membrane are of proprietary composition. However, the solutions recommended by laboratory manuals such as the one by Maniatis also yield excellent results.

> **Note**: Use gloves always and sterile techniques to handle solutions Do not allow the membrane to dry throughout the procedure.

1. Clean a small plastic container scrupulously with 2-3 ml of RNAse ZAP (a strong denaturing solution), kimwipes and milliQ doubly distilled water.
2. Place the membrane in the plastic container and wash it twice, 5 min each time in 25 ml of 1X Wash Buffer. Agitate gently on a gyratory shaker. Wash Buffer can be discarded in the sink at the end of every wash.

Note: The Wash buffer is provided as a 5X solution. Dilution with milliQ doubly distilled water is adequate. If necessary, at this point the membrane can be stored wet, wrapped in plastic wrap for several days at 4 °C.

3. Wash the membrane twice, 5 min each time in 25 ml of Blocking Buffer. Blocking Buffer can be discarded in the sink at the end of every wash.
4. Wash the membrane once for 30 min in 25 ml of Blocking buffer.
5. Discard Blocking Buffer and add to the membrane 10 ml of conjugate solution (1 µl Streptavidin/ Alkaline Phosphatase conjugate in 10 ml Blocking Buffer).
6. Incubate for 30 min on a gyratory shaker (room temperature).
7. Wash the membrane once for 10 min in 25 ml of Blocking buffer.
8. Wash the membrane three times, 5 min each time in 25 ml of 1X Wash Buffer.
9. Wash the membrane twice for 2 min each time in 15 ml of 1X Assay Buffer.

Note: The Assay buffer is provided as a 10X solution. Dilution with milliQ doubly distilled water is adequate.

10. Place the membrane in a plastic bag (such as a hybridization bag) and add directly to the membrane 1 ml of chemiluminescent substrate (CDP-Star).
11. Spread the substrate accross the entire membrane and close the bag eliminating excess substrate.
12. Seal the bag with a heat sealer and wash the outside with ethanol.
13. Expose membrane to X-ray film for 1-2 hours.