



**University of Wisconsin
Oshkosh**



Research Experience for Undergraduates

In Proteomics and Genomics

Techniques Workshop Manual

By

Lisa A. Dorn PhD

&

Beatrice Holton PhD

Summer 2007

TABLE OF CONTENTS

	Page
INTRODUCTION	1
 UV EXPERIMENT : PHENOTYPIC EFFECTS	
Day 1: Monday 4 June	2
Step 1: Streak for isolation	
Step 2: Prepare for UV treatment	
Step 3: UV treatments	
Step 4: Serial Dilution	
Step 5: Plating	
Day 2: Tuesday 5 June	10
Step 6: Examine plates	
 UV EXPERIMENT: MICROARRYS	
Day 1: Monday 4 June.....	12
Step 1: Stabilize RNA	
Step 2: RNA extraction	
Day 2: Tuesday 5 June	14
Step 3: cDNA synthesis	
Step 4: Clean up	
Step 5: Tailing	
Step 6: Ligate Capture Sequence	
Step 7: Clean up & combine samples	
Step 8: Hybridize cDNA to array	
Day 3: Wednesday 6 June	17
Step 9: Washes	
Step 10: 3DNA hybridization	
Step 11: Post 3DNA washes	
Step 12: Scanning	
Step 13: Data Analysis	
 MANIPULATING DNA	
Day 1: Monday 4 June	20
Step 1: Make O/N cultures	
Day 2: Tuesday 5 June	21
Step 2: Extract plasmid DNA.	
Step 3: Quantify plasmid DNA spec	
Step 4: Set up enzyme digest	
Day 3: Wednesday 6 June	23
Step 5: Pour agarose gel	
Step 6: Load and run Agarose gel	
Step 7: Take pictures	

SINGLE GENE EXPRESSION EXPERIMENT: RNA→ Proteins	
Day 4: Thursday 7 June	24
Step 1: Acquire cell types	
Step 2: Extract Proteins	
Step 3: Extract RNA and DNA	
Step 4: Quantify Proteins, DNA and RNA	
Step 5: Polymerase Chain Reaction of Genomic DNA	
Day 5: Friday 8 June.....	36
Step 6: Reverse transcription reaction	
Step 7: PCR of cDNA	
Day 6: Monday 11 June.....	39
Step 8: SDS-PAGE	
Step 9: Agarose gels	
Step 10: Immuno-blot Part 1	
Day 7: Tuesday 12 June	43
Step 11: Immunoblot Part 2	
FINAL RESULTS	
PASTE FIGURES	
 ALTERNATIVE METHODS STARTS	
Day 8: Wednesday 13 June.....	46
Step 1: Load isoelectric focusing strips	
Step 2: Separate proteins in 1 st dimension	
Day 9: Thursday 15 June.....	48
Step 3: Separate proteins in 2 nd dimension	
Day 10: Friday 16 June.....	49
Step 4: Silver stain the SDS-PAGE dimension	
Discussion	
Maldi-TOF	
Microchip	



**University of Wisconsin
Oshkosh**



National Science Foundation
WHERE DISCOVERIES BEGIN

Research Experience for Undergraduates

In Proteomics and Genomics

Protocols

By

Lisa A. Dorn PhD

&

Beatrice Holton PhD

Summer 2007

**PROTOCOLS
TABLE OF CONTENTS**

	Page
Protocol 1: Streak for Isolation	1
Protocol 2: RNA extraction for bacteria (Epicentre kit)	2
Protocol 3: Determination of yield and purity of nucleic acids.....	5
Protocol 4: Microarray Kit	6
Protocol 5: Promega Wizard Plasmid DNA Isolation	14
Protocol 6: Agarose Gel electrophoresis	16
Protocol 7A: DNA extraction from brain and liver	17
Protocol 7B: RNA extraction from brain and liver	21
Protocol 8: Protein Assay	27
Protocol 9: Polymerase Chain Reaction General & RT-PCR	28
Protocol 10: SDS PAGE.....	30
Protocol 11: Western (Immuno) Blotting	37

TENTATIVE SCHEDULE

Day 1: Monday 4 June.....

8:30 am: Meet and greet in Halsey Sciences Rm149

9:00 am: Introductory lecture Central Dogma

UV EXPERIMENT

9:30 am: Step 1: Streak for isolation

10:00 am: Step 2: Prepare UV treatment

10:45 am: Step 3: UV treatments

11:15 am: Rest period (10 min)

11:25 am: Step 4: Serial Dilution

11:45 am: Step 5: Plating

12:00 pm: LUNCH

1:00 pm:

MICROARRAY

Step 1: Stabilize RNA in the bacterial culture

LUNCH

Step 2: Extract RNA from bacteria, Freeze

MANIPULATING DNA STARTS

2:00pm: Step 1: Make O/N D

3:30pm: Keys

5:00 pm: Leave for Boat

Day 2: Tuesday 5 June.....

UV EXPERIMENT

8:00 am: Step 6: Examine plates

9:00 am: DISCUSS

MICROARRAY

MANIPULATING DNA (PLASMIDS)

10:00 am; Step 2: Extract plasmid DNA

11:00 am; Step 3: Quantify plasmid DNA

11:30 am; Step 4: Set up enzyme digest

12:00 pm: LUNCH

1:00 pm:

3:30 pm

4:00 pm

5:00 pm:

6:00 pm:

7:00 pm:

LUNCH

Step 3: cDNA synthesis

Step 4: Clean up,

Step 5: Tailing

Step 6: Ligate Capture Sequence

Step 7: Clean up & combine

Step 8: Hybridize cDNA to array

Day 3: Wednesday 6 June

**MANIPULATING DNA
(PLASMIDS)**

8:00 am:
8:30 am:
10:00 am: Step 5: Pour Agarose gels
10:30 am: Step 6: Load and Run gels
11:15 am: Step 7: Take pictures of gels
12:00 pm; LUNCH
1:00 pm
2:00 pm Library
2:30 pm
3:00 pm HS149: Discussions

MICROARRAY

Step 9: Washes
Step 10: 3DNA hybridization

LUNCH
Step 11: Post 3DNA washes
Step 12: Scanning
Step 13: Data Analysis (Due by Friday)

Day 4: Thursday 7 June.

SINGLE GENE EXPRESSION EXPERIMENT BEGINS

8:00 am: Step 1: Acquire cell types
9:00 am: Step 2: Extract Proteins
10:00 am: Step 3: Extract RNA and DNA
11:00 am: Step 4: Quantify Proteins, DNA and RNA
12:00 pm: LUNCH
1:00 pm: Step 5: Polymerase Chain Reaction Optimization
2:00 pm: :

Day 5: Friday 8 June

SINGLE GENE EXPRESSION EXPERIMENT

8:00 am: Step 6: Reverse Transcription of RNA into cDNA
10:00 am: Step 7: PCR of cDNA
12:00 pm: LUNCH
1:00 pm: Discuss results to date
4:00 pm: Fratello's

Day 6: Monday 11 June

GENE EXPRESSION EXPERIMENT

Step 8: SDS-PAGE

Step 9: Agarose gels

Step 10: Immuno-blot Part 1

Day 7: Tuesday 13 June

GENE EXPRESSION EXPERIMENT

Step 11: Immunoblot Part 2

Day 8: Wednesday 14 June

Step 1: Load isoelectric focusing strips

Step 2: Separate proteins in 1st dimension

Day 9: Thursday 15 June

ALTERNATIVE METHODS

Step 3: Separate proteins in 2nd dimension

Day 10: Friday 16 June

ALTERNATIVE METHODS

Step 4: Silver stain the SDS-PAGE dimension

Maldi-TOF

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

INTRODUCTION:

THE CENTRAL DOGMA

The Central Dogma is a rather succinct paradigm of genetics and cell biology. You can think of the Central Dogma as the transition steps between three important molecules for life: DNA, RNA and Proteins.

DNA stores information on how to make proteins as well as how to regulate proteins.

Transcription converts information encoded by DNA into information encoded by RNA.

RNA stores only the protein instructions minus regulatory instructions (more or less).

Translation synthesizes proteins using the instructions encoded in mRNA.

Proteins now build all cells, regulate cell growth and death, and run all cellular processes ultimately leading to the development of an individual organism.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

This lab manual progresses through the handling of all molecules and processes of the central dogma. We will explore the ways to acquire data at all levels starting with some standard laboratory techniques you may need to survive your 9 weeks with us and a foray into the emerging technology of microarrays.

At the bottom of each page of this lab manual, you will find a NOTES section that you can use to right notes and/or questions you may have. You can also use this space to answer the questions you find scattered about the worksheets typed in *bold italics*.

At the back of the manual is a protocols section. You can adapt these generalized protocols to any experiment. Use them as references through out the class and the summer.

The **Genetics Teaching Lab** in **Halsey Sciences Rm. 50** is open to you all summer (the keypunch code: 28367). You may use its computers any time, and the other equipment as long as you follow the rules for reserving their use if need be. **Please remember to shut all doors when you leave.** Most of all have fun!

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**UV EXPERIMENT 1:
DAY1
MARGARITAS BY THE POOL!**

BACKGROUND: It's the day after spring break and you've had a fabulous time on the beach, or you spent it outdoors every day baling hay on your family farm. You received a lot of sun time. You know from your biology class and endless TODAY show segments that sun exposure causes wrinkles and increases your risk of skin cancer. You decide you want to study how cells deal with UV irradiation for your senior thesis.

The effect of UV radiation is a good topic for exploring the Central Dogma because it is a mutagen that can alter DNA, which leads to a cascade of events including transcriptional changes, which in turn affect protein synthesis; ultimately affecting the behavior and viability of cells and the organism in general.

We designed an experiment to test the effect of UV irradiation on cells by exposing two strains of *E. coli* to UV light; one strain is an extra-sensitive mutant, the other has normal or wild type sensitivity. We chose bacteria for this first part because bacteria are single-celled organisms that can be used as a good stand-in for human cells (similar structural proteins and genetic processes) without exposing any of us to harmful UV rays.

We chose this particular UV sensitive strain because it lacks a functioning copy of a single gene that encodes a protein called *uvrA*. *UvrA* works with other proteins to repair mutations to DNA. We thought it might be interesting to compare the gene expression patterns of the insensitive strain after exposure to UV.

In this experiment, we will both measure the affect of UV on the phenotype of cell survival and use a microarray to see how UV affects global gene expression patterns as well.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

UV EXPERIMENT: DAY1

Preparation

There are 5 main steps to this experiment:

- Step 1:** Streak for Isolation
- Step 2:** Prepare for UV treatments
- Step 3:** UV treatments
- Step 4:** Serial Dilutions
- Step 5:** Plating

We will provide you with a liquid culture of each of two strains of *E. coli*. The DH5 α strain is the wildtype strain (i.e. resistant to UV) and N3305 is the mutant strain (i.e. sensitive to UV but tetracycline resistant).

These are overnight cultures. They consist of liquid media inoculated with cells from a single colony of the strain and allowed to multiply (i.e. grow) for 12 - 24 hours.

The skills you should learn:

1. Culturing microbes
2. Semi-sterile techniques
3. Serial Dilutions
4. Evaluating bacterial colonies

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

UV EXPERIMENT: DAY 1

Preparation

Step 1: Streak for Isolation

One good laboratory technique is to keep a reserve of your bacterial strains, genetic material, samples, or whatever in case something happens to your experiment and you have to repeat it. In this case, you will take some of the liquid media containing *E. coli* cells and transfer it to a Petri plate with solid growth media to isolate single colonies of each strain. ***Why streak for isolation of a single colony? Why not just keep a liquid culture or a lawn of bacteria?***

Assemble everything you need to follow Protocol 1.

:

- Loop
 - Petri plates with LB and w/wo appropriate antibiotic
 - DH5 α w/o tetracycline resistance
 - N3305 (UV sensitive & Tetracycline resistant)
 - Loop sterilizer
1. You will streak 2 plates, one for each strain using **Protocol 1**
 2. ***Why should your growth medium for antibiotic resistant strains always contain antibiotics?***

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

UV EXPERIMENT: DAY 1**Step 2: Prepare for the UV treatments**

Before you expose the cells to the UV treatments you need to dilute the cells. ***Why do you think you are doing this?***

Assemble everything you need:

- Sterile flask with 60 mls of sterile liquid LB
- Sterile flask with 60 mls of sterile liquid LB plus tetracycline 8-100mm Petri dishes
- P-1000 Pipettor and tips
- 10 ml Pipette
- The two overnight cultures

Dilution 1:

1. Put 1 ml of DH5 α overnight culture into one 60 ml flask of liquid LB w/o tetracycline.
 - i. Double check your settings on the P-1000.
 - ii. One way to detect pipettor errors as they happen is to keep in mind what the volume you are measuring looks like in a pipette tip. Check it the first time you do it (I'll come up with more as we go). ***Can you think of others?***
2. Put 1 ml of N3305's overnight culture into one 60 ml flask of liquid LB w/ tetracycline.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

UV EXPERIMENT: DAY 1

Preparation

You will place aliquots of dilution 1 (*what is an aliquot?*) into shallow Petri dishes in order to expose the cells suspended in liquid to UV. Before you do that, you should clearly label the plates so you can keep track of what you have done to each class of cells. Remember to label the **sides** of the dishes.

Now, label the dishes and place aliquots of Dilution 1 in the plates.

3. Label the sides of 6-100mm Petri dishes:
 - a. Write your group number on all dishes
 - b. Label 4 dishes:
 - DH5 α -, 0 min;
 - DH5 α -, 0 min; RNA
 - DH5 α -, 5 min;
 - DH5 α -, 5 min; RNA
 - c. Label the other 4:
 - N3305, 0 min;
 - N3305, 5 min;
4. Put **10 mls** of DH5 α 's dilution 1 into the 4 dishes labeled for DH5 α
5. Repeat for 2 dishes for N3305 (you should have 6 plates total, each with 10 mls of solution).

NOTES:

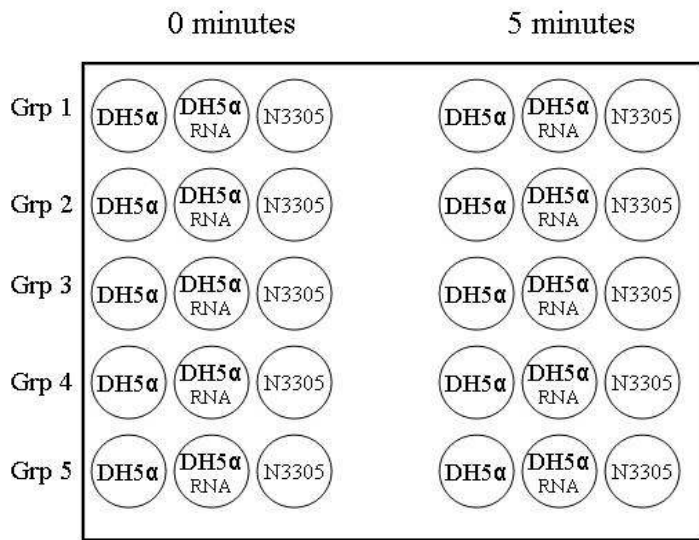
Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

UV EXPERIMENT: DAY 1

Step 3: UV Treatments:

1. To be done in the viral containment hood in the tissue culture room (HS 58):
Important: While setting up these dishes, make sure the UV light is off until everything is ready. Remember that we are investigating the effects of UV light on cells and that each one of you is a collection of cells.
2. Put the 4 DH5 α and 2 N3305 dishes on the shaker as shown in Figure 1 – *Why should we be concerned with conventions such as placing the samples in a certain order?*



3. Cover the 0-minute dishes with aluminum foil.
4. Turn on the shakers, then the UV lights.
5. After 5 minutes, stop the shakers.
6. Let the cells for RNA extraction rest for 10 minutes in the hood.
This step allows the cells to transcribe genes following the treatments.
7. Take the other plates back to HS50 and begin Step 4.

Figure 1. Placement of dishes for UV treatments

NOTES:

Answer the questions in bold italics (above) in this space.
Also note any concerns, mishaps, thoughts or questions of your own.

UV EXPERIMENT: DAY 1

Now we will begin the first steps for the microarray portion of this experiment in parallel with the steps to evaluate the effects of UV on the phenotype. You will have to coordinate both parts of this experiment starting now.

Step 4: Assessing treatment effects on survival: Serial Dilution

To evaluate the treatments on survival we will count the surviving cells. We will put a sample onto agar plates and count all of the colonies that grow from a single cell. First you have to make serial dilutions. *Why do we need this step?*

Assemble everything you need:

- 10ml Pipette
- 12 - 15 ml tubes (2 treatments x 2 strains X 3 dilutions) in a rack
- Fill each tube with 9mls liquid LB
- P-1000

1. For each strain and each treatment combination make the following 3 dilutions:
 - a. Dilution A: put 1 ml of treated culture into tube A; vortex, **get a new pipette tip**
 - b. Dilution B: put 1 ml of tube A dilution into tube B; vortex, **get new pipette tip.**
 - c. Dilution C: put 1 ml of tube B dilution into tube C; vortex.

What is the ratio of culture to diluent?

NOTES:

Answer the questions in bold italics (above) in this space.
Also note any concerns, mishaps, thoughts or questions of your own.

UV EXPERIMENT: DAY 1

Step 5: Assessing Treatment Effects: Plating

For each combination of UV treatment, strain and dilution you need to spread a sample onto a Petri plate with agar, so you can count the number of isolated colonies on the plate tomorrow.

Assemble everything you need:

- The tubes with the dilutions you just completed.
 - 6 plates without tetracycline to plate out DH5 α (the dishes not being used for RNA)
 - 6 plates with tetracycline to plate out N3305
1. Take 200 μ l from each tube and spread onto the media in its appropriately labeled plate.
 - a. Seal them with parafilm, make sure they are labeled accordingly, and put them in the 37°C incubator overnight.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

UV EXPERIMENT DAY 2

RESULTS:

There are a number of characteristics that could be examined to evaluate the effect of our treatments; in this case, we are going to use survivability. ***How will you quantify survivability? What other characteristics could you examine, if you were able (think wildly)?***

1. Examine the plates you removed from the incubator. .
2. Count colonies.
 - a. Each colony is a group of cells descended from a single surviving cell. Therefore, when we count colonies we are counting the number of cells that survived the UV treatment. ***What could you conclude if we had examined the cells by microscopy immediately after UV treatment?***

Table 1: Record your data.

		Dilutions		
Strain	treatment	A	B	C
DH5	0			
DH5	5			
N3305	0			
N3305	5			

NOTES:

Answer the questions in bold italics (above) in this space.
Also note any concerns, mishaps, thoughts or questions of your own.

**UV EXPERIMENT:
DAY 2**

RESULTS

1. Write a header for Table 1 describing what is in the table.
2. State your results generally referring to the table.

3. Graph your results (Figure 1).
 - a. Write a header for Figure 1.
4. State your results specifically referring to the graph.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

UV EXPERIMENT: MICROARRAY DAY 1

Microarrays are a tool that arose directly out of the efforts to sequence whole genomes. Once the nucleotide sequence of every known and predicted gene in a genome (*what is a genome?*) was determined then it became possible to ask about the expression patterns of every single gene in a genome at once. For instance, you might want to know which genes change their gene expression after a UV treatment. There are some obvious candidates, such as the genes that code for repair proteins, but there may be others we do not know about. In this experiment, we will attempt to answer that question. We will discuss microarray technology in more detail later. For now, let's get your experiment started.

There are 13 steps to this experiment:

- | | |
|---|---|
| Step 1: Stabilize RNA in culture | Step 8: cDNA hybridization to chip |
| Step 2: Extract RNA & quantify | Step 9: Washes |
| Step 3: cDNA synthesis | Step 10: 3DNA hybridization |
| Step 4: Clean up, and Freeze samples | Step 11: Post 3DNA washes |
| Step 5: Tailing | Step 12: Scanning |
| Step 6: Ligate Capture sequence | Step 13: Data Analysis |
| Step 7: Clean up and combine | |

Step1: Stabilize RNA

Unlike DNA, RNA is a highly unstable molecule, especially in prokaryotes. Fortunately, we can postpone the extraction of RNA for the microarray experiment by first adding a reagent that will stabilize the RNA.

1. Transfer the 10mls of liquid in the UV treatment dish to 15ml centrifuge tube.
2. Do the same for the non-UV treated dish.
3. Add ___ mls (depends on culture density)
4. Spin the culture down. Freeze the pellet.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**UV EXPERIMENT: MICROARRAY
DAY 1**

Step 2: Extract RNA and Quantify

1. Follow **protocol 2** for the Epicentre RNA extraction for bacteria.
2. Follow **protocol 3** for quantification of nucleic acids
 - a. Concentration RNA UV: _____
 - b. Concentration RNA NoUV: _____
3. Make two 5 μ l (can vary) aliquots of RNA totaling 1 μ g each.
4. Label correctly and freeze

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**UV EXPERIMENT: MICROARRAY
DAY 2**

Step 3: cDNA synthesis

1. Each group will take **two** 1µg samples of RNA and synthesize cDNA from just **one treatment type** in two reactions.

	UV RNA	No-UV RNA
	Group 1	Group 2
	Group 3	Group 4
	Group 5	Extra

2. Follow **Protocol 4: cDNA synthesis**

- a. **IMPORTANT** Use only the random primers
- b. Be aware of the volumes you are working with.
 - At the end of this you should have 50ul

Step 4: Clean up, and freeze

1. Follow **protocol 4: Clean up cDNA w/ Qiagen MiniElute PCR Purification Kit**
 - At the end of this protocol you should have 10ul

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**UV EXPERIMENT: MICROARRAY
DAY 2**

Step 5: Tailing

In eukaryotes, post-transcriptional processing adds a long tail of Adenines called the poly A tail to the end of mRNA transcripts. Microarray technology exploits this common characteristic of all mRNAs to target them for labeling. However, tailing of mRNA is not a feature of prokaryotic genomes so we have to do it ourselves.

1. Follow **protocol 4** for Terminal Deoxynucleotidyl Transferase (TdT) Tailing Reaction.

Step 6: Ligate capture sequence:

In this protocol, we are adding to the tail a sequence of nucleotides, to which the fluorescent dyes will attach. Each group will add both kinds of capture sequences to two different cDNAs from just one treatment.

Group	UV cDNA		No-UV cDNA	
1	Cy3 (green)	Cy5 (red)		
2			Cy3 (green)	Cy5 (red)
3	Cy3 (green)	Cy5 (red)		
4			Cy3 (green)	Cy5 (red)
5	Cy3 (green)	Cy5 (red)		
Extra			Cy3 (green)	Cy5 (red)

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**UV EXPERIMENT: MICROARRAY
DAY 2**

Step 7: Clean up & Combine

1. Use **protocol 4: Clean up tagged cDNA** to clean up your two samples separately.
2. Combine a UV and No-UV sample together as described in the table below.
 - a. Note the combinations of dyed samples each group will do. Group 1 does chip1, group2 chip 2 etc.

Chip	UV cDNA by sample & dye	No-UV cDNA By sample & dye
1	Group 1 sample Cy3 (green)	Group 2 sample: Cy5 (red)
2	Group 1 sample Cy5 (red)	Group 2 sample: Cy3 (green)
3	Group 3 sample Cy3 (green)	Group 4 sample: Cy5 (red)
4	Group 3 sample Cy5 (red)	Group 4 sample: Cy3 (green)
5	Group 5 sample Cy3 (green)	Extra sample: Cy5 (red)

Step 8: Hybridization (starts ~7pm)

1. Follow **protocol 4** for cDNA hybridization
 - a. Use Option 1.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**UV EXPERIMENT: MICROARRAYS
DAY 3**

Step 9: Post cDNA Hybridization washes

1. Pre-warm the buffers.
2. Follow **protocol 4** for post cDNA hybridization washes

Step 10: 3DNA Hybridization

1. Follow **protocol 4** for 3DNA hybridization.
2. **IMPORTANT:** thaw capture reagent in the dark (make sure it is wrapped in aluminum foil).
3. **Takes 4 hours**

Step 11: Post 3DNA Hybridization washes

1. Prior to the end of the hybridization pre-warm buffers
2. Follow **protocol 4** for post 3DNA hybridization washes

Step 12: Scan arrays

1. Be sure to use SAVE AS to save your scans.
2. Save your slide files in REU ARRAYS
3. Name your slide files with the following format:
 - a. Group # .sld
 - b. If you do not use SAVE AS, every new scan will simply write over the previous scan and only the last group to scan a slide will have an image.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**UV EXPERIMENT: MICROARRAYS
DAY 3**

Step 13: Analyze images

1. Open the Versarray Image Analyzer
2. Open your slide file
3. Set up a grid.
4. Save As a tiff file same naming format.
5. Export file to Excel
6. Add **UID**
7. **Sort by net intensity**
8. **Choose top 5 differentially expressed genes and anything else they find interesting at the top and bottom.**

DISCUSSION

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

MANIPULATING DNA EXPERIMENT DAY 2

This experiment has 5 steps:

- Step 1:** Making overnight cultures of each *E. coli* strain.
- Step 2:** Extracting plasmid DNA from *E. coli* with GFP.
- Step 3:** Quantify plasmid DNA extracted.
- Step 4:** Digest plasmid DNA with restriction enzymes.
- Step 5:** Agarose gel electrophoresis to separate fragments

BACKGROUND: Studying mutations that cause a visible change in a phenotype is a classical experimental method. We know there must be a gene to protect against the effects of UV light because we have found a mutated strain that is deficient in protecting itself. Eventually we can discover the exact chromosomal location of this gene and even the DNA sequence. But we want to know about every single gene in humans, or mice, or bacteria or a plant, that is its entire genome. Do researchers have to do this by mutation, one gene at a time? That question has inspired the development of the technologies that allow us to discover the nucleotide sequence of every single gene in a genome.

A basic technique called gene “cloning” is useful for studying a single gene at a time but is also the methodology at the heart of every genome project. With this method, you can study a single gene of interest by taking that gene and inserting it into a circular molecule of DNA called a plasmid. Plasmids are naturally present in bacteria and bacteria pass plasmids between them. To successfully insert a gene into a plasmid, you must cut the circular molecule with an enzyme that cuts just once and in a specific spot. That kind of enzyme is called a *restriction endonuclease*. There are hundreds of restriction enzymes, each cutting only at specific DNA sequences. To figure out which enzyme to use for cloning purposes you must build a map of all the restriction “sites”, i.e. the places where different enzymes cut.

NOTES:

Answer the questions in bold italics (above) in this space.
Also note any concerns, mishaps, thoughts or questions of your own.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

MANIPULATING DNA EXPERIMENT DAY 1

In this experiment, we will digest a plasmid that has a Green Fluorescent Protein (GFP) inserted into it. GFP is a protein that will fluoresce green under UV light. It is commonly used as a reporter gene. If another gene you want to study is on the same plasmid with GFP, you can verify that the plasmid (and therefore the gene you want to study) was successfully introduced or transformed into the bacteria, if the bacteria glows green in UV light.

Step 1: Make overnight cultures

Assemble what you need:

- 6 - 15 ml disposable centrifuge tubes
- LB medium without tetracycline
- LB medium with tetracycline
- Streaked plates from Day 1

Make overnight cultures

1. Select a few colonies from a plate with (DH5- α) **with GFP**. (We will provide you with plates with isolated colonies)
 - a. Start 3 small (3-5 ml) overnight cultures in LB medium w/ tetracycline.
2. Incubate both cultures O/N (12-16 h) with vigorous agitation at 37°C.

NOTES:

Answer the questions in bold italics (above) in this space.
Also note any concerns, mishaps, thoughts or questions of your own.

MANIPULATING DNA EXPERIMENT DAY 2

SOME MORE BACKGROUND: Gene cloning is really just a method of making many, many copies of a piece of DNA (an intact gene, a gene you construct, a piece of anonymous DNA). The gene must be contained in a vector, which is a DNA molecule that can enter a cell. When the vector with the gene on it is introduced into a cell, the cell copies the gene every time it copies itself. A common vector that is found naturally in bacteria is a **plasmid**. Plasmids are circular pieces of DNA that are not part of the bacteria's chromosomal DNA. In the bacterial strain DH5- α we have introduced a plasmid (pAlter) that carries a gene that encodes the green fluorescent protein (GFP). GFP is not normally found in bacteria, it is a gene found in a species of jellyfish. The genes carried on the plasmid are "cut" and "pasted" in and out of the plasmid using restriction enzymes, which cleave the DNA only at specific nucleotide sequences.

Assemble what you need:

- Overnight cultures that you set up yesterday
- SOC ??
- LB plates with and without tetracycline
- Promega Wizard extraction kit.

Step 2: Extract plasmid with GFP gene:

1. Take the O/N culture for DH5- α with GFP.
2. Follow **Protocol 5**, the Promega "Wizard" SV plasmid miniprep procedure to extract plasmid DNA from the O/N cultures.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**MANIPULATING DNA EXPERIMENT
DAY 2**

Step 3: Quantify Plasmid DNA

1. Follow **protocol 3** again for DNA quantification

Step 4: Digest Plasmid DNA with restriction enzymes

1. Make the enzyme master mix.
2. Add __ μ l master mix to __ μ l plasmid DNA
3. Place in 37°C incubator for 2 hours.
4. Stop the reaction and freeze

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**MANIPULATING DNA EXPERIMENT
DAY 3**

Step 5: Agarose Gel Electrophoresis

Follow **Protocol 6**

Step 6: Load & Run the gel

Step 7: Take pictures

RESULTS:

- 1. Paste in pictures of agarose gels.**

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

SINGLE GENE EXPRESSION EXPERIMENT DAY 4

BACKGROUND:

Manipulation of individual genes by cloning them into plasmids is a powerful tool. Not only has it been used to study individual genes and their products, but it has also been used as the first step in sequencing entire genomes, also known as genome projects. How valuable are genome projects? What information do they actually provide? Just knowing the sequence of nucleotides for every chromosome in a genome doesn't tell us much if we don't know which parts of that sequence encode a gene, and what these genes do.

It is obvious to all of us that we consist of different types of cells/tissues that perform different sorts of functions. Neurons send electrical signals; nerve supportive cells help support neurons metabolically; muscles allow us to move; bones support our structure, and so on. Each one of these cells and tissues contains the same genetic complement. But they function differently because gene expression is tightly regulated, so different cell types express different genes. Control of gene expression is central to understanding most fields of biology.

For the next series of exercises we will examine one difference between rat nerve supportive cells (specifically, glial cells) and rat liver cells: expression of *Glial Fibrillary Acidic Protein* (GFAP). We will also determine if GFAP synthesis is developmentally regulated in chick embryo brain.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**SINGLE GENE EXPRESSION EXPERIMENT
DAY 4**

1. First, think about the following questions:
 - a. Which classes of molecules would you expect to differ from one tissue or stage of development to another?*
 - b. Which of these molecule(s) should we work with?*
 - c. What questions can we answer with each class of molecule?*

2. Second, how will we identify specific molecules, say specific proteins or mRNA's, that give tissues unique characteristics? Cells/tissues contain many types of RNA's and proteins, but many are not tissue-specific.
 - a. What features of these molecules might we use to separate them into individual types of each kind of molecule so that we can see the differences?*

3. Third, let's consider techniques we can use.
 - a. What might be advantages/disadvantages of each technique?*

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**SINGLE GENE EXPRESSION EXPERIMENT
DAY 4**

This experiment has 11 steps (the first 4 will be done today):

- Step 1:** Acquiring cell types
- Step 2:** Protein extraction
- Step 3:** RNA and DNA extraction
- Step 4:** Quantifying each molecule
- Step 5:** PCR of DNA
- Step 6:** RT-PCR Reverse transcription reaction
- Step 7:** PCR of cDNA from step 6
- Step 8:** SDS-PAGE
- Step 9:** Agarose gels
- Step 10:** Immunoblotting (Western blotting) Part 1
- Step 11:** Immunoblotting Part 2

The first 3 steps have to be carefully coordinated. Make sure you have read and understand the protocols listed below **BEFORE** you start the procedure. We will verify that you have found everything you need.

Assemble everything you need for the first 3 steps, but keep them separated (the number of bullets does not indicate the number of items to be assembled):

- _____
- _____
- _____
- _____
- _____

NOTES:

Answer the questions in bold italics (above) in this space.
Also note any concerns, mishaps, thoughts or questions of your own.

**GENE EXPRESSION EXPERIMENT
DAY 4**

Step 1: Acquiring the two types of cells.

1. Brain will be the source of glial cells and liver the source of non-brain cells.
2. Dissect brain and liver tissue from rat.
3. Make sure you have read **Protocols 7A and 7B**
 - a. **IMPORTANT: PAY ATTENTION TO THE DIFFERENT QUANTITIES OF TISSUE AND TREATMENTS EACH EXTRACTION PROCEDURE REQUIRES.**

Step 2: Homogenization for protein extraction.

1. Transfer tissue to a tissue grinder
2. Add a minimal volume of PBS containing protease inhibitors, and homogenize the tissue.
3. Transfer the extract to a fresh tube.
4. Add DNAase $\mu\mu\mu$ and allow to incubate for 5 minutes.
5. Remove 100 μ l for a later protein assay and add 3X sample buffer to the remainder. (Add to your sample a volume of sample buffer that is 1/3 of the total sample volume.) ***How much sample buffer is that?***
6. Freeze all samples AFTER LABELING CLEARLY.

NOTES:

Answer the questions in bold italics (above) in this space.
Also note any concerns, mishaps, thoughts or questions of your own.

**GENE EXPRESSION EXPERIMENT
DAY 4**

Step 3: Extracting chromosomal DNA and mRNA.

1. Take a very small quantity of each type of tissue (no more than 25 mg).
2. Follow **Protocol 7A** for DNA extractions and **Protocol 7B** for RNA extractions.
 - a. Everything you need is in the kit.

Step 4: Assays: Quantify DNA, RNA and proteins

How will you know your extractions were successful?

1. Measure the success of each extraction and determine relative amounts of protein, RNA and DNA in each sample. DNA and RNA will be measured directly by spectrophotometry. Protein will be measured using a chemical assay (see below).
 - a. *Why bother with these protein, DNA and RNA assays? You simply want to know whether certain proteins/RNA's are expressed in nerve supportive cells and not in liver.*

RESULTS: ASSAYS-DNA/RNA

Despite the fact that the spectrophotometers we use do the calculations for you, study **Protocol 3** to understand the basis of this measurement.

	Liver	Brain
DNA concentration		
RNA concentration		

NOTES:

Answer the questions in bold italics (above) in this space.
Also note any concerns, mishaps, thoughts or questions of your own.

**SINGLE GENE EXPRESSION EXPERIMENT
DAY 4**

Make Working solutions of a known concentration. ***Do you remember how to dilute stock solutions to obtain a specified volume of liquid at a specific concentration?***

You need a 40 μl volume working solution of 40 $\text{ng}/\mu\text{l}$ DNA for PCR

You need a 40 μl volume working solution of 100 $\text{ng}/\mu\text{l}$ RNA for RT-PCR.

Liver:

Fill in the amount of DNA you need to add _____ and the amount of water _____.

Fill in the amount of RNA you need to add _____ and the amount of water _____.

Dilute the appropriate amount of DNA/RNA in a 0.5ml microfuge tube.

Brain:

Fill in the amount of DNA you need to add _____ and the amount of water _____.

Fill in the amount of RNA you need to add _____ and the amount of water _____.

Dilute the appropriate amount of DNA/RNA in a 0.5ml microfuge tube.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

GENE EXPRESSION EXPERIMENT

DAY 4:

RESULTS: ASSAYS- Proteins

Follow **Protocol 8**

Insert on the adjoining graph paper your standard curve and estimate protein concentration:

Protein concentration: Brain _____

Protein concentration: Liver _____

You need to load ~200 μg of protein per well. Well volume = ~20 μl .

What working concentration do you want to make?

To make the working concentration tube:

Fill in the amount of protein extract you need to add _____ (Consider the amount of sample buffer that you added).

Fill in the amount of diluent _____.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**SINGLE GENE EXPRESSION EXPERIMENT:
DAY 4**

Step 5: Polymerase Chain Reaction Optimization

Before you do rt-PCR, you have to determine the optimal conditions for the primers you will use to target the genes we are testing. You can do that with the double-stranded genomic DNA you extracted.

The goal of optimization is to find those conditions where the target sequence and only the target sequence will be amplified. There are 3 parameters that may affect the success AND specificity of your reaction: annealing temperature, MgCl₂ concentration and primer concentration.

Annealing temp: Low temps are permissive, High temps stringent.

MgCl₂ Concentration: Low concentrations are stringent, High concentrations permissive

Primer concentration: High concentrations causes self-annealing (i.e. primer dimer)

In this experiment, each group will test 2 primer conc. X 4 MgCl₂ concentrations at just **one** annealing temperatures for a total of 8 reactions per group. See table below(??)

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**SINGLE GENE EXPRESSION EXPERIMENT:
DAY 4**

	MgCl ₂ Concentrations					
Primer conc.	55.0 C Group 1	58.0 C Group 2	62 C Group 3	65 C Group 4	68 C Group 5	
20pM per µl	1.0 mM	1.0 mM	1.0 mM	1.0 mM	1.0 mM	
	1.5 mM	1.5 mM	1.5 mM	1.5 mM	1.5 mM	
	2.0 mM	2.0 mM	2.0 mM	2.0 mM	2.0 mM	
	2.5 mM	2.5 mM	2.5 mM	2.5 mM	2.5 mM	
80pM per µl	1.0 mM	1.0 mM	1.0 mM	1.0 mM	1.0 mM	
	1.5 mM	1.5 mM	1.5 mM	1.5 mM	1.5 mM	
	2.0 mM	2.0 mM	2.0 mM	2.0 mM	2.0 mM	
	2.5 mM	2.5 mM	2.5 mM	2.5 mM	2.5 mM	

1. To measure all these different variables you need 2 master mixes: **1 Master Reaction Mix**, and **2 Primer Master Mixes**, but you will **add MgCl₂ to each tube separately**.
2. Calculate **Master Reaction Mix** (Reagents for a single reaction in table ?? below)
 - a. Consists of all reagents **except primers and MgCl₂**
 - b. Calculate enough for 8 + 1 reactions
3. **IMPORTANT!** You must reserve some of the water for the primers and MgCl₂ Master Mixes.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

- a. Therefore, use 5.50 μl of water instead of 15.50ul; 5 μl will be used for the different MgCl_2 concentrations and the other 5 μl for primer mixes.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**SINGLE GENE EXPRESSION EXPERIMENT:
DAY 4**

Reagent	1X Volumes	1X Without Water for MgCl ₂ & primers	√	Master Mix Volumes 9 X
10x (Mg free) reaction buffer	2.50 µl	2.50 µl		
10 mM dNTP Mix	2.50 µl	2.50 µl		
Taq DNA polymerase (5 U/µl)	0.50 µl	0.50 µl		
DNA (40ng/ul)	1.00 µl	1.00 µl		
sterile ddH ₂ O	15.50µl	5.50µl		
25 mM MgCl ₂ Solution**	2.50 µl	2.50 µl		
Primer F @ 20pmol/µl	0.25 µl	0.25 µl		
Primer R @ 20pmol/µl	0.25 µl	0.25 µl		
Final volume	25.00µl	15.00µl		

** The volume of MgCl₂ is for a 2.5 mM MgCl₂ concentration.

- a. What is the final volume of your **Master mix**? _____
 - b. How much Master Mix will you add to each of your 8 tubes? _____
4. Calculate the two **4.5X Primer Master Mixes**
- One mix combines the forward and reverse primers at 80pmol/µl concentration.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

- The other combines the forward and reverse primers at 20pmol/ μ l concentration

**SINGLE GENE EXPRESSION EXPERIMENT:
DAY 4**

Primer Master Mix table

	80 pmol 1X	4.5X	\checkmark	20pmol 1X	4.5X	\checkmark
Forward Primer	0.25			0.25		
Reverse Primer	0.25			0.25		
Water	4.50			4.50		
Total	5.00			5.00		

c. How much **Primer Master Mix** will you add to each of your 8 tubes? _____

5. Add MgCl₂ & water to your 8 tubes directly as shown in the table below.

	MgCl₂ Concentrations			
	1.0 mM	1.5 mM	2.0 mM	2.5 mM
25mM MgCl₂	1.00 ul	1.50 ul	2.00 ul	2.50 ul
Water	4.00 ul	3.50 ul	3.00 ul	2.50 ul
Total Volume	5.00 ul	5.00 ul	5.00 ul	5.00 ul

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**SINGLE GENE EXPRESSION EXPERIMENT:
DAY 4**

6. **AFTER you finish your calculations: assemble everything you need:**
 - a. Get the reagents
 - b. Keep primers, dNTPs and TAQ on ice until the last minute, but begin thawing the other reagents.
 - c. **Keep your 8-tube strip on ice.**
7. Put your MgCl₂ and water aliquots into the appropriate tubes in the strip.
8. Make the **Primer Mix (KEEP IT ON ICE!!)**
9. Make the **Master Reaction Mix (KEEP IT ON ICE!!)**
 - a. Add 5µl of appropriate primer mix to the appropriate tube.
 - a. Aliquot __ µl of Master Mix into each of the 8 tubes
 - b. Turn on the machine and run the program *ICEBUCKET*
 - c. When everyone's reaction mixes are in the thermalcycler:
 - Put the lid down gently.
 - Stop *ICEBUCKET*
 - Run *REU-PCR*.
 - d. After the program finishes put your products in the -20° C freezer.
 - e. We will run gels later.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**SINGLE GENE EXPRESSION EXPERIMENT
DAY 5**

Step 6: Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The RNA extraction procedure extracted all RNAs including all mRNAs from every expressed gene, all rRNAs, and all tRNAs. Now that you want to determine whether the RNA extracts from rat livers, are different from those in rat brain with respect to the single gene GFAP. *What are your predictions?*

1. Calculate the Master Mix of Reverse transcription reagents without RNA.

Reagent	1X Volumes	Check	Master Mix Volumes ____ X
5x Reaction Buffer w/ MgCl	4.00 µl		
Reverse Primer @ 20pmol/µl	2.00 µl		
Control Reverse primer	2.00 µl		
100mM dNTP Mix	3.00 µl		
RNase inhibitor	0.50 µl		
MLV Reverse Transcriptase (200 U/µl)	1.00 µl		
RNA sample (100ng/µl)	2.00 µl		
RNase Free water	5.50 µl		
Final volume	20.00µl		

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**SINGLE GENE EXPRESSION EXPERIMENT
DAY 5**

Assemble everything you need for step 6

- _____
- _____
- _____
- _____
- _____

2. Make a Master Mix of RT reagents without RNA for the proper number of reactions (***How many is that?***) Add a fudge factor (usually 1 additional reaction).
 - a. See **Protocol 9 and the Table above.**
3. Put your different samples of RNA into separate RT-PCR tubes (**KEEP ON ICE!!**)
4. Put an aliquot (***How much is an aliquot?***) of Master Mix into each sample tube.
5. Run the program ***ICEBUCKET*** (Holds the block @ 4°C indefinitely)
6. Put tubes with reagent mix into the block
7. When everyone has added their sample, lock lid down.
8. Start the Program ***REU-RT***.
9. At the end of this program put cDNA in the freezer

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**SINGLE GENE EXPRESSION EXPERIMENT
DAY 5**

10. Calculate the Master Mix for the PCR step of RT-PCR.

Reagent	1X	Check	Master Mix
PCR Water	11.0 μ l		
cDNA from RT reaction	5.0 μ l		
10X Taq Buffer	2.0 μ l		
MgCl ₂	5.0 μ l		
20uM Forward primer	1.5 μ l		
Taq DNA polymerase	0.5 ul		
Total volume	25.0 ul		

11. Make the PCR reaction Master Mix.

12. Put tubes back in the machine.

13. Run the Program ***REU-RTPCR***

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**SINGLE GENE EXPRESSION EXPERIMENT:
DAY 6**

Main steps for Day 6

Step 8: Pour and run Sodium Dodecyl Sulfate – Poly Acrylamide Gel Electrophoresis gels

Step 9: Pour and run agarose gels

Step 10: Immuno-blotting

GENERAL BACKGROUND FOR SDS-PAGE: This technique will separate proteins by their size. A charged (ionic) detergent (usually sodium dodecyl sulfate) is added to protein extracts to denature the proteins and solubilize membranes. In addition, a reducing agent (β -mercaptoethanol or dithiothreitol) is added to break disulfide bonds. The protein loses all/most structure and therefore forms a capsule-shaped blob whose size is directly proportional to the mass of the protein. The detergent also coats the protein with charge (negative, in the case of SDS) and this serves to move the protein through the polyacrylamide matrix (gel) during the electrophoresis step. Even though large proteins should be coated with more charge than smaller ones, large proteins move slowly through the gel because they can't fit through the matrix easily. ***What do you predict we will see? What will we be able to conclude from this step?***

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**SINGLE GENE EXPRESSION EXPERIMENT: Proteins
DAY 6**

Assemble everything you need for Step 8

- _____
- _____
- _____
- _____
- _____

Step 8: Pour and run SDS-PAGE gels

1. Follow Protocol 10 **AFTER** we have discussed the theory of this protocol.
2. Discuss theory of one-dimensional SDS-PAGE
3. After your gels are finished, stain one gel, photograph and enter into the Results section (pg. 36??).

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**GENE EXPRESSION EXPERIMENT: RNA
DAY 6**

Assemble everything you need for Step 9

- _____
- _____
- _____
- _____
- _____

Step 9: Pour and run agarose gels

This technique will separate your PCR and RT-PCR products by size. ***What do you predict we will see? What will we be able to conclude from this step?***

1. Follow **protocol 6** for pouring agarose gels.
2. Discuss theory of agarose gel
3. Load Agarose gel with the PCR product (one reaction product per well) and RT-PCR products.
4. Photograph your results and enter into the Results section

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**SINGLE GENE EXPRESSION EXPERIMENT: Proteins
DAY 6**

Assemble everything you need for Step 10

- _____
- _____
- _____
- _____
- _____

Step 10: Immunoblotting (Western blotting): Part 1. Transfer of proteins to membranes

This technique allows identification and visualization of specific proteins through the use of protein-specific antibodies. Today we will do the blotting part of the protocol; tomorrow we will do the immunologic identification of proteins.

1. Follow THE FIRST PART OF **Protocol 11** (protein transfer to membranes) after we have discussed the theory of blotting.
2. Discuss theory of blotting:

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**SINGLE GENE EXPRESSION EXPERIMENT: Proteins
DAY 7**

Assemble everything you need for Step 11

- _____
- _____
- _____
- _____
- _____

Step 11: Immunoblotting (Western blotting): Part 2. Detection of antigens.

1. Follow the second part of Protocol 11 (detection of specific antigens) after we have discussed the logic of the protocol.
2. Discuss theory of immunologic identification of specific proteins.
3. After your blot has developed, place in Results section (pg. 36??).

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**GENE EXPRESSION EXPERIMENT: Protein level
DAY 7**

FINAL RESULTS:

Record data from agarose gel, protein SDS-PAGE gel and immunoblot, below. Make sure that you:

1. Label each image Figure 1, Figure 2 etc.
2. Write a header for each figure stating:
 - a. The hypothesis tested.
 - b. description of the figure if you want
3. Label all lanes so that 20 years from now you will still know what sample was loaded in each well.
4. Label the size of standard fragments/proteins.
5. Record the conclusions drawn from each gel.
6. Note whether the products seen are of the predicted size.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

GENE EXPRESSION EXPERIMENT: Proteins & RNA
DAY 7
PASTE FIGURES HERE

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**ALTERNATIVE METHODS IN EXAMINING GENE EXPRESSION
DAY 8**

BACKGROUND: Now we will practice/demonstrate/discuss some alternative techniques for examining protein expression: 2-dimensional gel electrophoresis (Day 8, 9, 10), Matrix-Assisted Laser Desorption Ionization (MALDI) mass spectroscopy (Day 10), and microarrays (Day 10).

2-D Gel Electrophoresis – You may have noticed that resolution of individual proteins by SDS-PAGE was good but did not yield many bands of well-purified protein. Even apparently purified bands may have contained multiple proteins of approximately the same size. 2-D gel electrophoresis is much more successful at purifying proteins, because it separates proteins by their charge as well as size. Purified, unknown proteins can be subjected to, for example, MALDI mass spectroscopy to identify the proteins. Remember, you may not always know what proteins you are after; you may not always be able to use antibodies or primers to identify individual proteins or their mRNAs.

There are 4 main steps in this experiment:

Step 1: Load protein on isoelectric focusing strips

Step 2: Separate proteins by isoelectric focusing point (1st dimension)

Step 3: Separate proteins by size (2nd dimension)

Step 4: Silver stain the SDS-PAGE dimension.

Assemble what you need for Steps 1-2:

- _____
- _____
- _____
- _____
- _____

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**ALTERNATIVE METHODS IN EXAMINING GENE EXPRESSION
DAY 8**

Steps 1-2: Loading and Running Isoelectric Focusing Strip

1. Follow the provided protocols for steps 1-2 after we have discussed the theory and advantages of 2-D gel electrophoresis.
2. Theory of 2-D gel electrophoresis: *When should one choose 2-D gel electrophoresis over 1-D SDS-PAGE?*

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**ALTERNATIVE METHODS IN EXAMINING GENE EXPRESSION
DAY 9**

Assemble what you need for Step 3:

- _____
- _____
- _____
- _____
- _____

Step 3: Run isoelectric focusing strip in the second dimension.

Follow the provided protocol.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

**ALTERNATIVE METHODS IN EXAMINING GENE EXPRESSION
DAY 10**

Assemble what you need for Step 4:

- _____
- _____
- _____
- _____
- _____

Step 4: Silver stain the SDS-PAGE dimension.

1. Follow the provided protocol for silver staining gels.
2. Photograph your gel and record below. Remember to label the photograph.

MALDI mass spectroscopy - The remainder of the day will be spent discussing this topic. **MALDI mass spectroscopy** is a means of identifying proteins (from your 2-D gels, for example) or other molecules by very precisely measuring their mass or the mass of their component fragments.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

PROTOCOLS

Protocol 1: Streak for isolation

- a. Dip a sterile loop into the liquid culture and spread cells in a zig-zag pattern in the first quadrant.
- b. Flame the loop, **LET IT COOL**, catch the end of the streak in quadrant1 and zig-zag into quadrant2.
- c. Repeat for the other 3 quadrants.
- d. Label around the edges of the bottom half of the plates with the strain name, the date and your name.
- e. Wrap the edges of the plates with parafilm.
- f. Put the plates upside down in the 37C incubator.
- g. Remove from incubator the next morning and put in the refrigerator.

Figure 1. Graphical illustration of the protocol

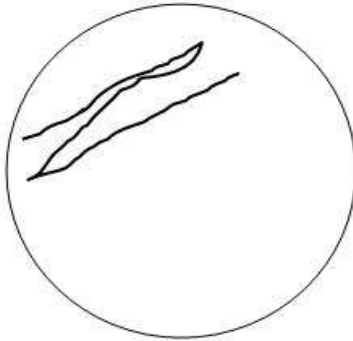
NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

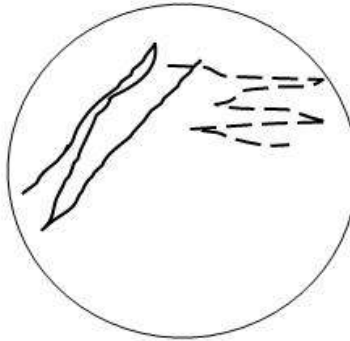
Streak 1:

- a. Flame loop, let cool**
- b. Streak cells in 1/3 of plate.**



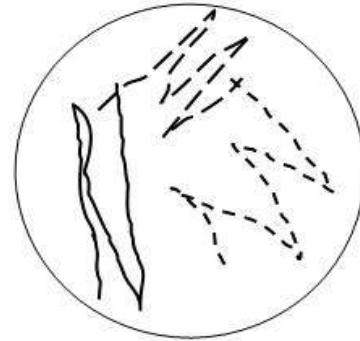
Streak 2:

- a. Flame loop again, let cool**
- b. Rotate plate,**
- c. Pick up from end of streak**
- d. Streak again**



Streak 3:

- a. Flame loop again, let cool**
- b. Rotate plate,**
- c. Pick up from end of streak**
- d. Streak again**



NOTES:

Answer the questions in bold italics (above) in this space.
Also note any concerns, mishaps, thoughts or questions of your own.

Protocol 2: RNA extraction for bacteria.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

RNA Purification Protocols

The following protocol is provided for the purification of RNA from several biological sources (see **General Considerations**, page 2). Lyse the fluid or tissue as outlined in Part A, and then proceed with the remainder of the protocol as outlined in Part B on page 4. If further purification of RNA (to remove contaminating DNA) is required, follow the protocol outlined in Part C on page 5. Use appropriate techniques to minimize degradation by exogenous ribonucleases. Additional purification protocols begin on page 6.

A. Lysis of Fluid or Tissue Samples

Thoroughly mix the various Lysis Solutions to ensure uniform composition before dispensing.

Fluid Samples (e.g., saliva, semen)

1. Collect samples and either process immediately or freeze at -70°C .
2. Dilute $1\ \mu\text{l}$ of $50\ \mu\text{g}/\mu\text{l}$ Proteinase K into $150\ \mu\text{l}$ of 2X T and C Lysis Solution for each sample.
3. Transfer $150\ \mu\text{l}$ of the fluid sample to a microcentrifuge tube and add $150\ \mu\text{l}$ of 2X T and C Lysis Solution containing the Proteinase K and mix thoroughly.
4. Incubate at 65°C for 15 minutes; vortex mix every 5 minutes.
5. Place the samples on ice for 3-5 min and then proceed with RNA precipitation in Part B (pg 4).

Cell Samples (e.g., mammalian cell culture, buccal cells, *E. coli*)

1. Dilute $1\ \mu\text{l}$ of $50\ \mu\text{g}/\mu\text{l}$ Proteinase K into $300\ \mu\text{l}$ of Tissue and Cell Lysis Solution for each sample.
2. Pellet cells by centrifugation ($0.5\text{-}1 \times 10^6$ mammalian cells; $0.1\text{-}0.5$ ml of an overnight culture of *E. coli*) and discard the supernatant, leaving approximately $25\ \mu\text{l}$ of liquid.
3. Vortex mix 10 seconds to resuspend the cell pellet.
4. Add $300\ \mu\text{l}$ of Tissue and Cell Lysis Solution containing the Proteinase K and mix thoroughly.
5. Incubate at 65°C for 15 minutes; vortex mix every 5 minutes.
6. Place the samples on ice for 3-5 min and then proceed with RNA precipitation in Part B (pg 4).

Tissue Samples (e.g., plant or animal tissues)

1. Collect 1-5 mg of tissue and either process immediately or freeze the samples at -70°C .
2. Dilute $1\ \mu\text{l}$ of $50\ \mu\text{g}/\mu\text{l}$ Proteinase K into $300\ \mu\text{l}$ of Tissue and Cell Lysis Solution for each sample.
3. Homogenize fresh tissue or grind frozen tissues in liquid nitrogen and transfer to a microcentrifuge tube.
4. Add $300\ \mu\text{l}$ of Tissue and Cell Lysis Solution containing the Proteinase K and mix thoroughly.
5. Incubate at 65°C for 15 minutes; vortex mix every 5 minutes.
6. Place the samples on ice for 3-5 min and then proceed with RNA precipitation in Part B (pg 4).

EPICENTRE**MasterPure™ RNA Purification Kit**

Whole Blood Samples (with RBC lysis)

1. Draw 5 ml of blood into an EDTA Vacutainer® tube. Transfer 200 µl of whole blood into a microcentrifuge tube.
2. Add 600 µl of Red Cell Lysis Solution. Invert 3 times to mix and then flick the bottom of the tube to suspend any remaining material.
3. Incubate at room temperature for 5 minutes and then vortex mix briefly. Continue incubating at room temperature for an additional 5 minutes followed again by brief vortex mixing.
4. Pellet the white blood cells by centrifugation for 25 seconds in a microcentrifuge.
5. Remove most of the supernatant, leaving approximately 25 µl of liquid. Vortex mix to suspend the pellet.
6. Resuspend the white blood cells in 300 µl of Tissue and Cell Lysis Solution by pipetting the cells up and down several times.
7. Place the samples on ice for 3-5 min and then proceed with RNA precipitation in Part B below.

RNA from Formalin-Fixed, Paraffin-Embedded (FFPE) Tissues

1. Remove section of tissue using a clean microtome blade; if possible trim excess paraffin.
2. Place 2-30 mg of 10-35 µm thick paraffin sections into an appropriate RNase-free tube. If using a larger amount of tissue, scale up the reagent volumes proportionally.
3. Per sample dilute 2 µl of 50 µg/µl Proteinase K into 300 µl of T and C Lysis Solution and mix.
4. Add 300 µl of the T and C Lysis Solution containing Proteinase K to the sample and mix.
5. Incubate at 65°C for 30 minutes.
6. Place the samples on ice for 3-5 min and then proceed with RNA precipitation in Part B below.

B. Precipitation of Total RNA (for all biological samples)

1. Add 175 µl of MPC Protein Precipitation Reagent to 300 µl of lysed sample and vortex mix vigorously for 10 seconds.
2. Pellet the debris by centrifugation for 10 minutes at $\geq 10,000 \times g$ in a microcentrifuge.
3. Transfer the supernatant to a clean microcentrifuge tube and discard the pellet.
4. Add 500 µl of isopropanol to the recovered supernatant. Invert the tube several (30-40) times.
5. Pellet the RNA by centrifugation at 4°C for 10 minutes in a microcentrifuge.
6. Carefully pour off the isopropanol without dislodging the RNA pellet. If removal of contaminating DNA is required, proceed with Part C (pg 5). Otherwise, complete the remainder of Part B.
7. Rinse twice with 75% ethanol, being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all of the residual ethanol with a pipet.
8. Resuspend the RNA in 35 µl of TE Buffer.

EPICENTRE

MasterPure™ RNA Purification Kit

C. Removal of Contaminating DNA from RNA Preparations (for all biological samples)

1. Remove all of the residual isopropanol with a pipet.
2. Prepare 200 μ l of DNase I solution for each sample by diluting 5 μ l of RNase-Free DNase I up to 200 μ l with 1X DNase Buffer.
3. Completely resuspend the nucleic acid pellet in 200 μ l of DNase I solution.
4. Incubate at 37°C for 10 min. **Note**, additional incubation (up to 30 min) may be necessary to remove all contaminating DNA.
5. Add 200 μ l of 2X T and C Lysis Solution; vortex mix for 5 seconds.
6. Add 200 μ l of MPC Protein Precipitation Reagent; vortex mix 10 seconds; place on ice 3-5 min.
7. Pellet the debris by centrifugation for 10 minutes at $\geq 10,000 \times g$ in a microcentrifuge.
8. Transfer the supernatant containing the RNA into a clean microcentrifuge tube and discard the pellet.
9. Add 500 μ l of isopropanol to the supernatant. Invert the tube several (30-40) times.
10. Pellet the purified RNA by centrifugation at 4°C for 10 minutes in a microcentrifuge.
11. Carefully pour off the isopropanol without dislodging the RNA pellet.
12. Rinse twice with 75% ethanol, being careful to not dislodge the pellet. Centrifuge briefly if the pellet is dislodged. Remove all of the residual ethanol with a pipet.
13. Resuspend the RNA in 10-35 μ l of TE Buffer.
14. Add 1 μ l of ScriptGuard RNase Inhibitor.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

Protocol 3: Determination of yield and purity of DNA and RNA**Principles:**

DNA yield is determined by measuring the concentration of DNA in the eluate by its absorbance at 260 nm. Absorbance readings at 260 nm should fall between 0.1 and 1.0 to be accurate. Sample dilution should be adjusted accordingly: e.g., an eluate containing 25–50 ng/ μ l DNA (A_{260} = 0.5–1.0) should not be diluted with more than 4 volumes of water. Measure the absorbance at 260 nm or scan absorbance from 220–330 nm (a scan will show if there are other factors affecting absorbance at 260 nm; for instance, absorbance at 325 nm would indicate contamination by particulate matter or a dirty cuvette). An A_{260} of 1 (with a 1 cm detection path) corresponds to 50 μ g DNA per milliliter water. Water should be used as diluent when measuring DNA concentration since the relationship between absorbance and concentration is based on extinction coefficients calculated for nucleic acids in water.* Both DNA and RNA are measured with a spectrophotometer at 260 nm; to measure only DNA in a mixture of DNA and RNA, a fluorimeter must be used. An example of the calculations involved in DNA quantification is shown below:

Volume of DNA sample = 100 μ l

Dilution = 20 μ l of DNA sample + 180 μ l distilled water (1/10 dilution)

Measure absorbance of diluted sample in a 0.2 ml cuvette A_{260} = 0.2
 Concentration of DNA sample = $50 \mu\text{g/ml} \times A_{260} \times \text{dilution factor} = 50 \mu\text{g/ml} \times 0.2 \times 10 = 100 \mu\text{g/ml}$
 Total amount = concentration \times volume of sample in milliliters = $100 \mu\text{g/ml} \times 0.1 \text{ ml} = 10 \mu\text{g}$ of DNA
 The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of DNA with respect to contaminants that absorb UV, such as protein. However, the A_{260}/A_{280} ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary greatly. Lower pH results in a lower A_{260}/A_{280} ratio and reduced sensitivity to protein contamination. For accurate values, we recommend measuring absorbance in 10 mM Tris·Cl, pH 7.5, in

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

which pure DNA has an A₂₆₀/A₂₈₀ ratio of 1.8–2.0. Always be sure to calibrate the spectrophotometer with the same solution.

Measure absorbance of diluted sample in a 0.2 ml cuvette

A₂₆₀ = 0.2

Concentration of DNA sample = 50 µg/ml x A₂₆₀ x dilution factor

= 50 µg/ml x 0.2 x 10 = 100 µg/ml

Total amount = concentration x volume of sample in milliliters

= 100 µg/ml x 0.1 ml

= 10 µg of DNA

The ratio of the readings at 260 nm and 280 nm (A₂₆₀/A₂₈₀) provides an estimate of the purity of DNA with respect to contaminants that absorb UV, such as protein. However, the A₂₆₀/A₂₈₀ ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A₂₆₀/A₂₈₀ ratio can vary greatly. Lower pH results in a lower A₂₆₀/A₂₈₀ ratio and reduced sensitivity to protein contamination. For accurate values, we recommend measuring absorbance in 10 mM Tris·Cl, pH 7.5, in which pure DNA has an A₂₆₀/A₂₈₀ ratio of 1.8–2.0. Always be sure to calibrate the spectrophotometer with the same solution.

NOTES:

Answer the questions in bold italics (above) in this space.

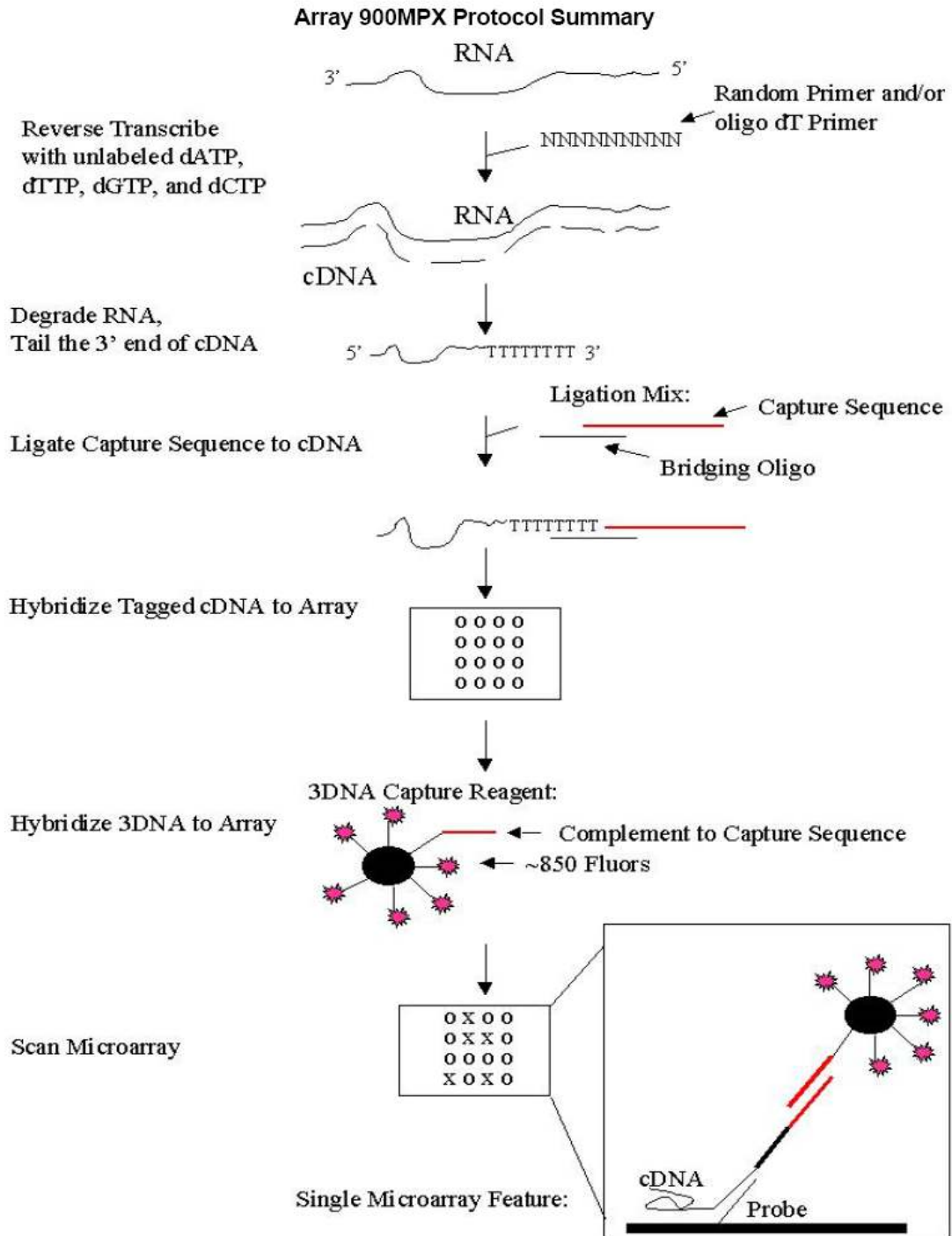
Also note any concerns, mishaps, thoughts or questions of your own.

PROTOCOL 4: MICROARRAY (GENISPHERE KIT)

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.



NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

Protocol 4 continued: cDNA synthesis

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

Procedure For Use

Preparation of cDNA

cDNA Synthesis (Reverse Transcription):

Since microarrays and RNA preparations vary in quality, the exact amount of RNA required for a given experiment will typically range from 0.5-2 μ g of animal total RNA or 1-4 μ g of plant total RNA. Larger or smaller amounts of RNA may be required to achieve optimal results. For new users, the following quantities of RNA are recommended as a starting point for cDNA synthesis:

Animal total RNA: 2 μ g

Plant total RNA: 4 μ g

Poly(A) RNA: 100ng

SenseRNA: 200ng-1 μ g; depending on 260/280 ratio. Refer to SenseAmp protocol.

The reverse transcription reaction can be performed with Random Primer (Vial 2), MPX dT Primer (Vial 8), or both primers. The primers are designed to be used differently: the MPX dT Primer (Vial 8) should be used at 1 μ l per reaction, whereas the Random Primer (Vial 2) should be used at 2X by mass of RNA; for example, use 2 μ l of Random Primer per 1 μ g of RNA. When using small quantities of RNA, the Random Primer may be diluted in Nuclease Free Water (Vial 10) for ease of pipetting. For example, when using 100ng of poly(A) RNA, dilute the Random Primer 1:10 and use 2 μ l (200ng) of this dilution in the reaction.

1. In a microtube, prepare the **RNA-RT primer mix**:
 - 1-9 μ l RNA
 - 1-4 μ l Random Primer (Vial 2) USE RANDOM PRIMER AT 2X BY MASS OF RNA.
 - 1 μ l MPX dT Primer (Vial 8) MAY BE OMITTED WHEN PRIMING PROKARYOTIC SAMPLES
 - Add Nuclease Free Water (Vial 10) to a final volume of 11 μ l
2. Mix the **RNA-RT primer mix** and microfuge briefly to collect contents in the bottom of the tube.
3. Heat to 80°C for 10 minutes and immediately transfer to ice for 2-3 minutes.
4. In a separate microtube on ice, prepare a **reaction mix** for every RT reaction:
 - 4 μ l 5X SuperScript II First Strand Buffer (or equivalent reaction buffer supplied with enzyme)
 - 2 μ l 0.1M dithiothreitol (if supplied with enzyme; otherwise use 2 μ l Nuclease Free Water (Vial 10))
 - 1 μ l dNTP mix (Vial 3)
 - 1 μ l Superase-In RNase inhibitor (Vial 4)
 - 1 μ l Superscript II enzyme, 200 units (or equivalent reverse transcriptase)
5. Gently mix (do not vortex) the **reaction mix** and microfuge briefly to collect reaction mix contents in the bottom of the tube.
6. Add the 9 μ l of **reaction mix** from step 5 to the 11 μ l of **RNA-RT primer mix** from step 3 (20 μ l volume).
7. Gently mix (do not vortex) and incubate at 42°C for 2 hours.
8. Stop the reaction by adding 3.5 μ l of 0.5M NaOH/50mM EDTA.
9. Incubate at 65°C for 15 minutes to denature the DNA/RNA hybrids and degrade the RNA.
10. Neutralize the reaction with 5 μ l of 1M Tris-HCl, pH 7.5.
11. Add 21.5 μ l of 1X TE buffer for a total volume of 50 μ l.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

Protocol4: Purification of cDNA

Purification of cDNA:

Purify the 50µl of cDNA using the Qiagen MinElute PCR Purification Kit (Catalog # 28004) as follows:

- a. Add 250µl Buffer PB to the 50µl cDNA sample and mix.
- b. Apply the cDNA mixture from step "a" to the MinElute column and centrifuge for 1 minute at 10-14,000 x g (~13,000 rpm) in a conventional tabletop microcentrifuge.
- c. Discard the flow-through. Place the MinElute column into the same collection tube.
- d. Add 750µl Buffer PE to the MinElute column and centrifuge for 1 minute.
- e. Discard the flow-through. Place the MinElute column back into the same collection tube and centrifuge for an additional 2 minutes to remove residual ethanol.
- f. Place the MinElute column into a clean 1.5mL microfuge tube.
- g. To elute cDNA, add 10µl Buffer EB to the center of the column membrane. Incubate at room temperature for 2 minutes. Centrifuge for 2 minutes. Discard column and save the 10µl eluted cDNA.

Protocol 4: Tailing & Ligation of Capture Sequence (Tagging)

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

Terminal Deoxynucleotidyl Transferase (TdT) Tailing Reaction:

1. To the purified cDNA, add 6.5µl of Nuclease Free Water (Vial 10), bringing the volume to 16.5µl.
2. Heat the 16.5µl of cDNA to 80° for 10 minutes and immediately transfer to ice for 2 minutes.
3. Add the following components for a volume of 25µl:
 - 2.5µl 10X Tailing Buffer (Vial 13)
 - 4µl 10mM dTTP (Vial 14)
 - 2µl Terminal Deoxynucleotidyl Transferase (Vial 15)
4. Incubate at 37° for 30 minutes.
5. Proceed immediately to ligation, below.

Ligation to 3DNA Capture Sequence (Preparation of “Tagged” cDNA):

1. Heat the 25µl of tailed cDNA to 95° for 10 minutes and immediately transfer to ice for 2 minutes.
2. Add the following components for a volume of 32µl:
 - 5µl appropriate 6X Ligation Mix (Vial 11: Cy3 / Alexa 546 or Cy5 / Alexa 647)
 - 2µl T4 DNA Ligase (Vial 12)
3. Mix gently, and incubate at 18-25°C (approximately room temperature) for 30 minutes.
4. Add 3.5µl of 0.5M EDTA to stop the ligation reaction, and vortex thoroughly (5-10 seconds).
5. Add 14.5µl of 1X TE buffer for a total volume of 50µl.

Protocol 4: Purification of Tagged cDNA

NOTES:

Answer the questions in bold italics (above) in this space.
Also note any concerns, mishaps, thoughts or questions of your own.

Purification of Tagged cDNA:

Purify the 50µl of Tagged cDNA using the Qiagen MinElute PCR Purification Kit (Catalog # 28004) as follows:

- a. Add 250µl Buffer PB to the 50µl Tagged cDNA sample and mix.
- b. Apply the cDNA mixture from step "a" to the MinElute column and centrifuge for 1 minute at 10-14,000 x g (~13,000 rpm) in a conventional tabletop microcentrifuge.
- c. Discard the flow-through. Place the MinElute column into the same collection tube.
- d. Add 750µl Buffer PE to the MinElute column and centrifuge for 1 minute.
- e. Discard the flow-through. Place the MinElute column back into the same collection tube and centrifuge for an additional 2 minutes to remove residual ethanol.
- f. Place the MinElute column into a clean 1.5mL microfuge tube.
- g. To elute cDNA, add 10µl Buffer EB to the center of the column membrane. Incubate at room temperature for 2 minutes. Centrifuge for 2 minutes. Discard column and save the 10µl eluted cDNA.

For single color assays: proceed to *Successive Hybridization of cDNA and 3DNA to Microarray*, pg. 13.

For dual color assays: combine the two 10µl purifications for a total of 20µl cDNA. If 20µl of cDNA exceeds the desired volume per the table on page 13, follow the *Concentration of cDNA* procedure in Appendix A. Otherwise, proceed to *Successive Hybridization of cDNA and 3DNA to Microarray*, pg. 13.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

Protocol 4: Hybridization of cDNA

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

cDNA Hybridization:

1. Thaw and resuspend the 2X Hybridization Buffer (Vial 5, 6 or 7) by heating to 65-70°C for at least 10 minutes or until completely resuspended. Vortex to ensure that the components are resuspended evenly. If necessary, repeat heating and vortexing until all the material has been resuspended. Microfuge for 1 minute. (See *Hybridization Conditions*, pg. 8, for help in selecting the appropriate buffer.)
2. For each array, prepare a **cDNA Hybridization Mix** according to the tables below.

Optional: 1µl of Cot-1 DNA may be added to the cDNA Hybridization Mix (denature at 95-100°C for 10 minutes prior to use).

Note: 2X Enhanced Hybridization Buffer (Vial 5) requires higher hybridization volumes due to its increased viscosity.

Option 1 (Recommended): Use of Enhanced Hybridization Buffer (Vial 5)

Glass Coverslip Size, mm	24x30	24x40	24x50	24x60
Final Hybridization Volume	30µl	38µl	48µl	58µl
cDNA (10µl if Microcon concentrated or 20µl from combined purifications)	10µl	10µl	20µl	20µl
LNA dT Blocker (Vial 9)	2µl	2µl	2µl	2µl
Nuclease Free Water (Vial 10)	3µl	7µl	2µl	7µl
2X Enhanced Hybridization Buffer (Vial 5)	15µl	19µl	24µl	29µl

Option 2: Use of Vial 6 or Vial 7 Hybridization Buffers

Glass Coverslip Size, mm	24x30	24x40	24x50	24x60
Final Hybridization Volume	26µl	34µl	43µl	50µl
cDNA (10µl if Microcon concentrated or 20µl from combined purifications)	10µl	10µl	20µl	20µl
LNA dT Blocker (Vial 9)	2µl	2µl	2µl	2µl
Nuclease Free Water (Vial 10)	1µl	5µl	0µl	3µl
2X Hybridization Buffer (Vial 6 or 7)	13µl	17µl	21µl	25µl

3. Gently vortex and briefly microfuge the **cDNA Hybridization Mix**. Incubate the **cDNA Hybridization Mix** first at 75-80°C for 10 minutes, and then at the hybridization temperature until loading the array (see the table located below step 5 for recommended hybridization temperatures). Pre-warm the microarrays to the hybridization temperature.
4. Gently vortex and briefly microfuge the **cDNA Hybridization Mix**. Add the **cDNA Hybridization Mix** to a pre-warmed microarray, taking care to leave behind any precipitate at the bottom of the tube.
5. Apply a glass coverslip to the array. Incubate the array overnight in a dark humidified chamber at the appropriate hybridization temperature:

Spotted DNA	Vial 5 or 6 Buffer	Vial 7 Buffer
Oligonucleotide	55-62°C	43-50°C
PCR Product (cDNA)	60-65°C	48-53°C

The hybridization temperatures recommended in this protocol are intended as a starting point and should be used as a guide. It may be necessary to adjust the temperatures to meet the stringency requirements dictated by the nature of the nucleic acids spotted on the array as well as the slide surface chemistry. In particular, increasing the hybridization temperature by 5°C may remove non-specific signal.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

Protocol 4: Post cDNA hyb washes

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

Post cDNA Hybridization Wash:

1. Prewarm the 2X SSC, 0.2%SDS wash buffer to:
 - 60-65°C for PCR product (cDNA) arrays
 - 42°C for oligonucleotide spotted arrays
2. Remove the coverslip by washing the array in prewarmed 2X SSC, 0.2% SDS for 2-5 minutes or until the coverslip floats off.* Additional time may be required to remove the coverslip when the 2X Enhanced cDNA Hybridization Buffer (Vial 5) is used.
3. Wash for 15 minutes in prewarmed 2X SSC, 0.2%SDS.
4. Wash for 10-15 minutes in 2X SSC at room temperature.
5. Wash for 10-15 minutes in 0.2X SSC at room temperature.
6. Immediately transfer the array to a dry 50mL centrifuge tube. Do this quickly to avoid streaky background on the slide. Orient the slide so that any label is down in the tube. Centrifuge without the tube cap for 2 minutes at 800-1000 RPM to dry the slide. Avoid contact with the array surface.

Further optimization of wash conditions may be required to achieve optimal array performance. If necessary to reduce background on the array, we recommend increasing the time of some or all of the washes to 15-20 minutes. Agitation during washing may also help to reduce background due to non-specific binding to the surface of the array.

***Note:** If the coverslip is difficult to remove, this may be an indication of drying. To prevent this problem from recurring in future experiments, increase the total volume of the **cDNA Hybridization Mix** by adding equal volumes of Nuclease Free Water (Vial 10) and 2X Hybridization Buffer (Vial 5, 6 or 7). In addition, ensure that the hybridization chamber is properly humidified and sealed.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

Protocol 4: 3DNA hybridization

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

3DNA Hybridization:

1. Prepare the 3DNA Array 900MPX Capture Reagent (Vial 1). It is necessary to break up aggregates that may form as a result of the freezing process.
 - a. Thaw the 3DNA Array 900MPX Capture Reagent (Vial 1) in the dark at room temperature for 20 minutes.
 - b. Vortex at the maximum setting for 3 seconds and microfuge briefly.
 - c. Incubate at 50-55°C for 10 minutes.
 - d. Vortex at the maximum setting for 3-5 seconds.
 - e. Microfuge the tube briefly to collect the contents at the bottom.

Be sure to check the sample for aggregates prior to use and repeat vortex mixing if necessary. Aggregates may appear as small air bubbles or flakes at the side of the tube below the surface of the solution. Repeat steps a-e if necessary.

2. Thaw and resuspend the 2X Hybridization Buffer (Vial 6 or Vial 7) by heating to 70°C for at least 10 minutes or until completely resuspended. Vortex to ensure that the components are resuspended evenly. If necessary, repeat heating and vortexing until all the material has been resuspended. Microfuge for 1 minute. **Caution: Do not use the 2X Enhanced cDNA Hybridization Buffer (Vial 5) in the 3DNA Hybridization step.**
3. For each array, prepare a **3DNA Hybridization Mix** according to the table below.

Optional: 1µl of Cot-1 DNA may be added to the cDNA Hybridization Mix (denature at 95-100°C for 10 minutes prior to use).

Note: For single channel expression analysis, use 2.5µl of Nuclease Free Water (Vial 10) in place of the second 3DNA Capture Reagent.

Glass Coverslip Size, mm	24x30	24x40	24x50	24x60
Final Hybridization Volume	26µl	34µl	42µl	50µl
3DNA Capture Reagent #1 (Vial 1)	2.5µl	2.5µl	2.5µl	2.5µl
3DNA Capture Reagent #2 (Vial 1)	2.5µl	2.5µl	2.5µl	2.5µl
Nuclease Free Water (Vial 10)	8µl	12µl	16µl	20µl
2X Hybridization Buffer (Vial 6 or 7)	13µl	17µl	21µl	25µl

4. Gently vortex and briefly microfuge the **3DNA Hybridization Mix**. Incubate the **3DNA Hybridization Mix** first at 75-80°C for 10 minutes, and then at the hybridization temperature until loading the array (see the table located below step 6 for recommended hybridization temperatures). Pre-warm the microarrays to the hybridization temperature.
5. Gently vortex and briefly microfuge the **3DNA Hybridization Mix**. Add the **3DNA Hybridization Mix** to a pre-warmed microarray, taking care to leave behind any precipitate at the bottom of the tube.
6. Apply a glass coverslip to the array. Incubate the array for 4 hours in a dark humidified chamber at the appropriate hybridization temperature:

<u>Spotted DNA</u>	<u>Vial 6 Buffer</u>	<u>Vial 7 Buffer</u>
Oligonucleotide	55-65°C	43-53°C
PCR Product (cDNA)	60-65°C	48-53°C

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

Protocol 4: Post 3DNA hybridization washes

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

Post 3DNA Hybridization Wash:

After hybridization the slides are washed several times to remove unbound 3DNA molecules. Perform these washes **in the dark to avoid photobleaching and fading** of the fluorescent dyes. To reduce fading of Cy5 post hybridization, it may also be beneficial to include DTT in the first two wash buffers at a final concentration of 0.5-1mM. Be sure to work with fresh DTT, as old or poor quality DTT may cause an increase in background visible as a "haze" in the Cy3 channel. Please refer to Appendix D for recommendations for reducing the degradation of Cy5 when performing microarray experiments.

Caution: In the preparation of wash buffers, avoid the use of water that may cause damage Cy5/Alexa 647. As noted in the Internet List Serve, MilliQ® water has been shown to damage Cy5 (<http://groups.yahoo.com/group/microarray/messages/2867>). Also, be certain that any DEPC treated solutions have had all of the DEPC fully removed (DEPC is a potent oxidizer). Alternatively, we recommend the use of non-DEPC treated nuclease free solutions. Commercially available solutions (water, buffers, etc.) from Ambion have been found to work well with Cy5 labeled microarrays. In addition to Ambion water (Cat. No. 9934), we also recommend DI water from VWR (Cat. No. RC91505). Water from Ambion and VWR have been validated for use with microarrays and do not contain components that will oxidize Cy5.

1. Prewarm the 2X SSC, 0.2%SDS wash buffer to 60-65°C.
2. Remove the coverslip by washing the array in prewarmed 2X SSC, 0.2% SDS for 2-5 minutes or until the coverslip floats off.*
3. Wash for 15 minutes in prewarmed 2X SSC, 0.2%SDS.
4. Wash for 10-15 minutes in 2X SSC at room temperature.
5. Wash for 10-15 minutes in 0.2X SSC at room temperature.
6. Transfer the array to a dry 50mL centrifuge tube, orienting the slide so that any label is down in the tube. Immediately centrifuge without the tube cap for 2 minutes at 800-1000 RPM to dry the slide (any delay in this step may result in high background). Avoid contact with the array surface.

Further optimization of wash conditions may be required to achieve optimal array performance. If necessary to reduce background on the array, increase the time of some or all of the washes to 15-20 minutes. Agitation during washing may also help to reduce background due to non-specific binding to the surface of the array.

Proceed to *Signal Detection*, pg.17, or first apply DyeSaver 2 coating (Genisphere Cat No. Q500500) to preserve fluorescent signal.

***Note:** If the coverslip is difficult to remove, this may be an indication of drying. To prevent this problem from recurring in future experiments, increase the total volume of the **3DNA Hybridization Mix** by adding equal volumes of Nuclease Free Water (Vial 10) and 2X Hybridization Buffer (Vial 6 or 7). In addition, ensure that the hybridization chamber is properly humidified and sealed.

Also note any concerns, mishaps, thoughts or questions of your own.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

Protocol 5: Plasmid DNA Isolation

(Promega “Wizard Plus SV minipreps” procedure)

The protocol below is an abbreviated version of that found in the Promega technical bulletin 225 (For more information, you may access this bulletin from the Promega web page).

Note: *Wear latex gloves, use sterile media and pipet tips, and take other precautions to avoid nuclease contamination.*

Use 1-5 ml overnight (O/N) cultures for strains carrying high-copy number plasmids and up to 10 ml cultures for strains carrying low-copy number plasmids.

Production Of A Cleared Lysate:

1. Centrifuge the 3 ml O/N culture.
2. If you grew the culture in a 15 ml snap-cap tube, pellet cells directly in that tube by centrifugation 5 min at 3,700 RPM in the Beckman table top centrifuge.
3. Pour off the supernatant and remove excess medium by blotting the inverted tube on a paper towel.
4. Add 250 μ l of ***Cell Resuspension Solution***. Completely resuspend the cell pellet by vortexing or pipetting.
5. Transfer the suspended cells to a sterile, 1.5 ml microcentrifuge tube.
6. Add 250 μ l of ***Cell Lysis Solution***. Mix by inverting the tube 4 times (**do not vortex**). Incubate until the suspension clears, about 1–5 min.

(Note: *It is important to observe partial clearing of the lysate before proceeding to the next step: but, do not incubate longer than 5 minutes.*)

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

7. Add 10 μ l of ***Alkaline Protease Solution***. Mix by inverting the tube 4 times. Incubate for 5 min at room temperature. (The Alkaline protease inactivates endonucleases and other proteins released during lysis.)
8. Add 350 μ l of ***Neutralization Solution*** and immediately mix by inverting the tube 4 times (do not vortex).
9. Centrifuge at 14,000 x g for 10 minutes at room temperature. (14,000 x g = top speed in a microcentrifuge or ca. 16,000 RPM in a Sorvall SS-34 rotor with microtube insert.)

Plasmid Dna Isolation And Purification:

1. Use one ***Spin column (filter)*** and one ***2 ml Collection tube*** per sample (= one ***DNA purification unit***).
 - a. Insert the ***Spin column*** into the ***Collection tube***.
2. Transfer the cleared lysate from above (about 850 μ l) into a ***DNA purification unit***. (Avoid disturbing or transferring any of the white precipitate with the supernatant.)
3. Centrifuge the supernatant at 14,000 x g in a microcentrifuge for 1 min at room temperature.
 - a. Remove the Spin Column from the tube and discard the flow-through from the Collection Tube.
4. Reinsert the Spin Column into the Collection Tube.
5. Add 750 μ l of Column Wash Solution to the Spin Column. (***Note that the Column Wash solution has been diluted with 95% ethanol. This has been done for you.***)
6. Centrifuge at 14,000 x g for 1 min. at room temperature.
7. Remove the Spin Column from the tube and discard the flow-through.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

- a. Reinsert the Spin Column into the Collection Tube.
 - b. Repeat the wash procedure using 250µl of Column Wash Solution.
8. Centrifuge at 14,000 x g for 2 minutes at room temperature.
 9. Transfer the Spin Column to a new, sterile 1.5ml microcentrifuge tube. (*Take care* not to transfer any of the Column Wash Solution with the Spin Column. If the Spin Column has Column Wash Solution associated with it, centrifuge again for 1 min. at 14,000 x g before transferring to the new 1.5 ml tube.
 10. Elute the plasmid DNA by adding 100µl of Nuclease-Free Water to the Spin Column. Centrifuge at 14,000 x g for 1 min. at room temperature.
 11. After eluting the DNA, remove the assembly from the 1.5 ml microcentrifuge tube and discard the Spin Column.
 12. The DNA is stable in water if stored at -20°C or below. For greater security, suspend in 10 mM Tris-HCl, 0.1 mM EDTA buffer (=10T/0.1E). This may be done approximately by adding 10 µl of 10 x 10T/0.1E buffer to the 100 µl of eluted DNA.
 13. Cap the microcentrifuge tube, ***label with a tough tag and a tough spot***, and store the purified plasmid DNA at -20°C or below.

Expected plasmid yield will vary depending on the plasmid, host strain, and growth conditions. For a high copy number plasmid, expect about 2-5 µg. At 2-5 µg per 100 µl, 5 µl (ca. 100 – 250 ng) is usually sufficient for restriction site analysis in one gel lane.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

PROTOCOL 6: AGAROSE GEL ELECTROPHORESIS

For a 3mm 1% gel:

1. Take 40mls 1X TBE in a 250 ml flask.
2. Weigh out .4 grams of high melting point agarose.
3. Slowly add it to the TBE while swirling.
4. Melt in the microwave (about 1 min)
 - a. Don't let it boil over but make sure it bubbles up.

These are the directions for pouring a 3mm thick gel in a horizontal gel box manufactured by Owl Separation Systems (model B-1). Because all gel boxes are different and different applications may require different gel thicknesses, or agarose concentration, you may have to re-calculate the volume of TBE and/or grams of agarose to add.

To estimate well volume: multiplying length, width and depth of a single tooth of the comb you will give you the cubic area of gel it will displace. Each 1mm of cubic area corresponds to 1ul of well volume. Remember the gel comb clears the bottom of the gel tray by about 2mm, so don't forget to take into account gel thickness.

Some applications will call for more or less concentrated gels than the 1% gel in this example.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

PROTOCOL 7A: DNA EXTRACTION FROM BRAIN AND LIVER

Principle and procedure as reported by the manufacturer of DNeasy kits.

DNeasy Tissue Kits use advanced silica-gel–membrane technology for rapid and efficient purification of total cellular DNA without organic extraction or ethanol precipitation. Following cell lysis the DNA is selectively bound to the DNeasy membrane. Centrifuging the samples then removes contaminants like proteins and divalent cations that are still in solution.

The DNeasy procedure is simple.

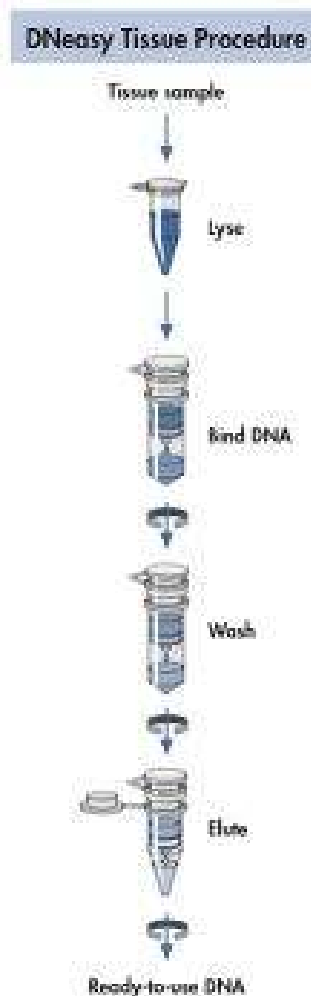
1. Cells are first lysed using proteinase K.*
2. The buffer provides optimal DNA-binding conditions and the lysate is loaded onto the DNeasy Mini spin column.
3. During a brief centrifugation, DNA is selectively bound to the DNeasy membrane and contaminants pass through.
4. Remaining contaminants and enzyme inhibitors are removed in two wash steps.
5. DNA is then eluted in water or buffer.
6. The manufacturer claims DNeasy purified DNA typically has an A260/A280ratio between 1.7 and 1.9, and is up to 50 kb in size, with fragments of 30 kb predominating.

a. *Is that what you get?*

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.



Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs,) available from the product supplier.

- Microcentrifuge tubes for lysis and elution (1.5 ml or 2 ml)
- Microcentrifuge with rotor for 1.5 ml and 2 ml tubes
- PBS (for use with cultured cells only)
- Ethanol (96–100%)*
- Shaking water bath
- Optional: RNase A

* Do not use denatured alcohol, which contains other substances such as methanol or methyl ethyl ketone.

NOTES:
Answer t]
Also note

Important points before starting:

- Before using the DNeasy Tissue Kit for the first time, read “Important Notes” on pages 10–17 (*These pages refer to the DNeasy Tissue Handbook pages*).
- If using fixed tissue, please refer to Appendices B and C (starting on page 30).
- All centrifugation steps are carried out at room temperature (15–25°C).
- Vortexing should be performed by pulse vortexing for 5–10 s.
- Optionally, RNase A may be used to digest RNA during the procedure. RNase A is not provided in the DNeasy Tissue Kit (see “Copurification of RNA”, page 14).

Things to do before starting:

- Buffers ATL and AL* may form precipitates upon storage. If a precipitate has formed in either buffer, incubate the buffer at 55°C until the precipitate has fully dissolved.
- Buffers AW1* and AW2 † are supplied as concentrates. Before using for the first time, add the appropriate amounts of ethanol (96–100%) to Buffers AW1 and AW2 as indicated on the bottles. ■ Prepare a 55°C shaking water bath for use in step 2 and a 70°C water bath or heating block for use in step 3.
- If using frozen material, equilibrate the sample to room temperature.

PROCEDURE

1. Cut up to 25 mg tissue (up to 10 mg spleen) into small pieces, place in a 1.5 ml microcentrifuge tube, and add 180 µl Buffer ATL.

Ensure the correct amount of starting material is used (see page 10). For tissues such as spleen with a very high number of cells for a given mass of tissue, no more than 10 mg starting material should be used. It is advisable to cut the tissue into small pieces to enable more efficient lysis.

NOTES:

Answer the questions in bold italics (above) in this space.
Also note any concerns, mishaps, thoughts or questions of your own.

2. Add 20 μ l proteinase K, mix by vortexing, and incubate at 55°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath or on a rocking platform.

Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1–3 h. If it is more convenient, samples can be lysed overnight; this will not affect them adversely. After incubation the lysate may appear viscous, but should not be gelatinous as it may clog the DNeasy Mini spin column. If the lysate appears very gelatinous, please see the “Troubleshooting Guide” on page 26 for recommendations. **Optional:** RNase treatment of the sample. Add 4 μ l of RNase A (100 mg/ml), mix by vortexing, and incubate for 2 min at room temperature (15–20°C). Transcriptionally active tissues such as liver and kidney contain high levels of RNA, which will copurify with genomic DNA. If RNA-free genomic DNA is required, carry out this optional step. If residual RNA is not a concern, omit this step and continue with step 3.

3. Vortex for 15 s. Add 200 μ l Buffer AL to the sample, mix thoroughly by vortexing, and incubate at 70°C for 10 min.

It is essential that the sample and Buffer AL are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. A white precipitate may form on addition of Buffer AL, which in most cases will dissolve during the incubation at 70°C. The precipitate does not interfere with the DNeasy procedure. Some tissue types (e.g., spleen, lung) may form a gelatinous lysate after addition of Buffer AL. In this case, vigorously shaking or vortexing the preparation before addition of ethanol in step 4 is recommended.

4. Add 200 μ l ethanol (96–100%) to the sample, and mix thoroughly by vortexing.

It is important that the sample and the ethanol are mixed thoroughly to yield a homogeneous solution. A white precipitate may form on addition of ethanol. It is essential to apply all of the precipitate to the DNeasy Mini spin column.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

5. Pipet the mixture from step 4 into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at $\geq 6000 \times g$ (8000 rpm) for 1 min. Discard flow-through and collection tube.*

6. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 μ l Buffer AW1, and centrifuge for 1 min at $\geq 6000 \times g$ (8000 rpm). Discard flow-through and collection tube.*

7. Place the DNeasy Mini spin column in a 2 ml collection tube (provided), add 500 μ l Buffer AW2, and centrifuge for 3 min at $20,000 \times g$ (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube. This centrifugation step ensures that no residual ethanol is carried over during the following elution. Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube and reuse it in another centrifugation step for 1 min at $20,000 \times g$ (14,000 rpm).

8. Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided), and pipet 200 μ l Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at $\geq 6000 \times g$ (8000 rpm) to elute.

Elution with 100 μ l (instead of 200 μ l) increases the final DNA concentration in the eluate, but also decreases the overall DNA yield.

9. Repeat elution once as described in step 8.

A new microcentrifuge tube can be used for the second elution step to prevent dilution of the first eluate. Alternatively, to combine the eluates, the microcentrifuge tube from step 8 can be reused for the second elution step. **Note:** More than 200 μ l should not be eluted

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

into a 1.5 ml microcentrifuge tube because the DNeasy Mini spin column will come into contact with the eluate.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

PROTOCOL 8B: RNA EXTRACTION

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

RNeasy Mini Protocol for Isolation of Total RNA from Animal Tissues

Determining the correct amount of starting material

It is essential to begin with the correct amount of tissue in order to obtain optimal RNA yield and purity with RNeasy columns. A maximum amount of 30 mg tissue can generally be processed with RNeasy mini columns. For most tissues, the binding capacity of the column (100 µg RNA) and the lysing capacity of Buffer RLT will not be exceeded by these amounts. Average RNA yields from various sources are given in Table 2 (page 17).

Some tissues such as spleen, parts of brain, lung, and thymus are more difficult to lyse or tend to form precipitates during the procedure. The volume of lysis buffer may need to be increased to facilitate complete homogenization and to avoid significantly reduced yields, DNA contamination, or clogging of the RNeasy column. See protocol for recommended amounts of lysis buffer to use.

Total RNA isolation from skeletal muscle, heart, and skin tissue can be difficult due to the abundance of contractile proteins, connective tissue, and collagen. The specialized protocol in Appendix C (page 93) includes a proteinase digestion and optimized RNA isolation procedure for these tissues.

If you have no information about the nature of your starting material, we recommend starting with no more than 10 mg of tissue. Depending on the yield and purity obtained, it may be possible to increase the amount of tissue to 30 mg.

Do not overload the column. Overloading will significantly reduce yield and quality.

Important notes before starting

- If using RNeasy or RNeasy Protect Kits for the first time, read "Important Points before Using RNeasy Kits" (page 16).
- If working with RNA for the first time, read Appendix A (page 88).
- For best results, stabilize animal tissues immediately in RNAlater RNA Stabilization Reagent following the protocol on page 47. Tissues can be stored in RNAlater RNA Stabilization Reagent for up to 1 day at 37°C, 7 days at 18–25°C, 4 weeks at 2–8°C, or for archival storage at –20°C or –80°C.
- Fresh, frozen, or RNAlater stabilized tissue can be used. To freeze tissue for long-term storage, flash-freeze in liquid nitrogen, and immediately transfer to –70°C. Tissue can be stored for several months at –70°C. To process, do not allow tissue to thaw during weighing or handling prior to disruption in Buffer RLT. Homogenized tissue lysates (in Buffer RLT, step 4) can also be stored at –70°C for several months. To process frozen lysates, thaw samples and incubate for 15–20 min at 37°C in a water bath to dissolve salts. Continue with step 5.
- β-Mercaptoethanol (β-ME) must be added to Buffer RLT before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 10 µl β-ME per 1 ml Buffer RLT. Buffer RLT is stable for 1 month after addition of β-ME.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

- **Buffer RPE is supplied 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.**
 - Generally, DNase digestion is not required since the RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA [e.g., TaqMan RT-PCR analysis with a low-abundant target]. In these cases, the small residual amounts of DNA remaining can be removed using the RNase-Free DNase Set (cat. no. 79254) for the optional on-column DNase digestion (see page 99) or by a DNase digestion after RNA isolation. For on-column DNase digestion with the RNase-Free DNase Set, prepare the DNase I stock solution as described on page 99 before beginning the procedure.
 - Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature.
 - Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. Guanidine is an irritant. Take appropriate safety measures and wear gloves when handling.
 - All steps of the RNeasy protocol should be performed at room temperature. During the procedure, work quickly.
 - All centrifugation steps are performed at 20–25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20°C.
1. **Excise the tissue sample from the animal or remove it from storage. Remove RNAlater stabilized tissues from the reagent using forceps.**
 2. **Determine the amount of tissue. Do not use more than 30 mg.**
Weighing tissue is the most accurate way to determine the amount. See page 50 for guidelines to determine the amount of starting material.
 3. **For RNAlater stabilized tissues:**
If the entire piece of RNAlater stabilized tissue can be used for RNA isolation, place it directly into a suitably sized vessel for disruption and homogenization, and proceed with step 4.
If only a portion of the RNAlater stabilized tissue is to be used, place the tissue on a clean surface for cutting, and cut it. Determine the weight of the piece to be used, and place it into a suitably sized vessel for homogenization. Proceed with step 4.
RNA in the RNAlater treated tissue is still protected while the tissue is processed at 18 to 25°C. This allows cutting and weighing of tissues at ambient temperatures. It is not necessary to cut the tissue on ice or dry ice or in a refrigerated room. The remaining tissue can be placed into RNAlater RNA Stabilization Reagent for further storage. Previously stabilized tissues can be stored at -80°C without the reagent.

For unstabilized fresh or frozen tissues:

If the entire piece of tissue can be used for RNA isolation, place it directly into a suitably sized vessel for disruption and homogenization, and proceed immediately with step 4.

If only a portion of the tissue is to be used, determine the weight of the piece to be used, and place it into a suitably sized vessel for homogenization. Proceed immediately with step 4.

RNA in tissues is not protected after harvesting until the sample is treated with RNA/later RNA Stabilization Reagent, flash frozen, or disrupted and homogenized in protocol step 4. Frozen animal tissue should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible.

Note: The remaining fresh tissue can be placed into RNA/later RNA Stabilization Reagent for stabilization (see Protocol for RNA Stabilization in Tissues with RNA/later RNA Stabilization Reagent, page 47). However, previously frozen tissue samples thaw too slowly in the reagent, thus preventing it from diffusing into the tissue quickly enough before the RNA begins to degrade.

4. Disrupt tissue and homogenize lysate in Buffer RLT. (Do not use more than 30 mg tissue.)

Disruption and homogenization of animal tissue can be performed by 4 alternative methods (a, b, c, or d). See pages 20–24 for a more detailed description of disruption and homogenization methods.

After storage in RNA/later RNA Stabilization Reagent, tissues may become slightly harder than fresh or thawed tissues. Disruption and homogenization of tissue samples using standard methods is usually not a problem. For easier disruption and homogenization it is recommended to increase the volume of lysis Buffer RLT to 600 μ l as recommended for tissues that are difficult to lyse.

Note: Incomplete homogenization will lead to significantly reduced yields and can cause clogging of the RNeasy column. Homogenization with rotor–stator homogenizers generally results in higher total RNA yields than with other homogenization methods.

a. Rotor–stator homogenization:

Place the weighed (fresh, frozen, or RNA/later stabilized) tissue in a suitably sized vessel for the homogenizer. Add the appropriate volume of Buffer RLT (see below). Homogenize immediately using a conventional rotor–stator homogenizer until the sample is uniformly homogeneous (usually 20–40 s). Continue the protocol with step 5.

Rotor–stator homogenization simultaneously disrupts and homogenizes the sample.

Note: Ensure that β -ME is added to Buffer RLT before use (see “Important notes before starting”).

Amount of starting material	Volume of Buffer RLT
<20 mg	350 μ l or 600 μ l*
20 to 30 mg	600 μ l

* Use 600 μ l Buffer RLT if preparing RNA from tissues that have been stabilized in RNAlater RNA Stabilization Reagent or that are difficult to lyse.

b. Mortar and pestle with QIAshredder homogenization:

Immediately place the weighed (fresh, frozen, or RNAlater stabilized) tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen-cooled, 2 ml microcentrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.

Add the appropriate volume of Buffer RLT (see below). Pipet the lysate directly onto a QIAshredder spin column placed in 2 ml collection tube, and centrifuge for 2 min at maximum speed. Continue the protocol with step 5.

Grinding the sample using a mortar and pestle will disrupt the sample, but it will not homogenize it. Homogenization is carried out by centrifugation through the QIAshredder spin column.

Note: Ensure that β -ME is added to Buffer RLT before use (see "Important notes before starting").

Amount of starting material	Volume of Buffer RLT
<20 mg	350 μ l or 600 μ l*
20 to 30 mg	600 μ l

* Use 600 μ l Buffer RLT if preparing RNA from tissues that have been stabilized in RNAlater RNA Stabilization Reagent or that are difficult to lyse.

c. Mortar and pestle with needle and syringe homogenization:

Immediately place the weighed (fresh, frozen, or RNAlater stabilized) tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen-cooled, 2 ml microcentrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.

Add the appropriate volume of Buffer RLT (see below), and homogenize by passing lysate at least 5 times through a 20-gauge needle fitted to an RNase-free syringe. Continue the protocol with step 5.

Grinding the sample using a mortar and pestle will disrupt the sample, but it will not homogenize it. Homogenization is carried out with the needle and syringe.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

Note: Ensure that β -ME is added to Buffer RLT before use [see "Important notes" before starting].

Amount of starting material	Volume of Buffer RLT
<20 mg	350 μ l or 600 μ l*
20 to 30 mg	600 μ l

* Use 600 μ l Buffer RLT if preparing RNA from tissues that have been stabilized in RNAlater RNA Stabilization Reagent or that are difficult to lyse.

d. **Mixer Mill MM 300:**

See "Appendix E: Disruption and Homogenization of RNAlater Stabilized Tissues Using the Mixer Mill MM 300" (page 101) for guidelines.

The Mixer Mill MM 300 simultaneously disrupts and homogenizes the sample.

5. Centrifuge the tissue lysate for 3 min at maximum speed in a microcentrifuge. Carefully transfer the supernatant to a new microcentrifuge tube (not supplied) by pipetting. Use only this supernatant (lysate) in subsequent steps.

In some preparations, very small amounts of insoluble material will be present, making the pellet invisible.

6. Add 1 volume (usually 350 μ l or 600 μ l) of 70% ethanol to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Continue without delay with step 7. If some lysate is lost during steps 4 and 5, adjust volume of ethanol accordingly.

A precipitate may form after the addition of ethanol, but this will not affect the RNeasy procedure.

7. Apply up to 700 μ l of the sample, including any precipitate that may have formed, to an RNeasy mini column placed in a 2 ml collection tube (supplied). Close the tube gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through.

Reuse the collection tube in step 8.

If the volume exceeds 700 μ l, load aliquots successively onto the RNeasy column, and centrifuge as above. Discard the flow-through after each centrifugation step.[†]

Optional: QIAGEN offers the RNase-Free DNase Set (cat. no. 79254) for convenient on-column DNase digestion during RNA purification. Generally, DNase digestion is not required since the RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). If using the RNase-Free DNase Set, follow the steps shown on pages 99–100 after performing this step.

[†] Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

8. Add 700 μ l Buffer RW1 to the RNeasy column. Close the tube gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the column. Discard the flow-through and collection tube.*

Skip this step if performing the optional on-column DNase digestion (page 99).

9. Transfer the RNeasy column into a new 2 ml collection tube (supplied). Pipet 500 μ l Buffer RPE onto the RNeasy column. Close the tube gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the column. Discard the flow-through.

Reuse the collection tube in step 10.

Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see "Important notes before starting").

10. Add another 500 μ l Buffer RPE to the RNeasy column. Close the tube gently, and centrifuge for 2 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to dry the RNeasy silica-gel membrane. Continue directly with step 11, or, to eliminate any chance of possible Buffer RPE carryover, continue first with step 10a.

It is important to dry the RNeasy silica-gel membrane since residual ethanol may interfere with downstream reactions. This centrifugation ensures that no ethanol is carried over during elution.

Note: Following the centrifugation, remove the RNeasy mini column from the collection tube carefully so the column does not contact the flowthrough as this will result in carryover of ethanol.

- 10a. **Optional:** Place the RNeasy column in a new 2 ml collection tube (not supplied), and discard the old collection tube with the flow-through. Centrifuge in a microcentrifuge at full speed for 1 min.

11. To elute, transfer the RNeasy column to a new 1.5 ml collection tube (supplied). Pipet 30–50 μ l RNase-free water directly onto the RNeasy silica-gel membrane. Close the tube gently, and centrifuge for 1 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to elute.

12. If the expected RNA yield is $>30 \mu$ g, repeat the elution step (step 11) as described with a second volume of RNase-free water. Elute into the same collection tube.

To obtain a higher total RNA concentration, this second elution step may be performed by using the first eluate (from step 11). The yield will be 15–30% less than the yield obtained using a second volume of RNase-free water, but the final concentration will be higher.

* Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach.

PROTOCOL 8: PROTEIN ASSAY

Protein measurement of intact cells or solubilized proteins (BioRad protein assay)

From Darryl Horn MS thesis (2005)

Protein Measurements on intact cells

Pelleted cells were resuspended in 1 ml of 20mM Tris-HCl pH 7.5 buffer. In a microfuge tube, 360 μ l of cells and 40 μ l 1M NaOH were mixed and heated to 90 °C for 10 minutes in order to lyse the cells and release proteins. To the lysed cells, 100 μ l of Bradford reagent (BIO-RAD; Hercules, CA) was added and allowed to incubate for 30 minutes at room temperature. The samples were read in a spectrophotometer at a wavelength of 595 nm. Protein concentrations were determined by comparison to Bovine Serum Albumin as a standard.

BioRad Microassay Procedure for solubilized protein samples

1. Prepare three to five dilutions of a protein standard which is representative of the protein solution to be tested. The linear range of the assay for BSA is 1.2 to 10.0 μ g/ml, whereas with IgG the linear range is 1.2 to 25 μ g/ml.
2. Pipet 400 μ l of each standard and sample solution into a clean, dry microfuge tube. Protein solutions are normally assayed in duplicate or triplicate.
(NB dilute proteins in an appropriate buffer (e.g. 50 mM ammonium bicarbonate pH 8) to a concentration of 1 – 10 μ g/ml. Overproduced protein preparations may have concentrations as high as 1 -10 mg/ml!)*
3. Add 100 μ l of dye reagent concentrate to each tube and vortex.
4. Incubate at room temperature for at least 5 minutes. Absorbance will increase over time; samples should incubate at room temperature for no more than 1 hour. *(go for 30')*
5. Measure absorbance at 595 nm. *(measure against a reagent blank, and against a wavelength (750 nm?) at which there is little absorbance from the reagent.)*

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

PROTOCOL 9: POLYMERASE CHAIN REACTION**STANDARD****Simple procedure with a pre-programmed method (Program: REU-PCR)**

- Step 1: 95°C; 2 minutes
 Step 2: 94°C; 1 minute
 Step 3: 55°C; 1 minute (range between 50°C and 70°C)
 Step 4: 72°C; 2 minutes
 Step 5: Repeat steps 2 thru 4, 40 times
 Step 6: 72°C 5 minutes
 Step 7: 4°C for 0 minutes (holds block at 4°C indefinitely)

50 ul reaction volume

10x (Mg free) reaction buffer	5.00 µl
25 mM MgCl ₂ Solution,	5.00 µl
2 mM each dNTPs	5.00 µl
Primer F @ 20pmol/µl	0.25 µl
Primer R @ 20pmol/µl	0.25 µl
Taq DNA polymerase (usually 5 U/µl)	0.50 µl
DNA (50ng/ul)	1.00 µl
sterile ddH ₂ O	33.00µl
Final volume	50.00 µl

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

RT – PCR

Protocols for RT-PCR are like those for PCR except that they begin with an incubation to allow reverse transcriptase activity. Reverse transcriptase elongates a strand of DNA from the primer that is complementary to the mRNA sequence. The DNA is complementary to the mRNA and, hence, is called cDNA. After cDNA has been made, the RT-PCR sample is placed in a thermalcycler and the cDNA is amplified. Reverse transcriptase will be destroyed by the first high temperature PCR incubation.

In a thin-walled 200 ul tube for each RNA sample you need the following:

Reaction Mix For RT Part Of The Reaction

5X Reverse Transcriptase Buffer w/ MgCl	4.0 µl
Reverse Primer (20pmol/ul) (100X)	2.0 µl
Control Primer	2.0 µl
100mM dNTP mix	3.0 µl
RNase inhibitor (40U/ul) need 20 U	0.5 µl
MLV Reverse Transcriptase (200U/ul)	1.0 µl
RNA sample (100 ng/ul)	2.0 µl
RNase free water	5.5 µl

Total Volume:	20.0 µl
---------------	---------

REU-RT program:

Step 1: 42° C for 60 min.

Step 2: 99° C for 5 min.

Step 3: 5° C indefinitely.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

Reaction Mix for PCR Part of the Reaction:

PCR Water (depends)	11.0 μ l
cDNA from RT reaction	5.0 μ l
10X Taq Buffer	2.0 μ l
MgCl ₂ (depends)	5.0 μ l
20uM Forward primer	1.5 ul
Taq DNA polymerase	0.5 ul
<hr/>	
Total volume	25.0 ul l

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

PROTOCOL 10:**Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis****Reagents List:**

Acrylamide/bis-acrylamide monomer stock solution (40% acrylamide/0.8% bis-acrylamide). (***WARNING: non-polymerized acrylamides are neurotoxins! Wear gloves, Avoid skin contact and inhalation.***)

65% sucrose

10x Resolving gel buffer = 3.75 M Tris-HCl pH 8.8, = 45.42 g Tris base/100 ml

ml 1% SDS = 10 ml 10% SDS/100 ml

Resolving gel overlay = 1x Resolving gel buffer

4X Stacking gel buffer = 0.5 M Tris-HCl pH 6.8, = 6.06 g Tris base/100 ml

ml 0.4% SDS = 4 ml 10% SDS/100 ml

4x Running buffer = 0.1 M Tris-HCl pH 8.3, = 12 g Tris base/liter
0.768 M glycine, = 57.6 g glycine/liter
0.4% SDS = 4 g SDS/liter

TEMED (stored at 4°C) (*Note: **TEMED is toxic.** We use only minute quantities, but avoid breathing vapors.*)

10% APS (ammonium persulfate): made fresh, stored up to a week at 4°C

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

4x SDS-PAGE Sample buffer = 40% glycerol,
200 mM Tris-HCl pH 6.8,
0.04% bromophenol blue
8% SDS

1.0 M DTT (dithiothreitol) in sterile ddH₂O.

(e.g. 5 ml of 1.0 M DTT. 100 µl aliquots stored at -20°C). *DTT is a reducing agent required to reduce disulfide bonds in proteins. DTT (or β-mercaptoethanol, another commonly used reducing agent) degrades rather easily and therefore DTT is added to samples from a freshly thawed stock tube.*

1.0 M Na₂CO₃ in sterile ddH₂O. (stored at -20°C, 4°C, or room temperature)

Preparation of 40% acrylamide/0.8% bis-acrylamide stock: *(This stock will be prepared for you, but if you ever need to prepare it, observe the following):* Avoid weighing acrylamide powder. Wear a dust mask if weighing is necessary.

The following is for preparation of the 30/0.8 stock solution from 100 g of acrylamide.

1. Pre-weigh a 500 ml flask. The final weight (= volume) of the solution will be 333.3 g.
2. Add about 100 ml of ddH₂O to the acrylamide reagent bottle.
3. Add a magnetic stir bar and stir gently until the crystals dissolve.
4. Pour this solution into the tared 500 ml flask.
5. Add 2.67 g bis-acrylamide (wear a dust mask and avoid breathing any dust from the reagent).
6. Add ddH₂O to a total weight of 333.3 g. Make sure the solution is well mixed but avoid vigorous stirring and aeration.
7. Use a vacuum filter apparatus to filter the solution through an 0.45 µm pore size filter.
8. Store aliquots in dark or foil-wrapped bottles at 4°C. *(Such solutions are usually good for several months to a year. Gas with argon to prolong shelf life.)*

Size standards for SDS-PAGE

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

Bio-Rad "Low" molecular weight standards

Figure 4 shows the six standards, 14.4 kD to 97.4 kD, in the Bio-Rad "Low Molecular Weight" SDS-PAGE standards preparation (Bio-Rad cat no. 162-0304). The concentrated standards (approximately 2 mg/ml of each protein) supplied by Bio-Rad are diluted 1:20 and used at 5 μ l per lane in a mini, vertical protein gel electrophoresis apparatus.

Note: we may also use different standards that may be handled differently. These will have special instructions.

Preparation of diluted standards (in SDS-PAGE sample buffer but without DTT or Na_2CO_3)

	For 4 loadings	For 40 loadings
Concentrated standard	1 μ l	10 μ l
ddH ₂ O	10 μ l	100 μ l
4x SDS-PAGE sample buffer	5 μ l	50 μ l
Total volume	16 μ l	160 μ l
(Diluted standards are stored in aliquots at -20°C)		

Treatment of diluted standards for loading onto the gel

	For one gel lane	For 2 gel lanes
Diluted SDS-PAGE standards	4 μ l	8 μ l
1.0 M DTT (freshly thawed)	0.5 μ l	1 μ l
1.0 M Na_2CO_3	0.5 μ l	1 μ l

1. Per gel lane, transfer 4 μ l of Diluted standards into a micro tube, add 0.5 μ l of fresh 1.0 M DTT and 0.5 μ l 1.0 M Na_2CO_3 as shown in the table above.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

2. Boil standards or samples for 50 seconds. Centrifuge a few seconds to collect the condensation.
 3. Load 5 μ l of the mixture per gel lane. (*This amounts to 2.5 μ g of protein per band.*)
-

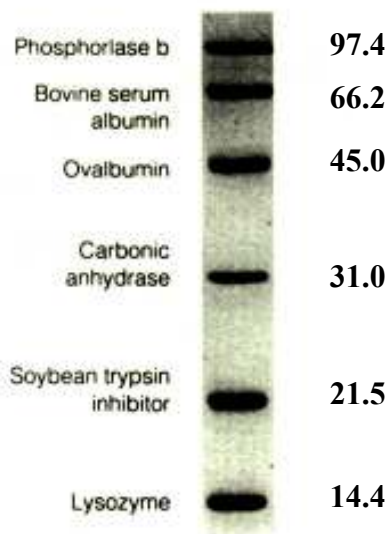


Figure 4. SDS-polyacrylamide gel showing Bio-Rad "Low" molecular weight standards. 4-20% gradient gel stained with Commassie R-250. Molecular weights are in kDa.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

Mixtures for casting gels in the Hoefer dual gel casting stand (for 30% acrylamide stock solution)

RESOLVING GEL

(volumes given are for two 0.75 mm thick gels)

	10% acrylamide	12% acrylamide	14% acrylamide
Stock solutions			
30% acrylamide-0.8% bis	3.35 ml	4.0 ml	4.67 ml
65% sucrose	0.75 ml	0.75 ml	0.75 ml
10x resolving gel buffer	1.0 ml	1.0 ml	1.0 ml
ddH ₂ O	4.9 ml	4.22 ml	3.55 ml
TEMED	8 μ l	8 μ l	8 μ l
10% APS	27 μ l	27 μ l	27 μ l
Total volume	10 ml	10 ml	10 ml

STACKING GEL

(volumes given are for two 0.75 mm thick gels)

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

Stock solutions	
30% acrylamide-0.8% bis	1.06 ml
4x Stacking gel buffer	2.0 ml
ddH ₂ O	4.89 ml
TEMED	6.4 μ l
10% APS	43.2 μ l
Total volume	8 ml

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

SDS-PAGE

(v.

03.3)

Mixtures for casting gels (e.g. BioRad Protean III system)

(40% acrylamide stock solution)

*RESOLVING GEL (volumes given are amply sufficient for two 0.75 mm thick gels)***Mixtures for 40% acrylamide (37.5:1 acrylamide:bis-acrylamide) stock:**

	10% acrylamide	12% acrylamide	14% acrylamide
Stock solutions			
40% acrylamide (37.5:1 acrylamide:bis)	2.5 ml	3.0 ml	3.5 ml
65% sucrose	0.75 ml	0.75 ml	0.75 ml
10x resolving gel buffer	1.0 ml	1.0 ml	1.0 ml
ddH ₂ O	5.69 ml	5.19 ml	4.69 ml
TEMED	10 μ l	10 μ l	10 μ l
10% APS	50 μ l	50 μ l	50 μ l
Total volume	10 ml	10 ml	10 ml

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

Note greater Temed and APS concentrations. Gels will polymerize more easily but also faster!

STACKING GEL

(volumes given are amply sufficient for two 0.75 mm thick gels)

Stock solutions	4% acrylamide
40% acrylamide (37.5:1 acrylamide:bis)	0.8 ml
4x Stacking gel buffer	2.0 ml
ddH ₂ O	5.14 ml
TEMED	10 µl
10% APS	50 µl
Total volume	8 ml

Preparation of SDS-polyacrylamide gels (Hoefler dual gel caster)

Pouring the resolving gel:

1. Clean gel plates, spacers, and combs with dH₂O and ethanol.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

2. Assemble gel “sandwiches” with one opaque (white notched) silica gel plate and one clear glass plate each. Note the T-shaped spacers. *See the Hoefer manual for detailed instructions.*
3. Clamp one or two gel ‘Sandwiches into the dual gel caster.
4. Prepare an appropriate volume of resolving gel solution. (Use a 12% acrylamide solution for separation of ca. 12 kD to 60 kD proteins.) Add reagents in order into a 25 or 50 ml flask. Wear gloves. Mix reagents by gentle swirling. Avoid aeration or introduction of air bubbles.
5. **Add TEMED and APS last!** After addition of the APS, you will have approximately 2-3 min to pour the gel before the acrylamide polymerizes. You may keep your acrylamide solution on ice if you wish to slow down the polymerization.
(Note that if you want more time before the gel polymerizes, keep the polyacrylamide solution cold!)
6. Once TEMED and APS is added, use a Pasteur pipet to gently allow the resolving gel solution to slide between the plates into the gel “sandwich.” **Avoid air bubbles! Add only enough resolving gel solution to allow for an adequate stacking gel layer on top.** *The depth of the stacking gel layer should be equivalent to the expected depth of your sample in the wells.*
7. After addition of the resolving gel layer to an adequate depth, gently add 1x resolving gel buffer to form a layer (about 0.5 cm deep) over the acrylamide. *Note that acrylamide requires relatively anaerobic conditions for polymerization.*

Note: *to test whether your polyacrylamide solution will polymerize, pull a small amount of the remaining solution into a Pasteur pipet. Polymerization should occur within 5 minutes.*

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

Pouring the stacking gel

1. After the resolving gel has polymerized (about 30 min), pour off the 1x resolving gel buffer layer and carefully wick dry with a piece of filter paper.
2. Insert a comb (of appropriate thickness) between the gel plates.
3. Prepare an appropriate volume of stacking gel buffer (see above). Again avoid air bubbles and aeration.
4. Once TEMED and APS are added, you will again have about 2-3 min to pour the stacking gel.
5. With a Pasteur pipet, carefully allow the stacking gel mix to flow between the gel plates and to surround the comb which will form loading wells in the polyacrylamide.
6. Allow the stacking gel to polymerize (about 30 min). If you do not wish to use the gel immediately, remove the gel from the casting stand, wrap in plastic wrap, and store at 4°C.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

Running SDS-PAGE gels

(Instructions are for the Hoefer "Mighty Small" apparatus. Bio-Rad instructions will be somewhat different.)

Sample preparation, gel loading, electrophoresis, and staining:

1. Each sample is mixed in a micro tube with 4x SDS-PAGE sample buffer, 1.0 M DTT, and 1.0 M Na₂CO₃ in ratios of 11 μ l sample + 5 μ l 4x buffer + 2 μ l DTT + 2 μ l Na₂CO₃ as described above. (Total sample volume is 20 μ l)
2. Samples and standards are boiled for 50 sec and then centrifuged briefly to collect condensed vapors.
3. Install the polymerized gel sandwich onto the electrophoresis unit as shown in the Hoefer manual (To aid loading, the "well template" may be clamped to the front of the gel plate). If only one gel sandwich is used, clamp a glass plate where the second gel would be installed. Dilute the 4X Running Buffer to 1X and add ca. 75 ml to the top and bottom buffer chambers. ***Avoid bubbles*** and make sure that the tops of the wells are covered.
4. Load samples carefully into the wells (use e.g. a micropipetor with capillary tips or a Hamilton syringe). ***Avoid bubbles***. The samples should sink to the bottoms of the wells. With the 10 slot comb (0.75 mm thick gel), a maximum of ca. 15 μ l may be loaded into each slot. Into one lane, load 5 μ l of BIO-RAD low molecular weight protein standards (equivalent to about 2.5 μ g protein per band).
4. Attach the cover and the electrical leads. SDS is negatively charged; therefore proteins migrate toward the positive (+) pole. Attach the positive lead to the bottom of the gel. For one gel use ca. 20 mA constant current (40 mA for two gels). Allow the blue dye front to migrate to the bottom (about 30-60 min).
5. Turn off the current, detach the leads, and remove the gel plates. Work over a plastic tray and gently pry the gel plates apart with a plastic spatula. Prepare the gel for staining with Coomassie blue (or with the fluorescent AllProTM (Promega) reagent) as described below:

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

SDS-PAGE:

Fixation and staining with Coomassie Blue R-250

Carefully place the gel into a tray or Petri dish that contains 0.125% Coomassie Blue R-250, 40% methanol, and 7% acetic acid. Stain with gentle agitation for 30 min to overnight. Pour the stain solution into the "Used Protein Stain" bottle and pour destaining solution (40% methanol, 7% acetic acid) over the gel. Agitate gently a few minutes to overnight. Change the solution if necessary (save the destain in the "used destain" bottle). Bands should become visible. Dry the gel between cellophane sheets as described separately.

(For "Western" (immuno) blotting, do not stain the gel with Coomassie blue but rather proceed as directed in the Western blot protocol.)

NOTES:

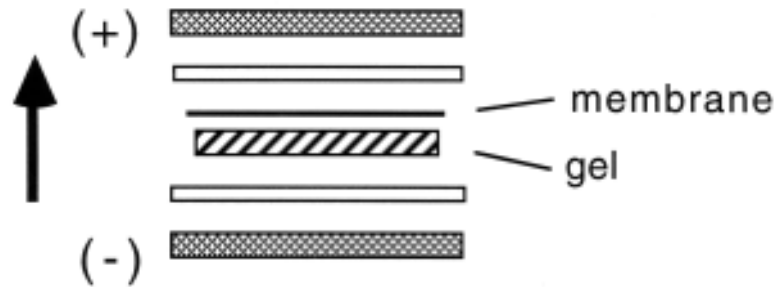
Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

PROTOCOL 11: WESTERN BLOTTING

Western (Immuno) blotting: electro-blot transfer

1. Separate protein samples by electrophoresis on an SDS-polyacrylamide gel (e.g. BioRad Protean III apparatus. See the protocol sheet for the specific apparatus.)
2. Wash gel 2-3x in dH₂O about 5 min. and then in 1 x TGM transfer buffer for 15 min.
3. Cut Immobilon-P (PVDF) membrane to size. Wear gloves, use a metal ruler and razor blade, do not waste any membrane (It's expensive!).
4. Wet membrane in 100% methanol 1-2 sec (*once wet do not allow the membrane dry*), then wash in about 500 ml dH₂O for 5 min, and soak in 1x TGM transfer buffer for about 15 min.
5. Mark the upper left corner of the membrane with a small diagonal cut and then set up the transfer stack according to the instructions for the Owl/Fisher Semi-Sry blotter. (*Do not allow the membrane to dry*). **OBSERVE THE CORRECT POLARITY!**



NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

6. Transfer conditions for the Owl/Fisher Semi-Dry Blotting Unit (FB-SDB-2020). Recommended conditions are **constant current** of 0.8 mA/cm² of gel surface. For two 10 x 8 cm mini-gels, try 160 mA constant current. ****Use the blotting power supply.*** Transfer should be completed in 30-120 min.
[For transfer in a tank-style apparatus: use 25 Volts for 30-60 min. Keep current at less than 1 Amp (the reading will be about 0.2-0.4 Amp). ***Caution, this is a potentially hazardous current. Do not transfer for more than 1 hour or damage may occur to the plate electrodes.***]
7. Turn off the power supply. Take down the blotting stack. Mark the positions of the wells. Mark the lane that contains the protein standards. You need to cut this off from the rest of the membrane for staining in Amido Black stain. ***Rinse the blotting apparatus in H₂O and dH₂O.***
8. Allow the membrane to dry. Store between blotting paper (envelope style) until needed for the antibody crossreaction step.
9. Amido Black staining for protein visualization (0.1% Amido Black in 10% Methanol, 2% acetic acid). Cut the lane(s) to be stained away from the rest of the membrane, stain in amido black solution for 10 min, destain with the Amido Black Destains I and II.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

Detection of specific antigens on protein ("Western") blots

Primary and secondary antibody binding reactions:

1. Wet blot in methanol (few seconds)
2. Rinse blot in ddH₂O
3. Place blot into a Petri plate or small plastic tray (*or a sealed e.g. 50 ml tube*) and equilibrate for 5 min in buffer A (20 mM Tris-HCl pH 7.5, 0.15 M NaCl, 0.05% Tween). Use e.g. 50 ml for a 100 cm² blot.
4. Incubate blot with gentle agitation 1 h at 37°C in buffer A + 5% nonfat dry milk powder (e.g. 50 ml for a 100 cm² blot). *Other temperatures are OK also.*
5. Incubate blot with gentle agitation ca. 2 h at room temperature (R/T) in a predetermined dilution of the primary antibody (anti glial fibrillary acidic protein) in buffer A + 1% nonfat milk. Use ca. 10 ml per 100 cm² blot (= 1µl of primary antibody per 10 ml buffer).
6. Wash 3-4 times 5 min each with buffer A + 0.1% nonfat milk.
7. Incubate blot with gentle agitation 1 h at R/T in a predetermined dilution of goat anti-rabbit horse radish peroxidase conjugate (anti-rabbit-HRP) in buffer A + 1% nonfat milk. (= 0.5µl of anti-chick-AP in 10 ml buffer).
8. Wash 3 times 5 min each in buffer A + 0.1% nonfat milk.
9. Wash 2 times 5 min in phosphate buffered saline (PBS: 136mM NaCl, 2.68 mM KCl, 10.1mM Na₂HPO₄, and 1.76mM KH₂PO₄).
10. Incubate the blot for about 5 min. in PBS and 0.3mg/ml diaminobenzidine and NiCl₂ (DAB; one 10mg tablet of DAB in 30ml PBS. Stir the solution for about 15min then filter through Whatman #1 filter paper. Add 0.3mg/ml NiCl₂.) **PROTECT FROM LIGHT.**
11. Add 0.03% H₂O₂ to the blots and DAB solution.. **PROTECT FROM LIGHT**, but check development of substrate every 5 min or so.
12. When sufficiently developed, wash the blot in several fast changes of dH₂O. Dry and store between pieces of paper towel or filter paper. It can also be mounted in your lab notebook.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

NOTE: DAB is a potent carcinogen. Wear gloves, work carefully, clean up spills. To inactivate its damaging effects, wipe with bleach or add bleach to liquid reagents.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

PROTOCOL 13: RESTRICTION ENZYME DIGEST OF PLASMID DNA

For a single digest:

1. Quantify your plasmid extraction.
2. Make a working solution of plasmid DNA that is 25ng/ul
 - a. You need to digest 1.0 – 2.0 µg of plasmid DNA
3. Add 40µl of plasmid DNA to a 1.5ml microfuge tube.
4. Add 5 ul of BSA (1mg/ml)
5. Add 5 ul of NEB buffer (depends on enzyme)
6. Add 0.5 ul of Enzyme.

Incubate for 2 hrs at 37°C then heat 5 min. at 65°C.

For a double digest:

1. Add 40µl of plasmid DNA to a 1.5ml microfuge tube.
2. Add 5 ul of BSA (1mg/ml)
3. Add 5 ul of NEB buffer (depends on enzyme)
4. Add 5 ul of NEB buffer (depends on enzyme)
5. Add 0.5 ul of Enzyme #1
6. Add 0.5 ul of Enzyme #2

Incubate for 2 hrs at 37°C then heat 5 min. at 65°C.

For both single and double digests

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.

NOTES:

Answer the questions in bold italics (above) in this space.

Also note any concerns, mishaps, thoughts or questions of your own.