# MCDB 140L
# Recombinant DNA Laboratory

## Spring 2013

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## Monday/Wednesday 9:00-11:50 and 2:00-4:50

## Tuesday/Thursday 2:00-4:50

# Course Information

Instructor: Seng Hui Low (shlow@lifesci.ucsb.edu)

Lab Coordinator: Dylan Peterson (dylan.peterson@lifesci.ucsb.edu)

Teaching Assistants: Erin Folchi Olsan (erin.folchi@lifesci.ucsb.edu)
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**Requirements:**

**You are expected to come to each scheduled class prepared for the day's experiments.** This means reading the procedures before coming to class, and being prepared to ask questions on procedures that you do not understand. Being well prepared will help you finish the day's work in a minimum of time and may prevent you from making careless mistakes. Please note that you will also be required to come in briefly at times other than the scheduled labs to check on experiments and start cell cultures growing.

The specific requirements for the course are:

| | points |
|---|---|
| 1. lab notebook | 40 |
| 2. quizzes | 30 |
| 3 final exam | 50 |
| 4. write-up | 40 |
| | 160 points total |

**1. Lab notebook:**

The purpose of the notebook is to record your daily activities in lab. Most of your entries into the notebook should be written in lab (not before or after). There should be sufficient detail in your notebook so that you, or someone else, could go back and repeat your experiments. It is best to avoid extraneous details in a notebook. You should not re-copy the lab manual into your notebook, nor should you keep lecture notes or your original works of art in your notebook. While this notebook may be more informal than other notebooks you have kept, it must still be neat and legible. You may use any permanently bound notebook. Your notebook should be organized as follows:

**a. Number all pages.**

**b. The first page should be a Table of Contents**.

The table of contents should have the date of each experiment, the title of each experiment, and the inclusive pages of each experiment. For example:

3/30 Streak out *E. coli* for plating library. 3-4

**c. Every day that you come to lab you should start a new entry, which includes:**

1) *Date*. Today's date.

2) *Title*. This should be, at the most, a one sentence statement of what you are doing that day and should match the title of each experiment from the table of contents.

3) *Purpose of the experiment.* This section should be several sentences on what that day's experiment is and how it relates to the preceding and following procedures (i.e. the big picture).

4) *Procedure.* The procedure in the notebook should only be a rough outline of the procedure in the manual and functions mainly to help you keep track of what you are doing. This should be a step by step listing of exactly what you did during the experiment. Any procedure you do that deviates significantly from the manual should be more thoroughly covered. Additionally, add any details or observations you make while doing the procedure. Important things to record are, for example, the time at which you started particular steps.

5) *Results and Discussion.* Not every procedure that we do will generate concrete/numerical results, but you must include any data that you generate as well as observations that you make. Everyday/every procedure you will end up with some result, for example, at the end of step 1, did you obtain a small pink pellet of nucleic acid or did you end up with an empty tube? You must include a discussion of the results you obtained that day. The discussion should present a clear understanding of what you did that day and why, as well as how the results fit with your expectations. Additionally, you should discuss reasons for any negative results.

The notebooks will be collected at the end of the **third week of lab (April 20$^{th}$ - must have all results up through competent cell test complete in lab notebook!),** at the end of the **sixth week of lab (May 11$^{th}$ – must have all results up through plasmid miniprep analysis complete and in lab notebook!)** and at the **end of the quarter**.

**2. Final Exam.**

There will be a final exam. It will cover both practical and background on the experiments we performed in lab.

**3. Write-up.**

At the end of the quarter you will be required to do a short write-up to tie together your work from the whole quarter and present your analysis of your unknown clone (what did you clone?). The write-up should be in the form of a mini-journal article (I will explain this in class). It should be no longer than 6 typed pages (double spaced). The 6-page limit does not include figures, figure legends, or the references. The format should be (in this order):

**a. abstract:** one paragraph summarizing what the write-up is about.

**b. introduction:** (≈ 1 page) In a normal journal article, one would summarize previously published observations, put forward the question that the present article is about to address, and briefly state how this question will be addressed. You need to give a little background on what is known about the biology of the genes you have isolated and its significance. Just briefly introduce the strategy (that is the techniques) we used to isolate and characterize the gene.

**c. materials and methods:** (≈ 1-2 pages) The objective here is to provide sufficient information so someone could repeat your experiments. You don't want to give all of the nitty-gritty details, rather you reference them. In general, one references other articles for the basic procedures used, and then gives details on the unique features of the present study.

**d. results**: (≈ 2 pages) This will probably be the most difficult section to write. You should include **4 figures** (put them at the end, and include **figure legends**). The figures I want are: (1) the photograph of the agarose gel from first round of the PCR, (2) the photograph of the agarose gel from the plasmid mini-preps, (3) a copy of the nucleotide sequences of your clones, with translation (this is the restriction map you printed out) and (4) a copy of the **Blast** results. The bulk of the Results section should be a description of what is in the figures.

**e. discussion:** (≈ 1 page) Here you want to wrap everything up, and tell the world what the results mean. Important topics to discuss are your interpretation of the BLAST search results, and what future experiments one might do.

**f. references:** You should have at least five references and at least 2 must be journal articles (not books). See the journal ***Cell*** for the proper formatting of references.

**Introduction:**

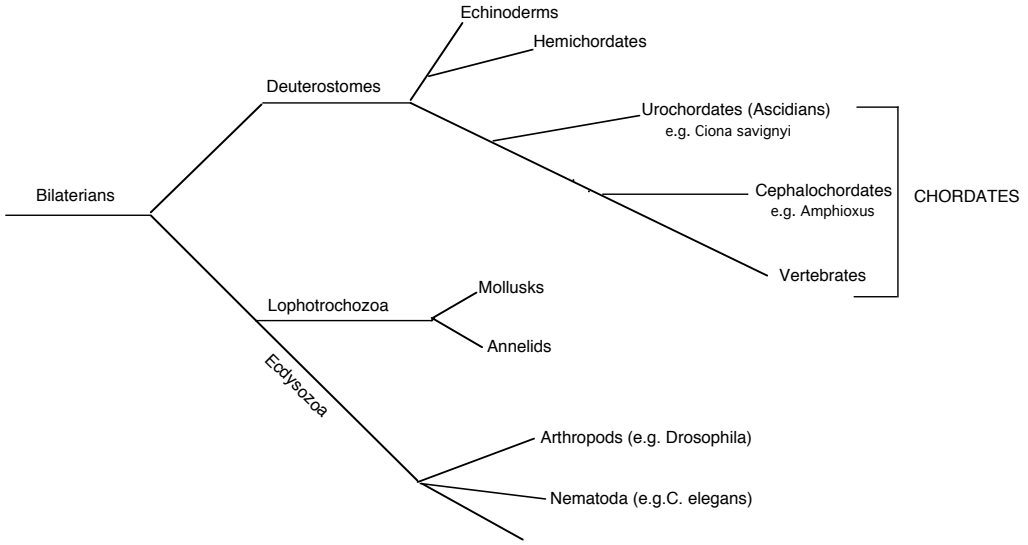
The purpose of this course is to familiarize the student with some of the basic techniques used in molecular biology. The use of molecular biology has become so widespread that it has found applications in almost all aspects of biological research from protein structure analysis to ecology. In this course we will learn the basic techniques used to manipulate **recombinant DNA**. Basic manipulations with DNA are most easily done in prokaryotic organisms, almost exclusively in *E. coli.* Most often when we want to isolate and study the genes of a higher organism (such as humans or plants), we transfer the genes to plasmids or phage that can be propagated and studied in *E. coli* (the DNA of interest has been recombined with prokaryotic plasmid or phage DNA, thus the term "recombinant DNA").

Molecular biology has given us a much deeper understanding of biology by allowing us to isolate genes that are important to cellular and physiological processes. With the isolated genes in hand we can begin to ask questions such as: What is the amino acid sequence encoded by the gene? What does the amino acid sequence tell us about the function and the mechanism of the protein? How is the transcription of the gene regulated? How might alterations in the gene (mutations) underlie disease? And finally, how might we treat genetic disease?

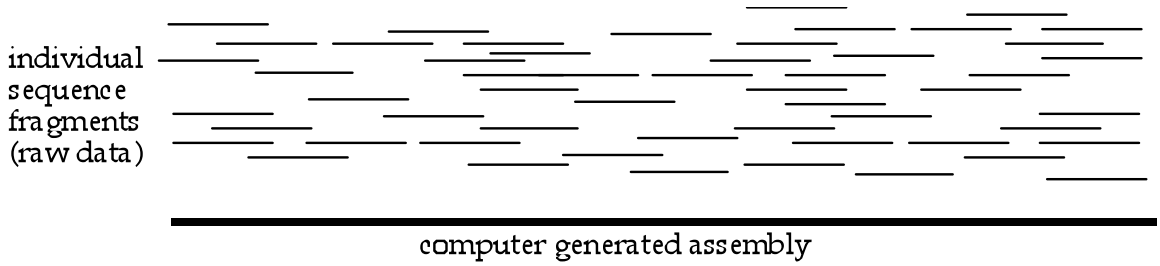
The first step in the molecular analysis of a gene is to isolate a segment of DNA comprising the gene (or a portion of the gene) of interest. Isolation means that we have separated the gene from a complex starting material (for example, the human genome or collection of cDNAs) and have ligated (i.e., combined) the DNA *fragment* into a plasmid that can be easily manipulated in the lab. Genes have been isolated using many different techniques, depending upon the particular situation. These techniques range from complex mapping of human disease genes, often involving hundreds of researchers and years of effort, to simpler techniques such as library screening and PCR amplification.

In this class you will participate in an ongoing project in the Smith laboratory at UC Santa Barbara. The Smith lab has been studying the embryology of a marine invertebrate called *Ciona savignyi*. *Ciona* are ascidians, primitive members of Phylum Chordata (they are urochordates), and they are commonly called sea squirts (see phylogenetic tree).

We are interested in ascidians because they hold many clues to the evolution of more complex chordates, vertebrates in particular. Although on the evolutionary time scale ascidians are very closely related to vertebrates, they have genomes that are ≈20 times smaller than those found in vertebrates. In order to identify potentially interesting genes and to study evolutionary changes between genomes of different species, it is extremely useful to have the entire genome sequence of an organism.




Genome sequencing projects are large multi-year efforts that require the collaborative input of many research groups. The initial phase of a genome sequencing project is determining the nucleotide sequence of the genome. This is done most commonly by the "shotgun" approach, in which large numbers of random fragments of the genome are sequenced and then assembled by computer into a complete and contiguous genome. It has been found that in order to fully assemble a genome, approximately 10 times the total number of nucleotides in the genome needs to be sequenced (referred to as "10X coverage", so that on average any given sequence in the genome will be represented ten times in the sequence data). The diagram below illustrates what such a set of sequence reads might look like, showing many regions of overlapping sequence:

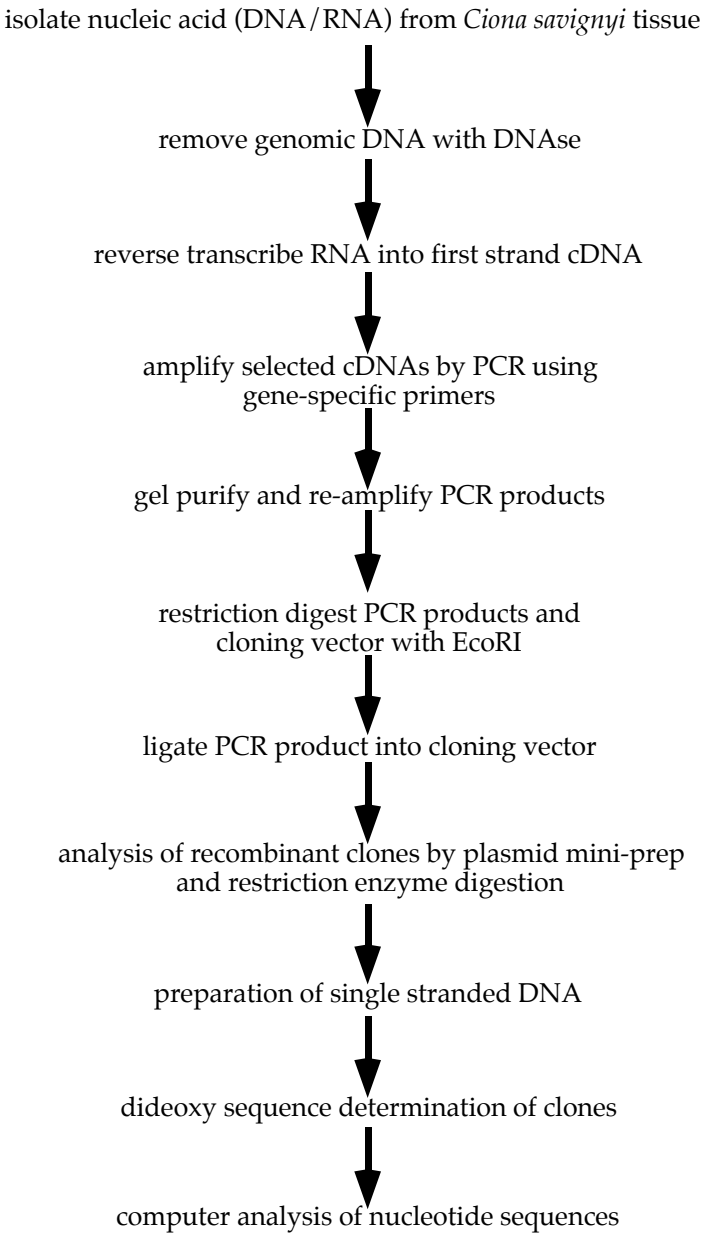



For the *Ciona* genome project, the initial sequencing and assembly have largely been completed. The second phase of the genome project is much more challenging, that is the "annotation" phase. The nucleotide sequence of the genome alone is of limited value; what we are interested in are the genes that are found in the genome. An annotated genome has information not only on the nucleotide sequence, but also on the transcribed and protein coding regions of the genome, as well as other features such as telomere attachment sites, transcriptional regulatory regions, and so on. You probably know from your genetics classes that most of a eukaryotic genome does not encode anything. The

genes consist of relatively small segments within the genome that are separated by vast untranscribed regions. Furthermore, in the protein-encoding genes (regions that are transcribed into mRNAs), the translated regions (exons) are usually separated by lengthy introns (spliced out during RNA processing). Thus, it can be very challenging to pick out the genes from the vast quantity of genomic data that result from the sequencing phase.

In this class we will use data from the *C. savignyi* genome project to isolate cDNAs that are of particular interest to the Smith lab. These cDNAs will in the future, be used by researchers in the Smith lab, to investigate developmental processes.

The overall scheme for isolating the cDNAs is presented below:



**Step 1. Start ascidian RNA isolation**

The first step in isolating the cDNAs is purifying RNA that will serve as the starting material. RNA is more difficult to work with than DNA, so you will be starting out with one of the more difficult procedures. The difficulty with RNA comes from the fact that all living tissues have a very high turnover rate for RNAs (i.e., they are continuously synthesizing new RNAs and breaking down old RNAs), and as a consequence are full of an RNA destroying enzyme called RNase. In order to isolate the RNA we need to lyse (break open) the cells. As soon as you do this, the RNAses in the tissue will go to work on the RNA, and if you are not careful the RNA will be chewed up into small pieces very quickly. To avoid this problem, the tissue will be lysed in a solution that simultaneously breaks open the cells and inactivates the RNAse. The key ingredient in the lysis solution is a detergent called sodium dodecylsulfate (SDS) that will denature the RNAses by causing them to unfold.

The procedure that we use here will first isolate the nucleic acid (DNA and RNA) away from the other cellular components. Briefly, the tissue is first quickly lysed in the SDS-containing solution and then incubated with a protease (proteinase K) which will digest all of the cellular proteins to short peptides, but leave the nucleic acids untouched. To separate the nucleic acids from the digested proteins, we will extract the solution with phenol. Phenol is a good protein solvent; the hydrophobic ring of the molecule sneaks into the core of globular proteins and the hydroxyl group will solubilize hydrophilic side groups of amino acids. Thus phenol is effective at both solubilizing and denaturing proteins. Phenol, being only slightly soluble in water, forms a separable phase into which proteins partition (the cellular lipids will also partition into this layer). When you do a phenol extraction you may see whitish material at the interface between the phenol and aqueous phase; you want to avoid this material. **When working with phenol you want to wear gloves and safety glasses (you don't want the phenol to do to the proteins in your hand what it does to the sea squirt proteins, much less to the proteins in your eye).** Phenol is often used on its own, but in this case we will use a 1:1 mix of phenol and chloroform. Chloroform is dense and will help to drag the organic phase to the bottom of the tube. The stock bottle of phenol/chloroform is overlaid with an aqueous layer (look at the side of the bottle, you should see two phases); you want to take only from the lower layer!

The nucleic acid is concentrated from the extracted aqueous phase by precipitation with salt/ethanol and resuspended in water. We can then selectively degrade the DNA with DNAse.

**One additional important point!!!** You hands are covered with RNAses, so you will need to wear gloves throughout this procedure.

**Procedure:**

1. You will be given a 1.5 ml tube containing *C. savignyi* embryos. Centrifuge the tube for 1 minute at 5,000 rpm. Use your pipetman to remove the sea water while not disturbing the orange pellet [remove as much fluid as

possible; you can briefly re-centrifuge the tube (10 sec) if the pellet becomes dislodged]. Place the tube on ice.

2. While keeping the tube with the embryos on ice, add 100 µl* **embryo lysis buffer** to the pellet**. Immediately** insert the plastic pestle and grind against the bottom and sides of the tube a dozen or more times. Do this step quickly. Add another 400 µl of **embryo lysis buffer** and 12.5 µl of 10 mg/ml **proteinase K** solution (250 µg/ml final concentration) and mix by inversion. Using a combination of smashing with the pestle and pipetting up and down with a 1 ml pipetman break up and homogenize the embryos as thoroughly as you can

3. Incubate the tube one hour at 42° C.

4. Add 0.5 ml of **phenol/$CHCl_3$**, vortex thoroughly (1-2 mins.), centrifuge 5 min., and transfer aqueous phase (upper) to new tube. *Do not transfer any of the white material at the interface.* (It's better to lose some of the sample than it is to carry-over some of the interface.)

5. Re-extract the aqueous (upper) phase from step 4 with a second 0.5 ml of phenol/CHCl3. Vortex and centrifuge as in step 4, and transfer the aqueous phase to a new tube as you did before.

6. To the aqueous phase add 1 µl of **Pellet Paint**, 50 µl of **3 M sodium acetate (DEPC treated)** and 1 ml of 100% **ethanol**, mix well and put tubes in a -20° C freezer for 10 minutes. The combination of the sodium acetate and ethanol will cause the DNA to precipitate. The Pellet Paint will precipitate along with the DNA, and make the precipitate red so it is easy to identify. Centrifuge 10-20 minutes at 10,000 rpm. You should see a small red/pink pellet at the bottom of the tube. **Keep your eyes on the pellet and do not lose it!!!** Remove fluid from tube with a pipetman, making sure not to disturb the pellet. Centrifuge the tube again briefly and remove any remaining traces of fluid.

7. Add 100 µl of 75% ethanol, cap tube and place in your labeled plastic cup in the freezer.

***see appendix A (at the end of this manual) for an explanation of units and calculations used in molecular biology.**

**Materials:**

Embryo lysis buffer:

100 mM NaCl
20 mM Tris, pH 7.8
10 mM EDTA
1% SDS

**Step 2. Finish RNA isolation and run RNA on gel.**

The final step in the RNA preparation is to remove the DNA with DNAse. Then, before we proceed with the reverse transcription, we want to ensure that the RNA you isolated is intact (not degraded). To check the RNA, we will run a small sample of it out on an agarose gel and visualize the RNA by staining with ethidium bromide.

DNAse treatment:

1. Find the tube of your nucleic acid in the freezer and centrifuge for 5 minutes at 10,000 x g. Look for the red/pink pellet. Remove all of the fluid and leave tube uncapped ≈ 5 minutes to allow remaining ethanol to evaporate (do not over dry).
2. Transfer the tube to an ice bucket, and keep the RNA on ice from now on.
3. Add 43 μl of RNAse-free water to the tube and help the nucleic acid dissolve by pipetting up and down.
4. To the tube add:
   - 5 μl 10X DNAse buffer
   - 1 μl ribonuclease inhibitor (RNAsin)
   - 1 μl of DNAse
5. Vortex tube to mix, and then incubate at 37$^{o}$ C for 30 minutes.
6. Add another 50 μl of RNAse-free water, mix.
7. Extract one time with 100 μl of phenol/$CHCl_3$.
8. Move aqueous phase to a new tube, add 10 μl of 3 M sodium acetate and 220 μl of ethanol. Mix by vortexing, put in the freezer for ten minutes and then centrifuge for 10 minutes at 10,000 rpm. Look for the red/pink pellet.
9. Remove fluid and add 100 μl of 75% ethanol (wash step).
10. Centrifuge for 5 minutes, remove fluid and allow pellet to air dry.
11. Dissolve pellet in 20 μl of RNAse-free water. **Keep on ice!!**

Quantity and quality of your RNA

1. We will use a spectrophotometer to quantify the RNA. We will take a small sample of the RNA (1 μl) and load it onto a special spectrophotometer called a Nanodrop. This sample will be lost in the process, but it will tell us the concentration of the remaining RNA sample. There will be a demonstration of how the spectrophotometer works. Nucleic acids absorb strongly at 260 nm, while proteins absorb most strongly at 280 nm (this is in the UV range). The readout on your sample will be in absorbance units (AU) at 260 and 280 nm.

   With the AU readings of your sample at 260 and 280 nm, first calculate the 260/280 ratio. For pure RNA this ratio should be ≈2. If it is lower than this, your RNA is likely contaminated with protein. From the OD at 260 nm you can calculate the concentration. The

absorbance of RNA at 260 nm is a known constant: a solution of RNA that has an AU of 1.0 at 260 nm is 0.04 mg/ml. Thus, in general, the formula below can be used to determine RNA concentration:

**$AU_{260}$ x 0.04 mg/ ml x dilution factor = RNA conc. in mg/ ml**

**example:** If your 1:50 dilution had an absorbance of 0.121 your RNA concentration is 0.242 mg/ml (i.e., 0.04 mg/ml X 0.121 X 50 = 0.242 mg/ml).

2. Finally, we will check the integrity of your RNA by running a small sample on an agarose gel. You will be running your own agarose gels later in the quarter, but now since we each have only one sample we will all run our samples together on one big gel. You will want to load 1 μg of RNA on the gel, so determine the volume of solution necessary (for the sample above with a conc. of 0.242 mg/ml, we need 4.1 μl: 1μg / .242μg/μl = 4.1 μl), and add this together with **RNA sample buffer** in a microfuge tube to make a total volume of 15 μl (for our example, that is 4.1 μl of RNA and 10.9 μl of RNA sample buffer). Give the sample to your TA.

**3. Put your remaining RNA sample back in the freezer before you leave!!!!!!!**

**Materials:**

DNAse buffer:
400 mM Tris pH 7.9
100 M NaCl
60 mM $MgCl_2$
1 mM $CaCl_2$
1 mM DTT

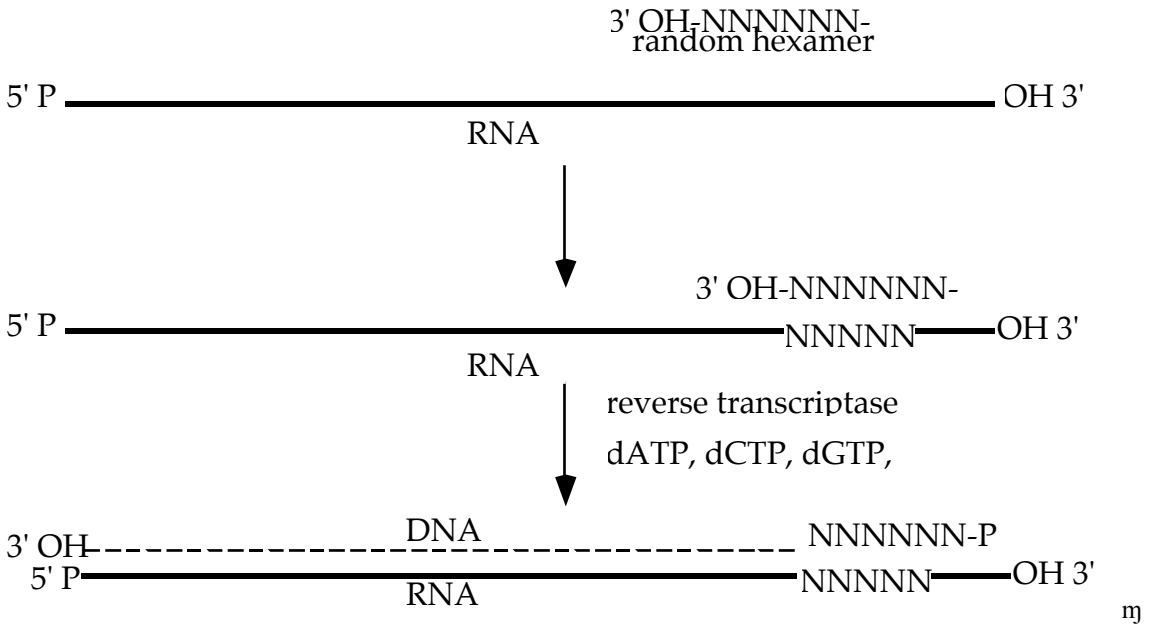
RNA sample buffer:
60% formamide
20 mM MOPS, pH 7.0
18% formaldehyde (from 37% stock)
0.2% bromphenol blue
0.2% xylene cyanol
40 μg/ml ethidium bromide

## Step 3. Reverse Transcription/ streak out E. coli

### A. Reverse transcription.

In the reverse transcription reaction, we use the RNA as a template to make a complementary DNA strand using the enzyme reverse transcriptase. Reverse transcription is normally a process done by retroviruses (such as HIV). The enzyme we will be using was isolated from Avian Myeloblastosis Virus (AMV). Reverse transcriptases, like all DNA polymerases, can only initiate polymerization from a free 3' OH group (i.e., it needs to be primed). To prime the reverse transcription we will mix the RNA with short oligonucleotides called random hexamers. The random hexamers are single stranded pieces of DNA six bases long. They were chemically synthesized and are random because all possible sequences of the four base pairs are present. Thus there are 4,096 different sequences ($4 \text{ bases}^6 = 4{,}096$) mixed together. The random hexamers will anneal at random locations on the RNA template and initiate reverse transcription at various locations. Remember that your RNA sample consists of tens of thousands of different RNAs (the transcription products of the tens of thousands of genes), and hundreds to thousands of copies of each RNA species.

The simple reverse transcription reaction is shown below. "N" indicates any nucleotide. Remember that there are millions of such reactions taking place in your reaction tube. The end product of the reaction is a DNA/RNA hybrid.




**Procedure:**

1. Calculate how much of your DNAse-treated RNA solution you need for 5 µg. Add this volume together with RNAse-free water to make a total volume of 10 µl. If the volume of RNA you need for 5 µg is greater than 10 µl, see your TA.

2. To the 10 µl of RNA (step 1), add 1 µl of 0.1 mg/ml random hexamer, heat to 65º for 2 min., then put on ice.

3. To the RNA/primer solution, add:

   4 µl 5X RTase buffer
   1 µl ribonuclease inhibitor
   2 µl 5 mM dNTPs
   0.5 µl AMV RTase
   1.5 µl RNAse-free water

   Mix well by pipetting up and down (centrifuge briefly if needed to gather all of the fluid to the bottom of the tube).

4. Incubate 30 min. at 42º.

5. Place tube in your freezer cup - make sure it is well labeled, you will need it again!!!

**B. Streak out *E. coli* XL-1-Blue MRF' for making competent cells.**

We are going to start something today that will be used later in the course. The goal of our project is to get our *C. savignyi* cDNAs replicating in *E. coli* on a plasmid. To begin the process of getting the cDNAs into E coli, we first have to make competent cells. They are called "competent" because they can take up foreign DNA. The first step in making competent cells is to streak the cells out on an agar plate and make sure they are the right strain (i.e., do they carry the proper antibiotic resistance). The *E. coli* strain we will be using is called XL-1-Blue MRF'. For us, the most important features are that it is recA minus *(recA)*, which eliminates a recombination activity, making the cloned genes more stable. The cells are also deficient in a number of restriction activities and other nucleases. Finally, they also carry the lacI$^{q}$ZΔm15 mutation that provides α-complementation for the β-galactosidase gene (more on that later).

**Procedure:**

1. You will be provided with a small liquid culture of XL-1-Blue MRF'.

2. Using a flamed inoculating loop (sterile) streak out the bacteria on an **LB/agar/tetracycline** plate.

3. Resterilize the loop and run it through the first streak of cells to spread them out, ensuring that single colonies will form.

4. Incubate the plate at 37$^{o}$ until the next day (greater than 12 hours); the plate at this time should have multiple colonies.

**Step 4. You will need to come in sometime the next day, IDEALLY allowing cells to grow for 16-18 hours (not more or less).**

Start an overnight culture of the XL-1-Blue MRF' cells.

**Procedure**:

1. Take a pre-made tube with 5 ml of LB media with tetracycline (12 μg/ml).

2. Pick a single colony from your plate of cells with a sterile toothpick and drop it into the culture tube.

3. Incubate the tube overnight with shaking at 37$^{o}$, for 16-18 hours.

4. Wrap the plate of XL-1-Blue MRF' cells in parafilm, and put it in the refrigerator (you will need it later).

**Step 5. We are going to do two different things today that will be brought together later in the quarter:**

**A. Make competent cells for plasmid transformation.**

**B. Start PCR reactions to amplify *C. savignyi* cDNAs**

**A. Preparation of competent cells.**

Experience has shown that cells which are competent for transformation by exogenous plasmid DNA must be prepared with care. Two of the most important tips are 1) start the culture the day you make the cells and do not grow beyond an OD of 0.5, and 2) keep the cells cold from the time you remove the culture from the incubator until the transformation is heat shocked. Therefore, you will need to pre-cool all solutions and tubes that will be used in the transformation.

**Procedure:**

1. Transfer 2.5 ml of the culture you started the previous day to 50 ml of fresh LB medium in a 500 ml flask.

2. Grow (~2hr, 37°, 200 rpm) until $A_{550\text{-}600}$ is about 0.5.

*(while the cells are growing we will proceed to step B, below)*

3. Transfer cells to a 50 ml centrifuge tube (on ice). Spin 4° C, 5 min. at 2500 rpm (just enough to pellet cells).

4. Drain supernatant and resuspend cells in 20 ml of chilled **TFB 1**.

5. Leave on ice for 30 min.

6. Spin cells down at 2500 rpm for 15 min. at 4° C.

7. Drain supernatant and resuspend in 2 ml **TFB2**. Be very gentle during resuspension. Try to suspend cells by swirling; failing that, very gentle pipetting.

8. Put 200 µl of cell suspension into 10 precooled 1.5 ml tubes (on ice). Freeze tubes on dry ice. Place tubes into labeled cups (keep on dry ice). The cells will be stored in a -80°C freezer.

**Materials:**

500 ml flask with 50 ml LB medium

TFB1:

30mM KOAc
100 mM KCl
50mM $MnCl_2$
15% glycerol (weight/volume)
adjust final pH to 5.8 with diluted acetic acid. Sterile filter.

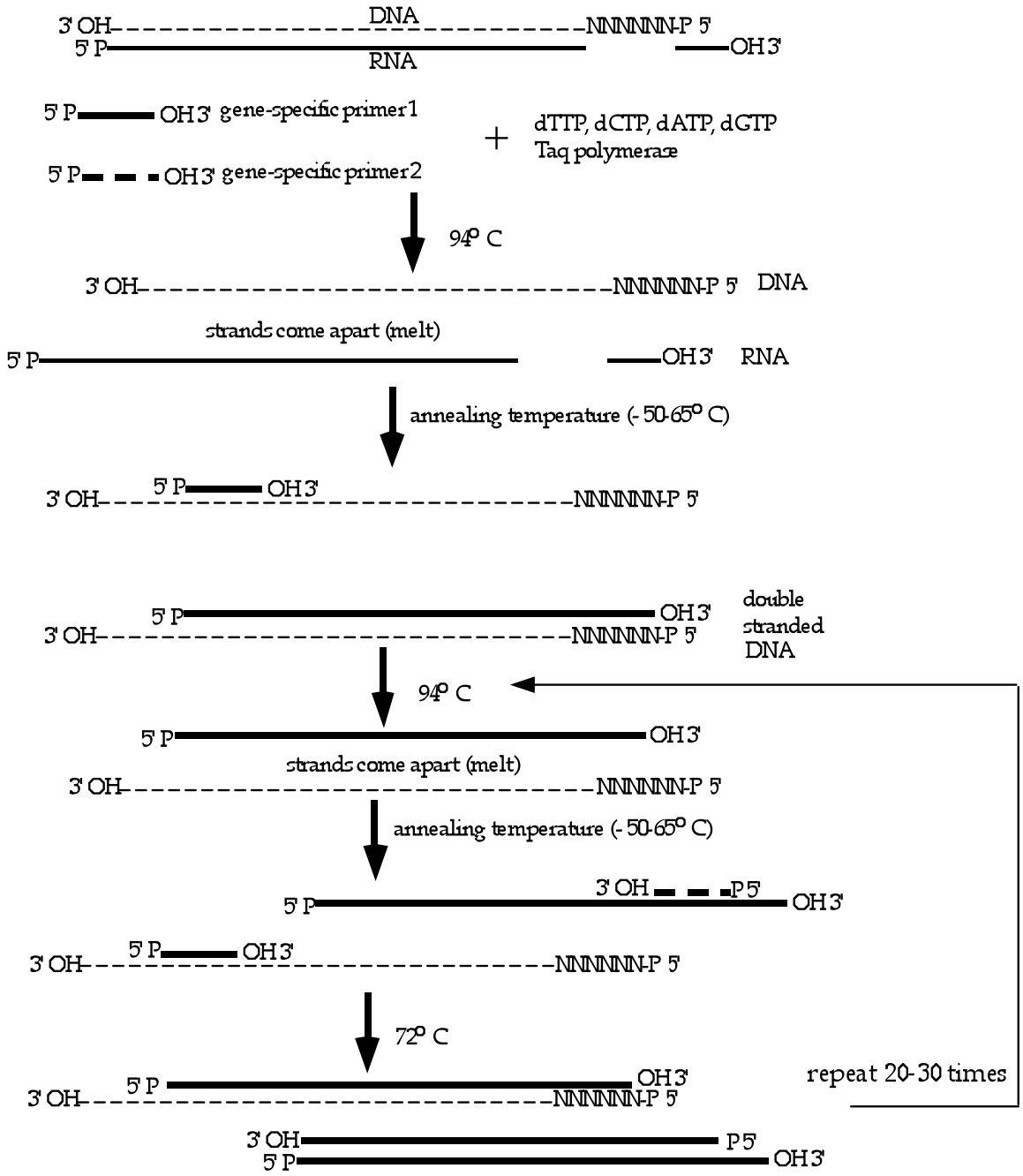
TFB2:

| | |
|---|---|
| 10 mM Na-MOPS pH 7.0 | 10 mM KCl |
| 75 mM $CaCl_2$ | 15% glycerol (w/v) |

**B. PCR amplification**

The PCR reaction is really very simple. We just add the template DNA (our DNA/RNA hybrid), two oligonucleotide primers, deoxynucleotide triphosphates (dATP, dCTP, dTTP, dGTP), the DNA polymerase (Taq polymerase), and some salts and buffer to make the enzyme work. The PCR machine does the rest. We will go over the workings of the PCR machine in class. Basically, it will cycle between three temperatures. First, it will heat the sample to 94$^o$ C to melt the two strands of the template. Second, it will go down to a temperature at which the oligonucleotides will anneal to the template. The specific annealing temperature depends upon the base composition of the oligonucleotides (a function of the length and the percent G/C content). If the annealing temperature is too high one will not get any amplification product; if it is too low one runs the risk of getting annealing at other, unwanted sites (due to "non-specific" binding). The third and final step is the elongation step. This is invariably done at 72$^o$ C, which is the optimum temperature for Taq polymerase.

The PCR reaction we are doing is outlined here. Note that in the first round of the cycle, the RNA/DNA hybrid is converted into a double-stranded DNA (the RNA strand can not be used as a template by Taq polymerase and does not contribute to the amplification). Thus in the first round there is no amplification. However, in the second and subsequent rounds the template doubles each cycle, giving a tremendous amplification of our specific gene after 20-30 cycles.




The design of the primers is a critical factor. Shorter DNA fragments amplify more readily than do longer fragments. We designed our primers to amplify fragments in the range of 500-1000 bases, and as a consequence we will not be amplifying the entire cDNA, just an internal segment.

For example, we designed the following primers for ETR-1 (ETR-U and ETR-D) based upon the underlined regions:

```
1   K  D  D  D  A  V  K  L  F  I  G  Q  V  P  K  N  W  D  E  K
1  AAAGACGACGACGCAGTAAAACTTTTCATCGGCCAAGTCCCGAAAAACTGGGACGAAAAA

21   E  L  R  Q  I  F  A  P  F  G  E  I  F  E  L  S  V  L  R  D
61  GAGCTTCGTCAGATTTTCGCGCCATTTGGAGAGATTTTCGAGTTATCAGTCCTAAGGGAT

41   K  Y  T  G  M  H  K  G  C  A  F  L  T  Y  A  H  K  T  A  A
121 AAATACACAGGCATGCATAAAGGCTGTGCCTTCCTCACCTACGCACACAAAACAGCAGCA

61   M  N  A  Q  N  S  L  H  E  R  K  T  L  P  G  M  N  H  P  I
181 ATGAACGCGCAGAACAGTCTCCACGAACGAAAAACGCTTCCTGGGATGAACCACCCAATC

81   Q  V  K  P  A  D  T  V  S  K  G  E  D  R  K  L  F  V  G  M
241 CAAGTCAAGCCAGCCGATACCGTTAGTAAAGGAGAGGACCGCAAGTTATTCGTTGGGATG

101  L  G  K  R  Q  N  E  E  D  V  R  V  L  F  E  P  F  G  T  I
301 TTGGGGAAACGACAAAACGAGGAGGATGTTCGCGTTCTCTTTGAACCGTTCGGAACGATT

121  E  E  C  T  I  L  R  T  P  E  G  Q  S  K  G  C  A  F  V  K
361 GAAGAATGCACGATTTTAAGAACGCCTGAGGGGCAGAGCAAAGGATGCGCATTCGTGAAG

141  L  A  C  H  S  E  A  K  T  A  M  D  A  L  H  G  S  Q  T  M
421 CTCGCCTGTCATTCGGAAGCTAAGACTGCGATGGACGCTCTGCACGGGAGTCAGACCATG

161  P  G  A  S  S  S  L  V  V  K  F  A  D  T  D  K  E  R  A  V
481 CCGGGGGCGTCCTCGAGCCTGGTTGTAAAGTTCGCGGACACGGACAAAGAGCGCGCCGTG

181  R  R  M  Q  Q  M  A

541 CGACGAATGCAGCAAATGGCG
```

We will have primer sets for the four different *Ciona* cDNAs. **Each student to try three of the primer sets.** The cDNAs we will attempt to amplify are:

**SET 1.** (EcoRI site is underlined)

```
   UP - 5' GGGGAATTCCGTCATGCGAGTTAAAATGG  3'
 DOWN - 5' GGGGAATTCTTCGGAGCAGTATTGGAACC  3'
```

**SET 2.**

```
   UP - 5' GGGGAATTCAGACGACGACGCAGTAAAAC-3'
 DOWN - 5' GGGGAATTCCCATTTGCTGCATTCGTC-3'
```

**SET 3.**

```
   UP - 5' GGGGAATTCATTCTCACGTGATGGGC 3'
 Down - 5' GGGGAATTCTTACTGGTGCAAAGGAG  3'
```

**SET 4.**

UP - 5' GGGGAATTCTGTGAACGATCAAACGA 3'

Down - 5' GGGGAATTCTCCAAATCCTTCCACTG 3'

Notice that only the last 18-21 bases of each primer corresponds to the genomic sequence. At the 5' end we have added a site for the restriction enzyme EcoRI. This should not interfere in the PCR and will be used later when we ligate the PCR products into a plasmid.

You will be setting up three PCR reactions. Choose three of the primer sets.

**Procedure:**

Keep all solutions, including your PCR reaction mix on ice.

In the special PCR tubes mix the following **on ice**. You will want to set up three different reactions. They will be same except for the primers you use. **Be careful that you are using the proper paired primers!!!**

10 μl of 5X PCR buffer
1 μl primer U (at 25 pmoles/μl)
1 μl primer D (at 25 pmoles/μl)
1 μl of 5 mM dNTPs (5 mM each dGTP, dCTP, dTTP and dATP)
3 μl reverse transcription reaction (from step 3)
1 μl Taq polymerase (add last)
33 μl $H_2O$

Mix contents well on the vortexer. Once you have all the components mixed together in your tube, take your ice bucket over by the PCR machine.

Keep your tube on ice until everyone is ready to use the PCR machine. Then, put your tubes in and start the PCR machine. When the reaction is finished, put the tubes (labeled with your name) in the freezer (this will be done for you).

**Materials:** (keep all excess reagents frozen in a cup labeled with your name)

5X PCR buffer:
100 mM Tris pH 8.4
500 mM KCl
7.5 mM $MgCl_2$
1 mg/ml gelatin

oligonucleotide primers at 25 pmoles/μl in TE
25 mM $MgCl_2$
5 mM dNTPS in TE

**Step 6. Test Competent Cells**

We will transform one tube of the competent cells with a test plasmid to make sure they work well. Good competent cells should give on the order of $10^6$ colonies per μg of plasmid. We will use the plasmid bluescript SK+ (see appendix B for map of plasmid). This plasmid carries the gene that confers resistance to ampicillin, so we can select for transformed cells by growing them on ampicillin-containing plates.

**Procedure:**

1. Retrieve one tube of competent cells from the freezer and place it immediately on ice (do not allow it to warm up); the cells will take about 5-10 minutes to thaw on ice. Put four LB/ampicillin plates (inverted) in the 37° incubator to warm up.

2. Split the thawed cells into two tubes of 100 μl each. Add 10 μl of the plasmid solution (containing 1 ng DNA) to one of the tubes. Add 10 μl of sterile water to the other tube. Leave the cells on ice for 30 min.

3. Heat shock the cells for 2 minutes in a 42° C water bath, then place back on ice.

4. Add 1 mL LB without ampicillin to each tube. Incubate at 37° C for 30 min.

5. Centrifuge briefly to form a soft pellet. Remove most of the supernatant; leave approximately 110 μl in the tube (use markings on side of microcentrifuge tube as a guide). Resuspend the pellet in the remaining 110 μl of LB.

6. For both of the tubes of cells (one with plasmid and one without), plate out two volumes of cells; one plate gets 10 μl of cells, the other 100 μl. (There will be a demonstration). Return the plates to 37° C (invert them).

**Step 7. Take the bacterial plates out of the 37° incubator and count colonies.** You need to take the plates out of the incubator before noon--otherwise they will be overgrown. Take the plate that has the most reasonable number of colonies to count and calculate the transformation efficiency (colonies per 1 μg of plasmid).

**Step 8. Isolation of PCR products and second PCR amplification**

In order to make a lot of the PCR products for ligating into the plasmid, we are going to perform a second PCR reaction. We will electrophorese samples of the three PCR reactions on an agarose gel and then isolate the bands that are of the expected sizes for the PCR products from the gel. We will then use these isolated DNAs as template for the second round of PCR. In the first PCR reaction most of the product should be of the expected size, but you will also notice a haze of DNA at various other sizes that result from the primers annealing to other sites. You started at least three different reactions - we hope that at least one worked.

**Procedure:**

1. First we need to make a 1.5% agarose gel (that means 1.5 grams of agarose for each 100 ml of gel). Weigh out 1.5 grams of agarose, add 100 ml of 1X TAE buffer. Melt the agarose in the microwave oven, make sure that all of the agarose is dissolved, and that the solution doesn't boil over. Allow the solution to cool on the bench top for 10 min. Add 10 μl of 1 mg/ml ethidium bromide, swirl to mix ethidium bromide into gel, then pour into the casting mold. Place the comb in the mold. The gel will be ready to use in about 1/2 hour.

****Ethidium bromide is a mutagen so you need to wear gloves**.

2. Thaw your PCR reactions (you should have 3), and place 20 μl of each in new microfuge tubes. Add 3.5 μl of 6X agarose gel sample buffer to the tubes and mix.

3. When the gel has solidified, remove the comb. Add enough TAE to cover the gel. Load each PCR sample into a separate well. To an adjacent well, add 10 μl of the molecular weight standards. Be sure to write down the loading order for your samples.

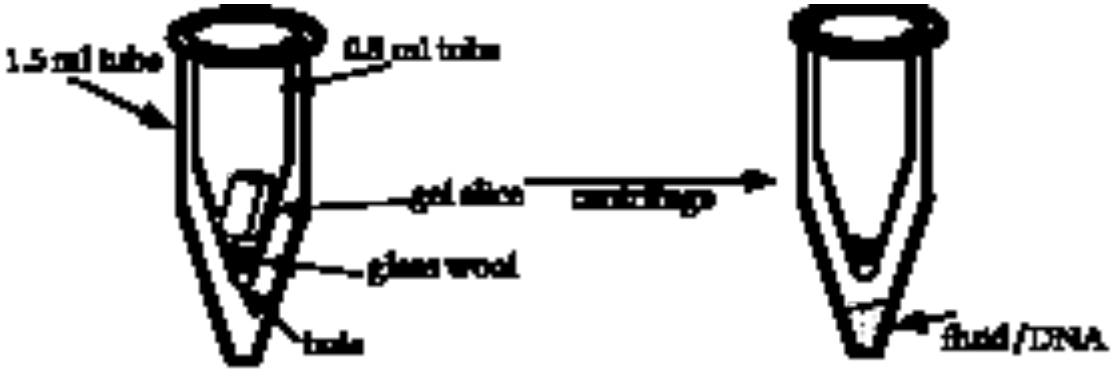
4. Connect the terminals of the electrophoresis tank to the power supply, turn it on, and set it to 100 volts. Run the gel for approximately 1 hr, or until the dye in the sample buffer (the phenol red) has migrated at least 5 cm.

5. To make a permanent record of the gel, take a Gel-Doc photograph. There should be a prominent band in the range of 400 to 600 bp.

6. To view the DNA, place the gel on the UV illuminator. **The UV illuminator is a very strong source of UV light - it is important to shield your face with the plastic face-mask, otherwise you can severely burn your skin and eyes**.

7. Using a razor blade cut out the desired bands from the gel, trim off all excess agarose. Place the excised band on a small piece of Saran wrap and take it to your bench. If all three worked, choose one at this point.

8. For the next round of PCR we need to isolate the DNA from the agarose. At this step we will use a relatively quick and dirty method. Take a 0.5 ml microfuge tube and cut the lid off. Poke a hole in the bottom of the tube with a syringe needle. Stuff a small piece of siliconized glass wool in the bottom of the tube to cover the hole, then place the gel slice on top of the glass wool. Take a 1.5 ml microfuge tube and cut the lid off. Place the 0.5 ml tube into the 1.5 ml tube. Finally, put the whole thing in the microfuge and centrifuge at maximum speed for 10 min. This will force most of the DNA along with the fluid out of the gel and into the bottom of the 1.5 ml tube, while the agarose will be trapped by the glass wool. Save the fluid in the bottom of the 1.5 ml tube—transfer it into a new tube.




9. Set up a new PCR reaction as before (step 5B), with the exception of using 1 μl of the recovered DNA from the step 8 as the template—instead of the "3μl of reverse transcriptase reaction"—and adjust the final volume accordingly.
**Make sure to use the same primer pair as in the first round**!!!
And remember to keep the **Taq polymerase on ice**!

10. At the end of the PCR reaction place the reaction tubes in the freezer.

**Materials:**

6 X agarose gel sample buffer: (keep excess at room temperature).

50% glycerol, 0.2% phenol red

low molecular weight DNA standards at 1 μg/10 μl in 1X agarose gel sample buffer (store excess in freezer)

siliconized glass wool

TAE: (instructions to make more TAE are on the 2 liter bottles you will find on your bench).

40 mM Tris-acetate, pH 8.5

2 mM EDTA

**Step 9. Gel purification of re-amplified PCR fragments.**

The quick and dirty method for isolating the PCR products used above is adequate for providing template for PCR, but DNA isolated by this procedure has proven difficult to ligate into plasmids (largely for unknown reasons). To prepare DNA for ligation, we are going to use a commercial kit from QIAGEN to isolate the fragment from the agarose gel. The objective here is to isolate as much of the PCR product as we can. The more we have to start with, the better the ligation will work.

**Procedure:**

1. Run the entire 50 μl PCR reaction on an agarose gel. Prepare a 2% agarose gel as described above, but use tape to mask over 3 teeth on the 5 well comb to make an extra wide well (the TA will demonstrate).

2. Add 10 μl of 6 X agarose gel sample buffer to the PCR reaction, mix and load the entire sample onto the solidified gel (and load molecular weight standards into an adjacent lane). Perform the electrophoresis and staining as before.

3. Cut out the PCR band, trying to minimize the amount of excess agarose that is NOT your band, and place into a pre-weighed 1.5 ml microfuge tube. Weigh the tube again to determine the mass of the gel slice.

4. For every 100 mg of gel, add 300 μl of Buffer QG. The maximum amount of gel slice per QIAquick column is 400 mg. If your gel slice is larger than 400 mg, then you will need to split the solution and use more than one QIAquick column. Place in a 50$^{o}$ C water bath for 10 min. Mix the tube every few minutes. The Buffer QG will dissolve the agarose and release the DNA into the solution. It is extremely important to solubilize the agarose completely; if it hasn't dissolved completely, increase incubation time.

5. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 μl of 3M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow. The adsorption of DNA to the QIAquick membrane is efficient only at pH $\leq$7.5. Buffer QG contains a pH

indicator, which is yellow at ≤7.5 and orange or violate at higher pH, allowing easy determination of the optimal pH for DNA binding.

6. For every 100 mg of gel, add 100 μl of isopropanol to the sample and mix immediately. Do not centrifuge sample at this stage.

7. Place a QIAquick spin column in a provided 2 mL collection tube.

8. Apply the sample to the QIAquick column, and centrifuge for 1 min. The maximum volume of the column reservoir is 800 μl. For sample volumes of more than 800 μl, simply load and spin again. If you had more than 400 mg of gel to begin with, then you will need to split your sample and use two columns. This step will bind the DNA to the column.

9. Discard flow-through and place QIAquick column back in the same collection tube. Buffer QG, which makes up most of your discarded flow-through, contains guanidine thiocyanate which liberates very toxic gas when mixed with acids.

10. To remove any remaining traces of agarose, add 500 μl of Buffer QG to QIAquick column and centrifuge for 1 min. Discard flow-through.

11. To wash the DNA, add 750 μl of Buffer PE to QIAquick column and centrifuge for 1 min. Buffer PE contains ethanol.

12. Discard the flow-through and centrifuge the QIAquick column for an additional 1 min. This removes any residual ethanol from the column.

13. Place QIAquick column into a **clean** 1.5 mL microcentrifuge tube.

14. To elute DNA, add 30 μl Buffer EB (10mM TrisCl, pH 8.5) to the center of the QIAquick membrane (do not touch membrane with pipette/do not poke through membrane), let the column stand for 1 min, and then centrifuge for 1 min.

15. The DNA should now be in the flow-through. Save this in the freezer. Discard the column.

## Step 10. Restriction digest PCR products/prepare plasmid vector

**Today we are going to do two things to get ready for ligating our PCR products:**

**A. Restriction digest the cloning plasmid with EcoRI**

**B. Digest the PCR products with EcoRI**

### A. Restriction digest the cloning plasmid vector with EcoRI

We will be ligating our PCR products into the plasmid bluescript SK+ (see appendix B for a map). First, we have to open up the plasmid so we can ligate in our PCR fragment. Some restriction enzymes produce staggered ends (also called sticky ends), others produce blunt ends. Ligation of two DNA pieces with compatible [complementary] sticky ends is the preferable route of ligation. We will cut open the plasmid with the enzyme EcoRI, which produces sticky ends.

**Procedure:**

1. When working with enzymes, it is important to **keep the enzyme stock on ice at all times**, otherwise it will lose activity.

2. To a 1.5 ml microfuge tube containing 3 μl of bluescript SK+ (1 μg/μl) add the following (in this order):

    21 μl of distilled water
    3 μl of 10X EcoRI enzyme buffer
    3 μl of EcoRI (10 units/μl)

    Put the reaction in the 37° C water bath for at least 2 hours

3. At end of incubation put the tube at 75° C for 10 min to inactivate the enzyme
4. Store the tube in the freezer

### B. Restriction digest PCR products with EcoRI

**Procedure:**

1. The PCR product DNA is not yet ready for ligation. Taq polymerase leaves the DNA with a 3' overhanging end. The PCR primers incorporated an EcoRI site that we will digest now to produce overlapping ends to ligate into the vector.

Move 20 μl of DNA from step 9.15 to a fresh tube and add:

22.5 μl $H_2O$
5 μl EcoRI reaction buffer
2.5 μl EcoRI enzyme

Incubate for 1.5 hours at 37° C.

2. We now need to remove the enzyme and concentrate the DNA. Add 50 μl of TE to the tube, and then incubate at 75° C for 10 min to inactivate the enzyme. Add 100 μl of 1:1 phenol/chloroform to the tube, vortex well, and centrifuge for 3 min. Remove the upper, aqueous phase to a new tube, and extract with another 100 μl of phenol/chloroform. Remove the aqueous layer to a new tube.

3. To the 100 μl in the tube, add 10 μl of 3 M sodium acetate (pH 5.2), 1 μl of Pellet Paint, and 275 μl of ethanol, mix well. The combination of the sodium acetate and ethanol will cause the DNA to precipitate. The Pellet Paint will precipitate along with the DNA, and make the precipitate red so it is easy to identify. Place the tube in the freezer for 10 min. to facilitate precipitation of the DNA. Centrifuge the tube for 10 min. at maximum speed. At the end of the centrifugation you should see a small red (pink) pellet. Use your Pipetman to carefully remove the supernatant, leaving the pellet undisturbed (keep the supernatant, just in case).

4. Add 100 μl of 70% ethanol to the tube containing the precipitated DNA. Don't mix, just return to the microfuge for a five minute centrifugation. Again, carefully remove the supernatant. Open the tube and allow it to dry completely on your bench. When there is no trace of fluid left in the tube, add 20 μl of TE to the tube, mix briefly, label the tube with your name and put it in the freezer.

**Materials:**

bluescript SK+ plasmid at 1 mg/ml
EcoRI
10X EcoRI buffer :
3 M sodium acetate, pH 5.2*
100% ethanol*
*store excess at room temperature

## Step 11. Dephosphorylate Plasmid / Quantify plasmid and PCR fragment / Start Ligation

**Today we are going to bring our plasmid vector and PCR products together and ligate them. There are several things we need to do today:**

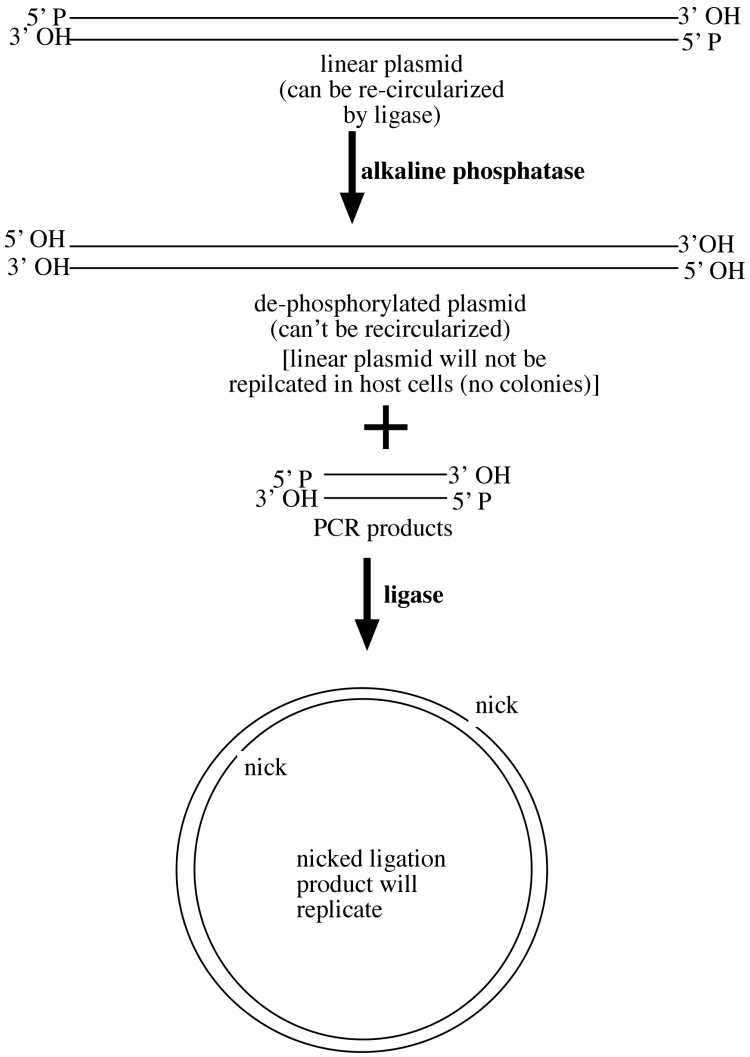
**A. Dephosphorylate the plasmid**

**B. Check our plasmid and PCR products on an agarose gel before we proceed.**

**C. Set up the ligation reaction.**

### A. Dephosphorylate Plasmid

The plasmid that we cut open at step 10.A with EcoRI will be the vector into which we ligate our PCR products. If we were to use the plasmid in its present form, the ligase could catalyze the simple recircularization of the plasmid without the introduction of the PCR fragment. We can prevent the recircularization of the vector by treating it with the enzyme alkaline phosphatase. This will remove the 5' terminal phosphate groups from the plasmid, and thus ligase cannot catalyze the closing of the plasmid. The PCR fragment that we are trying to ligate into the plasmid does have 5' phosphates that can be used to generate phosphodiester bonds. The resulting ligation product will have 2 nicks because only 2 of the possible 4 phosphodiester bonds can be made. This nicked plasmid, however, can be used to transform *E. coli*, which will quickly repair the nicks.




**Procedure:**

1. Thaw out the EcoRI digested plasmid from step 10.A.
2. Add the following to the tube:

    59 μl distilled water
    10 μl of 10X alkaline phosphatase buffer
    1 μl of calf intestinal alkaline phosphatase (1 unit/μl)
        incubate 30 min at 37° C

3. To stop the reaction, heat at 75° C for 15 minutes

4. Extract the sample 2 times with an equal volume of 1:1 phenol/chloroform (100 μl). Transfer aqueous (upper) phase to a new tube.

5. To the extracted aqueous phase, add 1 μl of Pellet Paint, 10 μl of 3 M sodium acetate, and 275 μl of ethanol to precipitate the DNA. Mix the tube well, and then set the tube in the freezer for 10 minutes. Centrifuge in the microfuge for 10 minutes on maximum speed. The purpose of adding the pellet paint is to improve the efficiency of the precipitation. The pellet paint will precipitate along with the DNA. Its presence in the DNA will not interfere with any of the subsequent steps (ligation, transformation).

6. At the end of the centrifugation there should be a small red pellet in the tube (if there isn't, worry). Remove the supernatant carefully, and wash the pellet with 70% ethanol. Allow the precipitated DNA to dry on your bench.

7. Resuspend the pellet in 30 μl of TE.

**B. Check our plasmid and PCR products on an agarose gel before we proceed.**

Before we set up the ligation reaction we are going to run a small agarose gel to examine the two pieces of DNA that we are going to ligate together. The purpose of this is to first make sure everything looks OK (e.g., was the plasmid digested to completion?), and from the staining of the DNA on the gel we can get a rough idea of the relative quantities of the two components.

**Procedure:**

1. While the DNA is being treated with the alkaline phosphatase prepare a 1% agarose gel (0.22 grams/22 ml 1X TAE), using an 8-slot comb.

2. To separate tubes add 1 μl of the PCR products (last seen at step 10.B), and 1 μl of the EcoRI cut and dephosphorylated plasmid (last seen at step 11.A) along with 8 μl of distilled water and 2 μl of 6X agarose gel sample buffer, mix. We also want to run a sample (0.2 μg) of uncut (circular) plasmid for comparison.

3. Load the samples onto the agarose gel. Load the molecular weight standards into an adjacent lane. Run and stain them as before. Take a Polaroid picture.

**C. Set up the ligation reaction**

You should now have the two DNA components ready for the ligation. We want to add the PCR fragment in approximately 3-fold excess over the plasmid to the ligation reaction. We started with 3 μg of the plasmid and ended up resuspending it in 30 μl of TE, so it should be about 100 ng/μl. The yield from the PCR will vary. We are only going to make an estimate of the amounts of each DNA by looking at the stained gel. Show the gel to the TA. When I did a pilot run on this experiment I did the following two reactions:

| positive reaction: | negative control reaction: |
|---|---|
| 9 μl distilled water | 15 μl distilled water |
| 2 μl ligase buffer | 2 μl ligase buffer |
| 2 μl cut plasmid | 2 μl cut plasmid |
| 6 μl PCR fragments | 1 μl ligase |
| 1 μl ligase | 20 μl |
| 20 μl | |

The reactions you set up will probably be the same, but you may need to make changes in the amount of plasmid or PCR fragments if your yields are significantly different (if you do make changes, be sure to adjust the amount of water to keep the volume at 20 μl). The purpose of the negative control reaction is to test the quality of our cut plasmid. If it was cut and dephosphorylated to completion it should not give any colonies on the ampicillin plates. Leave the ligation reaction at room temperature overnight.

**Materials:**

10X alkaline phosphatase buffer:
- 500 mM Tris-HCl, pH 9.3
- 10 mM $MgCl_2$
- 1 mM $ZnCl_2$
- 10 mM spermidine

alkaline phosphatase

20 mg/ml pellet paint

T4 ligase (20 units/μl)

10X ligase buffer:
- 300 mM Tris-HCl, pH 7.8
- 100 mM $MgCl_2$
- 100 mM DTT
- 5 mM ATP

**Step 12. Come in (preferably in the morning) and put your ligation reaction in the freezer).**

**Step 13. Transform ligations.**

Today will be very quick. We will transform the ligations into the prepared competent cells. You should have tested your competent cells, and if they were bad, you should have by this time re-made them. In plating the cells we are going to be doing two types of selection: first, by growing on ampicillin plates we will select only for transformed cells; second, we will grow the cells on plates that contain isopropyl thiogalactopyranoside (IPTG) and X-gal. IPTG is a non-metabolizable inducer of the lac operon, and X-gal is a chromogenic substrate of the *lac* locus gene product β-galactosidase. β-galactosidase has the special property that portions of the enzyme can be divided into separate polypeptides that will reform to make a functional enzyme. The N-terminal portion, called the alpha fragment, consists of the first 100 amino acids. Bluescript plasmids carry the gene for the alpha fragment (see appendix B). The multiple cloning site (MCS) of the plasmid (where EcoRI and a number of other restriction sites are found) is located inside the reading frame for the alpha fragment. The C-terminal portion of the enzyme is called the omega fragment and is provided by the ZΔM15 gene in the XL1-blue MRF' cells. Without the transfected plasmid, the XL1-blue MRF' cells cannot make a functional β-galactosidase. With the plasmid, the cells make functional β-galactosidase which will act on the X-gal to convert it from colorless to blue. However, if the plasmid has a piece of DNA ligated into the MCS this will usually disrupt the alpha fragment, and no functional β-galactosidase will be made. The result is that when we grow the cells on IPTG/X-gal plates we can distinguish between ampicillin resistant colonies having recircularized plasmid (blue colonies) and plasmids containing ligated fragments (white colonies).

**Procedure:**

1. Put your frozen ligation reactions in an ice bucket. Get one tube of your frozen competent cells from the freezer (do not let them warm up), and thaw them on ice for 5-10 minutes.

2. Add 10 μl of each of your two ligation reactions to 100 μl of competent cells. (Freeze the remaining 10 μl of each ligation reaction and hang on to it).

3. Incubate, heat shock, and spread the cells as before (step 6). The only difference is that we are going to use LB/ampicillin/IPTG/X-gal plates.

4. Label and put your plates at 37º C overnight. **Come in the next day before noon and put your plates in the refrigerator!**

**Materials:**

LB/ampicillin/X-gal/agar plates:

same as LB/ampicillin plates, but add to the cooled medium (no hotter than 60º C) X-gal to 40 μg/ml and IPTG to 0.5 mM.

## Step 14. Start overnight cultures of your recombinants

If everything went well you should see lots of white colonies (they may be light blue) on the plates from cells transformed with the positive ligation reaction, and just a few blue colonies (maybe some white also) on your plates from the negative control. The next step is to grow small cultures of several of the likely positive colonies (we will each grow up 5). The next day we will isolate plasmid DNA, cut the plasmids with restriction enzymes, and by agarose gel electrophoresis confirm that the plasmids contain ligated fragments of the predicted size.

**Procedure:**

1. Label 5 culture tubes pre-aliquoted with 3 ml of LB/ampicillin (100 μg/ml) 1 through 5 and your name or initials.
2. Pick a separate white (or light blue) colony using a toothpick from the positive plate into each culture tube.
3. Incubate the tubes with shaking at 37º C overnight.

**Step 15. Plasmid Minipreps.**

We now want to see which of the colonies you picked yesterday contain ligated DNAs of the appropriate size. Strange things can happen during ligation and plasmid propagation, so even though a colony was white it may not have an insert. The procedure we are going to use to isolate the plasmids is referred to as a "miniprep".

**Procedure:**

1. Transfer 1.5 ml of your overnight cultures to 5 appropriately labeled microfuge tubes. **Hang on to the remaining 1.5 ml of the culture, label it clearly, and store it in the refrigerator!**

2. Spin the tubes for 30 seconds to pellet the cells. Remove all of the medium, leaving the cell pellet intact.

3. Ensure that RNase A has been added to Buffer P1 (probably Tris, EDTA & sucrose). Resuspend the cell pellet in 250 μl Buffer P1. To achieve good yield it is essential that this step be thorough. After adding Buffer P1/resuspension solution continue to vortex until suspension is absolutely homogeneous. Hold the tube up to the light to check that there are no clumps of bacteria left.

4. Add 250 μl Buffer P2 (probably NaOH & SDS). Mix gently by inverting the tube 4-6 times (do not vortex as this will result in shearing of genomic DNA). The solution should become viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 minutes.

5. Add 350 μl of Buffer N3 (probably KOAc). Mix immediately and thoroughly by inverting the tube 4-6 times. Notice that the KOAc tends to go to the bottom of the tube, and because of the viscosity of the DNA mixing must be deliberate and thorough. Mix gently until the solution is homogeneous and has lost all traces of viscosity. Don't rush this step.

6. Centrifuge for 10 min. A compact white pellet will form. This is the genomic DNA and proteins bound by SDS and KOAc.

7. To bind the plasmid DNA to the column, apply the supernatants from step 6 to the QIAprep spin column by decanting or pipetting.

8. Centrifuge for 30-60 sec. Discard the flow-through.

9. Wash the QIAprep spin column by adding 750 μl Buffer PE and centrifuging for 30-60 sec. Discard the flow-through. Buffer PE contains ethanol.

10. Centrifuge for an additional 1 min to remove residual wash buffer/ethanol.

11. Place the QIAprep column in a **clean** 1.5 mL microcentrifuge tube. To elute DNA, add 50 μL Buffer EB (10 mM TrisCl, pH 8.5) or water to the center of each QIAprep spin column (do not touch the membrane/do not poke through the membrane), let stand for 1 min, and centrifuge for 1 min.

12. Place tube with plasmid DNA into freezer. Discard QIAprep column.

**Step 16. Analysis of plasmid minipreps**

In the previous step we prepared our plasmid minipreps. They are now ready for restriction enzyme digestion. Look at the map of bluescript SK+ (appendix B). Our PCR fragments should be ligated in at the EcoRI site. To cut our fragments back out of the plasmid, we will use two restriction sites that lie on either side of the EcoRI site. More-or-less at random I've chosen HindIII and BamHI.

**Procedure:**

1. For each of the plasmid minipreps, set up the following reactions in new tubes:

    14 μl distilled water
    2 μl plasmid DNA
    2 μl 10X HindIII/BamHI restriction enzyme buffer
    1 μl HindIII
    1 μl BamHI

    Make sure the components are mixed well and that all of the solution is at the bottom of the tube (if not, centrifuge them very briefly). Incubate for 1.5 hours at 37° C.

2. While the plasmids are incubating, pour a 25 ml 2% agarose/TAE gel. Use the 13 well comb.

3. At the end of the incubation add 4.0 μl of 6X agarose gel sample buffer to each of the tubes, mix. Load 10 μl per lane.

4. Run samples on the agarose gel along with molecular weight markers. Take a photograph of gel. If things worked correctly there should be a band of approximately 400 bp in some (or even all) of the clones you picked. It is also conceivable that some of the PCR clones could have internal HindIII and/or BamHI sites. In this case, multiple insert bands of lower molecular weight will be present.

**Materials:**

<u>10X HindIII/BamHI restriction enzyme buffer</u>:

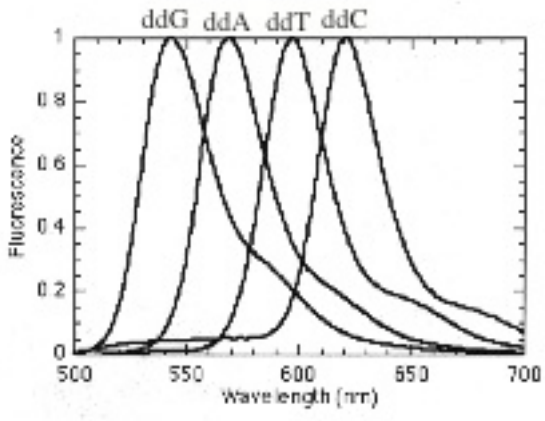
| | |
|---|---|
| 60 mM Tris-HCl, pH 7.5 | 1 M NaCl |
| 60 mM $MgCl_2$ | <u>BamHI</u> (10 units/μl) |
| 10 mM DTT | <u>HindIII</u> (10 units/μl) |

**Step 17. Set up sequencing reactions.**

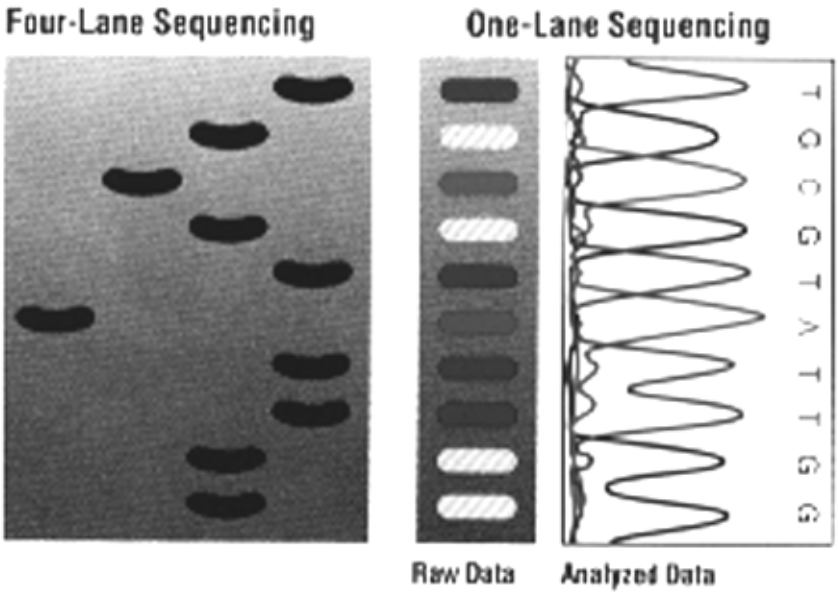
Now we are ready to determine the nucleotide sequence of the cloned DNA fragment. You've probably learned about both the Maxam-Gilbert and dideoxy (Sanger) methods of sequencing. I recommend that you re-read the section in your Genetics text that covers DNA sequencing (if you have Genetic Analysis by Griffiths, see pages 444-448).

Maxam-Gilbert sequencing is almost never done any more. Using radioactivity to label the sequencing products requires that you perform 4 separate sequencing reactions (one for each dideoxy nucleotide), and then electrophorese a sample of each reaction on a separate lane of a polyacrylamide gel. The whole process is very messy.

By using fluorescent dye-labeled dideoxy chain terminators (Sanger method) we are able to do the entire sequencing reaction in one tube. Each dideoxy nucleotide is coupled to a different fluorescent dye with its own absorbance maximum:




The sequencing reaction products that we generate today will be run on a capillary sequencing analyzer. In the Analyzer the sequencing products are separated according to their size on a matrix-filled capillary by electrophoresis. Remember that the dideoxy sequencing reaction generates a series of differently sized DNA molecules that have terminated because of the incorporation of a dideoxy nucleotide. As the sequencing reaction proceeds, products emerge from the capillary, the Analyzer scans the four wavelengths of the fluorescent dyes to determine which of the terminator was incorporated, and thus determines the sequence of the template DNA.




**Procedure:**

You are now ready to prepare your plasmid for sequencing. We are sending your DNA for sequencing to Eton Biosciences Inc. Determine your DNA concentration using the nano drop machine.

1. In a PCR tube for each of your templates, set up the following:

   600 ng of plasmid DNA template in 6 μl
   We are sending enough DNA for 2 reactions
   Therefore you need 1.2 μg DNA in **13 μl**
   The primers are supplied by the company

2. Mix well by pipetting up and down with your Pipetman.

3. Centrifuge tube briefly to collect everything at the bottom of the tube.

4. Place tubes in the tube rack at front.

**Materials:**

**Terminator Ready Reaction Mix** - this is sold as a "just add and mix" sequencing cocktail. It contains everything needed for the sequencing reaction except the template and the primer. It has all four dNTPs (dATP, dCTP, dTTP, dGTP), all four dye-labeled

ddNTP terminators, a modified form of Taq DNA polymerase, and various salts and pH buffers to make the polymerase function.

**Primer** - Like all DNA polymerases, Taq needs to be primed. In other words, it can only elongate the template from an existing 3' hydroxyl. To provide a primer for our sequencing reaction we will anneal a short (17-mer) oligonucleotide to the plasmid. Although we don't yet know the sequence of the inserted PCR fragment, the sequence of the entire plasmid is known. We can thus use one of the existing commercially available sequencing primers that are located just outside of the MCS of bluescript (see appendix B). The primers are for historical reasons called the M13 forward and reverse primers. These primers will prime the chain elongation inwards from either end of the MCS. If we wanted to sequence the other strand, we would have to either reclone the fragment into a plasmid with an inverted f1 origin (like bluescript SK-, which will give us the opposite strand in a single stranded preparation), or do "double stranded" sequencing.

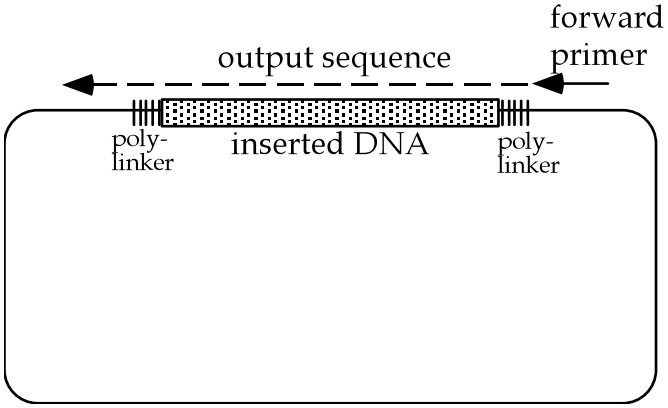
## 18. Catch Up Day (Doing Primer design)

It will take a while to run everyone's sequences on the sequencing machine. The next step is to meet in the Life Science Computing Facility (LSCF) to computer analyze our sequences. We want to ensure that everyone has a sequence to work with when we meet at the LSCF. Please come to class this day; we may or may not continue on to the next step.

## 19. Computer analysis of DNA sequence.

Hopefully your plasmid minipreps have worked well for sequencing. The raw sequence that we will get back from the ABI PRISM310 Genetic Analyzer will just be a string of ATCGGCTTAGGCA....., and so on. It is very hard to get much information out of the raw sequence data by just looking at it. We need to use computers to analyze our sequences. We are going to use a software package called Chromas to analyze our nucleotide sequences. We are going to be using the Chromas Lite program.

The goal of the computer analysis is to take your unknown sequence and have the computer compare it to a database of all known sequences to determine if it is a previously identified gene. Because we know that the region of the genes that we are analyzing encodes a protein, we are going to search the database not with the nucleotide sequence, but with a computer generated translation of the sequence. If your unknown has been previously sequenced, the computer will tell you the name of the gene plus some other information. If your unknown has not been previously sequenced, the computer will tell you to which gene your unknown is most similar. Before we can do much analysis with your sequences we need to figure out a few things. The sequencing primer we used (the reverse primer, see appendix B) primes outside of the polylinker. Thus, the first part of the raw sequence won't be your unknown - it will be plasmid. In addition, since our unknown inserts are around 400 bp, the very end of the sequence probably will run off the unknown, and back into the plasmid. We need to distinguish what part of the raw sequence is your unknown and what part is plasmid. We only want to compare your unknown to the database.

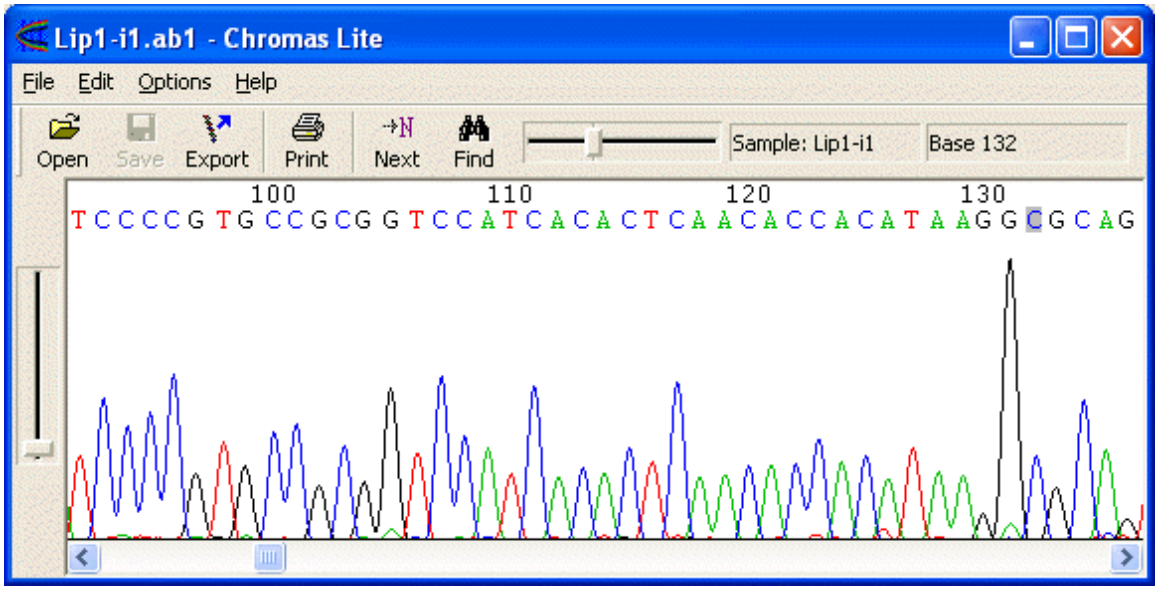


**Step 0 - Getting onto Chromas.**

Open Chromas Lite version
Open your sequence file.

**Step 1 - Fixing sequence ambiguities.**

Sometimes the ABI sequencer is not able to accurately determine the fluorescent signal that is passing by the detector for particular sequence positions. If the machine cannot determine a base it will insert an **N** into the sequence output. The **N** means that the position is ambiguous. By looking at the **sequence traces** we can often make a good guess. Ambiguities are more likely to be found near the ends (500 bases and further) of the sequence rather than at the beginning.




The file will open with a series of blue, black, green and red peaks which each correspond to one base.

**C= Blue**
**G= Black**
**A= Green**
**T= Red**

An unknown base is indicated by a **pink N**

A high narrow peak indicates a good read; there is no error in base calling

A broader, shorter peak can result in base calling errors.

Look through your sequence and find the Ns, examine the peak(s) beneath the N to determine what base it should be.

Click the "N" along the top of the view box to highlight it (no need to delete it), now type the letter of the base it should be.

Go through your sequence and change as many of the Ns as you can.
The beginning and end of the sequence are usually too messy to call bases. By the end you should have around 800bps of good sequence.
Once you have cleaned the sequence it is time to go on to the next step.

**Step 2- Deleting Plasmid Sequences**

Now it is time to remove the vector sequence. When we sequenced the insert, the reaction sequenced past the insert and sequenced some of the plasmid. We do not want this sequence in the next step so we need to remove it.

First go to "Edit" then to "copy sequence" then "plain text"
Now open a web browser and go to: http://tools.neb.com/NEBcutter2/index.php
This is a program from NEB which will scan your sequence for all known cut sites.
In our case we want to see where our EcoRI sites are, because everything between them is our sequence, and everything outside of them is the vector sequence.

"Paste" your sequence into the box and click "Submit"
If it tells you there are too many Ns do not worry, it is just the beginning and end of the sequence, so click "Continue".

Your "cut" sequence will load with all known cut sites shown. Look through all of the enzyme names until you see "EcoRI" mouse over but do NOT click (If you click on the name of the enzyme you will be sent to an informational page for that enzyme) the name of the enzyme and a box will pop up with the enzyme name, cut sequence, base pair cut

site and what is left at the end of the DNA after cutting. Look in that box and write down the base pair number where EcoRI cut. There should be two sites.

**Print this page. Click "print", select "Map Only" as display mode, format is PDF (this needs to be included in the write up).**

Go back to Chromas, find the first EcoRI cut site, by going to the right base pair number, click the letter G of the first cut site and hit "backspace" to delete all the sequence before the "G".
Repeat this by going to the VERY END of your sequence and deleting all the sequence back to the "C".

Now all that should be left is the primer sequences and the sequence of your insert. We need to remove the primer sequences as well, so delete the first and last 25 base pairs from your sequence.
(We remove the primer sequence as well because although it did match the base pairs in the template DNA we designed the primers, so any changes in the template DNA will be masked)

Now you have just the sequence of your insert.
**Print this out as a pdf (this needs to be included in the write up).**
Now it is time to analyze it using a database search.

**Step 3 - Database searching**.

The National Center for Biotechnology Information (NCBI) at the NIH in Bethesda, MD compiles all published protein and nucleic acid sequences information on a high speed computer. We are going to use a network connection to send our unknown sequence to this computer and have it compared to the known sequences.

There are two ways we can search the data base. The first is to compare our nucleic acid sequence to the database. The second is to compare the hypothetical translations from all six reading frames of our sequences. All of the nucleic acid sequences in the data base that encode proteins are also present in the data base as their

amino acid translations. If you know that your unknown sequence is in a coding region (as our's is), it is often more fruitful to search the data base with the amino acid translations (why might this be?).

To search the NCBI data base we will use a program called BLAST, which stands for Basic Local Alignment Search Tool. BLAST is an algorithm for comparing primary biological sequence information, such as the amino-acid sequences of different proteins or the nucleotides of DNA sequences. A BLAST search enables a researcher to compare a query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold. For example, following the discovery of a previously unknown gene in the mouse, a scientist will typically perform a BLAST search of the human genome to see if humans carry a similar gene; BLAST will identify sequences in the human genome that resemble the mouse gene based on similarity of sequence.

BLAST requires two sequences as input: a query sequence (also called the target sequence) and a sequence database. BLAST will find subsequences in the query that are similar to subsequences in the database. In typical usage, the query sequence is much smaller than the database, e.g., the query may be one thousand nucleotides while the database is several billion nucleotides.

BLAST searches for high scoring sequence alignments between the query sequence and sequences in the database using a heuristic approach that approximates the Smith-Waterman algorithm (the Smith-Waterman algorithm compares segments of all possible lengths and optimizes the similarity measure). The exhaustive Smith-Waterman approach is too slow for searching large genomic databases such as GenBank. Therefore, the BLAST algorithm uses a heuristic approach that is slightly less accurate than Smith-Waterman but over 50 times faster. The speed and relatively good accuracy of BLAST are the key technical innovation of the BLAST programs and arguably why the tool is the most popular bioinformatics search tool.

The BLAST algorithm can be conceptually divided into three stages.

1. In the first stage, BLAST searches for exact matches of a small fixed length W between the query and sequences in the database. For example, given the sequences AGTTAC and ACTTAG and a word length W = 3, BLAST would identify the matching substring TTA that is common to both sequences. By default, W = 11 for nucleic seeds.
2. In the second stage, BLAST tries to extend the match in both directions, starting at the seed. The ungapped alignment process extends the initial seed match of length W in

each direction in an attempt to boost the alignment score. Insertions and deletions are not considered during this stage. For our example, the ungapped alignment between the sequences AGTTAC and ACTTAG centered around the common word TTA would be:

```
..AGTTAC..
  |  |||
..ACTTAG..
```

If a high-scoring un-gapped alignment is found, the database sequence is passed on to the third stage.

3. In the third stage, BLAST performs a gapped alignment between the query sequence and the database sequence using a variation of the Smith-Waterman algorithm. Statistically significant alignments are then displayed to the user.

**Procedure:**

1. To perform the BLAST search, open a web browser and go to: "http://www.ncbi.nlm.nih.gov/"

2. On NCBI's homepage click on BLAST on the right.

3. Once the new page opens click on "blastx". "blastx" will translate your DNA sequence into amino acids using all six reading frames. It will then compare all six potential proteins to all the proteins present in the data base. We are comparing the amino acid sequence because proteins usually change less often than DNA and we know our sequence is expressed (remember we isolated it from RNA originally).

4. Paste your sequence into the box. Make sure that it is a "standard genetic code selected, and that you are looking in non-redundant sequences.

5. Click "Blast"

6. The program will return results. The top box is color coded matches showing how well and to what part of your sequence was matched. Scroll past this to the list of names and numbers. This shows you the summary of best alignments, what percentage of the two sequences overlapped, the E value and more. Further down the page are actual alignments showing you how and where the two amino acids lined up.

**7. Print the 4 best alignments to pdf; do NOT print all of them (this needs to be included in the write up).**

## 20. Computer Analysis of Sequences and Primer Design Lab

In order for you to gain confidence and experience in your new-found computer analysis skills, you will now be given the opportunity to design your own primers in order to hypothetically clone a gene using the NCBI database.

You will practice cloning the mouse STAT6 gene.
The **Signal Transducers and Activator of Transcription (STAT, also, called signal transduction and transcription) protein**s regulate many aspects of cell growth, survival and differentiation. The transcription factors of this family are activated by the Janus Kinase (JAK) and dysregulation of this pathway is frequently observed in primary tumors and leads to increased angiogenesis, enhanced survival of tumors and immuno-suppression. Knockout studies have provided evidence that STAT proteins are involved in the development and function of the immune system and play a role in maintaining immune tolerance and tumor surveillance.

The accession number for the mouse STAT6 mRNA is NM_009284
You will find this nucleotide sequence using the NCBI database and design primers to clone the coding sequence (CDS) of the mRNA into the MCS of the pBluescript II SK (+) plasmid, using only **TWO** out of three restriction enzymes, XbaI, PstI or EcoRI.

You must design two primers:

Forward primer – must contain a restriction enzyme cut site and ~18-20 bp including the ATG start codon at the beginning of the coding sequence.

Reverse primer – must contain a restriction enzyme cut site and ~18-20 bp including the stop codon at the end of the coding sequence.

You will be cloning this sequence into the plasmid **DIRECTIONALLY**, therefore you must determine where the promotor is on the plasmid and make sure that your forward primer/start of coding sequence is closest to the promotor once it is ligated into the plasmid. Use the *lac* promoter (P lac), NOT the T7 or T3 promoter (see appendix B).

Your TA will be available for consultation, but you must all try to design the primers as best you can. Use this website to help you select your restriction enzymes
http://tools.neb.com/NEBcutter2/

## 21. RNA gels/northern blot.

In the final experiment we will look at gene expression by northern blotting. Each cell type transcribes (expresses) a unique set of genes from its genome. This is what makes cells different from each other. The set of transcribed genes is being constantly modified as the cell (or organism that the cell is located in) responds to changing conditions. Whether in a single-celled prokaryote, or a complex animal like a mammal, it is the regulation of the gene expression that determines the behavior of cells. When certain genes are transcribed, cells can adapt to changing environments, differentiate into defined cells types, or even become cancerous. By determining what genes are being transcribed, and at what level, we can learn a lot about what is going on inside cells. Here is a typical experiment: let's say a pharmaceutical company has a new drug they suspect stimulates the transcription of the antiviral glycoprotein interferon. They would grow lymphocyte cells on culture plates. Some plates would get the drug and others would get a negative control (an inactive compound). After a reasonable wait to allow the drug to take effect (could be hours or days), they would test to see if the cells had the mRNA for interferon.

We can determine whether a gene is being transcribed by looking for the particular transcript (i.e., the mRNA) from that gene. There are a number of ways to assay for the presence of RNAs, they all have their own unique applications, and no one method is right for all situations. The most common methods are:

1. northern blotting
2. RNAse protection assay
3. RT-PCR or qRT-PCR
4. in situ hybridization

We will be doing this first technique (northern blotting) in class. The first step for all of these procedures is to isolate RNA from the experimental subject from which you want to assay gene expression. This could be bacterial cells grown under different conditions, cultured mammalian cells treated with different drugs, plant tissues from different stages of development, or just about any living tissue or cell in which you are interested. One has to start with a specific question: how will the expression of a particular gene change in response to a given set of conditions?

The procedure for isolating the RNA depends upon the particular cell or tissue type one is starting with. Working with RNA is typically much more difficult than

working with DNA because RNA is more labile due to the presence of ribonucleases just about everywhere, including the secretions from your hands. Because we are getting near the end of the quarter, and time is running out, we will not be isolating RNA. Instead you will be provided with two RNA samples. We will be looking at how the expression of the **muscle actin** gene changes during development of the sea squirt *Ciona savignyi*. One sample will be from *Ciona* eggs. The other will be from *Ciona* larva at tailbud stage. In the *Ciona* eggs, there are no differentiated muscles, so we expect to detect no muscle actin mRNA. By tailbud stage the muscles have formed so we should detect high levels of the muscle actin gene transcript.

The basic procedure for Northern blotting is:

1. Isolate RNA. The isolated RNA will be a complex mixture of rRNA, mRNA and tRNA. The particular mRNA that we want to detect (muscle actin) is only a minute fraction of this RNA mixture. The *Ciona* RNA samples you will be given contains ≈60,000 different mRNAs (each encoding a different protein), in addition to all of the tRNAs and rRNAs.

2. Separate the RNA species by molecular weight on an agarose gel.

3. Transfer the RNA from the gel to a nylon filter

4. Incubate the filter in a solution containing a labeled DNA probe that is complementary to (i.e., will base pair with) the RNA that you wish to detect.

5. Detect the presence of the label DNA probe.

**Procedure:**

Today we will run the RNA sample on the formaldehyde agarose gel and start the transfer procedure. We need to keep the single-stranded RNA free of secondary structures such as hairpins, as these will affect the mobility of the RNA through the gel. We will denature the RNA first.

1. Make a 1.2% formaldehyde agarose gel as follows: (this can be done ahead of class)

   0.6 grams agarose
   5 ml 10x formaldehyde agarose gel buffer
   RNase-free water to 50 ml

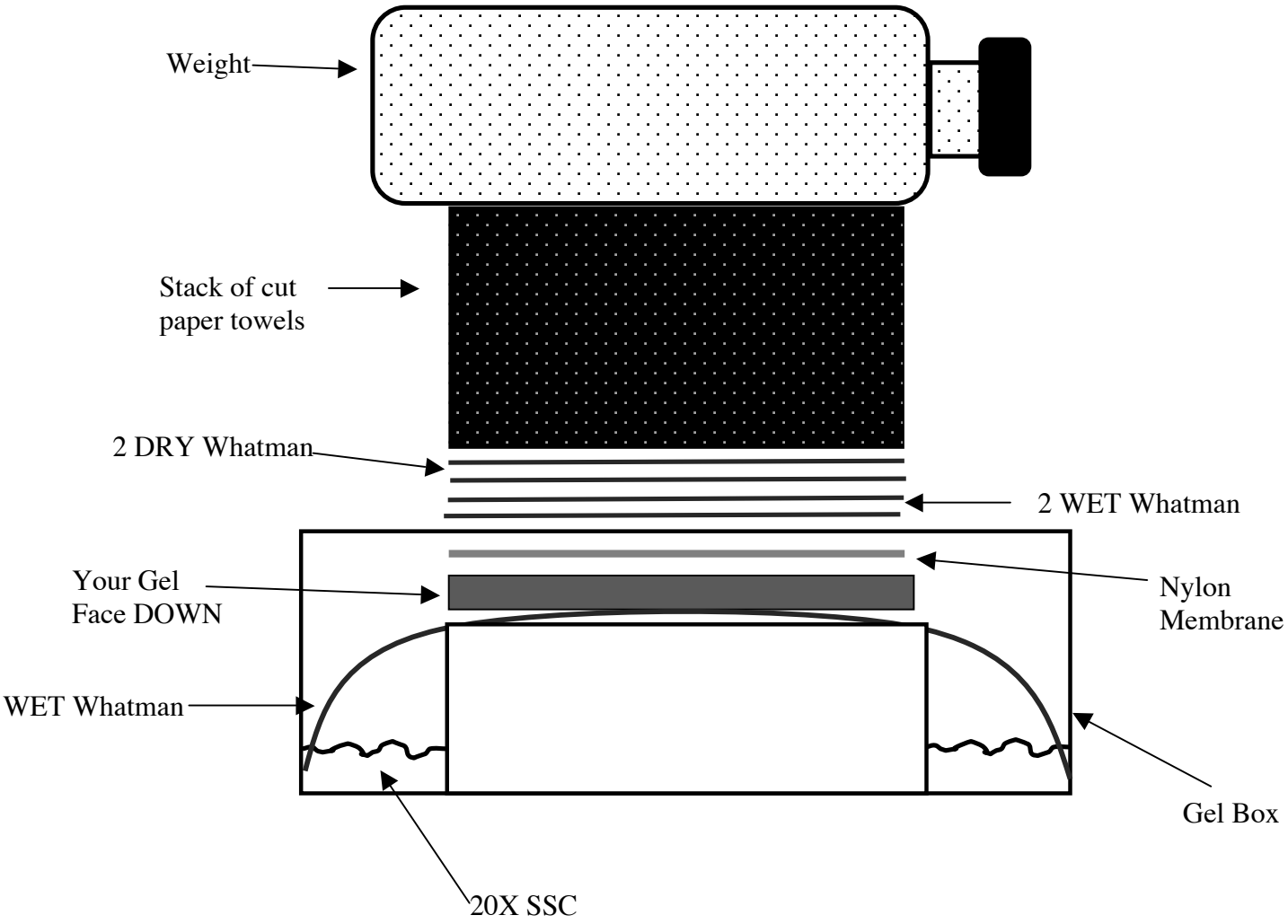
Heat the mixture to melt agarose. Cool to 65°C in a water bath. Add 0.9 ml of 37% (12.3 M) formaldehyde *(TOXIC!)*. Mix thoroughly and pour onto gel support. Prior to running the gel, equilibrate in 1x formaldehyde agarose gel running buffer for at least 30 min.

2. You will be given two *Ciona* RNA samples (unfertilized egg and tail bud larva). Dilute the samples with 5x RNA loading buffer.

3. Heat the samples at 65°C for 5 min then chill immediately on ice. Load the entire 20 μl sample QUICKLY on the gel. Run the gel in 1x formaldehyde agarose gel running buffer. The samples tend to look better if the gel is initially run at low voltage (30 volts) for the first 1/2 to 1 hour. Then turn the gel up to 100 volts. Transfer the RNA overnight to a filter by capillary action as described below:

4. **WHILE** the gel is running, cut a piece of nylon (Hybond-N) filter the size of the gel. Always wear gloves when handling nylon or nitrocellulose filters. The TA will show you how to set up the blot. Briefly, use a light touch with a razor blade, do not contaminate the filter with your fingers, **label the filter** with a ball point pen, and wet the nylon in distilled $H_2O$. Transfer the filter to **20X SSC**. The nylon filter is expensive, so be careful. **WHILE** your gel is running also start cutting paper towels and the Whatman 3MM filter paper to the correct size.

5. When your gel run is done, turn the gel over and place it gently on the Whatman paper in the transfer apparatus. **Place strips of Parafilm framing the gel** to insure that the buffer in the dish will wick up only through the gel. Flood the surface of the gel with 20X SSC. Gently place your cut and labeled filter on the gel and smooth it out, thereby removing both bubbles and excess buffer. **MAKE SURE YOU KNOW (AND HAVE MARKED) THE TOP FROM THE BOTTOM OF THE NYLON.** Check carefully that bubbles have been removed. Place two pieces of Whatman 3MM, which have been wet in 20X SSC, on top of the nylon, taking care not to disturb the nylon. Follow this with two pieces of dry Whatman 3MM. Add a 2 inch stack of paper towels (cut to size). Add a glass bottle filled with sand, TAPE DOWN. Leave on bench overnight.




**Materials:**

10x formaldehyde agarose gel buffer:
200 mM MOPS
50 mM sodium acetate
10 mM EDTA
pH to 7.0 with NaOH

1x formaldehyde agarose gel running buffer:
100 ml 10x formaldehyde agarose gel buffer
20 ml 37% (12.3 M) formaldehyde *(TOXIC!)*
880 ml RNase-free water

5x RNA loading buffer:
16 ul saturated aqueous bromophenol blue solution
80 ul 500 mM EDTA, pH 8.0
720 ul 37% (12.3M) formaldehyde *(TOXIC!)*
2 ml 100% glycerol
3084 ul formamide
4 ml 10x formaldehyde agarose gel buffer
RNase-free water to 10 ml
Stable for approximately 3 months at 4°C

20X SSC:
3 M NaCl
0.3 M $Na_3$Citrate, pH 7.0

Hybond-N nylon filter

Whatman paper

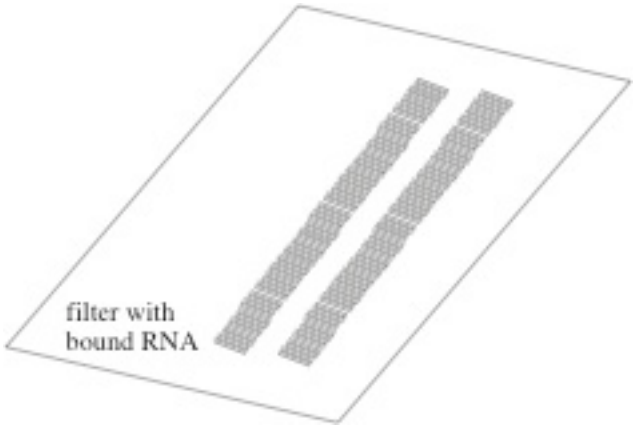
**22. Take down the blot.**

The RNA should now be transferred to the filter. Before we can do the hybridization we need to covalently bind the RNA to the filter. This is done with UV light in a machine called a Stratalinker.

1. In the morning remove the filter from the transfer apparatus, make sure to note which side of the filter was facing the gel. **Clip the top right corner**, so that later you can orient the filter. Place the filter **RNA-side up** in the Stratalinker. Set the Stratalinker to "auto cross link" and push start.
2. Wrap the blot in Saran wrap, label it and put it in the refrigerator.
3. Place gel box at front; lab coordinator will wash to remove all salt (waste must be collected and disposed of properly because of formaldehyde).

**23. Detection of muscle actin mRNA on the filter:**

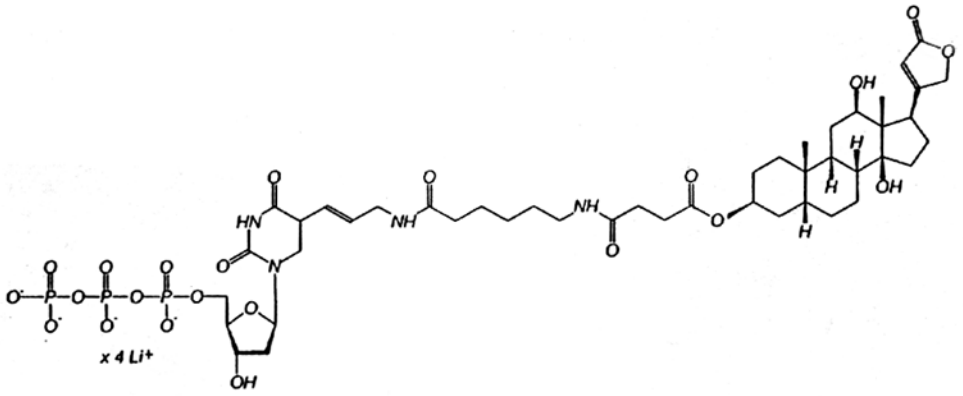
On our nylon filters we have the tens of thousands of RNA species spread out according to their molecular weight. One of these species is muscle actin (but don't get confused, there is not one muscle actin mRNA molecule on the filter - there are thousands of copies of each species of mRNA on the filter).



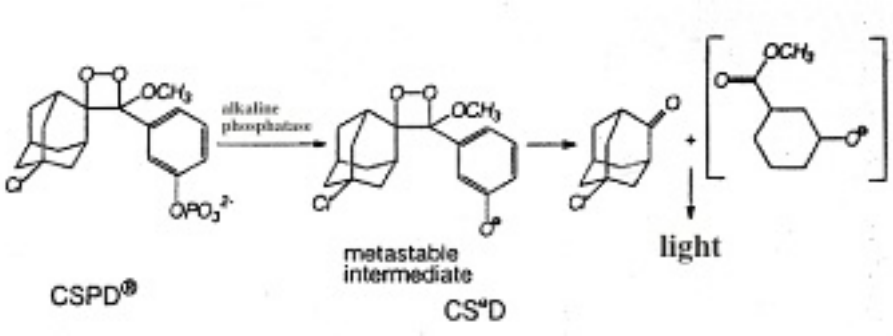

We want to specifically detect muscle actin mRNA in this complex mixture. To do this we will take advantage of the high specificity of base pairing. We will incubate the filter with a DNA probe that is complementary to the muscle actin mRNA sequence. The probe will hybridize only to the muscle actin mRNA. We can then assay for the presence of the DNA probe in order to detect the presence of the muscle actin mRNA on the filter.

There are several techniques for making DNA probes. The oldest and probably still the most common method is to make radioactive DNA probes by incorporating the radioactive isotope $^{32}P$. Although the amount of radioactivity used in such experiments is too small to pose a health risk, the use of radioactive probes presents many problems. The half-life of $^{32}P$ is only 14.3 days which means that $^{32}P$-containing probes have a short shelf-life. In addition, using radioactivity generates expensive waste products. Therefore, we will be using a more modern non-radioactive procedure. The way the probe works is a little complicated (as you will see below), but in principle we want our DNA probe to generate a strong enough signal to allow us to detect very small amounts of mRNA. [That is why radioactive probes work so well; an autoradiograph can be used to detect very small amounts of the probe by the decay of the $^{32}P$.]

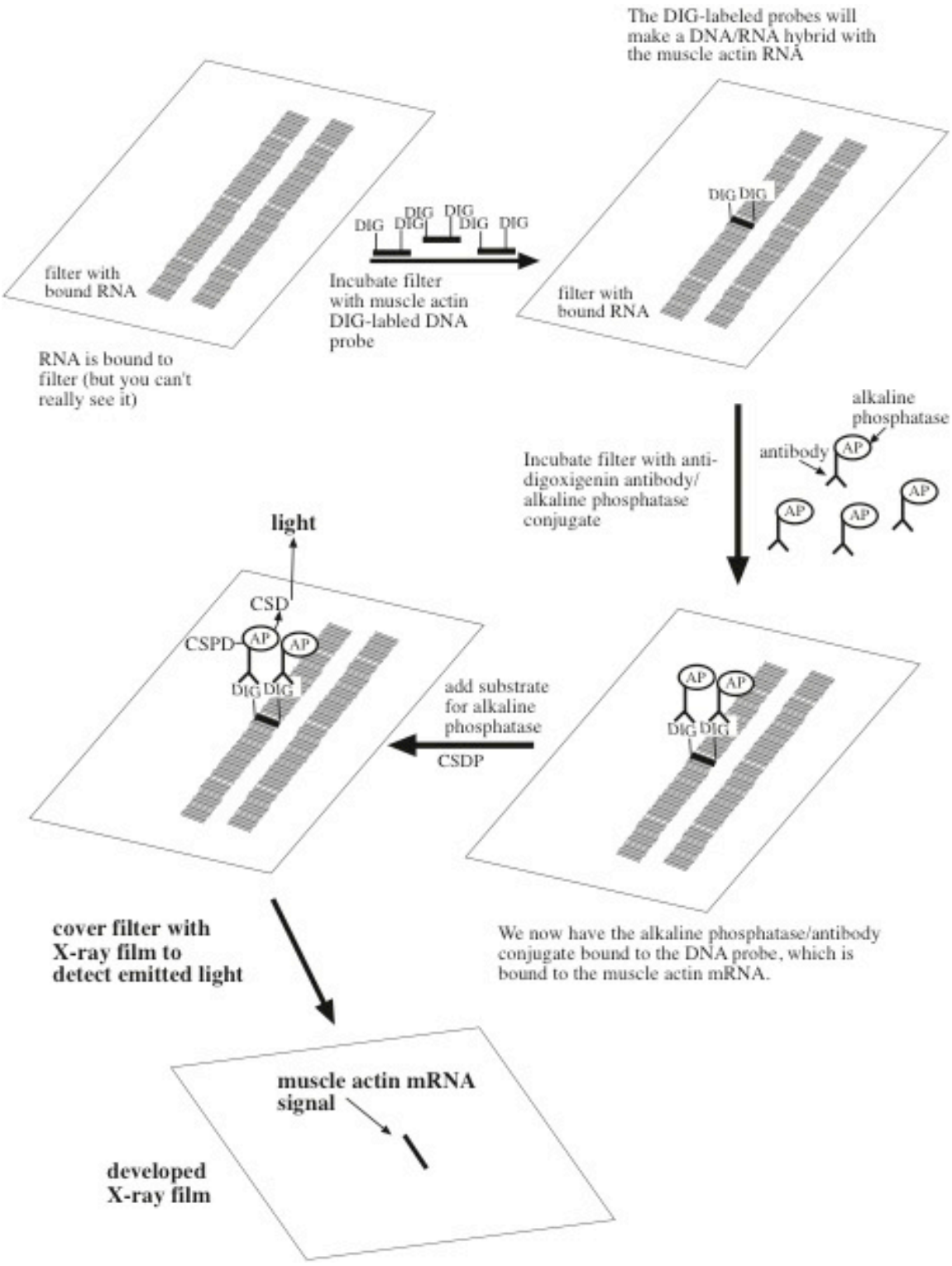
Here is how the non-radioactive method works. The actin DNA probe has a modified base incorporated into it. This base is digoxigenin-11-dUTP (shown here):




Believe it or not, DNA polymerase is able to recognize this modified base and incorporate it into DNA in place of dTTP. Our DNA probe thus has a string of digoxigenin (abbreviated DIG) molecules hanging off of it. Digoxigenin is actually a product of the plant *digitalis* (but that's not important). The important thing is that digoxigenin can be incorporated into DNA and it is very antigenic in animals. This means that antibodies can be made against digoxigenin that bind to it with very high affinity. Thus we can use an antibody against digoxigenin to detect the presence of the DNA probe. However, an antibody by itself does not give off a signal that we can detect. We will use a conjugate between the antibody and the enzyme alkaline phosphatase. The presence of the alkaline phosphatase can be detected by giving it a substrate that is converted into a light emitting product:




Because alkaline phosphatase is an enzyme, it can catalyze this reaction over and over again. The result is an amplification of our signal. The figure below puts all of these steps together and shows how this method is used to detect specific mRNAs on the filter:




**Procedure:**

The detection procedure is very long, so we're going to divide it between two class sections. Today we will hybridize our DIG-containing DNA probe to the muscle

actin mRNA on the filter. The next class meeting we will bind the antibody onto the DIG probe and perform the detection reaction.

1. Retrieve your filter from the refrigerator and unwrap. Wear gloves.

2. Wash filter for **1 minute** in a dish of distilled water at RT with shaking.

3. Slide filter, **RNA-side up**, into hybridization bottle.

4. Add 2 ml of QuickHyb solution to bottle and screw top on well.

5. Rotate the bottle in the hybridization oven for **5 minutes** at 68$^{o}$.

6. We will boil the double stranded DIG probe to separate the strands. **Do not** start this step until you are finished with step 5. Add 5 ul of sonicated salmon sperm DNA (10 mg/ml) to probe. Using the special holder, place the tube with the DIG-labeled muscle actin probe with salmon sperm DNA in a boiling water bath for **5 minutes,** and then immediately plunge the tube into ice water. Keep on ice.

7. Take your hybridization bottle out of the oven. Remove 1/2 ml of the QuickHyb solution from the bottle with a Pipetman and add it to the tube of the probe (step 6). Pipet up and down to mix, and then transfer the probe/QuickHyb back to the bottle.

8. Return the bottle to the hybridization oven and rotate for **45 minutes** at 68$^{o}$ C.

9. Remove filter from bottle, let as much QuickHyb drain from the filter as possible. Wash the bottle for reuse in step 12.

10. Put the filter in a dish with 50 ml of 0.1X SSC, 0.1% SDS at room temperature. Put on shaking platform for 5 minutes.

11. Pour off the 0.1X SSC, 0.1% SDS, and add a fresh 50 ml of 0.1X SSC, 0.1% SDS. Shake again for 5 minutes.

12. Put the filter back into the hybridization bottle and add 50 ml of 60ºC 0.1X SSC, 0.1% SDS. Rotate for 5 minutes at 60º C.

13. After this final wash remove filter from bottle and wrap well in Saran wrap. Make sure the filter will not dry out. Label with your initials and store in the refrigerator. Wash the bottle and return to the front bench.

**Step 24 - northern blot continued (immunological detection)**

**Procedure:**

1. Retrieve your filter from the refrigerator. Wash filter for 5 minutes in 50 ml washing buffer at room temperature with gentle shaking. While filter is washing, dilute 10x blocking solution in maleic acid buffer. Make 40 mls.

2. Incubate filter in 20 ml of diluted blocking solution at room temperature with gentle shaking for 30 minutes. While filter is blocking, dilute antibody 1:10,000 into diluted blocking solution. Make 20 mls. Check antibody amount with TA before adding.

3. Incubate filter in 20 ml of antibody solution at room temperature with gentle shaking for 30 minutes.

4. Wash filter 2 times for 15 minutes each with 50 ml washing buffer at room temperature with gentle shaking.

5. Incubate filter in 20 ml of detection buffer for 5 minutes at room temperature with gentle shaking.

6. Bring your filter to the front and place in the prepared autoradiograph cassette. The TA will image all the gels together after class using the following steps: add CSPD to filters and incubate for 5 minutes at room temperature; expose filters to x-ray film for 15-25 minutes at room temperature; develop film.

**Materials:**

Washing buffer:
0.1 M maleic acid, pH7.5
0.15 M NaCl
0.3% (v/v) Tween20

Detection buffer:
0.1 M Tris, pH 9.5
0.1 M NaCl

Maleic acid buffer:
0.1 M maleic acid (pH7.5)
0.15 M NaCl

Antibody solution:
diluted blocking solution
anti-DIG-AP conjugate

CSPD -light emitting substrate for alkaline phosphatase

## Appendix A. Calculations/Solution Making in Molecular Biology

**1. Units of measurement.**

In general the volumes and weights used in molecular biology are very small.

units of volume:

| | | |
|---|---|---|
| liter | l | |
| milliliter | ml | $10^{-3}$ l |
| microliter | μl | $10^{-6}$ l [often this unit is abbreviated as a "λ" (lambda)] |
| nanoliter | nl | $10^{-9}$ l |

units of mass:

| | | |
|---|---|---|
| gram | g | |
| milligram | mg | $10^{-3}$ g |
| microgram | μg | $10^{-6}$ g [often this unit is abbreviated as a "γ" (gamma)] |
| nanogram | ng | $10^{-9}$ g |
| picogram | pg | $10^{-12}$ g |

Examples. It is important to be able to quickly change from one unit of measurement to another. These examples will give you some practice.

a. You have a tube with 650 μl of water. If you add another 650 μl of water, how many ml will you have?

*650 μl + 650 μl = 1,300 μl*
*(1 ml = 1000μl )*
*1,300μl = 1.3 ml*

b. To test for the incorporation of an antibiotic resistance gene into a mouse cell line, you need to grow the cells in the presence of 100 μg/ml neomycin. The cells are currently growing in 10 ml of culture medium, and you have a stock solution of neomycin at 10 mg/ml. How many μl of neomycin should you add to the cells to get a final concentration of 100 μg/ml?

*First, 10 mg/ml = 10,000 μg/ml*

*So, if our stock is 10,000 μg/ml and we want 100 μg/ml, that means the stock is 100 -fold too concentrated (10,000/100 = 100). So if we add 1/100th the volume of the media (10 ml X 1/100 = 0.1 ml = 100 μl) we should get 100 μg/ml (actually, we'll get 99 μg/ml, if you take into account the added volume, but that's close enough)*

c. You want to microinject an antisense oligonucleotide into a frog egg. You know that the oligonucleotide will block translation if present at 100 pg/ml. The volume of the egg is 1.2 μl and you want to inject a volume of 10 nl. You are starting with an oligonucleotide stock solution at 5 μg/ml, and you want to make 1 ml of diluted oligonucleotide solution at a concentration such that 10 nl injected into the egg will give a final concentration of 100 pg/ml. How much of the stock oligonucleotide and how much water should you combine?

*First, we are going to be injecting 10 nl into a volume of 1.2 μl, and we want to get a final concentration of 100 pg/ml, This means that the injected oligonucleotide will have to be more concentrated than 100 pg/ml because it will become diluted out as it diffuses to fill up the egg. How much more concentrated does it need to be? The volume of the egg is 120 times the volume of the injection, so we need to have the oligonucleotide 120-fold concentrated (1200 nl/10 nl = 120). Therefore we want a concentration of 12,000 pg/ml.*

*How do we make 1 ml of 12,000 pg/ml oligonucleotide? First put everything in the same units. It usually doesn't matter what the units are, so long as everything is the same, so choose the most convenient units. In this case I'll put everything in ng/ml. Therefore our stock solution is 5,000 ng/ml and we want to make a solution that is 12 ng/ml. There are several ways to solve this type of problem. I'll demonstrate my way:*

*If we divide our desired concentration by our starting concentration we get the dilution factor we need: 12/5000 = 0.0024 .We then multiply the dilution factor by the volume we wish to make to give the volume of the stock solution to add: 0.0024 • 1ml = 0.0024 ml . Since there are 1000 μl per ml, 0.0024 ml is the same as 2.4 μl. The remaining volume needs to be water, which would be 1000 μl - 2.4 μl = 997.6 μl .*

**2. Moles and Molarity.**

Molarity is a measure of concentration and is in units of moles per liter.
Abbreviated as: M
mM = $10^{-3}$ M
μM = $10^{-6}$ M, ect.
Molecular weight is the number of grams of a substance in 1 mole. The unit of molecular weight is Daltons (abbreviated Da). In other words a protein with a molecular weight of 31,123 grams/mole is 31,123 Da (you will often see this shortened to 31 KDa).

Examples:

a. The molecular weight of sodium chloride is 58.44. You want to make 25 ml of a 500 mM NaCl solution; how many grams do you need?

*Remember that 58.44 grams in 1 liter of water would make a 1 M solution. 500 mM is the same as 0.5 M, and 25 ml is the same as 0.025 l. Now that everything is in the same units, we can set up the following equations:*

$$58.44\ g/mole = X/0.5\ \ mole\ (\ g_1/m_1 = g_2/m_2)$$

*X = 29.22 g for 1 l of 0.5 M, but we want 0.025 l, so we need to solve the next equation:*

$$29.22\ g/l = Y/0.025\ l\ \ (g_1/V_1 = g_2/V_2)$$

*Y= 0.731 grams*

*These two equations can be combined into the following single equation: (pay attention to the units)*
*58.44 grams/mole • 0.5 moles/liter • 0.025 liter = 0.731 grams*

b. You have a 1 M stock solution of $MgCl_2$. To make a 350 µl of a 17 mM solution how much water and how much of the 1 M stock solution would you combine?

$$17\ mM/1000\ mM \bullet 350\ \mu l = 5.95\ \mu l\ 1\ M\ MgCl_2$$
$$350\ \mu l - 5.95\ \mu l = 344.05\ \mu l\ H_2O$$

*If can't follow it this way , think of it in terms of moles. We want to make 350 µl of solution that is 17 mM $MgCl_2$. How many moles of $MgCl_2$ do we need? 17 mM means that we want a solution that is equivalent to 17 x $10^{-3}$ moles/liter. But we only want to make a fraction of a liter. What fraction? 350 µl is the same as 0.00035 liters. We can set up the following ratio:*

$$17 \times 10^{-3}\ moles/liter = x/0.00035\ liter\ ;$$
$$solve\ for\ x = 5.95 \times 10^{-6}\ moles$$

*The next step is to figure out what volume of our stock solution at 1 M would give 5.95 x $10^{-6}$ moles. We can solve another ratio:*

$$1mole/liter = 5.95 \times 10^{-6}\ moles/y$$
$$solve\ for\ y = 5.95 \times 10^{-6}\ liters = 5.95\ \mu l$$

*We now have the volume needed to get the required number of moles. By adding the remaining volume in water we get the final desired concentration*

$$350\ \mu l - 5.95\ \mu l = 344.05\ \mu l\ H_2O$$

c. The oligonucleotides we are going to use for PCR were ordered from a company called OPERON®. The oligonucleotides arrived as a dried powder at the bottom of the tube. I needed to resuspend them in water to make a 10X concentrated solution (1,000 pmoles/µl). The molecular weight of INT1 is 8,134

and the tube contained 1.194 mg. How much water should I add to the tube to get 1,000 pmoles/μl?

$$mole/8{,}134\ g \bullet 1.194 \times 10^{-3}\ g = 1.468 \times 10^{-7}\ moles\ of\ INT1$$

$$1.468 \times 10^{-7} \bullet 10^{12}\ pmoles/mole = 146{,}800\ pmoles$$

$$146{,}800\ pmoles/1{,}000\ pmoles/\mu l = 146.8\ \mu l$$

**3. Solutions as percentages.**

You will often find solutions used in molecular biology given as percentages. Frequently, some components of a solution are given in molarity and some in percentages. For example, SM buffer is: 100 mM NaCl, 10 mM Tris, 10 mM $MgCl_2$, and 0.02% gelatin. By convention, a 1% solution for a solution component that comes as a dry powder is defined as 1 gram/100 ml. For a component that comes as a liquid, a 1% solution is defined as 1 ml/100 ml (for example, a 10% ethanol solution would have 10 ml ethanol for every 100 ml).

a. To make 1 liter of SM buffer how many mg of gelatin should you add?

*0.02% means 0.02 grams/100 ml (the same as 20 mg/100 ml). 100 mls goes into a liter 10 times, so we would need 20 mg • 10 = 200 mg.*

**4. Protein/nucleic acid calculations.**

In routine molecular biology work, one usually expresses amounts of large molecules like plasmids or proteins in mass (μg for example), not in moles. But it is often important to calculate molar ratios.

Pieces of DNA or RNA are usually expressed in terms of bases (abbreviated b) for single stranded nucleic acids, and base pairs (bp) for double stranded nucleic acids. For example, the plasmid we are going to be using is called Bluescript SK+ and is a double stranded circular molecule of 2958 bp (abbreviated as 2.96 kb). The entire nucleotide sequence of the plasmid is known, so we could calculate its molecular weight by adding up the molecular weights of each of the bases:

dAMP = 331.2 Da
dCMP = 307.2 Da
dGMP = 347.2 Da
TMP = 322.2 Da

But this would be very laborious. Instead what is usually done for large nucleic acids (> 100 bp) is to make the assumption that all of the bases are present at equal amounts, and to then simply use that average molecular weight of a base.

a. What is the approximate molecular weight of Bluescript SK+?

*If we average the molecular weights of the above deoxyribonucleotide bases we get 326.9 Da. Because Bluescript SK+ is double stranded we want the average molecular weight of a base pair which would be 653.9 Da. Therefore, the molecular weight of the plasmid is 653.9 • 2,958 = 1,934,236 Da.*

b. You have restriction enzyme digested Bluescript SK+ with EcoRI to linearize it, and now want to ligate a 1.2 kb cDNA fragment into the EcoRI site. To optimize the ligation reaction, you want to have a 3-fold molar excess of the cDNA over the plasmid. If you are using 100 ng of the plasmid, how many ng of the cDNA should you add?

*First, how many moles of the plasmid are we adding?*

$$100 \times 10^{-9}\ g \bullet mole/1.93 \times 10^{6}\ g = 5.18 \times 10^{-14}\ moles$$

*Next, how many ng of the cDNA would equal 5.18 x 10-14 moles?*
*The approximate molecular weight of the cDNA is 784,680 Da*

$$7.84 \times 10^{5}\ g/mole = X/5.18 \times 10^{-14}\ mole, \text{ solve for X:}$$

$$X = 7.84 \times 10^{5}\ g/mole \bullet 5.18 \times 10^{-14}\ mole = 4.06 \times 10^{-8}\ g = 40.6\ ng$$

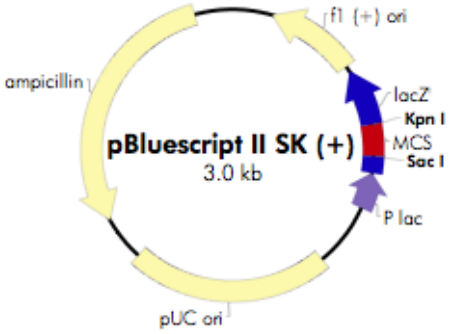
*Since we want a three fold excess, we should add 121.8 ng.*

c. You have determined the nucleotide sequence of a newly discovered 2.3 kd cDNA. You scan the sequence to try to deduce what protein it might encode. In doing this, you are looking for regions of sequence starting with the codon for methionine (ATG) that continue for long stretches without stop codons in the same frame as the ATG. These are called open reading frames (ORFs), and one normally uses a computer to find them. In the middle of the sequence you find a 1590 bp ORF. If the average molecular weight of an amino acid is 110 Da, what is the approximate size of the protein encoded by the ORF?

*Since the genetic code is in triplets, the ORF encodes a 530 amino acid protein, of approximate molecular weight 58,300 Da (530 x 110 Da).*

APPENDIX B

**f1 (+) origin** 135–441
**β-galactosidase α-fragment** 460–816
**multiple cloning site** 653–760
***lac* promoter** 817–938
**pUC origin** 1158–1825
**ampicillin resistance (*bla*) ORF** 1976–2833




**pBluescript II SK (+/–) Multiple Cloning Site Region (sequence shown 598–826)**