

University of Utah

Graduate Neuroscience Program

NEUSC 6250
Molecular Biology Laboratory

August 11-15, 2008

Instructors:	Shannon J. Odelberg, Ph.D. and Yukio Saijoh, Ph.D.
Office:	Wintrobe Building, Room 331
Telephone:	<u>581-7150</u>
E-mail:	odelberg@genetics.utah.edu
Teaching Assistants:	Eon Joo Park and Uchenna Emechebe

Valuable References

1. Current Protocols in Molecular Biology. (2000) 4 Volumes. FM Ausubel, R Brent, RE Kingston, DD Moore, JG Seidman, JA Smith, K Struhl, eds. John Wiley and Sons, New York.
2. Kornberg, A. and Baker, TA (1992) DNA Replication. W.H. Freeman and Company, New York.
3. New England Biolabs Catalog. Most recent edition, Beverly, MA.
4. Sambrook, J. and Russell, DW (2000) Molecular Cloning: A Laboratory Manual. 3 Volumes. Cold Spring Harbor Press, NY.

Acknowledgment

I would like to acknowledge Rosemary Gray for her help and for supplying me with the syllabus/manual for the course she teaches in basic laboratory techniques in the Bioscience Undergraduate Research Program at the University of Utah. Her manual served as a valuable resource while preparing for this course.

INTRODUCTION

This course is designed to equip incoming graduate students in the Neuroscience Program with the basic molecular biology skills required to successfully complete their laboratory rotations during the first year of graduate school. It is hoped that this accelerated course will give students with little or no molecular biology laboratory experience the confidence to choose rotations through laboratories that emphasize molecular biology approaches. For those students who already have extensive molecular biology experience, this course should provide a helpful review and may well introduce them to new techniques and approaches for solving biological problems. Lastly, this course will be integrated with the electrophysiology course that follows to demonstrate how molecular biology techniques can be used to help answer questions in electrophysiology.

Although the field of molecular biology encompasses the study of a wide variety of molecules, this course will only focus on the three types of biopolymers that are most widely encountered--DNA, RNA, and proteins. Other important molecules, such as lipids and carbohydrates, will not be included in this course and will have to be dealt with in later courses or while performing laboratory research. During this course you will learn how to prepare solutions, use basic lab equipment, amplify DNA by the polymerase chain reaction, run DNA and protein gels, purify and clone DNA fragments, perform plasmid preps, and make RNA by *in vitro* transcription.

It is important students recognize that this course is for them. This is the time to ask those questions that you might be embarrassed to normally ask. Don't try to impress your classmates by only asking profound questions. Ask those basic "I-should-already-know" questions. What is molarity? How do I make a 1 in 10 dilution of this solution? What is a plasmid? How do I pour a gel? These are all legitimate questions in this class. My goal as your instructor is to ensure that this course gives you the basic knowledge and tools to confidently enter your first year of graduate school and to be able to choose your rotations from a wide variety of laboratories, not just those with which you currently feel comfortable. This should be an enjoyable and informative experience designed to reduce your anxiety as you face your first year of graduate school.

This course is organized as a continuous 4 day experiment (almost) with the ultimate goal of preparing RNA that could be injected into *Xenopus* (African clawed frog) oocytes for electrophysiological study. On the fifth day, the students will perform an unrelated experiment where they will learn how to run protein gels. On this final day, students will also be exposed to computer sequence analysis programs that are useful for molecular biology research.

You will be graded using three criteria. First, you will be expected to keep a laboratory notebook that you will turn in at the end of the course. This will count for 60% of your grade, so please carefully follow the instructions below:

1. Everything should be written in pen.
2. The notebook must be detailed enough that another scientist would be able to

- understand your experiments, why you did them, and how you did them.
3. You should state your interpretation of the results and conclusions.
 4. Your notebook must also be legible so that others could read and interpret your results.
 5. You must date all of your work so that it is obvious on which date you performed the experiments or thought of a particular idea.
 6. If you make a mistake, place a **SINGLE** line through the error and replace it with the correct version. **INITIAL THE CHANGE. DO NOT OBLITERATE THE ERROR BY SCRIBBLING THROUGH IT WITH THE PEN. DO NOT USE WHITE-OUT. DO NOT TRY TO ERASE THE ERROR.** Anyone reading the notebook should be able to read the error and know that you intentionally made the correction.
 7. Your laboratory notebook should be prepared in real time or close to real time. Do not keep notes on a separate piece of paper and then neatly copy these notes into an official, “super-organized” lab notebook. Your notebook should be as neat and organized as a “real time” notebook can be, but it should not resemble a Rembrandt painting. You can write up protocols and notes before class to help you prepare for the experiments. Anything that changes during the actual experiment can then be corrected as described above in your notebook.
 8. Everyone in each group should keep a separate lab notebook.

Second, you will be graded on your problem set. This will count for 10% of your grade. Try to get the correct answers, because you will need to be able to do these types of problems for the written exam on Friday. However, your grade for the problem set will not be dependent upon whether you get the correct answer. Instead, it will be dependent on the effort you gave to try to get the correct answer. The problem sets are due Wednesday and we will discuss them in class that day. You can work together on the problem set but remember, you will need to be able to do these problems on your own for the exam on Friday.

Third, you will have a written exam on Friday. The exam will count for 30% of your grade. This test will cover the important points that the instructor thinks you should know to successfully complete this course. Make sure you read this syllabus/manual and are able to do the problem set. Also, pay close attention to items typed in **red** or in **BOLD** type (excluding headings). If you do this, you should have no problem with the exam. If you have any questions concerning the syllabus/manual or problem set, please ask the instructor(s) or a Teaching Assistants (TAs).

The instructors hope there are no substantive errors in this syllabus/manual (other than typographical errors). However, bonus points will be awarded to the first individual who notifies the instructor of each substantive error. Substantive errors include errors in calculations and errors in fact.

Attendance is mandatory unless you have been excused by the instructor. If you get sick, please contact the instructor to make appropriate arrangements.

You will be guaranteed an A if your weighted average for all three graded items is 90% or higher. You will also be guaranteed at least a B if your weighted average is at least 80%. If required, the instructor reserves the right to grade more subjectively; however, this subjective grading will always favor the student and will not negate the guarantees above.

This syllabus is organized by information that will be covered on each of the five days. The student should read the material to be covered for a given day before coming to class. Words that are written in black contain background information. Words written in blue are experimental protocols. As mentioned above, words that are written in red or in bold type are especially important.

SCHEDULE

Day	Date	Time	Subject or Experiment	Pages
1	Monday, 8/11/08		Introduction to the course Measurement units Solution Preparation dNTPs and NTPs; α , β , γ phosphates PCR amplification of an ORF Lunch Discussion--PCR, RT-PCR, and gel purification of DNA fragments Gel Electrophoresis Extract PCR products and precipitate Discussion--Restriction enzymes and ligation Overnight restriction digest	2 7 8, 18 11 12, 19 14, 19 21 16, 22
2	Tuesday, 8/12/08		Purify digested PCR product and digested plasmid Discussion--Bacterial transformation Ligate PCR product into vector Lunch Transform bacteria Start overnight bacterial cultures Discussion--Plasmid preps	23 24, 27 24, 27 28
3	Wednesday, 8/13/08		Check plates for transformed bacteria Perform plasmid midipreps Release inserts and linearize plasmids Lunch Discussion of the Problem Set and Question/Answer Period Extract linearized plasmids and run gel to confirm linearization and insert size	29 25, 29 30 31
4	Thursday, 8/14/08		Discussion-- <i>In vitro</i> transcription and formaldehyde gel electrophoresis <i>In vitro</i> transcription and RNA extraction Lunch Run transcription products on an RNA formaldehyde gel	 32, 35 34, 35
5	Friday, 8/15/08		Discussion--PAGE and western blotting Run a protein PAGE gel Exam Lunch--Pizza! Computer programs for sequence analysis	37, 37 39

			BLAST ClustalW and T-COFFEE SignalP TMpred	
--	--	--	-----------------------------------------------------	--

DAY 1

Measurement Units

Molecular biologists, like all scientists, work in metric units. The basic unit of mass is the gram (g)*, the basic unit of length is the meter (m), and the basic unit of volume is the liter (L or l). The metric system is convenient because it is based on powers of 10. Therefore, the system is quite amenable to scientific notation. The following chart provides the prefixes that can be attached to the basic units to describe the various amounts of mass, length, or volume. The ones shown in red are used often enough that you should commit them to memory. The ones shown in black are not used as often and can be used to impress your friends at parties (or probably more likely to brand you as a geek or nerd--nothing wrong with that!).

<u>Prefix</u>	<u>Factor</u>	<u>Symbol</u>	<u>Prefix</u>	<u>Factor</u>	<u>Symbol</u>
deci-	10^{-1}	d	deka-**	10^1	da
centi-	10^{-2}	c	hecto-	10^2	h
milli-	10^{-3}	m	kilo-	10^3	k
micro-	10^{-6}	μ	mega-	10^6	M
nano-	10^{-9}	n	giga-	10^9	G
pico-	10^{-12}	p	tera-	10^{12}	T
femto-	10^{-15}	f	peta-	10^{15}	P
atto-	10^{-18}	a	exa-	10^{18}	E
zepto-	10^{-21}	z	zetta-	10^{21}	Z
yocto-	10^{-24}	y	yotta-	10^{24}	Y

*Although the gram is not an official SI unit (the official SI unit of mass is the kilogram or kg), it is still used as the base word for mass with all prefixes attached to it.

***deka-* is sometimes spelled *deca-*.

Solution Preparation

A. Concentrations

The concentration of a solution can be determined in several ways. Some methods are based on the *number of moles* of solute in a solution, while other methods are based on the *percentage* of solute in a solution. Both methods are described below:

Methods based on the number of moles of solute in the solution

Molarity Molarity (M) is by far the most useful and widely used measure of concentration for molecular biology research. **It is defined as the number of moles of solute in 1 liter of solution.** Almost invariably, the solvent will be purified water (such as doubly-distilled or deionized water). One mole of a chemical is equal to the formula weight (FW)(also known as the molecular weight) in grams. Therefore, to make 1 liter of a 1 M solution of a particular reagent, one would perform the following steps.

- 1) look up its formula weight (found on the container)
- 2) weigh out the formula weight of the chemical in grams
- 3) add the chemical to less than 1 liter (usually about 900 ml) of ddH₂O (or other solvent).
- 4) stir the solution until the solute is in solution and then bring the volume up to 1 liter.

Note: One does not mix the chemical with 1 liter of water but rather the final volume of the solution is 1 liter.

To calculate the number of grams required to make any desired concentration of solution at any desired volume, the following formula can be used:

$$\text{g of chemical} = M (\text{moles/liter}) \times \text{formula weight (g/mole)} \times \text{liters of solution}$$

Example: Assume you want to make 250 ml of a 3 M NaCl solution. The FW for NaCl is 58.44.

$$3 \text{ moles/liter} \times 58.44 \text{ g/mole} \times 0.25 \text{ liters} = 43.83 \text{ g NaCl}$$

Normality Normality (N) is an alternative measure of solution concentration that is not often used in molecular biology research, except when dealing with acids and bases. Normality is equal to the number of equivalents/liter. When dealing with acids and bases, this refers to the number of H⁺ or OH⁻

groups available for a reaction. Some acids and bases, such as HCl and NaOH, have only one mole of H⁺ or OH⁻ to contribute to the reaction per mole of acid or base, while others, such as sulfuric acid (H₂SO₄), can contribute more than one mole of H⁺ or OH⁻ per mole. Sulfuric acid can contribute 2 moles of H⁺ per mole of H₂SO₄. **Therefore, the normality of a solution is equal to number of equivalents/mole x the molarity of the solution.** A 0.25 M solution of HCl or NaOH would be 0.25 N, while a 0.25 M of sulfuric acid would be 0.5 N.

Molality Molality (m) is another measure of solution concentration that is rarely encountered in molecular biology research. **It is defined as the moles of solute per kg of solvent.** The density of water is ~1 g/ml (which equals 1 kg/liter), so the molality of an aqueous solution is roughly equal to the molarity (although the two concentrations are not quite equivalent, because the amount of solvent is slightly different).

Methods based on the percentage of solute

w/v Weight/volume concentrations are often encountered in molecular biology research. In this measure of concentration, 1 g of solute is set equivalent to 1 ml of solvent and then the percentage of solute is calculated.

Example: A 15% w/v solution of glucose would contain 15 g of glucose per 100 ml of solution. To prepare such a solution, one would weigh out 15 g of glucose, mix it with 80 ml of ddH₂O, and then bring the volume up to 100 ml with ddH₂O.

v/v Volume/volume concentrations are also encountered in molecular biology research. In this measure of concentration, a liquid solute is mixed with a solvent to produce final concentration of solute.

Example: A 70% v/v ethanol solution is prepared by mixing 7 parts 100% ethanol with 3 parts ddH₂O. Alternatively, a 70% v/v ethanol solution could be prepared from 95% v/v ethanol.

w/w weight/weight concentrations are similar to v/v concentrations, except that the solute and solvent are measured in mass rather than volume. A 15% w/w solution would contain 15 g of solute and 85 g of solvent per 100 g of solution.

B. Preparing buffers

For preparing most solutions used in molecular biology research, the above discussion provides adequate detail. However, extra information is required for preparing buffers. Buffers are ionic solutions that are designed to resist changes in pH. They are comprised

of a weak acid and its salt or a weak base and its salt. Several buffers are routinely encountered in molecular biology research. One such buffer is Tris-HCl. We will use Tris-HCl as an example of how to prepare a buffer.

Caution: Unlike most solutions, you cannot prepare a 1 M Tris-HCl solution by simply looking up the formula weight of Tris-HCl and adding the appropriate amount of Tris-HCl to ddH₂O. Instead, you have two options. We will cover only one of these options in this class and briefly mention the other option.

The first option is to determine the formula weight for Tris base (Sigma calls this Trizma base) and weigh out the appropriate amount for a 1 M solution. The tris base is dissolved in a volume of ddH₂O that is 80% the final volume. Once the Tris base is in solution, the desired pH for the buffer is achieved by adding 6 M HCl drop-wise to the solution, while stirring and monitoring it with a pH meter. **Before performing this procedure, be sure that your pH electrode is compatible with Tris. Many of the old electrodes were not. Also, be careful to do this procedure slowly enough so that you do not overshoot the desired pH. If you overshoot the desired pH, you have two options: 1) start over or 2) make a larger volume of buffer by adding more Tris base and water and then adjusting the pH, again. DO NOT attempt to “correct” the error by adding a base, such as NaOH.** Once the solution is within 0.3 pH units of the desired pH, check the temperature of the solution. Tris-HCl buffers are highly sensitive to temperature and therefore the pH should be measured at $25 \pm 2^\circ\text{C}$. As HCl is added to the solution, the temperature will rise. If it exceeds 27°C , stop the procedure and allow the solution to cool. Once cooled, continue adjusting the pH to the desired level using 1 M HCl. Another way of compensating for small temperature fluctuations is to understand that for every 1°C increase in temperature in the Tris solution, the pH will decrease 0.03 pH units. Therefore, if the temperature of your Tris solution is 27°C , you should adjust the pH to 0.06 pH units lower than the desired final pH. Once the desired pH has been achieved, add ddH₂O to the appropriate final volume.

An alternative method for making Tris buffers is to use the Henderson-Hasselbalch equation to calculate the required concentrations of Tris base and Tris-HCl. There are charts based on this calculation that will indicate the precise amount of each chemical to use for a particular concentration and pH.

If you are interested in learning more about buffers and buffer preparation, you can access an informative booklet that Calbiochem has prepared. The link to the Calbiochem site can be found at <http://www.neuro.utah.edu/courses/biochem/index.php>.

Preparing complex solutions

Often a solution will contain multiple solutes in fairly dilute concentrations. One convenient way to prepare such solutions is to make high concentration stock solutions of each of the components and then mix them together at the appropriate dilutions. For example, one might want to prepare 100 ml of the following complex solution:

20 mM Tris-HCl, pH 7.5

150 mM NaCl
1 mM EDTA
0.05% SDS (sodium dodecyl sulfate)

One could prepare such a solution from the following stock solutions: 1) 1 M Tris-HCl, pH 7.5; 2) 3 M NaCl; 3) 500 mM EDTA; and 4) 10% SDS. To do this, one simply calculates the dilution factor required for each reagent to achieved the final desired concentration and then divides 100 ml by the respective dilution factors to determine how many ml of each reagent should be added. The total number of ml are then summed and the sum is subtracted from 100 ml to determine how much ddH₂O must be added. The amount of 1 M Tris-HCl that must be added to this solution is calculated as follows:

1 M = 1,000 mM
1,000 mM/20 mM = 50-fold dilution factor
100 ml/50 = 2 ml of 1 M Tris-HCl

Your problem set contains an exercise in which you determine the amounts of each reagent needed to prepare the above complex solution. You should know how to do these calculations.

C. Important tips for making solutions

1. Some chemicals and reagents are expensive or have a short shelf life in solution. If you are preparing a solution that contains an expensive reagent or is labile, think carefully about how much of the solution you will need. Don't make an excessive amount.
2. If you are preparing only a small volume of a particular solution and you need very small quantities of solute (mg or less), you may want to consider weighing a small portion of the solute and then calculating the appropriate volume for the amount of solute you have weighed. This is often easier than weighing out the precise amount you need for a predetermined volume. In practice, when one is dealing with small solution volumes, the solutions are often made by mixing an appropriate amount of solute with the final volume of solution. In these cases, if the solute is measured in moles and the solvent is water, the solution is technically a molality solution, rather than molarity. However, the difference between these two types of concentrations is usually negligible and for purposes of molecular biology research, these differences can usually be ignored.

Use of dNTPs and NTPs (rNTPs) in molecular biology

dNTPs are the building blocks of DNA, while NTPs (rNTPs) are the building blocks of RNA. Make certain you use the correct building blocks when synthesizing DNA and RNA. Also, when using radiolabeled nucleotides, you must know the difference between

the α , β , and γ phosphate groups of dNTPs and NTPs. The phosphate group closest to the sugar is the α phosphate, the next phosphate group is the β phosphate, and the one furthest from the sugar is the γ phosphate. Therefore, when radioactively labeling DNA or RNA during DNA or RNA synthesis, one should choose a nucleotide that has a labeled α -phosphate group, such as $[\alpha\text{-}^{32}\text{P}]\text{-dCTP}$. When labeling the 5'-end of a primer or nucleic acid strand where there will be a transfer of the labeled phosphate group, one would choose a nucleotide with a radiolabeled γ -phosphate group, such as $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$.

Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) can be used to make millions of copies of a particular DNA sequence *in vitro*. This is accomplished by mixing the DNA to be amplified (DNA template) with specific primers (defines the region to be amplified), dNTPs (building blocks for making the DNA copies), a thermostable DNA polymerase, and a Mg^{2+} -containing buffer (DNA polymerases require Mg^{2+} for function). The mixture is then cycled through a series of temperature changes. Very high temperatures (near the boiling point of water) will denature DNA, which produces two complementary single-stranded DNA (ssDNA) molecules. When the temperatures are lowered, the primers can anneal to their specific targets and the DNA polymerase can extend these primers using the available dNTPs, thus making complementary copies of each of the single DNA strands. This procedure is repeated usually 25-35 times. Theoretically, each cycle could double the amount of target DNA sequence. However, in practice, the efficiency of the reaction is not 100%, so doubling does not occur every cycle. Regardless, this method allows for the rapid production of millions of copies of the target DNA sequence.

It is important for students to know how to convert measurements of mass to moles and vice versa. I also think it is useful for students to become familiar with chemical and enzymatic reactions at the level of the molecule. For example, how many DNA template molecules are in your PCR reaction when you start? How many primer molecules? How many dNTP molecules? Researchers do not calculate these numbers for every reaction, but they should know how to do these calculations. It is also useful to perform these calculations at least once to get a better understanding of the magnitude of the chemical and enzymatic reactions that are occurring during PCR.

These calculations require the following known constants. These are very useful constants to remember so you will not have to look them up when you need to perform a similar calculation. Memorize the ones in **red**.

1 mol of a base pair of DNA \approx 660 g
Avagadro's number = 6.023×10^{23} particles/mol

Your problem set contains an exercise in which you perform such a calculation. Make sure you are comfortable with performing such calculations.

Calculating oligonucleotide concentrations

There are several methods that can be used to calculate the concentration of an oligonucleotide. Two of them will be discussed here. First, the absorbance of the oligonucleotide solution should be determined by spectrophotometric analysis at 260 nm. Only trust “spec” readings between 0.1 and 1.0. If the reading is higher than 1.0, dilute a *portion* of the sample until the reading falls within this range. This often means that DNA or RNA samples, including oligonucleotides, must be diluted 10- to 1,000-fold before they are within this range. Once the “spec” reading is within the appropriate range, the two methods can be used to determine the oligonucleotide concentration. The first method is a quick and dirty method that is often useful when great accuracy is not needed. It involves the use of a constant. **It is one of three OD constants that you should memorize. The other two will be discussed later.**

OD of 1 at 260 nm = 33 $\mu\text{g/ml}$ of oligonucleotide

If you have diluted a sample of your oligonucleotide 100-fold and get an absorbance of 0.2356 at 260 nm, you can calculate the concentration as follows.

$$0.2356 \times 100 \times 33 \mu\text{g/ml oligonucleotide} = 777 \mu\text{g/ml}$$

Oligonucleotides are usually used in μM concentrations. Assume your oligonucleotide is 22 nucleotides in length. If 1 bp has a molecular weight of 660, a single nucleotide should have a molecular weight of about 330. Using this information, the above concentration can be converted to μM as follows:

$$\text{MW of oligonucleotide} = 330 \text{ g/mole} \times 22 \text{ nucleotides} = 7,260 \text{ g/mole oligonucleotide}$$

$$777 \mu\text{g/ml} \times 1 \text{ g}/10^6 \mu\text{g} \times 1000 \text{ ml/l} \times 1 \text{ mole}/7,260 \text{ g} = 1.07 \times 10^{-4} \text{ M} = 107 \mu\text{M}$$

A more accurate method for determining oligonucleotide concentration is to use extinction coefficients. Again, there are at least two methods used to calculate concentrations based on extinction coefficients. We will only cover one of them but be aware that another, possibly more accurate, method exists based on a nearest neighbor approach. To perform the calculations using extinction coefficients, you must know the specific nucleotide sequence of the oligonucleotide. Assume the above oligonucleotide has the following sequence: 5'-GCATGTCTGACCAATTTGCACT-3'. You can calculate the concentration using the following extinction coefficients:

$$\text{pdG} = 11.7 \text{ ml}/\mu\text{mole}/\text{cm}$$

$$\text{pdC} = 7.3 \text{ ml}/\mu\text{mole}/\text{cm}$$

$$\text{pdA} = 15.4 \text{ ml}/\mu\text{mole}/\text{cm}$$

$$\text{pdT} = 8.8 \text{ ml}/\mu\text{mole}/\text{cm}$$

The number of Gs, Cs, As, and Ts are counted. Each of these numbers is then multiplied by the corresponding extinction coefficient (ϵ) and the totals are summed to arrive at an extinction coefficient for the entire oligonucleotide. Beer's law is then used to determine the concentration.

4 Gs	$4 \times 11.7 \text{ ml}/\mu\text{mole-cm} = 46.8 \text{ ml}/\mu\text{mole-cm}$
6 Cs	$6 \times 7.3 \text{ ml}/\mu\text{mole-cm} = 43.8 \text{ ml}/\mu\text{mole-cm}$
5 As	$5 \times 15.4 \text{ ml}/\mu\text{mole-cm} = 77.0 \text{ ml}/\mu\text{mole-cm}$
7 Ts	$7 \times 8.8 \text{ ml}/\mu\text{mole-cm} = 61.6 \text{ ml}/\mu\text{mole-cm}$

Oligonucleotide Extinction coefficient (ϵ) = 229.2 ml/ μ mole-cm

Beer's Law (Beer-Lambert Law)

$$A = \epsilon bC$$

where A = absorbance at 260 nm

b = path length = 1 cm (in most cases)

C = concentration

$$23.56 = 229.2C$$

$$C = 0.1028 \mu\text{mole/ml}$$

$$C = 102.8 \mu\text{M}$$

Note: Extinction coefficients for each of the nucleotides vary slightly depending on the source. Remember that all of these calculations give you estimates of the oligonucleotide concentration.

Agarose Gel Electrophoresis

One of the convenient methods for separating DNA or RNA according to size is by agarose gel electrophoresis. Agarose is one of the constituents of agar. It gels at a lower temperature than agar and the pore sizes in agarose are more uniform than those in agar. To prepare an agarose gel for DNA analysis, one usually places powdered agarose in the electrophoresis buffer and then heats the solution until the agarose goes into solution. Once the solution cools to about 60°C, additives such as ethidium bromide can be added so that the DNA can be visualized under UV light. The gel is then poured into a gel tray and the solution is allowed to solidify by cooling. Once the gel has solidified, it can be covered by the electrophoresis buffer (containing ethidium bromide) until used to prevent the gel from drying. Alternatively, ethidium bromide can be omitted from both the gel and buffer until after gel electrophoresis is completed. The gels can then be stained in electrophoresis buffer containing ethidium bromide for 15-30 minutes and then visualized under UV light.

Agarose gels can be poured at different concentrations. These concentrations are usually determined by percentage w/v. For example, a 1% gel would be equivalent to 1 g of agarose in 100 ml of running buffer. Different percentages of gels are useful for different

Cloning DNA

DNA cloning refers to a technique in which a specific DNA fragment is replicated to produce multiple copies of the identical DNA sequence. Under this definition, PCR is a modern method for cloning a DNA fragment. However, in this section we will be dealing with the more traditional method of producing DNA clones by placing the DNA fragment of choice (in our case, a known open reading frame, or ORF, of a gene) into a plasmid and then transforming bacteria with this recombinant plasmid so that the bacteria can make multiple copies of the ORF DNA sequence. We will prepare our insert by PCR and then clone this insert into a plasmid that has the necessary components for making RNA *in vitro*. We will clone the insert by taking advantage of the restriction sites we have placed on the 5'-ends of the amplification primers. These same restriction sites are present in our plasmid vector. By digesting both the insert and plasmid with the same two restriction enzymes, we will be able to ligate the insert into the plasmid in a particular orientation. The corresponding sticky (cohesive) sites on the insert and plasmid will be ligated together, forcing the insert into the plasmid in a specified orientation. The recombinant plasmid will then be transformed into *E. coli* and because the plasmid contains an antibiotic resistance gene, bacteria containing the plasmid will be able to grow in the presence of the antibiotic, while those bacteria that do not contain a plasmid will not be able to grow and will die. Clones of bacteria can be isolated on bacterial plates containing the antibiotic. These bacteria can then be grown in liquid culture to high density in the presence of antibiotic. Plasmid preps are made from such cultures so that the final product is a large amount of cloned plasmid DNA that contains the insert of interest. Following a cloning procedure that uses PCR to amplify the insert, it is necessary to sequence the entire insert if the clone is going to be used for functional analyses. Although error correcting thermostable polymerases should be used for amplifying the insert in such cases, these enzymes can still make mistakes and the insert needs to be sequenced to determine whether any errors have been introduced. Time does not permit us to include this step in this laboratory class but remember that it is important when the insert will be used for functional analyses.

Restriction Enzyme Digests

Restriction digests are usually performed in volumes varying between 25-100 μ l. BSA is often required for some enzymes but not for others. However, restriction enzymes are not inhibited by BSA, so it should be added whenever a double digest is being performed and one of the enzymes requires BSA. The amount of enzyme added to a reaction depends upon the amount and complexity of DNA being digested, the cost of the enzyme, and how quickly a complete digest is needed. It also depends upon whether the investigator desires a complete or partial digest. As a general practical rule, if a complete digest is needed, 10 units of enzyme should be able to digest 1 μ g of almost any DNA in about an hour. However, longer incubations often ensure complete digestion, especially of large DNA molecules or genomic DNA. For extremely expensive enzymes, use fewer units of enzyme per μ g of DNA and compensate for the lower enzyme concentration with longer incubation times.

There are several important points to consider when one is working with restriction enzymes:

1. Although restriction enzymes are quite specific, they can cleave DNA sequences that vary slightly from their normal recognition sequence when the enzymes are used inappropriately. This noncanonical cleavage is known as “star” activity and can usually be avoided by following four rules:
 - a. Always use the appropriate buffer for the enzyme. Not enough salt in the buffer, high pH, or divalent ions other than Mg^{2+} can lead to star activity.
 - b. Never allow the concentration of glycerol in the reaction to exceed 5% v/v. Remember that stock enzyme solutions from the manufacturer contain 50% v/v glycerol. This means that the total volume of enzyme added to a restriction digest cannot exceed 10% of the total volume.
 - c. Do not use extremely high units of enzyme to μg of DNA ratios (>100 units/ μg).
 - d. Avoid the use of organic solvents (DMSO, ethylene glycol, dimethylformamide, etc.) in the restriction digest.
2. Some restriction enzymes recognize the same DNA sequence. These enzymes are known as isoschizomers. Isoschizomers can cleave the recognition sequence between the same nucleotides or they can cleave the recognition sequence between different nucleotides. Different isoschizomers can have different digestion conditions so it may be useful to use one isoschizomer over another when digesting DNA with two restriction enzymes simultaneously (double digest).
3. Remember that different restriction enzymes can produce compatible sticky-ends that can be used for ligating and cloning DNA.
4. Some restriction enzymes can be easily heat inactivated, while others are resistant to this treatment.
5. Restriction enzymes vary in their abilities to cleave close to the ends of DNA fragments. This is important to know when designing primers containing restriction sites that are to be used for subsequent cloning following PCR amplification.
6. Some restriction enzymes are inhibited by methylation. This is important, because CpG sequences can be methylated in genomic DNA from eukaryotes, while bacteria often methylate the A in GATC sites and the second C in CCWGG sites. Cleavage of a known restriction site might not occur when the site has been methylated.
7. One of the most important single sources of valuable and practical information concerning restriction enzymes is the New England Biolabs Catalog. It contains all the information given above and indicates which enzymes are in each of the above categories. Make sure you have access to a current version of this valuable resource if you plan to work in a molecular biology laboratory.

Experiment 1. Preparation of buffers

For this first experiment, you will prepare a 10x *Taq* DNA polymerase buffer that could be used in a PCR reaction. For the actual PCR experiments you will be doing later, you will use a special buffer that is specific for the error-correcting thermostable DNA polymerase you will use to amplify your target DNA. This special buffer is prepared by the manufacturer. However, for routine PCR amplifications using *Taq* DNA polymerase, the following 10x buffer is routinely used. Often 500 mM KCl is substituted for the 400 mM NaCl and the MgCl₂ concentration can be varied from 5 mM to 30 mM to optimize amplification of specific DNA templates.

Prepare 100 ml of each of the following solutions:

1 M Tris-HCl, pH 8.8

3 M NaCl

1 M MgCl₂

Most often, you would sterilize these solutions by autoclaving them; however, today you will sterilize them by filtration through a 0.22 μm filter.

1. Attach the Steritop filter to the sterile bottle.
2. Attach the vacuum hose that is connected to a vacuum trap and house vacuum.
3. Pour your sample into the Steritop.
4. Apply the vacuum until all the contents have filtered into the sterile bottle.
5. Remove the Steritop and immediately screw on the bottle lid.
6. Be sure the bottle is labeled.

Using the above stock solutions, prepare 1 ml of 10x *Taq* DNA Polymerase Buffer with the following final concentrations of reagents:

10x *Taq* DNA Polymerase Buffer

100 mM Tris-HCl, pH 8.8

400 mM NaCl

15 mM MgCl₂

Experiment 2. PCR amplification of an ORF-containing cDNA and extraction of the PCR product

Note: For all of your agarose gel electrophoresis experiments, two groups will team up together to run a single gel. The groups will load their samples in different lanes of the same gel. Both groups should split the work load when pouring the gels so that all members of each team can learn the basic techniques. When required, make sure enough photographs are taken of each gel so that each student can have a photograph for his/her notebook.

PCR Amplification

1. Prepare two 50 μ l PCR reactions as shown below. Keep on ice during preparation.

<u>Stock Solutions</u>	<u>Final Amount</u>
pCDH1-3/10b DNA template (50 pg/ μ l)	50 pg
5x Buffer	1x
dNTP Mix (2.5 mM each)	200 μ M each
5 μ M 5'-CDH1 fwd primer	0.2 μ M
5 μ M 3'-CDH1 rev primer	0.2 μ M
Takara PrimeSTAR DNA pol (2.5 U/ μ l)	1.25 U
ddH ₂ O	calculate

2. Program the thermocycler with the following cycling profile.

30 cycles	94°C	30 sec minutes for initial denaturation
	98°C	10 seconds
	55°C	5 seconds
	72°C	90 seconds
	72°C	5 minutes
	4°C	hold

3. Load the thermocycler with your samples, close the lid and start the reaction.
4. Prepare a 1% w/v agarose gel as follows.
5. Weigh 0.35 g of agarose and place in a 125 ml Erlenmeyer flask.
6. Prepare 500 ml of 1x TAE solution from a 20x TAE stock solution.
7. Add 35 ml of 1x TAE to the Erlenmeyer flask and mix by swirling.
8. Mark the level of the liquid on the side of the flask with a marker and microwave for 1-2 minutes, while stopping the microwave occasionally and swirling the flask. Once the agarose has gone into solution, check liquid level, add ddH₂O to mark, and swirl. Let the agarose cool until you can barely hold the flask in the palm of your hand without burning it. Add 1.75 μ l of 10 mg/ml ethidium bromide (20,000-fold dilution; final concentration 0.5 μ g/ml) and gently swirl the solution to mix.

NOTE: Ethidium bromide is a suspected carcinogen so do not get it on your

skin. Make sure you wear gloves.

9. Insert comb (B1A-12, 1.5 mm) in gel tray and gently pour the agarose solution into tray. Try to avoid bubbles. If you get a bubble, you can remove it with a pipet tip.
10. Allow the gel to solidify (15-30 minutes) and add 1x TAE (containing 0.5 $\mu\text{g/ml}$ ethidium bromide—your stock is 10 mg/ml) to the gel box until it covers the gel by about 0.5 cm.
11. When the PCR is completed, pulse spin the tubes and transfer 5 μl of each reaction to a new tube. Add 1 μl of loading dye to each 5 μl sample. Place the two tubes containing the remaining 45 μl of each sample on ice to be used later for DNA extraction as described below.
12. Gently remove the comb from the gel by slowly pulling it directly up and out of the gel.
13. Add the 6 μl samples to adjacent wells. Add 10 μl (1 μg) of a molecular weight standard into an adjacent lane.
14. Place the lid on the gel box and make sure the leads are plugged into the power supply and gel box.

IMPORTANT! NEVER FORGET! Always make sure the red lead is attached to the positively-charged electrode (anode) that is on the opposite end of the gel away from the loading wells. The black lead should be attached to the negatively-charged electrode (cathode) on the same end as the loading wells. DNA, which is negatively charged due to its phosphate-containing backbone, will migrate through the gel towards the anode. Also, make sure that the red lead is plugged into the red outlet on the power box and that the black lead is plugged into the black outlet on the power box. Failure to do so will cause the DNA to migrate backwards out of the gel and into the buffer, resulting in the loss of the DNA sample if the mistake is not caught in time!

15. Turn on the power supply and adjust the voltage to ~100 V. Check to make sure there is a current. Two methods: 1) look for bubbles coming up from the electrode wires and/or 2) turn the power supply to milliamps and see if a current is registered (55-65 mA).
16. Allow electrophoresis to continue until the bromophenol blue band is about 1 cm from the bottom of the gel. Turn off the power supply.
17. View the gel under UV light and take a photograph. Estimate the concentration of the band. The instructor and TA will help you with this task.

IMPORTANT! NEVER FORGET! Always wear UV protective goggles, glasses, or face shields when viewing DNA with UV light. UV light can burn the retina of the eyes very quickly and can give you a nasty “sunburn” if you stare at the gel too long without a complete face shield. This is especially true of short wavelength UV light but long wavelength UV light is also dangerous.

DNA products will appear as orange bands. You should see a series of bands in the molecular weight lane. Each of these DNA bands corresponds to a known molecular

weight and can be used to estimate the size of your PCR amplification product. In each PCR lane, you should see a primary band of approximately 1.2 kb and you may see a minor band of about 100 bp or less. The larger 1.2 kb band is your PCR product and the small, minor band is either unused primers or primer dimers that are sometimes formed during PCR. You should estimate the size of your PCR product using the molecular weight standards. If it is the correct size, proceed to the next step. If you do not see a band or it is the incorrect size, talk to one of the instructors or TAs.

Extraction of PCR products

NOTE: WEAR GLOVES DURING THIS PROCEDURE. You will be working with an organic solvent containing phenol. Phenol can severely burn you if it gets on your skin and can cause DEATH if too much of your skin gets contaminated. Please be extremely careful when handling reagents containing phenol. If you happen to get phenol on your skin, get it off immediately by flushing the area with copious amounts of cold water for 15-20 minutes. Inform instructor immediately. After flushing the area, an aqueous solution of 50% PEG300 (if available) can be used to wash the solution for an additional 30 minutes. Seek medical attention.

1. For each PCR sample, follow the procedure below.
2. Add 55 μl of TE to the remaining portion of your PCR sample (final volume 100 μl).
3. Add 100 μl of phenol:chloroform:isoamyl alcohol and replace the cap. **Make sure the cap is tightly closed.**
4. Vigorously shake the tube keeping a **GLOVED** finger over the cap of the tube to prevent leakage. Alternatively, vigorously vortex the tube. The mixture should turn cloudy.
5. Centrifuge the tube for $\geq 14,000 \times g$ for 2 minutes.
6. Carefully transfer the upper aqueous layer (containing the DNA) to a microfuge tube. Make sure you do not transfer any of the lower organic layer. It is better to lose some of the aqueous layer (and therefore DNA) than to contaminate the solutions with the organic layer. Save this tube for step 6. Transfer the lower layer to an organic waste container and discard the tube.
7. Add 100 μl of chloroform:isoamyl alcohol (24:1) to the aqueous phase and again shake vigorously or vortex as described above. Again, the mixture will turn cloudy.
8. Centrifuge the tubes at $\geq 14,000 \times g$ for 2 minutes.
9. Again, carefully transfer the upper aqueous layer to a sterile microfuge tube.
10. Add 10 μl of 3 M sodium acetate and mix by vortexing.
11. Add 275 μl of ice cold ethanol and mix well, first by inversion and then by vortexing. Place in the -20°C freezer for 15 minutes.
12. Centrifuge at $\geq 14,000 \times g$ for 20 minutes.
13. Carefully remove the ethanol supernatant with a pipetman. Do not disturb the precipitate at the bottom of the tube.
15. To remove residual salt, add 200 μl of 70% ethanol and centrifuge the sample again for 5 minutes at $\geq 14,000 \times g$.
16. Remove the ethanol using a pipetman. Do not disturb the pellet but try to remove as

much ethanol as possible.

17. Resuspend the DNA pellet in 5 μl of TE.

Note: In dry places like Utah, you should not let the DNA pellet dry. You should resuspend the pellet soon after removing the ethanol. In more humid climates, some investigators allow the pellets to dry a considerable amount of time. If the DNA dries too much it will be very difficult to resuspend.

Experiment 3. Restriction digestion of amplified PCR products and plasmid vector

1. Perform the following digests of your purified PCR product (insert) and plasmid vector:

Double digest 3 μg of plasmid DNA using the restriction enzymes *SalI* and *NotI*.
Digest 500 ng of plasmid DNA using *SalI* only.
Digest 500 ng of plasmid DNA using *NotI* only.
Double digest all of the remaining PCR product from one reaction with *SalI* and *NotI*.
Digest 1/3 (15 μl) of your PCR product with *SalI* only.
Digest 1/3 (15 μl) of your PCR product with *NotI* only.
Save the other 1/3 (15 μl) for Experiment 5.

10x Buffer D (Promega) will be used for these digests. Use acetylated BSA at a final concentration of 0.1 mg/ml. Use 5-fold more units of enzyme for each digest than you have μg of DNA. Your final digestion volumes should be 35 μl .

<u>Component</u>	<u>DD plas</u> <u>Volume</u>	<u>SalI plas</u> <u>Volume</u>	<u>NotI plas</u> <u>Volume</u>	<u>DD ins</u> <u>Volume</u>	<u>SalI ins</u> <u>Volume</u>	<u>DD NotI</u> <u>Volume</u>
DNA						
10x Buffer D						
BSA-ac 10 mg/ml						
<i>SalI</i> (10 U/ μl)						
<i>NotI</i> (10 U/ μl)						
ddH ₂ O						
Total	35 μl	35 μl	35 μl	35 μl	35 μl	35 μl

2. Digest the DNA samples overnight at 37°C.

DAY 2

Experiment 4. Gel electrophoresis and purification of the digested PCR products and plasmid

1. Pour two 1% agarose gels as you did yesterday, except today you should use the comb with 6 x 1.5 mm teeth (B1A-8).
2. Add 7 μ l of loading dye to each restriction digest, mix, and place on ice. Place the single-digested samples in the refrigerator at 4°C to be used in Experiment 5.
3. Once the gel has solidified, gently remove the comb and add all of your double-digested PCR sample to lane 3 (groups 1 and 3) or 4 (group 2 and 4) and all of your double-digested plasmid DNA to lane 6 (group 1 and 3) or 7 (group 2 and 4). Add 10 μ l of a molecular weight standard to lane 1.
4. Run the gels at 100 V as you did yesterday.
5. Allow electrophoresis to continue until the bromophenol blue band is about 1 cm from the bottom of the gel. Turn off the power supply.
6. Normally you would view the gel under long wavelength UV light. However, we only have short wavelength UV light available, so you want to work quickly. UV light can do all sorts of nasty things to DNA molecules, such as nicking them and introducing thymine-dimers. **Remember to wear a UV face shield.** The digested PCR products should be about 1.2 kb, while the digested vector DNA should be about 2.9 kb. You may also see a faint band that is about 65 bp in length. This is the released portion of the polylinker of the vector. This is the piece of DNA you wanted to eliminate during gel purification, because it could interfere with the ligation of the PCR insert into the vector.
7. Using the slot maker, quickly cut a slot in the gel just anodal to each of the DNA samples. Hold Takara recochips in the buffer to fill up their recovery chambers and then insert one recochip into each slot. Make sure the **BLACK** side of the chip is adjacent to the PCR product. If you place the recochip in backwards, the DNA will not enter the recochip chamber and your sample will be lost.
8. Place the gel back into the gel box, and electrophorese the sample 10 minutes until the amplified DNA has migrated into the recochip.
9. Turn off the power supply and transfer each recochip into a separate microcentrifuge collection tube.
10. Spin for 15 seconds at 5,000 rpm. During this short spin the recochip will break and release its contents (DNA solution) into the microcentrifuge tube. When the spin is completed remove the broken recochip and discard. Keep the microcentrifuge tube with the liquid in the bottom (approximately 30-50 μ l).
11. Dilute the sample up to 500 μ l with TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA).
12. Place in a YM50 microcon tube and spin for 12 minutes at 14,000x g.
13. Invert the filter unit and place it in a fresh collection microcentrifuge tube and spin at 1,000x g for 3 minutes. The desalted purified solution containing the amplified DNA fragment will now be in the collection tube.

Experiment 5. Agarose gel to determine concentration of digested vector and PCR product

1. Follow the directions above from Experiment 2 to make a 1% agarose gel.
2. Use 1/20 of your linearized plasmid sample and 1/5 of your digested PCR sample for this gel. Add 1/6 volume of loading dye. Also run 14 μl (including dye in the volume) of each of your four single-digested samples on this gel.
3. Load samples in adjacent lanes and remember to run a DNA marker lane as well.
4. Run the products at 100 V until the bromophenol blue is about 1 cm from the end of the gel.
5. Photograph the gel. Print enough photographs of the gel, so that each student can have a copy for his/her notebook.
6. Estimate the concentration of your DNA products. If you need help, ask an instructor or a TA for assistance. Confirm that the double-digests worked for the PCR products (inserts).

DNA Ligation

DNA ligation involves the formation of a phosphodiester bond between nucleic acid chains. **The reaction requires either ATP (T4 DNA ligase) or NAD^+ (*E. coli* DNA ligase).** The reaction occurs in three reversible steps: 1) the adenylyl group from the ATP or NAD^+ is transferred to the $\epsilon\text{-NH}_2$ of a lysine on the DNA ligase (either PP_i or NMN are also produced depending on whether ATP or NAD^+ was used in the reaction); 2) the adenylyl group is transferred from the enzyme to the 5'-P group of the DNA strand; and 3) the phosphodiester bond between the two DNA strands is produced by an attack of the 3'-OH group on the activated 5'-P group, with a release of AMP.

T4 DNA ligase is usually preferred for DNA ligation, because it can ligate both cohesive (sticky) ends and blunt ends together, while *E. coli* DNA ligase can only ligate sticky ends, unless volume excluders such as polyethylene glycol (PEG) or Ficoll are added to the reaction.

Transformation of Bacteria

Bacteria can be transformed with foreign DNA by making them “competent.” Competent cells have been prepared in such a way so that they will readily take up DNA from outside the cell. For example, the membranes of chemically competent cells have been damaged, allowing these cells to take up DNA when heat shocked. Electroporation competent cells can be transformed with foreign DNA when shocked with an electric field. We will be transforming bacteria using chemically-competent cells and the heat shock method.

Once the DNA has been taken up by the cells, culture medium is added so that the damaged cells can begin repair. The cells are grown in medium without antibiotic usually for about 1 hour to allow completion of the repair process and the expression of the antibiotic resistance gene. Then the cells are plated onto agar plates containing the selection antibiotic.

Plasmid Preps

Plasmid preps are used to isolate pure plasmid DNA from the rest of the bacterial contents, including bacterial genomic DNA and proteins. Once the liquid cultures have grown overnight, the bacteria are pelleted by centrifugation. Depending on the type of medium used and the application, it is sometimes wise to wash the bacterial pellet once in buffer and then pellet the bacteria a second time. This is not usually necessary for routine plasmid preps, however. The bacteria are then resuspended in buffer and lysed. During the lysing step both plasmid and genomic DNA are denatured in the presence of base (high pH). It is important that this step not last any longer than 5 minutes. If anything, err on the side of a shorter lysis period. If the lysis step is too long, the plasmid can become irreversibly denatured, leading to a decrease in the yield of supercoiled double-stranded plasmid DNA and a concomitant increase in single-stranded plasmid DNA that migrates slightly faster than supercoiled plasmid DNA. Once the lysis step is completed, the solution is immediately neutralized with a high concentration of KOAc solution. This allows the plasmid DNA to renature and form double-stranded DNA (dsDNA) once again. Also, a fluffy white precipitate will form consisting of genomic DNA, proteins, cell debris, and KDS (potassium dodecyl sulfate). The mix will be passed through a filter to remove the precipitate and the plasmid DNA will then be passed through a column. The plasmid DNA will be washed while in the column and then eluted.

Spectrophotometry is performed at 260 nm and 280 nm to determine the quantity and purity of the plasmid prep. Pure DNA should have a 260/280 ratio of about 1.8 ± 0.1 . Ratios below 1.7 suggest that the sample is contaminated with protein, while those above 1.9 suggest that the sample may contain substantial amounts of RNA. The amount of plasmid DNA can be calculated using the following constant. This is a constant that you will want to know without looking it up, so memorize it.

OD of 1 at 260 nm = 50 $\mu\text{g/ml}$ dsDNA

Assume you have diluted a portion of your plasmid prep 1/100 in TE buffer. You blank your spectrophotometer with TE and then get a reading of 0.1934 at 260 nm and 0.1048 at 280 nm. The following calculations can be made.

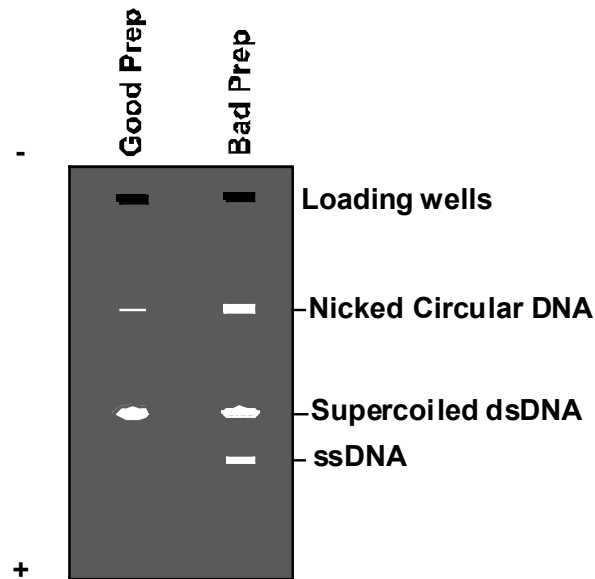
$$260/280 \text{ ratio} = 0.1934/0.1048 = 1.845$$

$$\text{Estimated yield} \quad 0.1934 \times 100 \text{ (dilution factor)} \times 50 \mu\text{g/ml DNA} = 967 \mu\text{g/ml}$$

Assuming that you have a volume of 100 μ l of plasmid DNA, your total yield would be calculated as follows:

$$967 \mu\text{g/ml DNA} \times 0.1 \text{ ml} = 96.7 \mu\text{g of plasmid DNA}$$

Once the DNA concentration has been determined by spectrophotometry, the integrity of the plasmid DNA can be determined by agarose gel electrophoresis. 100-200 ng of plasmid DNA can be run on a 1% agarose gel. This allows the researcher to confirm DNA concentration and determine whether the DNA is supercoiled, nicked, or permanently denatured. A successful plasmid prep will result in most of the DNA being in the supercoiled form with a minor fraction being nicked. There should be no irreversibly denatured ssDNA. An example of how the gel should and should not look is shown below:



Experiment 6. Ligate insert and plasmid DNAs together

1. Use 50 ng of plasmid DNA and a 3:1 molar ratio of insert to plasmid in the ligations.
2. Set up the ligations in a final volume of 20 μ l:

Vector + Insert Ligation

Plasmid DNA (50 ng)	calculate
Insert DNA (3:1 molar ratio to plasmid)	calculate
5x Buffer (contains ATP)	4 μ l
T4 DNA Ligase (1 U)	1 μ l
ddH ₂ O to 20 μ l	calculate

Vector Ligation

Plasmid DNA (50 ng)	calculate
5x Buffer	4 μ l
T4 DNA Ligase (1 U)	1 μ l
ddH ₂ O to 20 μ l	calculate

3. Leave ligation reactions at room temperature for 1 hour.

Experiment 7. Transform bacteria with recombinant plasmid

1. Thaw competent cells on ice.
2. Transfer 100 μ l of competent cells to four prechilled Falcon 2059 tubes and place on ice.
3. Add 2 μ l of 1.22 M β -mercaptoethanol to each tube. For future reference, β -mercaptoethanol ordered from Sigma is 14.3 M and must be diluted with H₂O to 1.22 M. This step enhances transformation efficiency but is not required.
4. Swirl the cells gently and place on ice for 10 minutes. Swirl gently every 2 minutes during this incubation time.
5. Add 10-25 ng (2-5 μ l) of DNA from your ligation mixes to two of the tubes and swirl gently. To the third tube, add the same amount of linearized unligated vector DNA. To the fourth tube, add 1 ng of supercoiled BS II plasmid DNA.
6. Place on ice for 30 minutes.
7. Heat shock for 45 seconds in a 42°C water bath.
8. Incubate the cells on ice for 2 minutes.
9. Add 900 μ l of SOC or NZY⁺ broth to each tube and incubate at 37°C for 1 hour with shaking at 225 rpm.
10. Plate 100 μ l of the transformed cells on S-Gal agar plates containing ampicillin. Plates should either be prewarmed to 37°C or be room temperature. Spread the cells and let the plates stand upright for about 5 minutes. Invert the plates and place in 37°C incubator overnight.

Recipes for Broth

NZY ⁺ Broth	1% w/v	NZ amine (casein hydrolysate)
	0.5% w/v	Difco yeast extract
	0.5% w/v	NaCl
		Adjust to pH 7.5 and autoclave
	12.5 mM	MgCl ₂ Add after autoclaving
	12.5 mM	MgSO ₄ Add after autoclaving
	20 mM	glucose Add after autoclaving
SOC	2% w/v	bacto-tryptone
	0.5% w/v	bacto-yeast extract
	0.05% w/v	NaCl
		Mix well until solutes are dissolved
	2.5 mM	KCl
		Adjust pH to 7 with 5 M NaOH
		Autoclave
	10 mM MgCl ₂ Add after autoclaving	
	20 mM glucose Add after autoclaving	

Experiment 8. Plasmid Prep

1. Pick a colony from the bacterial plate using a sterile toothpick or pipet tip and place it in 25 ml of NYZ broth containing 100 $\mu\text{g/ml}$ ampicillin. The cultures will be grown in 125 ml culture flasks.

These colonies contain plasmids that have previously been characterized and are known to contain the *hERG* ORF.

2. Place the flasks in the 37°C shaking incubators and grow overnight.

DAY 3

Experiment 7. Transformation (Continued)

11. Check bacterial plates for colonies. You should see very few, if any, colonies in your control nonligated and control ligated vector alone plates. You should see numerous black colonies in your supercoiled BS II plasmid plates. You should see many colonies in your vector + insert plates. Most of these colonies should be white and these should contain your insert. Although you should see very few, if any, black colonies, there may be a few. These represent bacteria that have been transformed with the original plasmid that does not contain an insert. If you were to perform the following experiments using these colonies, you would want to select several of the white colonies and grow them up overnight. You would then perform minipreps and determine which one of the colonies had your insert by restriction digest. The inserts would be sequenced to ensure no errors had been generated during PCR. Larger plasmid preps would then be prepared for subsequent steps. In our experiment, we have by-passed the miniprep procedure and have gone directly to a larger prep using colonies that have been previously characterized as described above. However, we will still perform a restriction digest to confirm the presence of the insert so that you can see how this procedure is done.

Experiment 8. Plasmid prep (Continued)

Prepare the reagents from the Sigma Genelute HP Plasmid Midiprep Kit by adding 750 μ l of the RNase Solution to the Resuspension Solution (then store at 4°C) and 120 ml of 100% ethanol to Wash Solution 2. Mark the tops of the bottles, once these solutions have been added. Only one person in the class should perform this step.

3. Remove your liquid cultures from the incubator and transfer the contents to a 50 ml centrifuge tube.
4. Centrifuge at 3,000-5,000 x g for 10-15 minutes at 4°C.
5. Discard supernatant. Invert tubes and let the residual culture medium drain for a couple of minutes.
6. Resuspend the bacterial pellet in 4 ml of Resuspension Solution. Make sure the cells are completely resuspended. This will probably require that the samples be vortexed or resuspended by pipetting the cells up and down.
7. Once the cells are completely resuspended, add 4 ml of Lysis Solution and replace the cap. Immediately mix the contents thoroughly by GENTLY inverting the tube 6-8 times. Incubate the tube at room temperature for 3-5 minutes. **NOTE: DO NOT exceed 5 minutes. This is the lysis/denaturing stage and if the reaction proceeds for more than 5 minutes you run the risk of permanently denaturing your plasmid. Also, please replace the cap on the Lysis Solution bottle immediately when you are through dispensing the solution to prevent acidification of the buffer from CO₂ in the air.**

8. Neutralize the reaction by adding 4 ml of ice cold Neutralization Solution. Replace cap and immediately mix thoroughly by GENTLY inverting the tube 6-8 times.
9. Add 3 ml of Binding Solution and gently invert 1-2 times to mix.
10. Immediately add the mix to the barrel of the filter syringe and let sit for 5 minutes.
11. While incubating the mix for 5 minutes, place a binding column onto the vacuum manifold and apply the vacuum.
12. Add 4 ml of Column Preparation Solution to the column and allow it to pass through. Do this procedure towards the end of the 5 minute incubation period, so that the column is prepared just prior to use.
13. Hold the filter syringe over the column and gently insert the plunger to expel the cleared lysate. Allow the lysate to pass through the column.
15. Add 4 ml of Wash Solution 1 to the column and allow it to pass through.
16. Add 4 ml of Wash Solution 2 to the column and allow it to pass through.
17. Leave vacuum on for 10 minutes to dry the column.
18. Transfer the column to the collection tube provided.
19. Add 1 ml of Elution Solution and centrifuge in a swinging bucket rotor at 3,000x g for 5 minutes.
20. Gently vortex the plasmid solution and determine DNA yield as follows.
 - a. Blank the spectrophotometer with 50 μ l of TE.
 - b. Transfer 5 μ l of plasmid solution into 45 μ l of TE.
 - c. Mix and transfer to a spectrophotometric cuvette.
 - d. Determine absorbance at 260 nm and 280 nm.
 - e. Calculate yield and purity as described above.

Experiment 7. Insert confirmation and preparation of DNA template for *in vitro* transcription

1. Using the knowledge you have already gained in this course, linearize 3 μ g of your plasmid with *EcoRI* in a 100 μ l reaction. This linearized plasmid will be used for preparing RNA for *Xenopus* oocyte injections. Also confirm that the plasmid contains the appropriate sized insert by performing a 30 μ l double digest of 200 ng of the plasmid using *HindIII* and *BamHI*. Use Promega Buffer E for this digest and a 10-fold excess of restriction enzyme. Incubate the reactions for several hours at 37°C.

Note: In this case, the insert size is 3.5 kb and the vector is 3 kb. If we had been using the recombinant plasmid you prepared, we would have digested it with *SalI* and *NotI* and the expected insert size would have been 1.2 kb and the vector would have 2.9 kb.

2. During linearization of the plasmid, pour a 1% agarose gel.

Wear gloves for the following procedure and remember the precautions one must take when working with phenol. Perform the following DNA extraction on the LINEARIZED PLASMID ONLY. There is no need to extract the DNA from the reaction designed to check insert size. Just leave this reaction at 37°C until you are ready to run the gel.

3. Add 100 μ l of an organic extraction solution containing phenol:chloroform:isoamyl alcohol (25:24:1) to the reaction containing the linearized plasmid. Thoroughly mix by rapid shaking or vortexing.
4. Spin the tube at $\geq 14,000 \times g$ for 2 minutes.
5. Carefully transfer the upper aqueous layer (containing the DNA) to another tube. Make sure you do not transfer any of the organic layer. It is better to lose some of the aqueous layer (and therefore DNA) than to contaminate the solutions with the organic layer.
6. Add 100 μ l of chloroform:isoamyl alcohol (24:1), thoroughly mix by rapid shaking or vortexing.
7. Spin the tubes at $\geq 14,000 \times g$ for 2 minutes.
8. Again, carefully transfer the upper aqueous solution to a new tube.
9. Add 10 μ l of 3 M sodium acetate and mix by vortexing.
10. Add 275 μ l of ice cold ethanol and mix well, first by inversion and then by vortexing.
11. Centrifuge at $\geq 14,000 \times g$ for 20 minutes.
12. Carefully remove the ethanol supernatant with a pipetman. Do not disturb the precipitate at the bottom of the tube.
13. To remove residual salt, add 200 μ l of 70% ethanol and centrifuge the sample again for 5 minutes at $\geq 14,000 \times g$.
14. Remove the ethanol using a pipetman. Do not disturb the pellet but try to remove as much ethanol as possible.
15. Resuspend the DNA pellet in 6 μ l of Nuclease-free water.
16. Determine the DNA concentration using spectrophotometry. Dilute 0.5 μ l of DNA into 49.5 μ l of TE and then determine absorbance at 260 nm and 280 nm.
17. Confirm complete digestion of the plasmid and DNA concentration by running a 1% agarose gel. Transfer 0.5 μ l (~250 ng) of digested DNA to 9.5 μ l of TE and add 2 μ l of loading dye. Run the digested plasmid on the gel along with 100 ng of undigested plasmid DNA. The undigested plasmid DNA serves two purposes: 1) it will allow you to assess the integrity of the plasmid DNA (*i.e.*, how good your plasmid prep was) and 2) it can act as a control. Be sure to add 1/6 volume of loading dye to the undigested plasmid control before loading it onto the gel.
18. Photograph the gel.

DAY 4

Working with RNA

In general, RNA is less stable than DNA due to its 2' OH group, which can act as a nucleophile on the adjacent phosphodiester bond. This is especially true in basic solutions. Unlike DNases, many RNases do not require divalent metal ions for activity and instead take advantage of the 2'-OH to act as a reactive species. RNA is also more sensitive to repeated freeze/thaw cycles.

RNases are ubiquitous, being found on your skin, lab benches, etc. Unlike DNA, RNases are extremely stable and many types are resistant to prolonged boiling and autoclaving. Because of these properties of RNA and RNases certain precautions should be followed when working with RNA. These are listed below.

1. Use commercially available RNase-free solutions whenever possible. When unavailable, you can prepare many solutions using the RNase inhibitor diethyl pyrocarbonate (DEPC). When treating solutions with DEPC, add 0.1% v/v to the solution, stir for at least 1 hour, and autoclave for 1 hour. Be careful when using DEPC. It is a suspected carcinogen. Autoclaving destroys unreacted DEPC, which could otherwise inactivate other proteins or RNA found in a treated solution. Solutions containing primary amines, such as Tris solutions cannot be DEPC-treated directly, but can be prepared in DEPC-treated water that has already been autoclaved to inactivate the residual DEPC. When doing this, use Tris that has been set aside for RNA work alone and has therefore been treated appropriately. To adjust the pH of such solutions, clean the pH electrode first with electroZAP and then adjust the pH. Do not use active DEPC to clean polycarbonate or polystyrene containers such as gel electrophoresis tanks. DEPC can destroy these materials.
2. Always wear powder-free gloves. Treat these gloves with RNaseZAP or RNase-OFF. These solutions can be sprayed directly onto the gloves, rubbed in, and allowed to dry.
3. Treat your bench space with RNaseZAP or RNase-OFF.
4. You can assume that sterile, disposable plasticware is RNase-free.
5. Glassware can be baked at $\geq 180^{\circ}\text{C}$ for several hours to destroy RNases.

In vitro Transcription

In vitro transcription is a convenient method for making microgram quantities of RNA. This process can be used for many purposes including RNA *in situ* hybridization, blot hybridizations, nuclease protection assays, aRNA amplification, *in vitro* translation to

make proteins, RNA-protein structural analyses, ribozyme analyses, and for injection into cells to test the function of the gene. Next week, you will be injecting frog oocytes with RNA made by *in vitro* transcription. One of the RNAs you will be injecting will be prepared from the same clone you are using today. This clone contains the *hERG* cDNA, which encodes a sodium channel. Mutations in this gene can cause long QT syndrome, which is often a silent disease that can cause sudden death. The RNA you are making today could be used in these frog oocyte injections.

To perform *in vitro* transcription, the plasmid must contain a phage promoter, such as T7, T3, or SP6. Transcription reactions using the T7 or T3 are usually faster and require less time than SP6 reactions. The promoter must be upstream of the cDNA and the plasmid must be linearized downstream of the cDNA to prevent run-on of the transcription products. The linearized plasmid is then mixed with the appropriate phage RNA polymerase, NTPs, and a buffer containing Mg^{2+} and dithiothreitol (DTT). Transcription is allowed to proceed for 1-2 hours depending on the yield of RNA required and the RNA polymerase that is being used. SP6 reactions are generally performed for 2 hours, while T7 and T3 reactions can be run for 1-2 hours. Using such a method, 15-30 μg of RNA can be synthesized.

Spectrophotometry is performed at 260 nm and 280 nm to determine the quantity and purity of the RNA. Pure RNA should have a 260/280 ratio of about 2.0 ± 0.1 . The amount of RNA can be calculated using the following constant. This is a constant that you will want to know without looking it up, so memorize it.

OD of 1 at 260 nm = 40 $\mu g/ml$ RNA

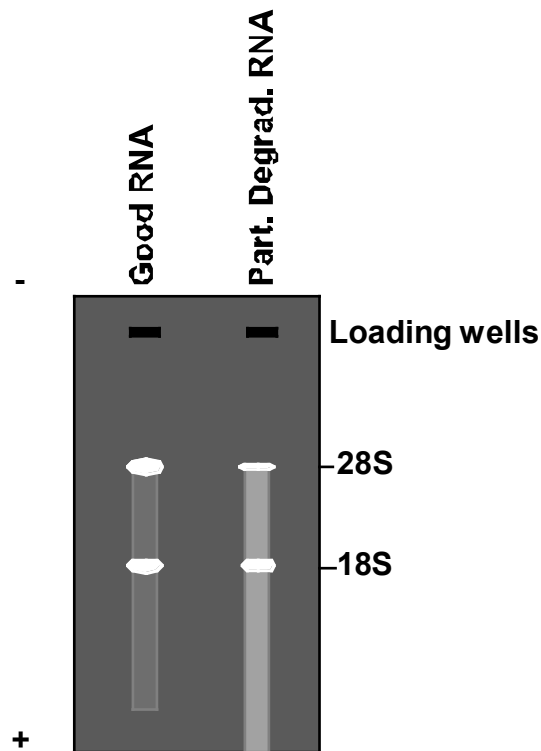
The use of organic solvents during nucleic acid purification

An extremely effective method for separating nucleic acids from protein is through the use of organic solvents such as phenol and chloroform. One solution commonly used for this purpose is a phenol:chloroform:isoamyl alcohol solution with the ratios of 25:24:1. Phenol denatures proteins and facilitates the removal of proteins from nucleic acids. Chloroform is also a protein denaturant that stabilizes the interphase, reduces the amount of aqueous phase retained in the organic phase, increases the mixture's density, and facilitates the removal of lipids. Isoamyl alcohol is added because it helps prevent foaming, enhances phase separation, and helps in the collection of denatured proteins at the interphase. The pH is highly critical. When the solution is at pH 7.0 or greater, both DNA and RNA will be in the aqueous phase. If the solution is acidic, DNA will partition into the organic phase leaving only RNA in the aqueous phase. This dependence on pH can be used to help separate RNA from DNA.

Formaldehyde agarose gel electrophoresis

RNA is often run on formaldehyde agarose gels. These gels maintain RNA in its completely denatured single-stranded form and allow a fairly accurate estimate of the size of an RNA molecule. When performing northern blot analysis, formaldehyde gels are used to separate the RNA according to size before transferring it to a membrane to be hybridized with a labeled probe. Also, formaldehyde gels can be used to assess the integrity of RNA extracted from cells or tissues or RNA made from *in vitro* transcription. We will be running a formaldehyde gel for the latter purpose.

When running total RNA that has been extracted from cells or tissues, the researcher looks for the prominent 28S and 18S ribosomal bands. If the RNA is intact the 28S band should be at least as intense as the 18S when stained with ethidium bromide. Most often the 28S band is slightly more intense. The mRNA will form a light smear throughout the gel. If the RNA is partially degraded, the 18S band will be more intense than the 28S band and there will be a greater background smear. See the example below.



Experiment 8. *In vitro* transcription using Ambion's High Yield Capped RNA Transcription Kit

1. Place the RNA Polymerase Enzyme Mix on ice. Vortex the 10x Reaction Buffer and 2x NTP/CAP until they are completely in solution. Once thawed, store the 2x NTP/CAP solution on ice (this solution contains the ribonucleotides) but store the 10x Reaction Buffer at room temperature (RT). Pulse spin just before use.
2. Add the following reagents at RT in the order given to avoid precipitation:

Nuclease-free water	~4 μ l (up to 20 μ l final volume)
2x NTP/CAP	10 μ l
10x Reaction Buffer	2 μ l
1 μ g linearized DNA template	~2 μ l
Enzyme Mix	2 μ l

3. Mix the tube by gently flicking the tube and then pulse spin.
4. Incubate the tube at 37°C for 2 hours.
5. Add 115 μ l of Nuclease-free water and 15 μ l of Ammonium Acetate Stop Solution (5 M ammonium acetate, 100 mM EDTA). Mix thoroughly.
6. Extract once with 150 μ l of phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1) as described above.
7. Precipitate the RNA by adding an equal volume of isopropanol (130-150 μ l) and mixing well.
8. Chill the mixture for 15 minutes at -20°C.
9. Centrifuge at 4°C for 15 minutes at $\geq 16,000 \times g$.
10. Carefully remove the supernatant using a pipetman and resuspend the RNA in 15 μ l of nuclease-free water.
11. Dilute 0.5 μ l of the RNA into 49.5 μ l of TE and determine the absorbance at 260 nm and 280 nm. Calculate RNA concentrations as described above.

Experiment 9. Formaldehyde agarose gel electrophoresis

1. Prepare a 1% agarose formaldehyde gel as follows.

0.35 g agarose
31.8 ml of ddH₂O

Melt agarose and allow to cool as you normally would before adding EtBr. However, do not add EtBr. Instead, quickly add the following reagents and gently swirl, trying to avoid air bubbles. Immediately pour the gel. Use the B1A-8, 1.5 mm comb. **CAUTION: Do not inhale with your face directly over the flask. Turn away to inhale. Formaldehyde fumes are dangerous and will irritate your lungs and eyes.**

1.75 ml 20x MOPS
1.42 ml 12.3 M formaldehyde (final concentration 0.5 M)

2. Allow the gel to solidify for no more than 30 minutes. During this time period, prepare 500 ml of the following electrophoresis buffer.

1x MOPS
0.5 M formaldehyde

3. Once the gel has solidified, submerge it in electrophoresis buffer.
4. Prepare the RNA samples for electrophoresis as follows. Also prepare RNA markers. Mix the following reagents together in two separate tubes:

1.25 μ l 20x MOPS buffer (from frozen stock)
4.47 μ l 37% formaldehyde
12.5 μ l formamide
1.8 μ l 1 mg/ml EtBr

Add 500 ng of RNA to one of the tubes and 5 μ g of RNA marker to the other tube

Bring volume up to 25 μ l in nuclease-free water

Heat at 65°C for 5-10 minutes

5. While the samples are incubating, remove the comb from the gel and rinse the wells with buffer. **CAUTION: Do not inhale while you are directly over the sample. Turn away to take a breath. Remember the buffer contains formaldehyde. Do this procedure rapidly.** Prerun the gel at 70 V for 5 minutes.
6. After the 5-10 minute 65°C incubation, place the samples on ice, spin briefly, and add 5 μ l of loading buffer.
7. After the prerun, turn off the power supply, rinse the wells again and add the samples. **CAUTION: Remember the formaldehyde!**
8. Turn on the power supply and run the gel at 70 V until the bromophenol blue dye is about 1 cm from the bottom of the gel.
9. Photograph the gel.

DAY 5

Protein gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) can be used for a variety of purposes including the separation of proteins, DNA, and RNA. The separated molecules can either be pre-labeled, stained directly while in the gel, or blotted onto a membrane and probed with labeled DNA, RNA, protein, or antibodies. PAGE is routinely used in DNA sequencing and western blotting. Western blotting involves the identification of specific proteins by transferring the separated proteins to a membrane and then probing the membrane with an antibody that recognizes the specific protein. The bound proteins are usually visualized through enzymatic reactions or chemiluminescent techniques. Today, we will simply separate proteins by PAGE and then stain the separated proteins with a dye that binds to all proteins.

PAGE

1. Fill the lower buffer chamber with 180 ml of 1x Running buffer.
2. Assemble the precast gel (15%, 15-well Bio-Rad gel) into the gel holding apparatus and place it in the lower buffer chamber.
3. Partially fill the upper chamber with 1x Running buffer so that the sample wells are filled with buffer. Be sure there are no buffer leaks from the chamber.
4. Straighten the wells if needed and wash them with Running buffer.
5. Pipet 5 μ l (200 ng) of protein into a microcentrifuge tube. Pipet 10 μ l of the molecular weight marker into another tube.
6. Add 5 μ l of loading dye of the protein sample. Immediately vortex and heat the protein samples and molecular marker for 5 minutes at 95°C.
7. Load 10 μ l of each sample and the molecular weight marker into separate lanes. Add blank samples containing loading dye to the lanes containing no protein.
8. Carefully fill the upper chamber with buffer. Make sure the loaded samples are not disturbed.
9. Run at 200V for 30-45 minutes.
10. Turn off the power and disassemble the apparatus.
11. Pop the plates apart with a spatula and place the gel into ddH₂O.
12. Wash the gels 3 times with ddH₂O for five minutes each wash.
13. Place the gel in GelCode Blue for 1 hour.
14. Rinse thoroughly in ddH₂O.
15. Examine the gel for protein bands. Determine the molecular weight of your protein by comparing it to the molecular weight standards.

4x Running Buffer

57.6 g glycine
12.11 g Tris base
40 ml 10% w/v SDS
Bring up to 1 liter in ddH₂O
no need to pH. When diluted to 1x, pH will be 8.3
Can be stored for up to 1 month at 4°C

2x Sample Buffer

4 ml 10% SDS
2 ml glycerol
4 ml 0.5 M Tris-HCl (pH 6.8)
1 mg bromophenol blue
0.2 ml β-mercaptoethanol
Freeze and store in aliquots at -70°C

Computer Programs for Sequence Analyses of DNA and Proteins

There are several computer programs that are useful for sequence analyses of DNA, RNA, and proteins. In this course, you will be introduced to five such programs that can be accessed through the internet. These programs include BLAST searches, ClustalW, T-COFFEE, SignalP, and Tmpred. There are many other programs that may also be useful to you in your research, but we do not have time to cover these in this course.

BLAST searches

BLAST searches can search for homology between DNA or protein samples. BLAST searches come in several varieties including nucleotide-nucleotide BLAST (blastn), translated query vs. protein database (blastx), protein query vs. translated database (tblastn), translated query vs. translated database (tblastx), align two sequences (bl2seq), and protein-protein BLAST (blastp). All of these programs can be accessed at the following web site: <http://www.ncbi.nlm.nih.gov/BLAST/>. You should become familiar with these programs. Start by going to <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>. In the *Search* dropdown box select Nucleotide. In the *for* box type in bmp-4. Select the first sequence you see, e.g., [NM_130850](#). This will give the DNA and protein sequence of this gene. Go to the DNA sequence and select the entire sequence by clicking and dragging the mouse over it. Copy the sequence. Go to <http://www.ncbi.nlm.nih.gov/BLAST/>. Choose tblastx. Paste in the sequence and hit search. In several minutes you will observe numerous hits. These hits are from translated sequences of DNA that are in the database that have homology to the translated bmp-4 sequence you selected. You can continue to explore the other BLAST programs.

ClustalW and T-COFFEE

These programs help you align multiple protein sequences. They can be accessed at <http://www.ebi.ac.uk/clustalw/> and <http://www.ch.embnet.org/software/TCoffee.html>, respectively. To see how these programs work, go back to the <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi> site and select several bmp-4 sequences by copying and pasting and moving back and forth between the PubMed and ClustalW sites or PubMed and T-COFFEE sites. Once you have pasted the sequences into these sites and have them in the proper format hit Run button to start the alignment process.

SignalP

The SignalP program allows you to determine whether your protein has a signal peptide. Signal peptides are found in secreted proteins and cell membrane proteins with extracellular N-term ends. To access this program go to the following web site: <http://www.cbs.dtu.dk/services/SignalP/>. Use the bmp-4 protein sequence and run the SignalP program to determine whether this protein has a signal peptide. Do you think it will? What do you know about bmp-4?

TMpred

The TMpred program predicts whether a protein has transmembrane regions. Proteins with transmembrane regions are usually embedded in cell membranes. Many receptors have transmembrane regions. This program can be accessed at the following web site: http://www.ch.embnet.org/software/TMPRED_form.html. Determine whether bmp-4 has any predicted transmembrane regions. Do you expect it to have any? Go to PubMed and search for ALK-3. Determine whether it has any predicted transmembrane regions.

Primer3

Primer 3 is a very popular primer design tool for designing primers for PCR and oligonucleotides for hybridization. It was developed at the Whitehead Institute/MIT Center for Genome Research. PCR primer design requires the researcher to consider many factors, such as the T_m of the primers, avoidance of self-complementarity and primer-dimers, etc. Therefore, a computer program, such as the web-based Primer3 program, is useful for rapidly identifying suitable primer sets for your DNA templates. When you provide your DNA sequence, the program generates the five best candidate primer sets with information on T_m , primer length, GC content, primer-dimer possibilities, and PCR product size. This program can be accessed at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi.