Polymerase Chain Reaction

The development of the polymerase chain reaction (PCR) ushered in an unprecedented era of control over the DNA molecule. The polymerase chain reaction allows for the production of large quantities of identical DNA molecules. It is that simple.

Prior to the use of the polymerase chain reaction, the synthesis of DNA was largely the domain of organisms. Certainly you could chemically synthesize short oligonucleotides in the laboratory, but these were rarely longer that fifty base pairs. To work with longer molecules, you were forced to isolated DNA from living organisms. With the exception of some viruses, the DNA one purified was a conglomeration of many molecules of varying length and sequence. The polymerase chain reaction changed that. Using PCR, fantastically large quantities of identical DNA molecules could be produced in several hours using simple and inexpensive materials. The science of molecular biology was never the same.

The fatefully dawn of the PCR era came on a warm Friday evening in May of 1983 (*1*). Kary Mullis, staff scientist at Cetus Corporation, was thinking about a method for DNA sequencing using the short oligonucleotides Cetus could synthesize faster than they could use as he drove from Berkeley, CA to his mountain home in Mendocino County. What he fixated on during that drive was the power of sequence amplification that came from repeating his reaction. If he started with one molecule, after one cycles he would have two molecules, after the second cycle, he would have four. Then eight, sixteen, thirty-two, sixty-four ... until, after just twenty cycles, there would be more than one million identical molecules (2^{20}) produced. An example of this exponential amplification is shown below in Figure 1. As with many novel ideas, few people were interested in the idea and the first reaction wasn't completed until December of 1983. The first paper using the polymerase chain reaction was published in 1985 and the definitive papers describing the method were published in 1987 and 1988 (*2,3,4*). The ideas about sequencing with this method never worked out, but Kary Mullis was awarded the Nobel Prize in chemistry in 1993 for his work on the polymerase chain reaction.

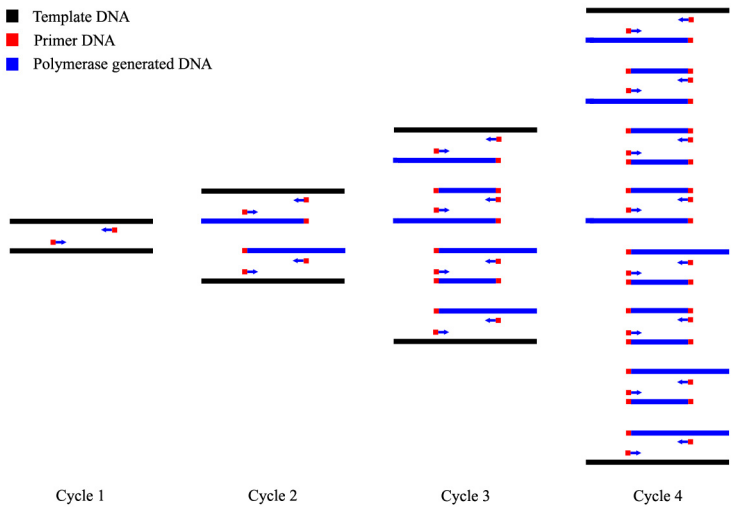

Figure 1: Exponential growth of the small product in the polymerase chain reaction. Note that amplification continues from the first products as well as the template in the second and subsequent rounds. However, after only a few cycles, the small product predominates.

The Polymerase Chain Reaction

The polymerase chain reaction is built from just a few readily available components. The key reagents are a buffering system, deoxynucleotide triphosphates, DNA polymerase, template DNA to copy and a pair of short oligonucleotide primers to be extended. The first three reagents are common to every reaction and are often prepared together as the "master mix" in order to simplify reaction preparation. Template DNA can be isolated from any source (DNA is fairly stable—the source does not need to be fresh), and only a vanishingly small quantity is required due to the amplification power of the chain reaction. The keys to primer design are well characterized and the cost of PCR primers is negligible.

Primers The polymerase chain reaction requires two oligonucleotide primers. These oligonucleotides are designed to flank the region of DNA to be amplified, one complimentary to the coding strand (often referred to as the forward or + primer) and one complimentary to the non-coding strand (referred to as the reverse or − primer). Also, single strand DNA sequences are *always* written in the $5'\rightarrow3'$ direction; the sequence of the reverse primer must be reversed to illustrate it in binding orientation relative to a double strand DNA template. These primers serve both to recognize the DNA sequence to be amplified as well as serve as the primer extended by DNA polymerase. Oligonucleotides are readily synthesized in any sequence for 30-60 cents per base on the scales necessary for several hundred reactions. Typically amplification primers are 18-24 nucleotides long (lengths of 15-35 nucleotides span the functional range for PCR primers). Assuming a 24 nucleotide primer at \$0.35 per base and a moderate yield (enough primer for 125 reactions), the cost of a single primer in each reaction would be about \$0.07. While primers are a rather inexpensive component of the reaction, the design of these sequences can have dramatic effect on the accuracy (amplifying the correct sequence) and efficiency (producing robust amplification) of the reaction.

Melting temperature (T_m) of duplex DNA is the temperature at which one half the molecules are in the double stranded helical conformation and one half are single stranded (also known as the random coil conformation). For the polymerase chain reaction, melting temperature will define the temperature used in the second step (annealing) of the thermal cycle. For any primer pair, being able to predict the melting temperature is critically important. Many have developed empirical and theoretical equations for the calculation of primer annealing temperatures. An early (and often applied due to its simplicity) suggestion for the estimation of T_m was suggested by Suggs, *et al.*

$$T_m\ (^oC) = 4^oC \times (G + C) + 2^oC \times (A + T), \tag{1}$$

where G, C, A and T represent the numbers of these nucleotides in a primer (*5*). This estimation of T_m is simple arithmetic using base the composition of the primer. The initial suggestion of this formula was DNA sequences shorter than 14 base pairs, under the size of the typical primer used in PCR.

Another empirical suggestion for the calculation of T_m using composition was given by Chester and Marshak

$$T_m(^oC) = 69.3 + 0.41 \times (\%GC) - 650/n, \tag{2}$$

where %GC is the GC percentage and n is the length of the primer (*6*). This estimation is only slightly more complicated (enough so that you'd probably want to use a calculator), yet it is still tied directly to the base composition of the primer. While these methods are relatively easy to apply, they do not consider the theoretical view of nucleic acid dimerization.

The nearest-neighbor model is the best theoretical description of duplex nucleic acid stability (*7*). In brief, the nearest neighbor model suggests that duplex stability is based not on composition but on sequence. The equation derived from this model to calculate T_m is

$$T_m(^oC) = \frac{\Delta H^o}{\Delta S^o + R\ln(\frac{C_T}{4})} - 273.15, \quad (3)$$

where ΔH^o is the standard enthalpy change for helix formation in the primer, ΔS^o is the standard entropy change for helix formation in the primer, R is the gas constant (1.987 cal/mol·K), and C_T is the primer concentration. This formula assumes that short, random DNA sequences melt *via* a two-state transition. This assumption is typically valid for synthetic oligonucleotides, but would not apply for longer DNA molecules with natural sequences that populate many intermediate structures during melting. In order, then, to calculate T_m, ΔH^o and ΔS^o must first be calculated. The values for both of these parameters have been measured several times and combined into a unified set of parameters (see reference *8* for review and the table of unified parameters).

The values of ΔH^o and ΔS^o are typically measured in 1 M NaCl. This high concentration of salt is used to screen charges in the DNA backbone and promote double helix formation. It is, however, very uncommon to conduct PCR is such high concentrations of sodium chloride (or, more generally, monovalent cations). Thus, there has been interest in correcting the value of ΔS^o (where the salt effects predominate) for the considerably lower salt concentrations found in typical polymerase chain reactions. The typical correction equations for the effects of salts on ΔS^o were determined empirically by von Ahsen *et al.* (*8*). Their method consists of the application of two equations to correct ΔS^o. The first equation calculates the total *effective* sodium cation concentration

$$[Na^+_{eq}] = [Monovalentcations] + 120\sqrt{[Mg^{2+}] - [dNTPs]},$$

where all concentrations are given in units of mmol/L and the monovalent cation concentration term encompasses Na^+, K^+ and $Tris^+$ concentrations (the net effect of each of these cations seems to be similar). The correction for ΔS^o then is given as

$$S^o[Na^+] = S^o[1M\ Na^+] + 0.847 \times n \times log[Na^+_{eq}],$$

where $S^o[1M\ Na^+]$ is ΔS^o in 1 M NaCl, n is the total number of phosphate groups in the duplex divided by 2 (or, more easily, equal to the length of the primer - 1) and $[Na^+_{eq}]$ is the effective sodium concentration as calculated above (*9*). The net effect of this correction is to raise the value of ΔS^o, thereby lowering the calculated T_m of the primer, as would be expected in solutions of lower salt concentration.

Closely linked to the T_m of the oligionucleotide is the GC content. As is evident from equation 2 above, GC content is directly related to T_m. Thus, optimizing primer length and GC content is an easy way to achieve T_m values which are very similar between primers, both as a functional pair as well as between primers designed for different experiments. The latter is often a goal, designing primers which always function at the same set of reaction conditions. In this way primer design is controlled between experiments and various primers, even those designed years apart, can be used together in novel amplifications. Typically, GC content in a primer is around fifty percent (the usual target range during design is forty to sixty percent). Radically different GC content leads to primers with unusual properties that may not function optimally under typical PCR conditions.

Another concern in primer design is the selection of nucleotides at the $3'$ end. As GC base pairs are stronger than AT base pairs, many primers are designed with one or two GC base pairs within the final five nucleotides at the $3'$ end of the primer, or a single GC base pair at the terminal $3'$ end. This added stability at the site where DNA polymerase begins extension is thought to aid in the priming of the reaction as $3'$ terminal mismatches have the largest effect on polymerase chain reaction yields (*10*). In contrast to the requirement for good base pairing at the $3'$ end, a mismatch (or multiple mismatches) is well tolerated in the middle of the primer or toward the $5'$ end. Remembering that the primer is extended to form the product and that the primer sequence, then, is part of the product, this observation can be utilized in order to incorporate specific nucleotide changes into the amplified sequence.

This might be done in order to introduce a restriction site or change a gene sequence. The GC base pair or two, near the $3'$ terminal end of the primer is often referred to as the GC clamp in order to emphasize the importance of good binding.

While the $3'$ end sequence is key for effective priming, it can also be catastrophic. If the pair of primers contains complementarity at their $3'$ ends, the primers can hybridize or anneal leading to the amplification of very short products. This structure is known as a "primer dimer" and is evidenced as products of very small molecular weight (these PCR products will be less than the sum of the primer lengths). Primer dimer can easily be avoided by checking primer pairs for complementarity, and the $3'$ ends are the most crucial region to examine.

In addition to simple complementarity in the primer, primers designed with internal secondary structure may not work efficiently. Oligonucleotides are able to form secondary structures, such as a stem loop, like RNA. These structures, if they are stable, or even marginally stable, at the annealing temperature of the reaction, will compete with productive priming of template DNA. When primers are involved in other structures, they are not available to bind to the target sequence of the template DNA. Thus, secondary structure, or primer dimer, is largely just lowering the effective concentration of these components in the reaction and lowering the efficiency of the reaction.

With each of the above functional criteria for primers, it is very important that primers be matched in their proprieties in order to function well as a pair. If, for example, GC content or T_m is radically different between primers, it may be challenging to find ideal thermal cycling conditions where under which both primers are accurate and efficient. With all of these considerations, it might seem that it would be challenging to design a single primer, let alone a functional pair, in less than one hour. It turns out that, if you follow the general rules, it isn't that challenging to design functional primers is very short order. Also, if your problem involves the design of a large number of primers, or you'd like confirmation of your designs, there are many software tools available to assist in the process. Some of these tools simply provide calculations of T_m, other go so far as to design complete primer set based on a few (or more) criteria about your sequence. The "rules" for primer design are very easy to set out and can be applied either by the researcher or, deterministically, by algorithm.

DNA Polymerase At the dawn of the polymerase chain reaction era, the DNA polymerase used was the Klenow fragment from *E. coli* DNA polymerase. This polymerase is heat labile and required re-addition of enzyme following each denaturation cycle. The tedium of replacing enzyme after each denaturation was ended with the application of DNA polymerase from *T. aquaticus* (*4*). This bacterium is found in hot springs, and the DNA polymerase from *T. aquaticus* (often called Taq polymerase) is thermostable to 95^oC. It survives the thermocycling procedure and does not need to be replenished during the reaction. To recognize this great contribution, Taq polymerase was named "molecule of the year" by **Science** in 1989.

Like *E. coli* DNA polymerase, Taq polymerase is member of the DNA polymerase I or A polymerase family (*11*). DNA polymerases share a common general structure which is often described *via* comparison to a right hand. The three classic domains are referred to as the fingers, palm and thumb. The fingers primarily interact with the incoming nucleotide triphosphate, the palm is the primary catalytic site for phosphodiester bond formation and the thumb is involved in binding and positioning the template DNA. Sequence identity is high between the two structures, almost fifty percent (*12*).

While the two enzymes share many features in common, Taq polymerase differs in two fundamental respects. Taq polymerase has much greater thermal stability (the optimal temperature of activity of Taq polymeraseis near 80^0C, for *E. coli* DNA polymerase optimal temperature is just under 40^oC). In the amino acid difference between *E. coli* DNA polymerase and Taq polymerase, seventy-nine involve charged residues, and many of these substitutions function to optimize long-range electrostatic interactions, a strategy known to be effective for increasing protein stability (*12, 13*). Also, Taq polymerase lacks the $3'$ proof-

reading activity of the *E. coli* DNA polymerase (*14*). Taq polymerase is then more prone to base substitution errors (1 in 9000 nucleotides at 70^{o}C). than is *E. coli* DNA polymerase.

Deoxynucleotide Triphosphates The deoxynucleotide triphosphates (dNTPs) molecules are the monomer units used by Taq polymerase to extend the primers. These reagents were, classically, individual solutions, each added separately to the reactions. It is currently most common to purchase a mixture of dNTPs (dATP, dCTP, dGTP and dTTP). This mixture is then added as a single component to the reactions. The dNTP mixture is typically provided in water so that they can be added to the reactions without concern for altering the buffer conditions.

Reaction Buffer The Taq polymerase polymerase is supplied with a matching reaction buffer, or, more typically, a suite of buffers from which to select based on your application. The exact details of any PCR buffer supplied by a manufacturer are closely guarded trade secrets. In general these buffers are a pH values slightly higher than neutral, pH 8.0 to 8.5 in Tris buffer is common. Along with the buffering system, these buffers usually contain various ions (typically the ionic strength is 50 mM) and possibly other small molecules found to enhance the activity of Taq polymerase.

The major buffer feature of interest to the experimenter is the absence or presence (and concentration) of the divalent cation Mg^{2+}, as $MgCl_2$. Remembering that both the template DNA and the primers are polyanions, their binding is electrostatically unfavorable. The inclusion of Mg^{2+} ion helps to relax that repulsion. PCR buffer with Mg^{2+} typically produces a final [Mg^{2+}] in the reactions of 1.5 mM. PCR buffers which do not contain Mg^{2+} usually supply a concentrated solution of $MgCl_2$ in order to allow the experimenter to easily vary the [Mg^{2+}] in the reactions, optimizing conditions for the best accuracy and efficiency.

Thermal Cycle The polymerase chain reaction is built around a thermal cycle of denaturing, annealing and extension. A typical thermal profile is shown in Figure 2.

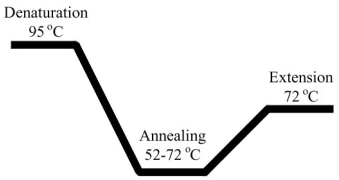

Figure 2: Typical thermocycling profile for each round of the polymerase chain reaction.

During denaturation, all double strand DNA is separated into single strands. The denaturation temperature of 95^{o}C is sufficiently high to denature both template DNA and any primer structure. The annealing step is designed to allow the primers to bind to template DNA in the first cycles and products there after. The temperature chosen for annealing is the melting temperature of the lowest melting primer in the reaction. Following the rules of typical primer design, primer melting temperatures are, at the outside, 52-72^{o}C. A more typical range might be 56-68^{o}C, with the full range reserved for some extreme primers. The extension temperature of 72^{o}C is a balance between the optimal temperature of Taq polymerase activity (closer to 80^{o}C) and the stability of the primer–template duplex.

The length of time spent at each temperature, and the time required to adjust temperatures, depends largely on the instrument and the sample volume. The smaller the volume of liquid in the reaction, the quicker the sample can be heated or cooled. The quickest PCR reactions are completed in capillary-like tubes where the total volume of the reaction is a small fraction (often 0.1 or 0.2) of a microliter. With these volumes, and concomitantly quick sample heating and cooling, reactions of 20–30 cycles can complete in 20 minutes. For more traditional volumes, $\approx$50 μL, typical thermocyclers will complete 20–30 cycles in 60–120 minutes. While this is certainly slower, it isn't typically disruptive to a graduate

student's workday. In traditional volumes, each temperature will be maintained for 15–120 seconds. Thirty to sixty seconds are most typical and longer times may be used for certain applications.

In addition to the 20–30 cycles illustrated in Figure 3, there are usually cycles in a program both before and after the amplification cycles. Most PCR protocols will begin with an initial denaturation. This cycle heats to and holds the reactions at 95^{o}C for a couple minutes. This cycle is designed to be an initial denaturation in which all double strand DNA (the amplification template) is melted in order to prepare for the first annealing step. This initial denaturation cycle should not be confused with hot start polymerase chain reaction in which a component is withheld (usually the polymerase) or compromised (again the polymerase) until the reaction reach a temperature sufficient to denature double-strand DNA (usually 60–80^{o}C) (*15*). Hot start polymerase chain reaction is designed to limit non-specific reactions while the complete reactions wait at room temperature before thermal cycling begins.

Following the completion of thermal cycling, there is often a long hold at 72^{o}C. This hold is often 3–10 minutes and is designed to allow the polymerase to finish extending any single strand DNA that may not yet be fully extended. This is just a period for DNA polymerase to clean up its work. The final hold is at ≈15^{o}C, and the timer is set to infinity. This cold soak is set to stop (or greatly slow) the reactions and preserve them until the experimenter returns. It is not uncommon for this hold to run all night for reactions prepared at the end of the day. Most thermocyclers will provide a detailed history of each run so that you can identify machine failures and distinguish them from protocol, design or reagent failures. It isn't particularly risky to allow reactions to run in the thermocycler when you are not in the laboratory.

Experiment—Week 1

The first polymerase chain reaction experiment will be a standard amplification of template DNA. As template, we will use the cloning plasmid pBR322. Plasmid pBR322 was developed by Bolivar and Rodriguez in 1977 and has proven to be a very useful and popular plasmid (*16*). The synthetic plasmid pBR322 was derived from transposon Tn3, and portions of plasmids pSC101 and pMB1 and contains two selectable markers. Those markers are the *tet* gene, conferring resistance to tetracycline and the *bla* gene, conferring resistance to ampicillin, both derived from pSC101. In this experiment, you will amplify the *tet* and *bla* genes from pBR322.

Reactions In preparing for polymerase chain reactions, the first thing to consider is exactly which reactions you will be performing. This week you will amplify both marker genes from pBR322 in duplicate. This repetition allows for confirmation of a result (and added material, if needed).

In addition to your reactions of interest, it is common to preform control reactions. One type of control reaction is the positive control. The positive control is a reaction which is known to work under at the annealing temperature of your reactions. The positive control is prepared with the same common reagents (buffer, dNTPs, DNA polymerase) but uses a different template DNA and primer set. If the positive control reaction produces the appropriate product, you can be sure that the common reagents in your reactions are all performing well. If the positive control works, but your reactions fail, you know to check template DNA quality and quantity or the design of your primers. If the positive control fails, there is likely to be some problem with your reagents or technique.

For a positive control, you will amplify a section of bacteriophage λ DNA. This is a common positive control reaction because λ DNA is easily purified and there are several sets of primers targeting λ DNA which have been well tested and are robust over a wide range of annealing temperatures. You should complete the positive control reactions in duplicate.

In addition to the positive control, it is also advisable to include negative controls in your experiment. As the name suggests, the negative control is a reaction designed to,

all things going correctly, fail. The standard template for a negative control is to setup a complete reaction, but withhold template DNA from the negative control. This control has a dual purpose. If your primers are in any way complimentary to one another, small products in your negative control will alert you to the primer dimer (seeing the product you hope for in your experimental reactions might cause you to overlook some smaller, fainter bands on your gel and thereby miss an opportunity to refine your primer design). The other purpose of the negative control is the make sure that your reagents are free of contaminating DNA. If your sterile water has accidentally collected DNA, you might be amplifying from that DNA instead of, or in addition to, your desired template. This situation is very dangerous in a laboratory that works heavily with very similar DNA molecules; producing products from unintended templates can lead to erroneous experimental conclusions.

There is one other error that the negative control reaction checks. Unless your reactions are prepared by robot, the experiment has his or her *own* DNA. The negative control reaction is a good, all-around check of cleanliness for the reagents involved in these reactions. You should perform a negative control reaction using each of your experimental primer sets (the λ primers are known to be well behaved, so you don't need to use them in a negative control reaction).

Master Mix The master mix is a combination of all reagents that are common to each reaction. The common components are the reaction buffer, additional Mg^{2+} if required, dNTPs, Taq polymerase polymerase and sterile, distilled water to bring the mixture to the correct concentration (2X *(hint: this is molecular biology shorthand for "two times the working concentration." Thought of another way, this reagent will need to be diluted two fold in order to dilute it to the "working concentration" for the reaction, 1X)*. This mixture then will be used as one half the volume of each reaction. Preparing a master mix serves two purposes. First it greatly reduces the number of components added to each reaction; when adding the master mix to each reaction you are adding three or four separate components at once, not individually. The second benefit is that the master mix will be added as a large volume, if you added DNA polymerase directly to each reaction, it would only be in a small fraction of a microliter, a much more challenging volume to measure accurately.

Your reactions will be 50 μL in total volume. Begin with the total number of reactions you'll be performing (see *Polymerase Chain Reaction Worksheet* for an alternate presentation) and add one reaction to that number. In this way you'll be making master mix for one extra reaction. That extra volume might come in handy if you drop a reaction, or it might be necessary to cover small errors in micropipetting. You can then calculate the total volume of master mix you'll need to make by multiplying the number of reactions, plus one 'extra', by one half the total volume of each reaction (remember that master mix is made at 2X and thus must be half the total volume of each reaction (*hint: This is the beginning of the Polymerase Chain Reaction Worksheet*)).

With the volume of the master mix, you can begin to calculate what volumes of each reagent become part of the master mix. The commercial reaction buffer you will be using is prepared at 5X. The calculation of the volume of 5X PCR buffer required follows the theme of $concentration_1 \times volume_1 = concentration_2 \times volume_2$. You can calculate the volume of 5X reaction buffer you need to add to the master mix by answering the question, what volume of 5X buffer do I need to use in order to make the total volume of the master mix at a concentration of 2X? Using a similar method, you can calculate the needed volumes of dNTPs (the stock is 50X) and Taq polymerase (the stock is 200X). The remainder of the volume of the master mix is sterile distilled water.

Primer Sets As with the master mix, you will prepare primer sets in order to ease the preparation of each PCR reaction. Since you would never use just one primer, the two amplification primers can be combined together as a primer set and introduced to each reaction as a single solution.

The sequences of the oligonucleotide primers for this reaction, along with melting temperatures, are given in Table 1 below. Each primer is named according to the template

DNA, gene to be amplified (if applicable) and the template strand the primer binds. Also, the calculated melting temperatures are given for each primer. In a motley crew of melting temperature such as this set of primers, it would be most prudent to run the reactions at the lowest annealing temperature in the bunch.

Table 1: Primer sequences for the amplification of AMP and TET genes from pBR322 along with the sequences of the λ control primers.

Primer	Sequence	T_M (^{o}C)
pBR322-AMP+	GGTCTGACAGTTACCAATGC	60
pBR322-AMP−	ATGAGTATTCAACATTTCCG	54
pBR322-TET+	ATGAAATCTAACAATGCGC	52
pBR322-TET−	TCAGGTCGAGGTGGCCCGGC	70
lambda+	GATGAGTTCGTGTCCGTACAACTGG	76
lambda−	GGTTATCGAAATCAGCCACAGCGCC	78

Primer sets are made from both the forward and reverse primer in a pair and sterile distilled water. The primer stock solutions are prepared at a concentration of 100 μM, or 100X. The primer sets are prepared for PCR at a concentration of 5X, and, like the master mix, prepare enough of each primer set for one more reaction than you are planning to do. The mechanics of the calculations are identical to those for preparing the master mix.

Reactions With the calculations complete for the master mix and the various primer sets, you can now prepare the table of reactions. This table details what volume of each reagent should be added to each reaction and is the primary guide used while preparing each reaction. Remembering that each reaction has a total volume of 50 μL, the master mix (2X) will be the first 25 μL. Each reaction will also include one, and only one, primer set. Since the primer sets are prepared at 5X, they will be the next 10 μL of each reaction. The template DNA is prepared at the standard concentration of 25X ($\approx$0.005 mg/mL), and thus will be added to each amplification reaction and positive control in a volume of 2 μL. Each amplification reaction will use pBR322 as the template and the positive control reactions will use λ DNA as a template. No template is added to the negative control reactions. The final component of each reaction is water, and the volume of water added is the total volume of the reaction minus the volume of the other components.

Protocol

The first thing you should do is don gloves (to protect your reagents from you). You'll wear these gloves for everything involved with the polymerase chain reaction; it is a good idea to become comfortable working in gloves. A proper fit should be tight enough that the glove doesn't foul in the micropipettes or get caught in tubes, but not so tight as to limit circulation at the wrist.

Next, you'll want to find all your reagents, except DNA polymerase and template DNA, in the freezer and thaw them. All of these reagents should be in the blue freezer box on the second shelf (from the top) of the door in freezer in STEM 176 (there are also some freezer racks in that freezer which will keep reagents frozen outside of the freezer). These reagents are all dilute aqueous solutions and will be frozen solid. Be sure to thaw them complete so that they are at their proper concentrations when you use them (as dilute aqueous solutions freeze, water nucleates to form ice at the center and tends to exclude solute particles. If a solution is only partially thawed, the concentration of the solute is usually much greater as the remaining block of ice is largely water). A good method to thaw microfuge tubes is to place them between your fingers so that you can still go about working while they thaw.

With thawed buffer and dNTPs, begin preparation of the master mix. The typical vessel for the master mix is a 2 mL screw cap tube . Add buffer, dNTPs and water (sterile, distilled water) according to the calculations you have already made. The final component

of the master mix is Taq polymerase, but you haven't thawed that reagent yet, is everything alright? Yes, Taq polymerase is stored in a solution which is close to fifty percent glycerol—this functions to stabilize the enzyme for extended storage, and prevents the solution from freezing at -20^{o}C. The enzyme is in the same freezer box and can be used directly from the freezer. The standard protocol is to fetch the enzyme in the cold block, use it and immediately return it to the freezer.

The challenge of adding a solution in heavy glycerol to your growing master mix is that the enzyme solution will sink directly to the bottom of the tube, and, unless mixed, remain there. Remembering that you've made one extra portion of master mix, it could remain entirely in the portion you discard at the end. And with no enzyme in any of your reactions, you'll not get much yield. Thus, it is imperative that after returning the enzyme to the freezer, you mix the master mix. The easiest method is to set a large micropipette to about half the volume of the master mix and pipette up and down, being sure to reach the bottom, several times.

With the master mix complete, the next solutions you should prepare are the primer sets. You'll be making one solution for each primer set. This solution is made from forward primer, reverse primer and water. Typically these reagents are combined in a 0.65 mL microcentrifuge tube (about half the size of a 'normal' microcentrifuge tube). Prepare each of these solutions according to your calculations. When you are finished making the master mix and the primer sets, you are finished with all the reagents and can return them to the freezer.

The PCR reactions are prepared in 0.2 mL thin-walled tubes. These are found in the hood and do not have attached lids. Set out as many as you need in the appropriately sized section of the tube rack. The major caution here is that these tubes have very thin walls in order to maximize heat transfer from the thermocycler block to your reactions. If you squeeze these tubes firmly from the sides, they will split along their long axis. It is best to hold them gently from the top and bottom. The lids (which you don't need yet) are in a separate bag and attached in strips of ten—do *not* detach these lids from one another when the time comes to cap your reactions.

Assemble your reactions according to your calculations adding master mix, primer sets and water. When it is time for the template DNA, retrieve it from freezer box and begin to thaw it. Add template to each reaction according to your reaction prototypes. Return the template DNA to the freezer.

Nestle your reactions up next to one another and add a strip of lids. Feel free to remove an extra lid or two from the strip, but leave the strip intact over your reactions. This will eliminate a major problem if you drop your reactions on the way to the thermocycler—you'll need only to pick up one rather large thing instead of many very small things.

The thermocycler is to the left of the whiteboard in STEM 155. A method is already programmed for your reactions `CHEM 352 standard` with an annealing temperature of 52 ^{o}C. Sign the log book next to the thermocyclers noting your identity, samples and method. Run your samples using the `CHEM 352 standard` method.

The thermocycler will require ≈seventy-five minutes on the wall clock to complete your reactions. Use the early portion of that time to clean up the hood, replace the cover and re-strike the ultraviolet lamp to sterilize the hood. Toward the end of your wait, you should begin to prepare for gel electrophoresis by gathering a gel, loading dye and molecular weight ladder from the freezer box.

When your reaction is complete, prepare your sample for electrophoresis, run the gel and photograph your gel.

With your data (the photograph of the gel) in hand, you can clean any areas which still have your reagents or hardware, discard your gel and PCR samples. That is it for the day.

Analysis The first step in the analysis is to construct a standard curve relating the molecular weights of your standards to their migration distance. This curve is usually constructed by plotting the base 10 logarithm of the molecular weight of each molecule as a function

of the migration distance of that molecule. The molecular weights of the DNA ladder are given as a supplement (*note: the 500 bp band will be the brightest on your gel*) and the migration distance can be measured in any units which are convenient (cm or pixels are the likely units). The resulting plot should be a straight line with a negative slope.

Use your standard curve to estimate the molecular weights of your products.

Questions

Question 1: Use Primer-BLAST to determine the sizes of the products. Primer-BLAST is a tool that incorporates primer design software (primer3, particularly) and BLAST searching through any NCBI database to establish the specificity of a primer set (or to design a specific primer set). You will be using just the primer design features in this question. For the two products from pBR322, use J01749 as the accession number for the PCR template and the appropriate sequences for the forward and reverse primers. To turn off the BLAST searching, disable the specificity check by unchecking "Enable search for primer pairs specific to the intended PCR template." The results will provide some information about the primers; the final final line of the output is the length of the product in base pairs. Repeat this process for the second set of primers used against pBR322. Calculate the product size of the bacteriophage λ positive control. Use NC_001416 as the accession number for the bacteriophage λ chromosome.

Question 2: With your measured product sizes and the expected product sizes, did you amplify what you were expecting? If yes, congratulations, you are finished with this question. If no, what went wrong? To answer this question, please be sure to analyze each of your control reactions (*note: it isn't always possible to identify the failure explicitly*).

Question 3: Design a pair of primers that generate an *exactly* 500 bp from pBR322. The 500 bases you amplify are your choice. You should aim to design primers that melt at similar temperatures, lack self-complimentary or high likelihood of finding primer dimer, that is, you should follow the "rules." Try it first by hand. Once you have a set of primers, try it again using Primer-BLAST. You can just ask Primer-BLAST to design primers to amplify a 500 bp fragment and let it do all the work. Since you've already selected a region to amplify, try giving Primer-BLAST a forward (or reverse) primer as well and allow it to select the matching primer. Report two pairs of primers: sequences, melting temperatures and locations relative to the numbering of pBR322. Distinguish which pair you designed solely by hand and which pair had algorithmic assistance.

Experiment—Week 2

Having completed a polymerase chain reaction amplification, it is now time to turn your attention to the interactions you are exploiting within your reactions. The most fundamental feature of the system which you control is the annealing of your primers to the template DNA. That is, you have complete freedom in designing primers, and the quality of your design, when matched with selected annealing temperature, will largely dictate the success of your amplification.

With the central role of good primer design, there has been a great deal of effort placed on automating this process (*17, 18*). The goals of automation are two-fold. First, with an automated process, the design of primers will adhere strictly to the "rules" as codified in the program. When primers are designed by hand, the research has the opportunity to ignore or modify "rules" that the computer will religiously follow. The second major advantage is that the computer is quite happy to apply every rule in every situation and never, like the scientist, tires of checking every detail of every possible primer (*note: there may be, in some experiments, tens of thousands of potential primers. If you think checking each one sounds like a enjoyable pastime, we should talk about opportunities for graduate education*). The typical primer design program will check all of the properties listed above in the 'primers' section (and many more).

Since we have only one week, and more practically, time for one more run in the thermocycler, we will limit our study to the role of annealing temperature in primer function. Using pBR322 as a template, there are two primers listed in Table 2 below that amplify a random 300 bp fragment. The design of this primer set, after selecting a product size, centered on designing a functional primer set with wildly different melting temperatures. The forward primer, pBR322-300+, forms a very stable duplex that doesn't melt until the temperature is raised well above 60 ^{o}C.

Table 2: Primer sequences for the amplification of a 300 base pair product from pBR322.

Primer	Sequence
pBR322-300+	`CCCTCGTGCGCTCTCCTGTTCCG`
pBR322-300−	`TCAAGAACTCTGTAGC`

The reverse primer, pBR322-300-, however, is designed to form a double helix which readily melts. It is your goal to describe this melting by estimating the melting temperature of pBR322-300-. The experiment to estimate the actual melting temperature is rather straight-forward; the melting temperature of the primer is the annealing temperature in an actual PCR reaction which gives the most product from the reaction. If the reaction is run with an annealing temperature that is too high, the primer will not bind to its target sequence. If the annealing temperature is too low, the primer will bind, with lowered specificity, to a great variety of sequences, lowering the overall efficiency of the reaction. To measure a melting temperature, then, you need only to repeat identical reactions under a variety of annealing temperatures and compare the results.

But we only have one afternoon, eh? Well, the problem of optimizing annealing temperature is a much more universal problem in PCR. Often it is the best approach to simply find the optimal conditions for a particular reaction *via* testing different annealing temperatures. In order to optimize the solution to this problem, many modern thermocyclers are equipped with a gradient feature (often referred to simply as gradients). This feature allows the thermocycler to generate a temperature gradient across the thermal block during the annealing stage of the thermal cycle. The particulars of the gradient function are tied directly to the instrument implementing the function. Our thermocycler has a 96 well block. The rows are designated A–H and these can be thermally controlled independently of one another. Our instrument, at a the maximum, will establish a thermal gradient of 25^{o}C from row A to H (with A being the high temperature side and H the low temperature side). An illustration of the thermal gradient across the thermocycler block is shown below in Figure 3.

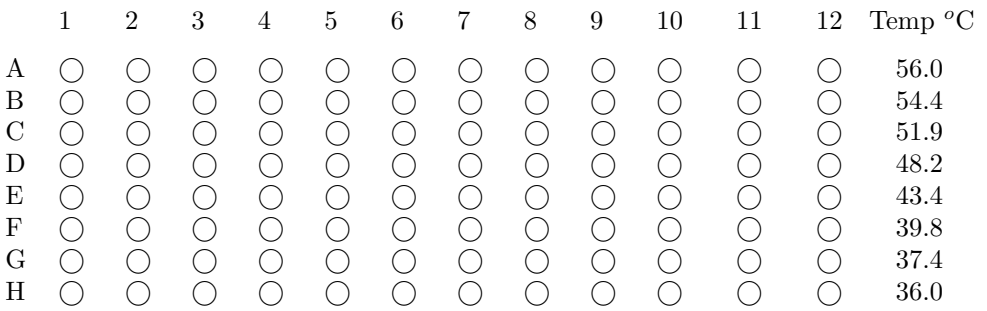

Figure 3: Schematic illustration of the 96 well thermocycler plate. The thermal gradient feature of the instrument creates the gradient across rows A to H, from warmest to coolest (*note: the designations A–H and 1–12 are found only on the plastic insert, the metal block is without labeling*). The temperatures to the far right are the actual annealing temperatures in the `CHEM 352 grad` protocol.

With this instrument then, we are able to compress eight trials into one experiment and collect a range of eight annealing temperatures across 20^{o}C in a single experiment. We'll then use this capacity to amplify this 300 bp fragment with primers pBR322-300+ and pBR322-300- at annealing temperatures between 36 and 56 ^{o}C. The only other wrinkle different from the standard amplification you ran last week is that the `CHEM 352 grad` program only runs for 15 cycles. This run is significantly shorter (remember the powers of two involved) than the typical amplification protocol of 20–30 cycles. This is just to make sure that our reaction doesn't reach completion, which would make it significantly more challenging to see the differences in yield.

Prepare 8 reactions using the pBR322-300± primer set and pBR322 as the template. You do not need to prepare any control reactions. The calculations for preparing master mix, primer set and reactions are performed identically to the calculations you preformed last time, just adjust the total number of reactions and the primer set volume. Prepare these reactions, load them in the thermocycler being sure that one is in each row from A to H and run the `CHEM 352 grad` program. This program should take ≈90 minutes on the wall clock (*hint: if you are wondering why these 15 cycles take longer than the 20 cycles last week, it turns out that the gradient function takes just a little longer to reach thermal equilibrium in the annealing step than a single annealing temperature somewhere within the range of the gradient*).

When your program is complete, run your samples on a gel using 100 bp ladder as molecule weight markers. When your gel is finish, collect an image of the gel and perform densitometry on your gel, collecting average densities for each band.

Analysis Begin by plotting a standard curve and verifying that your products are 300 bp in length. To address the question of the annealing temperature of the pBR322-300- primer, plot the average density of each band collected by densitometry as a function of annealing temperature. The melting temperature of this primer is the annealing temperature with the highest average density. If two annealing temperatures give very similar results, the ideal value lies somewhere in between those two temperatures, report the average of those two annealing temperatures.

Questions
Question 4: What is optimal annealing temperature of pBR322-300-?

Question 5: Calculate the melting temperature of pBR322-300- using the three methods described in the introduction to PCR primers *hint: assume the effect sodium concentration is 30 mM*). Which method gives results closest to the measured melting temperature? Which method is easiest to use?

Question 6: Many laboratories post their protocols on the Internet, either simply for their own ease of use or to share with the world at large. The laboratory of John Roth (Distinguished Professor of microbiology) at U.C. Davis is one of those laboratories. From their documents on PCR primer design, evaluate the second suggestion, the suggestion about annealing temperature and T_M. Do you agree with this recommendation? Why or why not?

References

(1) Mullis, K. 1998. **Dancing Naked in the Mind Field**. Random House, Inc. New York, NY.
(2) Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N. 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science*. **230:** 1350-4.
(3) Mullis KB, Faloona FA. 1987. Specific synthesis of DNA *in vitro via* a polymerase-catalyzed chain reaction. *Methods Enzymol*. **155:** 335-50.
(4) Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*. **239:** 487-91.
(5) Suggs SV, Hirose T, Miyake EH, Kawashima MJ, Johnson KI, Wallace RB. 1981. **In DD Brown (ed), ICN-UCLA Symp. Dev. Biol. Using Purified Genes**. Academic Press, Inc, New York, NY.
(6) Chester N, Marshak DR. 1993. Dimethyl sulfoxide-mediated primer T_m reduction: a method for analyzing the role of renaturation temperature in the polymerase chain reaction. *Anal Biochem*. **209:** 284-90.
(7) Borer PN, Dengler B, Tinoco I Jr, Uhlenbeck OC. 1974. Stability of ribonucleic acid double-stranded helicies. *J Mol Biol*. **86:** 843-53.
(8) SantaLucia J Jr. 1998. A unified view of polymer, dumbell, and oligonucleotide DNA nearest-neighbor thermodynamics. *Proc Natl Acad Sci USA*. **95:** 1460-5.
(9) von Ahsen N, Wittwer, CT, Schütz E. 2001. Oligonucleotide melting temperatures under PCR conditions: nearest-neighbor corrections for Mg(2+), deoxynucleotide triphosphate, and dimethyl sulfoxide concentrations with comparison to alternative empirical formulas. *Clin Chem*. **47:** 1956-61.
(10) Kwok S, Kellogg DE, McKinney N, Spasic D, Goda L, Levenson C and Sninsky JJ. 1990. Effects of primer–template mismatches on the polymerase chain reaction: Human immunodeficiency virus type 1 model studies. *Nucleic Acids Res*. **18:** 999-1005.
(11) Steitz TA. 1999. DNA polymerases: structural diversity and common mechanisms. *J Biol Chem*. **274:** 17395-8.
(12) Korolev S, Nayal M, Barnes WM, Di Cera E and Waksman G. 1995. Crystal structure of the large fragment of *Thermus aquaticus* DNA polymerase I at 2.5-Å resolution: structural basis for thermostability. *Proc Natl Acad Sci USA*. **92:** 9264-8.
(13) Grimsley GR, Shaw KL, Fee LR, Alston RW, Huyghes-Despointes BM, Thurkill RL, Scholtz JM and Pace CN. 1999. Increasing protein stability by altering long-range coulombic interactions. *Prot Sci*. **8:** 1843-9.
(14) Tindall KR and Kunkel TA. 1988. Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Biochemistry*. **27:** 6008-13.

(15) Chou Q, Russell M, Birch DE, Raymond J and Bloch W. 1992. Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications. *Nucleic Acids Res.* **20:** 1717-23.
(16) Bolivar F, Rodriquez RL, Greene PJ, Betlach MC, Heynecker HL and Boyer HW. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene.* **2:** 95-113.
(17) Rychlik W and Rhoades RE. 1989. A computer program for choosing optimal oligonucleotides for filter hybridization, sequencing and *in vitro* amplification of DNA. *Nucleic Acids Res.* **17:** 8543-51.
(18) Rozen S and Skaletsky HJ. 2000. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol.* **132:** 365-86.

Prepared @ 11:36 on 03–11–2024.