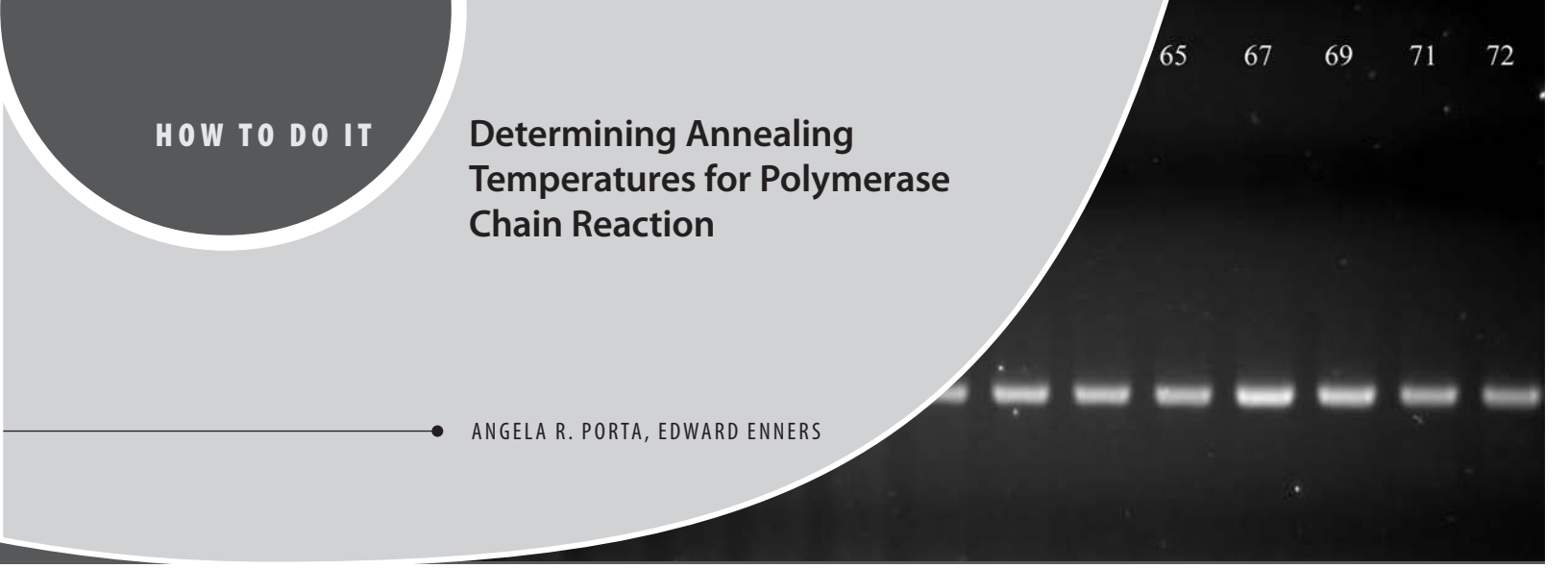
HOW TO DO IT

Determining Annealing Temperatures for Polymerase Chain Reaction



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Abstract

The polymerase chain reaction (PCR) is a common technique used in high school and undergraduate science teaching. Students often do not fully comprehend the underlying principles of the technique and how optimization of the protocol affects the outcome and analysis. In this molecular biology laboratory, students learn the steps of PCR with an emphasis on primer composition and annealing temperature, which they manipulate to test the effect on successful DNA amplification. Students design experiments to test their hypotheses, promoting a discovery-based approach to laboratory teaching and development of critical-thinking and reasoning skills.

Key Words: *Polymerase chain reaction; PCR; annealing temperature; DNA primers; inquiry-based; critical thinking.*

The analysis of DNA by the polymerase chain reaction (PCR) is a remarkably simple technique that allows for amplification of minute quantities of DNA. The commercial availability of kits has made laboratories utilizing PCR more common in high school and undergraduate science classes. Students use PCR to determine DNA typing and fingerprints (Baker et al., 2002), to identify bacterial contaminants (Baker et al., 1999), and to clone for a particular gene of interest (Dong et al., 2008). Parameters for these experiments are often standard and preset. Students run the reactions without having a true appreciation for the critical experimental details required to amplify a specific segment of DNA.

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The PCR cycle involves three steps: denaturation, primer annealing, and primer extension. Each of these steps requires incubation of the reaction mixture at different temperatures. In the first step, denaturation, the DNA is incubated at 93–95°C from 30 seconds to 2 minutes. This breaks the hydrogen bonds between the nucleotide base pairs (bp) and separates the two strands of DNA. In the second step, primer annealing, the reaction is incubated at 45–65°C for 45 seconds to 1 minute; the presence of excess primers allows the complementary primers to hybridize to target DNA. The third step, primer extension, is conducted at 72°C from 15 seconds to 1 minute and involves DNA synthesis, in which the primers are used to synthesize two new daughter strands complementary to the original mother strands. Subsequent PCR cycles will replicate each PCR product in the reaction mixture, resulting in the exponential amplification of the DNA target sequence.

The early innovators of PCR needed to optimize this procedure. Initially, fresh DNA polymerase had to be added after each denaturation step. Eventually, a thermally stable form was discovered in the hot springs bacteria *Thermus aquaticus* (*Taq*), hence the term *Taq* DNA polymerase. Each incubation period required the transfer of test tubes by hand from one temperature to another until the advent of the thermal cycler, which regulates cycling temperatures automatically.

Even in the "real world" of scientific research, commercially available PCR kits are used, but two critical PCR components are usually provided by the scientist. Researchers supply their own primers, which are designed to anneal to a specific DNA sequence, and the DNA template to be amplified. An ideal PCR will be specific, generating one and only one amplification product, be efficient, yielding the theoretical two fold increase of product for each PCR cycle, and have fidelity, reproducing the exact sequence of the template. Each of these parameters is affected by variables within the PCR reaction mixture such as buffer components, cycling number, temperature, and duration of each cycling step, primer composition, and DNA template. In this laboratory exercise, students use two sets of primers to determine optimal annealing temperature on PCR product formation to optimize for efficiency of amplification. We use this exercise in a cell physiology laboratory course for upper-division undergraduates. It is also appropriate for

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AP Biology courses, where funding for more advanced laboratory exercises may be available.

○ Overview of Experiments

This set of experiments focuses on the amplification of two PCR products: one for claudin-2 and one for claudin-12. The claudins are components of tight junctions found between intestinal cells and are involved in creating a permeability barrier so that substances cannot pass from the lumen of the intestine to the blood. In these experiments, students

1. study general parameters that influence PCR,
2. calculate and estimate optimal annealing temperature for primers of claudin-2 and claudin-12 DNA sequences,
3. run PCR using a range of annealing temperatures that students determine,
4. visualize the PCR products in a horizontal agarose gel electrophoresis,
5. determine the quantity and size of the PCR product for each set of primers, and
6. compare observed annealing temperature to calculated annealing temperature.

○ Parameters that Influence PCR

Specificity, efficiency, and fidelity: optimization of these three parameters requires knowing the purpose of your PCR reaction (Cha & Thilly, 1993). An ideal PCR possesses high specificity (one and only one product), efficiency (good exponential yield), and fidelity (an accurate product). These parameters are influenced by a number of variables including buffer conditions such as Mg^{++} concentration, cycling time, annealing temperature, and time duration. Adjusting these variables will maximize one parameter over another, and thus there is a compromise depending on your purpose. Fidelity is of primary importance when the purpose is to sequence a particular DNA. In quantitative PCR, used for evaluation of gene expression, specificity and efficiency are also important.

○ Optimal Annealing Temperature & Primer Design

Primer length and sequence are critical in amplifying PCR products with specificity and efficiency (Dieffenbach et al., 1993). The stability of the primer-DNA template duplex is measured by its melting temperature (T_m), the temperature at which half of the primer-DNA duplex dissociates to become single-stranded DNA. The length of the primer and the G and C content of the primer-DNA template duplex play critical roles in determining T_m, which is calculated by the formula 4(G+C) + 2(A+T), and this is the rule of thumb for calculating annealing temperature. The observed annealing temperature is usually a few degrees below the calculated annealing temperature and is influenced by other variables of the PCR, such as Mg^{++} concentration and K^+ concentration. Primer length is typically between 18 and 22 nucleotides.

○ Experimental Setup

Table 1 shows the primers used in this exercise to amplify intestinal cDNA for claudin-2 and claudin-12. Students can calculate the optimal annealing temperature on the basis of primer compositions and design an experiment to test different temperature ranges in order to determine the optimal annealing temperature. The experimental protocol to test actual annealing temperature is described below, and variations are suggested so that instructors can guide students to create their own hypotheses and adapt the experiment to test other variables that students can manipulate. Students can be grouped to test different hypotheses, or a consensus can be reached whereby one hypothesis will be tested by all groups.

○ Methods

The laboratory is divided into three modules. In the first module, the class uses RNA to synthesize cDNA by the enzyme reverse transcriptase (RT). In the second module, cDNA is used in PCR to amplify cDNA for claudin-2 and claudin-12 at varying annealing temperatures. In the third module, the PCR products are analyzed by separation through agarose gels. The class is divided into groups of two or three students, depending on class size. The entire laboratory exercise takes 3–4 weeks, assuming a 3-hour laboratory per week, but each module can be carried out separately so that time can elapse between modules. A schematic diagram of the exercise is represented in Figure 1.

Module 1: Preparation of first-strand cDNA using reverse transcriptase (RT)

We prepare our own RNA from mouse intestinal tissue, using guanidinium isothiocyanate (Chomczynski & Sacchi, 2006). Alternatively, mouse intestinal RNA can be purchased from suppliers (Amsbio, catalog no. M1334226; or Zyagen, catalog no. MR-307). A kit from Invitrogen (catalog no. 18080-051) that provides all the reagents required for 50 reactions is used to make cDNA. RNA (2 µg) is added

Table 1. Relationship of primer composition and annealing temperature.

cDNA Amplified	Primer Sequence	G + C/L	% GC	T_m (°C)	T_o (°C)	Product Size (base pairs)
Claudin-2	FP 5'-ATG GCC TCC CTT GGC GTT CA-3'	12/20	60	64		
	RP 5'-TCA CAC ATA CCC AGT CAG GC-3'	11/20	55	62	60	692
Claudin-12	FP 5'-CTT TGC GGG GAC TCT GCT TCC-3'	13/21	62	68		
	RP 5'-ATG AAT AGG GCT GTG AGT AAG TGT-3'	10/24	42	68	67	604

Abbreviations: FP = forward primer; RP = reverse primer; L = length; T_m = calculated annealing temperature; and T_o = observed optimal annealing temperature.

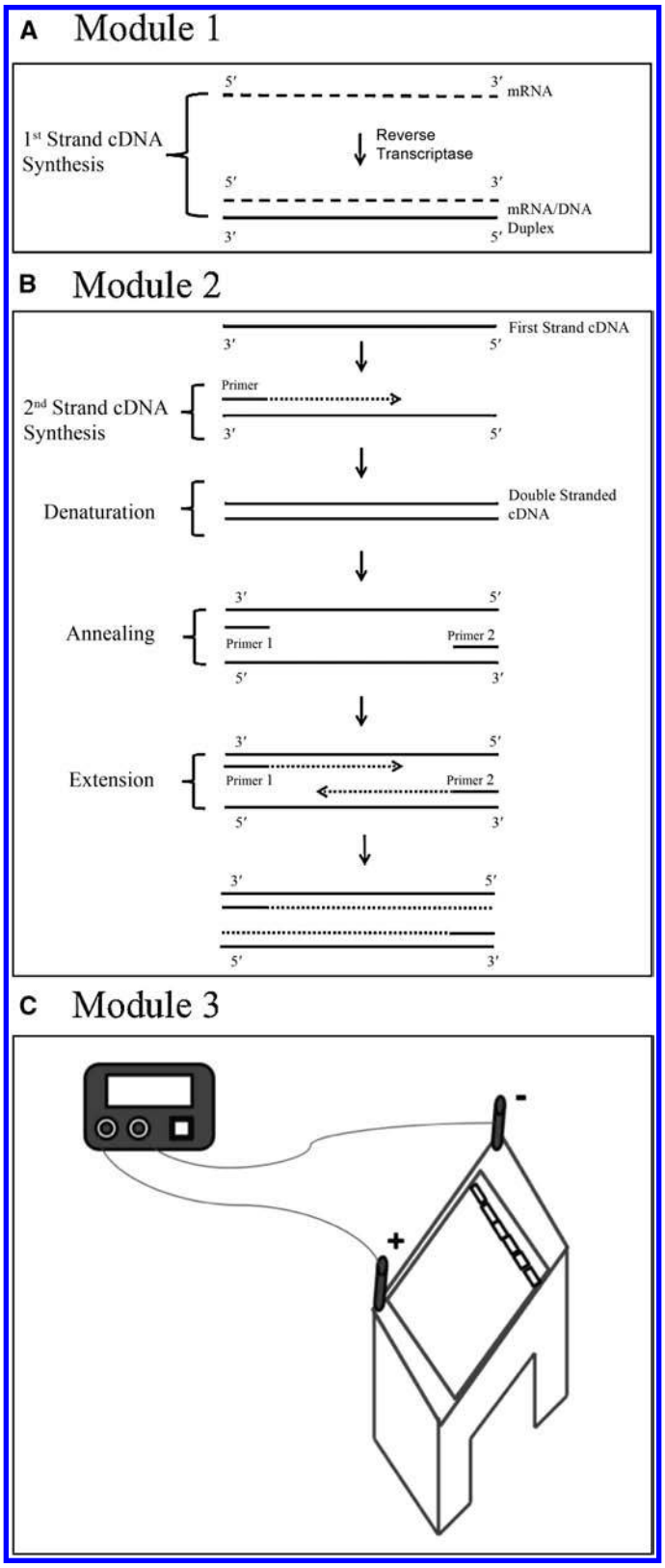

Figure 1. Schematic diagram of experiments used in each module. (A) Module 1: preparation of cDNA using reverse transcriptase. (B) Module 2: amplification of gene-specific cDNA using PCR. (C) Module 3: agarose gel electrophoresis of PCR products.

to 1 µL oligo dT and 1 µL dNTP mix and brought up to 10 µL with DEPC-treated water. DEPC water contains diethyl pyrocarbonate, which degrades any trace of RNases. The entire mix is then heated to 65°C for 5 minutes, followed by 4°C for 7 minutes. This allows oligo dT to anneal to mRNA and to serve as the primer from which the first strand of cDNA is synthesized. In the second step, a cDNA synthesis mix (10 µL) is added to the RNA sample, and the sample is heated at 50°C for 50 minutes, followed by 85°C for 5 minutes. This results in extension of the primer by reverse transcriptase (RT) and synthesis of first-strand cDNA using mRNA as the template (Figure 1A). The cDNA is stored at 4°C for immediate use or at –20°C for future use. Alternatively, the instructor can opt to skip module 1 and purchase mouse cDNA (Zymogen mouse cecum dDNA, catalog no. MD-310; or mouse colon cDNA, catalog no. MD-311) and use at a concentration of 10 ng per PCR mixture.

Module 2: Preparation of second-strand cDNA and amplification of gene-specific cDNA using PCR

The cDNA generated from the RNA is used in standard PCR with *Taq* polymerase and gene-specific primers for claudin-2 and claudin-12. Primer sequences for claudin-2 and claudin-12 are shown in Table 1, with details concerning composition and annealing temperatures. Students can be given the composition of the primers and calculate %GC and T_m. On the basis of class discussions, they can set up the range of annealing temperatures to be tested to determine actual annealing temperature of each set of primers. PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html) is a public source of primers for >300,000 human and mouse genes, and a very useful tool for searching different primer sets for specific genes of interest (Spandidos et al., 2010). The database can be used to give students a list of primers to calculate annealing temperatures for, and can also be used to choose different sets of primers to be tested by different groups of students.

Synthesis of second-strand cDNA and amplification of gene-specific cDNA (Figure 1B) were performed by adding 2 µL of the RT-cDNA, 1 µL forward primer, 1 µL reverse primer (50 pmoles each), and 12.5 µL *Taq* polymerase (Premix *Taq* Polymerase; TaKaRa catalog no. RR003) into a 0.2-mL PCR tube and enough DEPC-treated water to bring the reaction to 25 µL. In our exercise, we tested 12 different annealing temperatures, so we prepared a 12X reaction mix in a 1.5-mL microfuge tube and dispensed 25-µL aliquots into 12 PCR tubes. The cDNA was amplified using an Eppendorf-gradient PCR thermal cycler using the following parameters: initial DNA denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C denaturation for 1 minute, annealing temperatures ranging from 51°C to 71°C for 1 minute, elongation at 72°C for 1 minute, and final elongation of 72°C for 7 minutes, followed by cooling at 4°C. The PCR products can be stored at 4°C until future analysis. Students can adjust the number of annealing temperatures chosen, depending on the primers used. If a gradient cycler is not available, the reaction can be run several times in a regular PCR cycler by changing the annealing temperature for each run. After studying the basics of DNA synthesis and PCR, there are many variations on the exercise that instructors might challenge students to examine. In addition to annealing temperature, variables such as length of primer, concentration of primers and cDNA, and cycle number can also be tested.

Module 3: Agarose gel electrophoresis of PCR products

A 1% agarose gel (Figure 1C) is prepared by adding 1 g agarose (BioRad catalog no. 161-3104) to 100 mL 1X TAE buffer (40 mM Tris, 1 mM EDTA, pH 7.6) (BioRad catalog no. 161-0743) and boiling to dissolve the agarose. The solution is cooled to about 60°C and poured into the gel holder with comb to form wells. The PCR samples to be run on the gel are prepared by adding 5 µL of 6X DNA loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water) to the 25 µL PCR reactions and vortexing. After the agarose gel has solidified, it is placed in the buffer chamber filled with 1X TAE buffer so that the gel is submerged, and the comb is gently removed. DNA ladder (5 µL; Phenix Research Products 100-bp DNA ladder) is loaded in the first well; in the subsequent wells, 20 µL of the PCR and loading dye mixtures are loaded. The cover is placed on the chamber, the electrodes appropriately connected (positive–red, negative–black), and the gel is run at 90 V for 90 minutes or until the blue dye is three-quarters of the way toward the end of the gel. We stain the gel using InstaStain Ethidium Bromide paper (Edvotek) because this reduces potential exposure to ethidium bromide and is safer for student use. The agarose gel is layered over an ethidium bromide sheet, a second sheet is place over the gel, and a light weight is placed over the gel. After 10–15 minutes, the sheets are removed and the gel is visualized under ultraviolet light.

○ Data Analysis

Separating the PCR products through an agarose gel and staining with ethidium bromide (Figure 2) shows one clear band at the expected length for each primer set: 692 bp for claudin-2 and 604 bp for claudin-12. Each set of primers shows an enhanced PCR product just below the calculated annealing temperature (60°C for claudin-2 and 67°C for claudin-12). As the temperature deviated from the observed optimal annealing temperature, either decreasing or increasing, the amount of product decreased proportionally. The claudin-12 primers were able to produce the expected PCR product over a wider range of annealing temperatures than the claudin-2 primers because the claudin-12 primers have a higher T_m, which allowed for a more stable primer-DNA duplex than the claudin-2 primers, thus supporting primer elongation at higher temperatures.

○ Evaluation of Student Understanding

To assess student learning, the first laboratory begins with a pretest consisting of 20 multiple-choice questions designed to test the student's knowledge of DNA and PCR. An in-depth discussion ensues to teach students the basics of DNA synthesis and how specific gene sequences of DNA can be amplified using PCR. The details of each laboratory module are described, and variables that affect PCR are listed by the class. This allows students to understand the importance of optimization in experimental protocols. Students often perform laboratory exercises without giving thought to the painstaking work involved in development of the protocol, and without fully comprehending and analyzing the outcomes of their experiments (Phillips et al., 2008). The instructor and students should explore what variables each group will test and the basis of their hypothesis. A posttest consisting of the same questions is given at the beginning of module 1 to assess both understanding of the concepts and preparation for the lab exercise. The questions include numerical calculations for CG ratio and annealing temperature. In addition, at the end of module 3, each lab group is required to submit a lab report written in scientific format that includes calculated data on the primers used and images of the agarose gels.

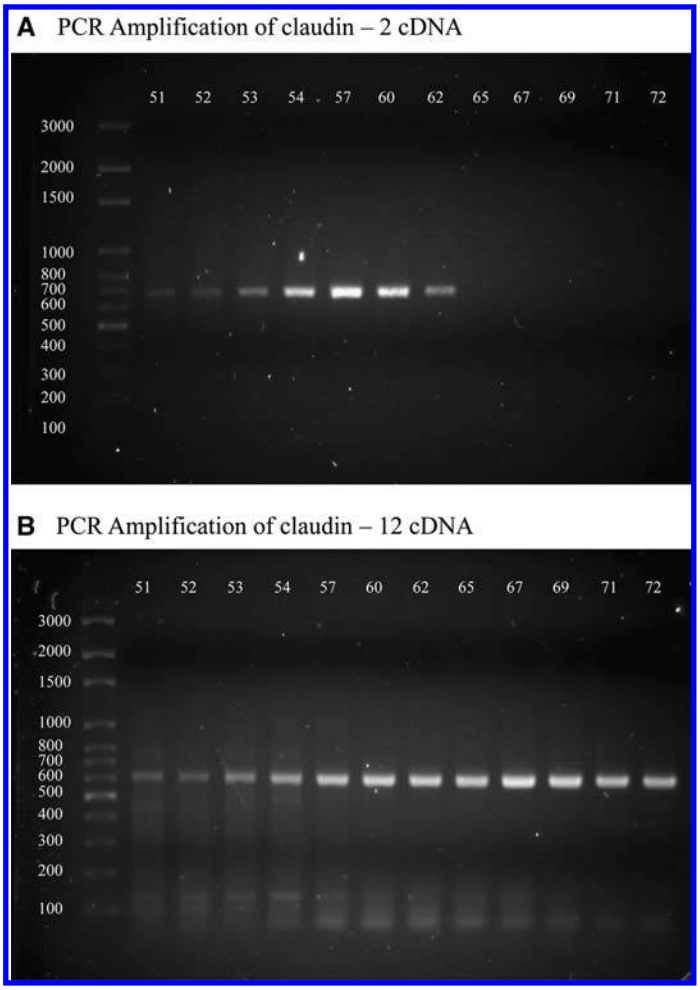

Figure 2. Typical patterns of PCR product analysis by ethidium bromide staining of agarose gels produce one band of amplified cDNA for each set of (A) claudin-2 primers and (B) claudin-12 primers when using annealing temperature ranging from 51°C to 72°C.

○ Conclusion

This set of laboratory exercises introduces students to DNA amplification using PCR in a way that demonstrates the underlying principles of PCR with emphasis on parameters that influence it. Students learn how primer design influences annealing temperature and how this is only one of many parameters that can significantly change the outcome of the experiment. Highlighting these variables encourages students to think outside the standard "cookbook" protocol for PCR and thus promotes critical-thinking skills necessary for lifelong learning and success.

Acknowledgments

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