Which came first: the chicken or the finch?

How we extract DNA and use sequences to determine relatedness of organisms

BiGe105 Lab for Jan. 21st 2020

Contents

1 Extracting DNA from Produce

DNA serves as the molecular fingerprint of an organism, allowing us to uniquely identify different species and even different individuals based solely on the sequence of As, Ts, Cs, and Gs found in their DNA. We can now readily access these sequences by sending out DNA samples to companies who specialize in large-scale sequencing technologies. For short sequences (around 1000 basepairs or fewer), we are even able to get sequencing results overnight. But the first key step to this process is to extract the DNA in the first place. To illustrate how amazingly convenient sequencing has become, we will be extracting DNA from produce, and by Monday we can have sequencing results that will reveal the identity of our samples. While in this case we already know the identity of the samples we are working with (e.g. chicken or turkey), we hope you'll see the utility of being able to determine the identity of a unknown sample with just an afternoon of lab work.

For extracting DNA, you will be doing a column-based purification, so named because of the spin columns that are used (see Figure 1). These column act as microscopic filters that allow certain cellular components through while capturing others on the white membrane of the column. Specifically, we will be using materials from the Qiagen DNeasy Blood and Tissue Kit. Kits like this are extremely common for many molecular biology techniques in the lab since they come with all the needed reagents conveniently packaged together. An overview of the protocol is shown in Figure 2, but we will walk though the details more carefully in the step-by-step protocol at the end. For now, you will need to familiarize yourself with some new pieces of lab equipment:

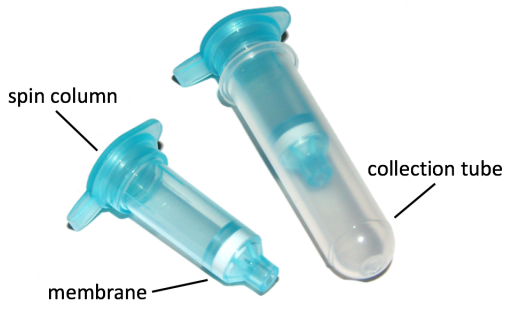

Figure 1: Spin columns. On the left is shown a spin column by itself, and on the right is a spin column in a collection tube. When enough force is applied, a liquid sample will flow through the white membrane and collect in the clear collection tube. These membranes are designed so that certain cellular components are captured or bind to the membrane while others are allowed to flow through. Such selectivity is essential for isolating DNA from samples.

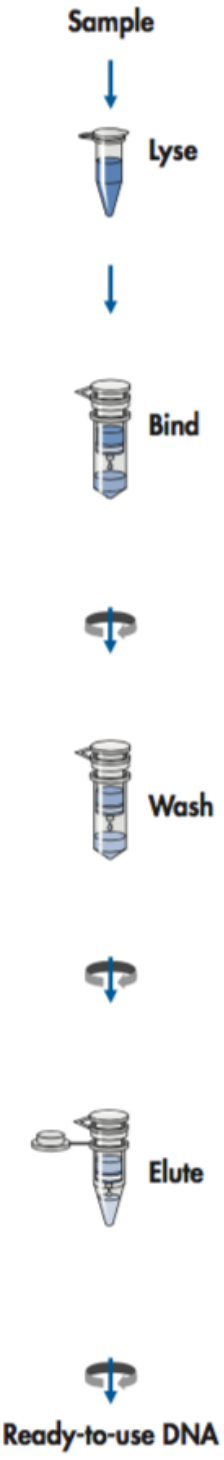

Figure 2: Overview of the Qiagen DNeasy Blood and Tissue protocol. This protocol consists of four main steps: 1) lysing or breaking open the cells, 2) binding DNA on the DNeasy column membrane, 3) washing the DNA sample to ensure that only DNA is retained, and 4) collecting the DNA off the column by eluting it.

- First, we have a **vortexer** (shown in Figure 3), which is a device that is used to vigorously shake samples. Vortexers are used to mix samples quickly and thoroughly when set at the highest speed.
- Next, we have a **heat block** (shown in Figure 4), which simply maintains a block of metal at a specified temperature. The heat block has holes in it for placing tubes, allowing us to incubate samples at a desired temperature.
- Lastly, we have a tabletop **centrifuge** (like the one show in Figure 5), which is used to spin samples at high speeds. Centrifuges like this can spin at an incredible speed of over 13,000 rotations per minute, corresponding to a force that is 16,000 times the force of gravity. Such forces can be used to separate different components in a liquid suspension based on their density, and is in fact how plasma and red blood cells are separated from donated blood. Given how quickly these centrifuges spin, it is essential to make sure that the samples are balanced in the centrifuge before spinning. For an even number of samples, this simply means making sure that each tube in the centrifuge has a corresponding tube placed directly across from it (for example, having a tube placed at position 1 and position 13 in the centrifuges we will be using). Refer to Figure 6 for examples of how to balance a centrifuge for different numbers of samples.

Figure 3: Vortexer. A device used to vigorously shake samples. Simply press your tube on the black rubber platform, and the platform will begin to shake. The speed of the vortexer can be changed by turning the knob, but we will be using it at the maximum speed (indicated as 10 on the vortexer).

Figure 4: Heat block. An instrument used to keep samples at a particular temperature.

Figure 5: Tabletop centrifuge. Used to spin samples up to 13,000 rotations per minute (rpm). These high speeds allow for the separation of different components of an initially homogenous solution.

Figure 6: How to balance a centrifuge. Depending on the number of samples you are spinning (indicated by the black circles), you will need to place the tubes in a different orientation to make sure the centrifuge is balanced.

1.1 Materials

At your lab bench, you should find a rack, with all the tubes, columns, and reagents you'll need placed from left to right in the order that you will use them:

- A tube labeled "lysis"
- A tube labeled "proteinase"
- A tube labeled "binding"
- A tube labeled "ethanol"
- A spin column labeled "DNeasy," placed in a collection tube
- An empty collection tube
- A tube labeled "wash 1"
- Another empty collection tube
- A tube labeled "wash 2"
- An empty 2 mL tube, labeled "DNA"
- A tube labeled "elution"

A TA will come by to give you your produce sample from which you'll extract DNA.

1.2 Protocol

1.2.1 Preparing the samples for DNA extraction

Before the bootcamp, the TAs cut up and weighed out 25 mg of either chicken, Cornish game hen, turkey, or duck for you to work with. While it may not look like much, there are actually around 10 million cells in your sample! This means there are 10 million copies of DNA for you to potentially extract. We've cut the sample into as small of pieces as possible to make it easier to break open the cells in the following steps.

1.2.2 Chemically breaking open the cells

In order to extract the DNA, we first need to break open the cells through a process known as lysis. The following steps will facilitate lysing the cells:

1. Take your produce sample and add 180 μL of lysis buffer (red tube). This buffer serves as a detergent that helps to chemically break open the cells,
2. Next, add 20 μL of proteinase (red tube) to your sample. Proteinase is an enzyme that will degrade any protein in the sample while leaving the desired DNA intact.
3. Make sure the lid on your tube is closed securely, and vortex your sample at the maximum speed for 10 seconds by pressing the tube down on the rubber platform. Your sample should be well mixed after vortexing.
4. Incubate the mixture for about one hour at 56 °C in the heat block. The sample will slowly become clearer as cells are broken open and the proteinase breaks down the unwanted protein in the sample. The TAs will periodically vortex your samples for you to keep the samples well-mixed.

1.2.3 Binding DNA to the spin column membrane

At this point, your sample should completely lysed, resulting in a homogeneous solution. We ultimately want to isolate just the DNA by itself, but there are still a lot of other cellular components in the sample. The following steps will bind the DNA to the spin column, while allowing other cellular material to pass through.

5. Vortex your sample for 15 seconds before proceeding to ensure the sample is well-mixed.
6. Add 200 μL of binding buffer (green tube) to your sample. Vortex your tube for 10 seconds.
7. Add 200 μL of ethanol (green tube) to your sample. Vortex your tube for 10 seconds.
8. Transfer your solution (a total of around 600 μL) into the DNeasy Mini spin column. Note that this spin column is already placed in a collection tube.
9. Coordinate with other groups to balance your DNeasy Mini spin columns in a centrifuge. Centrifuge your samples for 1 min at 13,000 rpm. This column will capture DNA while allowing other cellular components to flow through into the collection tube below.
10. Transfer your spin column to a new collection tube. You can discard your old collection tube as well as the liquid flow-through.

1.2.4 Isolating the DNA

You have now removed many extraneous cellular components while capturing the desired DNA on the white membrane of the spin column. The following steps serve to wash the DNA a couple of times to ensure that DNA, and only DNA, is bound to the column membrane:

11. Pipette 500 μL of of wash buffer 1 (blue tube) into the spin column.
12. Centrifuge your spin column for 1 min at 13,000 rpm.
13. Once again transfer your spin column to a new collection tube. You can discard your old collection tube as well as the liquid flow-through.
14. Add 500 μL of wash buffer 2 (purple tube) to the spin column.
15. Centrifuge you spin column for 3 min at 13,000 rpm. This spin step is longer than the previous ones to ensure that the membrane is thoroughly dried and no wash solution is carried over in the final step.

16. **Carefully** transfer your spin column to the final empty 2 mL tube labeled "DNA". Once agin, we don't want to carry over any wash solution, so make sure the spin column doesn't touch the liquid flow-through in this step. You can discard your collection tube as well as the liquid flow-through.

1.2.5 Eluting the DNA

With your purified DNA bound to the column membrane, you are nearly done! The last step is to release the DNA from the membrane so we can collect it in a tube. That is achieved by doing the following:

17. Pipette 100 μL of the elution buffer (yellow tube) directly onto the DNeasy membrane.
18. Leave the column for about 1 min at room temperature, and centrifuge for 1 min at 13,000 rpm. In this step, the DNA will now be released from the column and will be collected in the tube.
19. Throw away the the spin column and keep your tube of DNA.

Congrats! You should now have a clear and colorless liquid that contains your DNA. Take your sample to a TA, and they will show you how to determine how much DNA you have in your sample.

2 Polymerase Chain Reaction (PCR)

Objective: Learn to set up a reaction that will replicate the DNA you just extracted

Previously in the lab, we extracted DNA from various types of produce by removing all other substances that compose the cell. Now, you have a tube containing the genome of a particular species of bird suspended in water. The next thing we hope to do in determining the relationship between our various samples is to sequence the DNA. By looking at the sequences of vital genes, we can directly compare various organisms (as in Figure 7) to determine how they relate to one another.

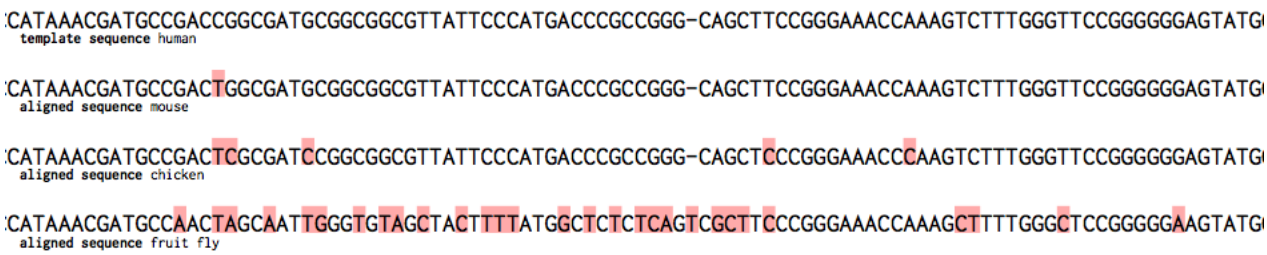

Figure 7: Sequence alignment of DNA encoding for ribosomal RNA. Here, segments of DNA from four different organisms are compared. The highlighted letters indicate where the organisms differ relative to the human sequence on the top. As can be seen, different organisms have various differences in their DNA sequence, but more importantly, these difference recapitulate what we know about how these organisms are related. The human and mouse sequences differ at only one basepair as we might expect from two relatively closely related mammals. Chicken however, as a more distantly related vertebrate has accumulated several more mutations, while the most distantly related fruit fly (an invertebrate) has many many more mutations.

While advancements in DNA sequencing allow us to read out entire genomes for an ever-decreasing price, we will be looking at a particular gene which encodes for ribosomal RNA (rRNA). As previously discussed, the ribosome is an essential piece of machinery in the cell, making it a great gene for comparing different species since we can confidently assume that all of our samples will contain this gene. However, considering that this small piece of DNA which we are interested in lies among a dense tome of other genes, we want to make that piece stand out. In other words, we want to amplify the presence of that sequence of DNA by making many copies of the region so it is easier to read the sequence. How does one go about it?

As the story goes, one day in the early 1980s, Kary Mullis realized that the best way to amplify up a piece of DNA is to enlist the microscopic factory of DNA replication. By using the key molecular players in DNA replication and with a programmable machine that changes the temperature of its internal compartment at specific times, Mullis managed to imitate DNA replication in a small tube and subsequently make a segment of DNA over and over again. The result of his work earned him the Nobel Prize in 1993 and has become ubiquitous in biology labs everywhere: polymerase chain reaction (PCR), illustrated in Figure 8 and described in more detail below.

The basis for amplifying, say, the portion of the DNA that encodes for rRNA involves two short strands of DNA and one enzyme used in three steps. First, the two strands that make up the DNA must be separated. Here, we simply need to raise the temperature. In the second step, the two short segments of DNA called primers, which complement the starting and ending locations of the desired region of DNA for amplification, attach to the separated strands. Finally, the molecular machine behind DNA replication, DNA polymerase, uses the primers as a launchpad for writing in complementary nucleotides, creating two new double-stranded DNA pieces. This amplified DNA can

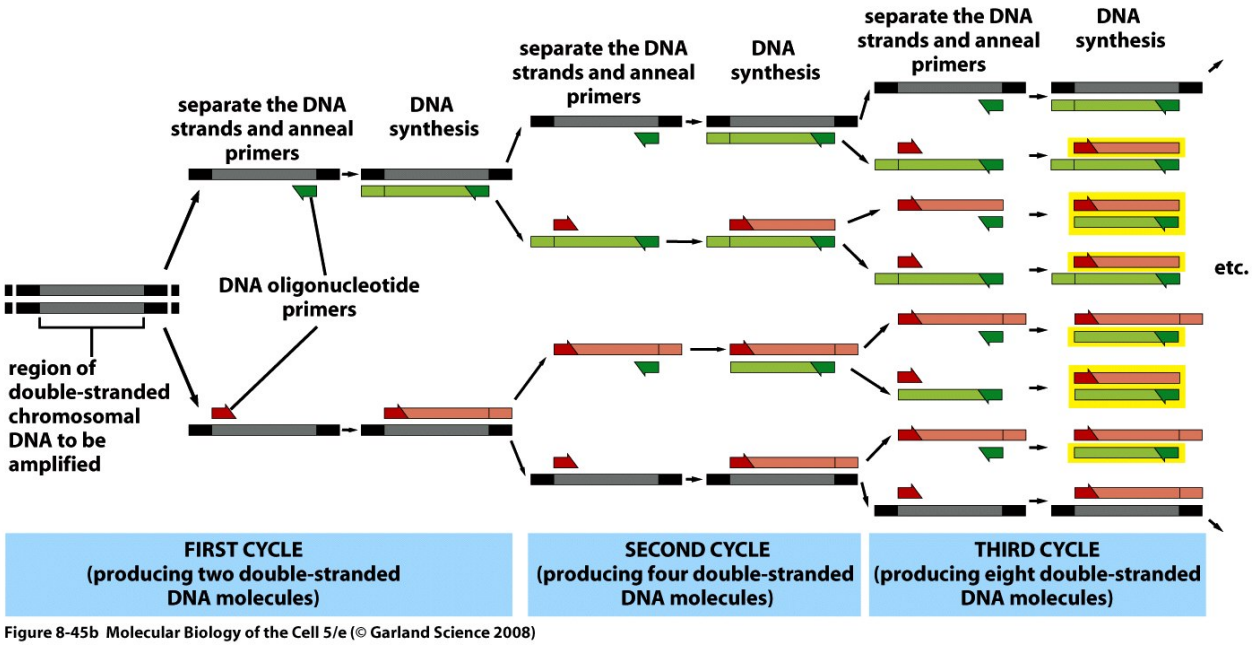

Figure 8: Overview of polymerase chain reaction. We can imagine the region of DNA that encodes for our rRNA to be the gray highlighted portion of the DNA to the left. The short red and green arrows are the primers; their sequences are specifically designed such that they attach onto opposite strands of DNA and at opposite ends of the gene. Eventually, as the DNA is replicated more times, the short segment of DNA is amplified so much that all other DNA is found at negligible concentrations. Figure taken from Molecular Biology of the Cell, 5th edition.

now be sent out to a sequencing company, where the sequence can be read out, letter by letter, using clever biochemistry.

For this portion of the lab, we will set up the reaction necessary to amplify the rRNA gene. Standard PCR reactions will involve five components:

1. The DNA sample, which we extracted,
2. The two primers designed to interface with the ends of the amplified region, which we specifically designed and asked a company to synthesize for us,
3. A mixture containing the DNA polymerase, nucleotides and any buffers necessary for the reaction,
4. Water to ensure that the contents of our reaction are at the right concentration.

Once we have combined all of the key pieces into a small tube, we will put the tube into a machine called a thermocycler (Figure 9). The thermocycler is specifically designed to hold this tube and can be programmed to provide a protocol for when we want to change the temperature of the reaction and for how long the chamber holding the tube remains at this temperature.

Figure 9: Thermocycler – The thermocycler has two chambers (labeled A and B) into which we can insert your PCR tubes. Using the buttons and display on the front, we can then program the thermocycler to incubate the tubes at specific temperatures for specified lengths of time.

2.1 Materials

At your lab bench, you should find the following items:

- A clear tube about five times smaller than the tubes we have been using, into which you will put all of the ingredients for your PCR reaction,
- your tube of extracted DNA,
- a tube labeled with a yellow sticker "water,"
- a tube labeled with a purple sticker "18S," containing the two primers mixed together
- and a tube labeled with a red sticker "Q5," containing the specific type of polymerase we will be using, along with nucleotides and any necessary buffers.

2.2 Protocol

1. Pipette 18 µL of water into the the small tube. The addition of this water ensures that all the components of the reaction are at the appropriate concentrations.
2. Pipette 5 µL of the 18S primer mixture into the small tube.

3. Pipette 2 µL of your extracted DNA into the small tube.
4. Lastly, pipette 25 µL from the Q5 tube into the small tube. This mixture contains the polymerase enzyme itself along with other components needed for the reaction to occur. Mix your sample by pipetting it up and down a few times.
5. Make sure all the liquid in your tube is collected at the bottom and that there are no droplets on the side. The TAs can help you centrifuge your sample if needed.
6. Hand your tube to one of the TAs. When all the tubes have been collected, we will demonstrate how the thermocycler is set up to start the PCR.

The thermocycler will then carry out the necessary steps to replicate your DNA. Here, the thermocycler will have the following program:

Step	Purpose	Temperature	Time
1	initial denaturation	98°C	2 min.
2	denaturation	98°C	10 sec.
3	annealing	48°C	30 sec.
4	elongation	72°C	30 sec.
5	repeat steps 2-4 29 times		
6	final elongation	72°C	2 min.
7	suspend reaction	4°C	forever

3 Gel Electrophoresis

Our next objective is to determine whether or not our polymerase chain reaction (PCR) from last worked. To this end, we will be using agarose gel electrophoresis, a powerful method for separating DNA by size. An agarose gel consists of microscopic pores that act as a molecular sieve. Samples of DNA are loaded into wells made in the gel during casting, as shown in Figure 10. Since DNA has a strong negative charge at neutral pH, it migrates through the gel towards the positive electrode. This is called **electrophoretic motion**. The DNA molecules are separated in the gel according to their size and shape. Linear DNA molecules are separated almost exclusively according to their size. The smaller the linear fragment, the faster it migrates due to less entanglement with the gel mesh. If the sizes of two fragments are similar or identical, they will migrate together in the gel.

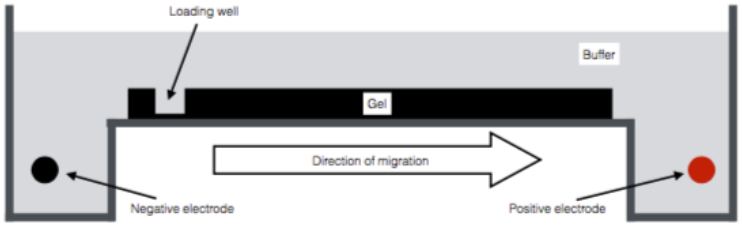

Figure 10: A schematic of gel electrophoresis. DNA is pipetted into the sample wells at the top of the gel, and since DNA is negatively charged, the DNA will move through the gel toward the positive electrode once a current is applied to the gel box.

The samples are loaded into the wells of the gel with a **loading dye**. The loading dye contains glycerol, which helps the solution sink into the wells of the gel and dyes that provide visualization. After the DNA has migrated and the power is shut off, the final positions of the DNA are found by taking a picture using a **gel scanner**. The DNA will be separated by size, with each size appearing as a line (referred to as a "band") on the gel. The approximate lengths of the DNA fragments in the cell are determined by comparing the positions of the bands to those of a reference set of DNA strands called a **ladder**. The ladder consists of pre-cut DNA that has been cut into fragments of known length. Images from a gel scanner of the two ladders we use in the lab are shown in Figure 11.

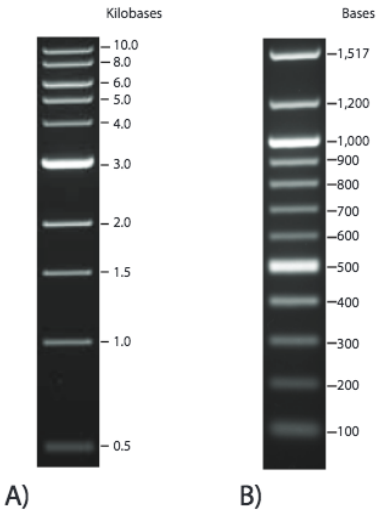

Figure 11: Left, a 100 bp ladder. Right, a 1000 bp ladder. The tick marks to the right of the DNA ladders indicate the number of base pairs in the DNA in the respective bands. These images came from New England Biolabs, the supplier of our DNA ladders.

3.1 Gel electrophoresis of PCR products.

We will now examine your PCR product using gel electrophoresis. PCR is supposed to produce large amounts of identical pieces of DNA. Using a nanodrop or other device to quantify the amount of DNA is not sufficient to insure your PCR worked properly. For example, your PCR reaction can amplify DNA contaminates, your PCR primers can bind to the wrong place, or they can bind to multiple places on your DNA template. The only way to completely guarantee your PCR worked properly is to sequence the DNA, but this is expensive and time consuming. Gel electrophoresis is a simple quality control measure that can ensure that the resulting DNA from your PCR is of the right length. A sample gel image of what we expect to obtain is shown in Figure 12.

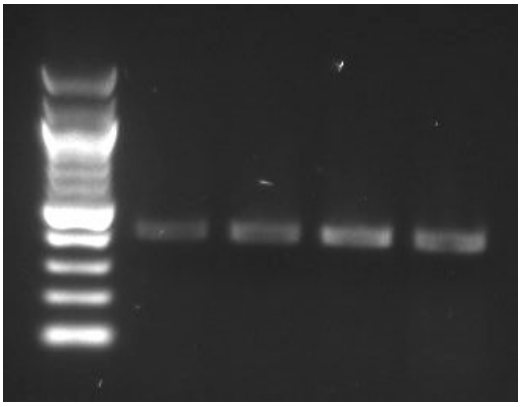

Figure 12: Example PCR results. The first lane is a 100 bp ladder used for reference and the next four lanes are the results of running a PCR with chicken, duck, hen, and turkey DNA. We see that the amplified segment of DNA that we expect to find is around 400 bp.

3.2 Making the gel

We first need to make the gel through which our DNA samples will run.

1. For this gel, we will be making a 1% agarose gel in TAE buffer. For a 30 mL gel, this corresponds to adding 0.3 g of agarose. Mix the TAE buffer and agarose together in a flask.
2. Microwave the agarose mixture, watching carefully to make sure it doesn't boil over, and stirring it occasionally. Continue until the agarose has completely dissolved and is no longer visible.
3. Allow the heated gel to cool for a few minutes and add 3 μL of SYBR Safe. This chemical binds within the double stranded part of DNA, making it possible to visualize the DNA bands when the gel is exposed to UV or blue light.
4. Pour your molten gel into the gel cast, with the comb in place. This comb will make divots in the gel into which you can pipette your DNA sample once the gel has solidified.
5. Now we wait . . . Once the gel has solidified, cover the gel in the TAE buffer.

3.3 Running the gel

1. For running your PCR product from last week out on a gel, you'll need to combine 20 μL of your PCR and 4 μL of loading dye. The loading dye makes your aqueous PCR product denser, allowing your sample to sink to the bottom of the sample well when you load it into the gel.
2. Similarly, a DNA ladder with loading dye has already been prepared for you.

Figure 13: Mini centrifuge to use in step 4. Note that your centrifuge may be a different model.

3. Gently vortex to mix your samples. You should set the vortexing device to setting 7 and vortex for 2-3 seconds.

4. Spin down your samples briefly to get rid of any bubbles. The mini centrifuge to use for this is shown in Figure 13. Press the cover of the centrifuge down for approximately 1 second to spin the tubes.

5. Load your samples into your agarose gel, coordinating with other groups, as shown in the table below. First load 10 µL of your DNA ladder. Then load 10 µL of your PCR product and loading dye mixture into each the next two adjacent lanes.

Lane	**Group #**	**Sample**
1	Group 1	100 bp DNA ladder
2	Group 1	PCR product
3	Group 1	PCR product
4	—	—
5	Group 2	100 bp DNA ladder
6	Group 2	PCR product
7	Group 2	PCR product
8	—	—
9	Group 3	100 bp DNA ladder
10	Group 3	PCR product
11	Group 3	PCR product

   To load the gel, place your pipette beneath the buffer surface layer, part-way into your target well. Very slowly depress the pipette plunger to load your sample. As you depress the plunger, you will notice the sample fall into the well. Do not rush as it may cause the sample to flow out of the well.

6. After your gel is loaded, ask your TA to help set up the power supply. Run the gel at 100V for 45 minutes.