Student Manual

Background

With the world population exploding and farmable land disappearing, agricultural specialists are concerned about the world's ability to produce enough food to feed the growing population. Environmentalists are concerned about the overuse of pesticides and herbicides and the long-term effects of these chemicals on the environment and human health. Might there be a solution to both of these problems? The biotechnology industry thinks so. Its proponents believe genetically modified organisms (GMOs), particularly genetically modified (GM) crop plants, can solve both problems. This proposed solution, however, has met with great opposition throughout the world. Dubbed "frankenfoods" by opponents and restricted in most European countries, GMOs are widely produced and sold in the United States. Currently in the US, foods that contain GMOs do not have to be labeled as such.

Genetic manipulation of crop plants is not new. Farmers have been genetically modifying crops for centuries and crop breeding to encourage specific traits, such as high yield, is still an important part of agriculture today. However, there is now the option to place genes for selected traits directly into crop plants. These genes do not have to originate from the same plant species—in fact, they do not have to come from plants at all. One popular class of GM crops has a gene from the soil bacterium *Bacillus thuringiensis* (Bt) inserted into their genomes. Bt crops produce a protein called delta-endotoxin that is lethal to European corn borers, a common pest on corn plants. Farmers who plant Bt crops do not have to apply pesticide because the plants produce the toxic protein inside their cells. When the corn borers feed on the genetically modified plant, they die. Other GMOs include those that are herbicide-resistant delayed for fruit ripening, are resistant to fungi or drought, have increased crop yield, or bear improved fruits.

Many people object to the use of GM crop plants. They argue that there is a potential to create super-weeds through cross-pollination with herbicide-resistant crops or that super-bugs will evolve that are no longer resistant to the toxins in pest-resistant crops. Many are concerned with potential allergic reactions to the novel proteins or antibiotic resistance arising from the selectable markers used to develop the crops or other unforeseen effects on public health. Proponents of GM foods argue these crops are actually better for the environment. Fewer toxic chemicals are put into the environment and thus fewer toxic chemicals can harm the environment and human health. In addition, these crops can preserve arable land by reducing stresses on the land, improve the nutritional value of food in developing countries, and allow crops to be grown on previously unfarmable land.

Whatever position one takes in the GMO debate, it would be beneficial to be able to test foods found in the grocery store for the presence of GMO-derived products. This can be done in several ways. One would be to use an antibody-based test such as the enzyme-linked immunosorbent assay (ELISA), which can detect the proteins that are produced specifically by GM crops. However, the ELISA is not useful for testing foods that have been highly processed, because the proteins have most likely been destroyed and different GM foods produce different proteins. Another method is to use the polymerase chain reaction (PCR) to look for a DNA sequence common to GM foods. DNA is more resistant than proteins to processing and can be extracted from even highly processed foods. It is these GMO DNA sequences that we will be testing for in this laboratory.

In the first lesson you will extract genomic DNA from food samples, in the second lab you will run PCR reactions to amplify GMO and natural plant sequences from the DNA, and in the third lab you will electrophorese the amplified samples to visualize the DNA.

Let's see if your favorite food contains GMOs!

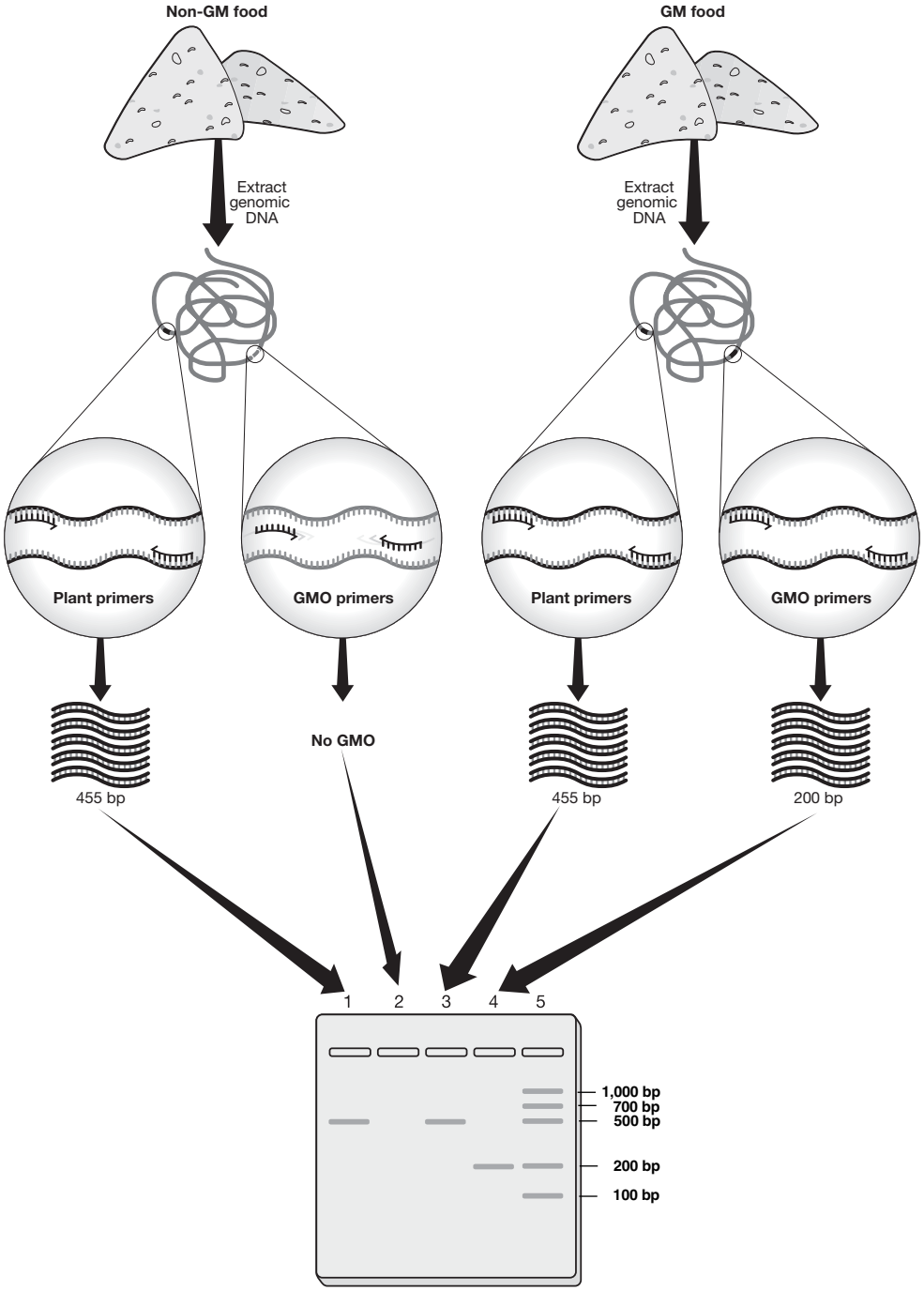


Fig. 1. Detecting GM foods by PCR. Genomic DNA is extracted from test foods (Lesson 1) and then two PCR reactions are performed on each test food genomic DNA sample (Lesson 2). One PCR reaction uses primers specific to a common plant gene (plant primers) to verify that viable DNA was successfully extracted from the food. No matter whether the food is GM or not, this PCR reaction should always amplify DNA (See lanes 1 and 3 of the gel above). The other PCR reaction uses primers specific to sequences commonly found in GM crops (GMO primers). This PCR reaction will only amplify DNA if the test food is GM (See lane 4). If the test food is non-GM, then the GMO primers will not be complementary to any sequence within the test food genomic DNA and will not anneal, so no DNA will be amplified (see lane 2). To find out whether DNA has been amplified or not, the PCR products are electrophoresed on a gel and stained to visualize DNA as bands (Lesson 3). A molecular weight ruler (lane 5) is electrophoresed with the samples to allow the sizes of the DNA bands to be determined.

Lesson 1 Extraction of DNA From Food Samples

In this lesson you will extract DNA from a control non-GMO food and a grocery store food item that you will test for the presence of GMOs. The most commonly modified foods are corn and soy-based, and so the test food could be fresh corn or soybeans, or a prepared or processed food such as cornmeal, cheese puffs, veggie sausage, etc. You will process the non-GMO control first.

You will first weigh your food sample, then grind it with water to make a slurry. You will then add a tiny amount of the slurry to a screwcap tube containing InstaGene matrix and boil it for 5 minutes.

The cellular contents you are releasing from the ground-up sample contain enzymes (DNases) that can degrade the DNA you are attempting to extract. The InstaGene matrix is made of negatively charged microscopic beads that "chelate" or grab metal ions out of solution. It chelates metal ions such as Mg^{2+}, which is required as a cofactor in enzymatic reactions. When DNA is released from your sample in the presence of the InstaGene matrix, the charged beads grab the Mg^{2+} and make it unavailable to the enzymes that would degrade the DNA you are trying to extract. This allows you to extract DNA without degradation. Boiling the samples destroys these enzymes.

After you centrifuge the samples to remove the InstaGene matrix and debris, the supernatant will contain intact extracted DNA. This extracted DNA will be used in the next laboratory as your target DNA.

Lesson 1 Extraction of DNA From Food Samples

Focus Questions

1. How can you test a food to find out if it contains material derived from a genetically modified organism (GMO)?

2. In what organelles is plant DNA located?

3. Many foods containing GM crops are highly processed. Can you suggest how DNA from whole plants may differ from that extracted from processed foods, e.g., corn chips, cornmeal, etc.?

4. What molecules are present in the cell that might interfere with DNA extraction?

5. Why do you also perform analysis on food that is known to be a non-GMO food control?

6. Why was the non-GMO food control prepared prior to your test food sample?

Student Protocol – Lesson One

Materials and supplies required at the workstation prior to beginning this exercise are listed below.

Student Workstation

Material	Quantity
Screwcap tube with 500 µl InstaGene matrix	2
Beaker of distilled water	1
Food samples	1 or 2
Disposable plastic transfer pipets (DPTP)	2
2–20 µl micropipet (if preparing non-GMO food control)	1
2–20 µl pipet tips, aerosol barrier	1 rack
Mortar and pestle	1
Marking pen	1

Common Workstation

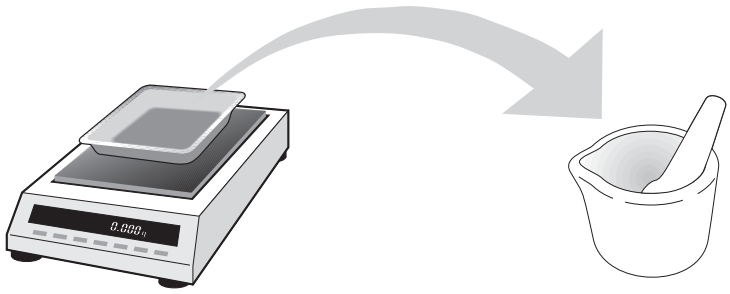
Material	Quantity
Water bath set to 95–100°C	1
Microcentrifuge or mini centrifuges	3–4
Balance and weigh boats	1

Protocol

Note: ALWAYS process the non-GMO control before the test sample to reduce the risk of contamination.

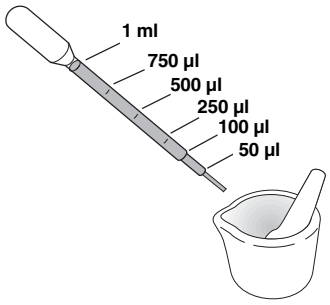
Grind non-GMO food control (your instructor may perform this step for you)

1. Find your screwcap tubes containing 500 µl of InstaGene matrix and label one “non-GMO” and one “test”.
2. Weigh out 0.5–2 g of the certified non-GMO food control and place in mortar.

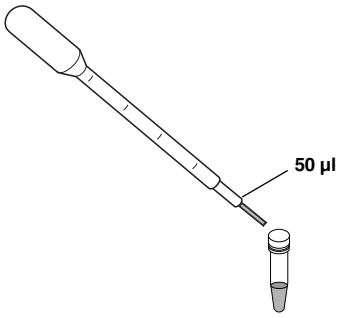


2. Using the transfer pipet, add 5 ml of distilled water for every gram of food using the graduations on the transfer pipet. To calculate the volume of water you need, mulitply the mass in grams of the food weighed out by 5 and add that many millimeters.

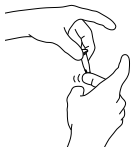
   Mass of Food = _____ g x 5 = _____ ml



3. Grind with pestle for at least 2 min until a slurry is formed.
4. Add another 5 ml of distilled water for every gram of food. Mix or grind further with the pestle until the slurry is smooth enough to pipet.
5. Add **50 µl** of ground slurry to the screwcap tube containing 500 µl of InstaGene matrix labeled “non-GMO” using a transfer pipet.



6. Recap tube and shake well.



7. Wash mortar with detergent and dry.

Grind Test Food

1. **Weigh out 0.5–2 g of test food and place in mortar.**
2. Using the transfer pipet, add 5 ml of distilled water for every gram of food using the graduations on the transfer pipet. To calculate the volume of water you need, mulitply the mass in grams of the food weighed out by 5 and add that many millimeters.

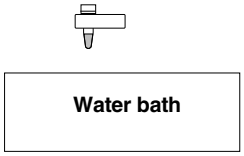
   Mass of food = _____ g x 5 = _____ ml

3. Grind with pestle for at least 2 min until a slurry is formed.

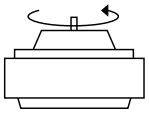
4. Add another 5 ml of distilled water for every gram of food and mix or grind further with pestle until the slurry is smooth enough to pipet.
5. Add **50 µl** of ground slurry to the screwcap tube labeled “Test” using the 50 µl mark on a transfer pipet.
6. Recap tube and shake well.

Process Samples to Extract DNA

1. Place non-GMO food control and test food sample tubes in 95°C water bath for 5 min.



2. Place tubes in a centrifuge in a balanced conformation and spin for 5 min at max speed.



3. Store tubes in refrigerator until the next lesson.

Lesson 2 Set Up PCR Reactions

In the last laboratory, you extracted DNA from a certified non-GMO food sample and a test food sample that you are analyzing for the presence of GMO DNA sequences. In this lab you will prepare those two samples and a positive control (GMO-positive template DNA) for the polymerase chain reaction (PCR).

PCR is DNA replication in a test tube. PCR allows you to amplify specific sections of DNA and make millions of copies of the target sequence. Your experiment is to determine whether or not the DNA you extracted from food in Lesson 1 contains or does not contain the target sequences of interest typically found in genetically modified (GM) foods.

PCR Review

PCR is such a powerful tool because of its simplicity and specificity. All that is required are minute quantities of the DNA template you want to amplify, DNA polymerase, two DNA primers, four DNA base pair subunits (deoxyribonucleotide triphosphates of adenine, guanine, thymine, and cytosine) and buffers.

Because PCR identifies a specific sequence of DNA and makes millions of copies of (or amplifies) that sequence, it is one of the most useful tools of molecular biology. Scientists use PCR to obtain the large amounts of a specific sequence of DNA that are necessary for such techniques as gene cloning, where DNA is physically moved from one genome to another. You are using the property of PCR that allows identification of a specific sequence, namely, the ability of PCR to search out a single sequence of a few hundred base pairs in a background of billions of base pairs. For example, the corn genome has 2.5 billion base pairs of DNA. This sequence is then amplified so that there are millions of copies of it so that it stands out from the few copies of the original template DNA.

PCR locates specific DNA sequences using primers that are complementary to the DNA template. Primers are short strands of DNA (usually between 6 and 30 base pairs long) called oligonucleotides. Two primers are needed to amplify a sequence of DNA, a forward primer and a reverse primer. The two primers are designed and synthesized in the laboratory with a specific sequence of nucleotides such that they can anneal (bind) at opposite ends of the target DNA sequence on the complementary strands of the target DNA template. The target DNA sequence is copied by the DNA polymerase reading the complementary strand of template DNA and adding nucleotides to the 3' ends of the primers (see fig 2). Primers give the specificity to the PCR, but they are also necessary because DNA polymerase can only add nucleotides to double-stranded DNA.

During PCR, double-stranded DNA template is separated by heating it, then each primer binds (anneals) to its complementary sequence on each of the separated DNA strands, and DNA polymerase elongates each primer by adding nucleotides to make a new double-stranded DNA (see fig 2).

The DNA polymerase used in PCR must be a thermally stable enzyme because the PCR reaction is heated to 94°C, which would destroy the biological activity of most enzymes. The most commonly used thermostable DNA polymerase is *Taq* DNA polymerase. This was isolated from a thermophillic bacterium, *Thermus aquaticus*, which lives in high-temperature steam vents such as those in Yellowstone National Park.

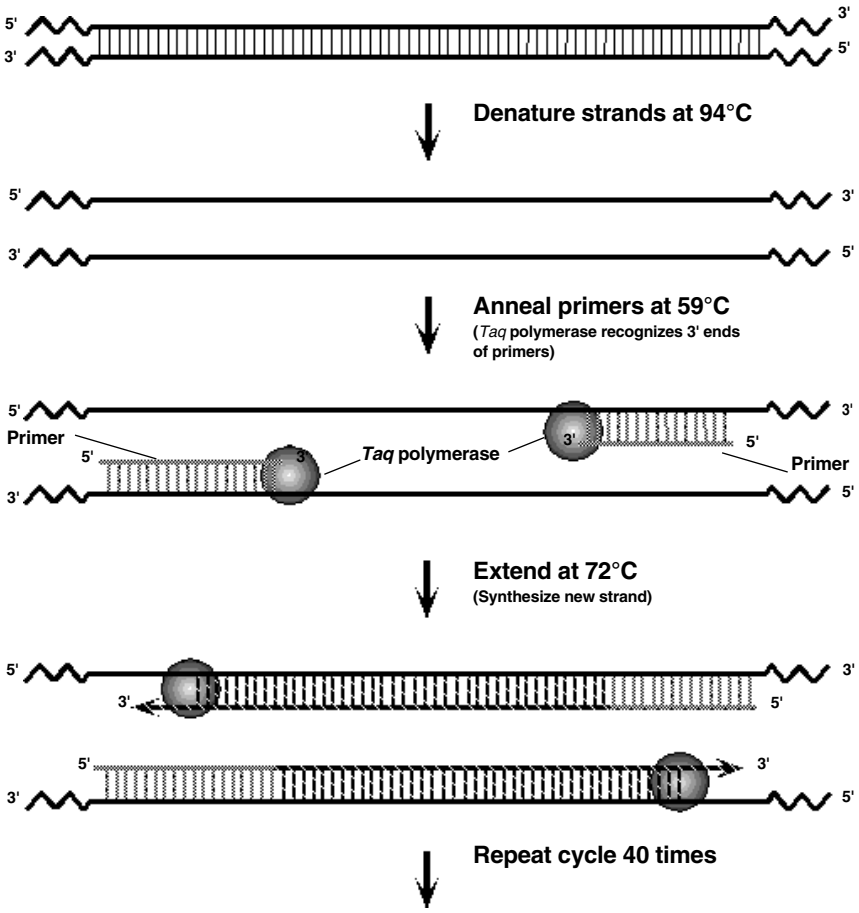


Fig. 2. A complete cycle of PCR.

PCR Step by Step

PCR has three steps, a denaturing step, an annealing step, and an elongation step. During the denaturing step, the DNA template is heated to 94°C to separate (or denature) the double-stranded DNA molecule into two single strands. The DNA is then cooled to 59°C to allow the primers to locate and anneal (bind) to the DNA. Because the primers are so much shorter than the template DNA, they will anneal much more quickly than the long template DNA strands at this temperature. The final step is to increase the temperature of the PCR reaction to 72°C, which is the optimal temperature for the DNA polymerase to function. In this step the DNA polymerase adds nucleotides (A, T, G, or a C) one at a time at the 3' end of the primer to create a complementary copy of the original DNA template. These three steps form one cycle of PCR. A complete PCR amplification undergoes multiple cycles of PCR, in this case 40 cycles.

The entire 40 cycle reaction is carried out in a test tube that has been placed in a thermal cycler or PCR machine. This is a machine that contains an aluminum block that can be rapidly heated and cooled. The rapid heating and cooling of this thermal block is known as thermal cycling.

Two new template strands are created from the original double-stranded template during each complete cycle of PCR. This causes exponential growth of the number of target DNA molecules, i.e., the number of target DNA molecules doubles at each cycle; this is why it is called a chain reaction. Therefore, after 40 cycles there will be 2^{40}, or over 1,100,000,000,000 times more copies than at the beginning. Once the target DNA sequences of interest have been sufficiently amplified, they can be visualized using gel electrophoresis. This allows researchers to determine the presence or absence of the PCR products of interest.

Your Task for This Lesson

For this experiment you will set up two PCR reactions for each DNA sample, which makes 6 PCR reactions in total. One PCR reaction, using the plant master mix (PMM), is a control to determine whether or not you have successfully extracted plant DNA from your test food. This is done by identifying a DNA sequence that is common to all plants by using primers (colored green in the kit) that specifically amplify a section of a chloroplast gene used in the light reaction (photosystem II). Why is this necessary? What if you do not amplify DNA using the GMO primers? Can you conclude that your test food is not GM or might it just be that your DNA extraction was unsuccessful? If you amplify DNA using the plant primers, you can conclude that you successfully amplified DNA, therefore whether or not you amplify DNA with your GMO primers, you will have more confidence in the validity of your result.

The second PCR reaction you carry out will determine whether or not your DNA sample contains GM DNA sequences. This is done by identifying DNA sequences that are common to most (~85%) of all GM plants using primers specific to these sequences. These primers are colored red and are in the GMO master mix (GMM).

Why do you have to set up a PCR reaction with DNA from certified non-GMO food? What if some GMO-positive DNA got into the InstaGene or master mix from a dirty pipet tip or a previous class? This DNA could be amplified in your test food PCR reaction and give you a false result. By having a known non-GMO control that you know should not amplify the GMO target sequences, you can tell if your PCR reactions have been contaminated by GMO-positive DNA.

Lesson 2

Focus Questions

1. What chemicals and molecules are needed for PCR, and what is the function of each component?

2. Examine the 150 base promoter sequence below.

5'TAGAAAAGGA AGGTGGCTCC TACAAATGCC ATCATTGCGA TAAAGGAAAG

GTATCATTC AAGATGCCTC TGCCGACAGT GGTCCCAAAG ATGGACCCCC

ACCCACGAGG AGC ATCGTGG AAAAAGAAGA CGTTCCAACC ACGTCTTCAA3'

Write in the sequence of the complementary strand and mark the 3' and 5' ends of the complementary strand.

Remembering that DNA polymerases can only add nucleotides to the 3' end of DNA, design a forward primer and a reverse primer, each 10 bases long, to amplify a target sequence of the DNA that is at least 100 bp long. Write the sequence of the primers below, with their 3' and 5' ends indicated. Also indicate on the sequence above which strand they are complementary to (will anneal to).

Forward primer sequence:

Reverse primer sequence:

4. Why are you performing two PCR reactions on each DNA sample?

5. What is the purpose of the GMO-positive control DNA?

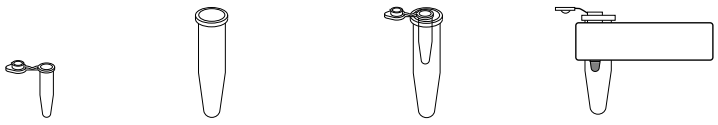
Student Protocol – Lesson Two Set Up PCR Reactions

Student Workstations

Material	Quantity
Ice bath containing 3 tubes	1
GMO master mix (red) (on ice)	1
Plant master mix (green) (on ice)	1
GMO positive control DNA (on ice)	1
Test food DNA (from previous lab)	1
Non-GMO food control DNA (from previous lab)	1
PCR tubes	6
PCR adaptors	6
Foam microtube holder	1
Marking pen	1
2–20 µl adjustable-volume micropipet or fixed-volume 20 µl micropipet	1
2–20 µl pipet tips, aerosol barrier	1 rack

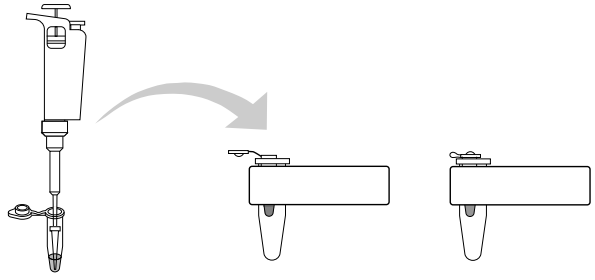
1. Number six PCR tubes 1–6 and label them with your initials. The numbers correspond to the following tube contents:

Tube Number	DNA	Master Mix
1	20 µl Non-GMO food control DNA	20 µl Plant master mix (green)
2	20 µl Non-GMO food control DNA	20 µl GMO master mix (red)
3	20 µl Test food DNA	20 µl Plant master mix (green)
4	20 ul Test food DNA	20 µl GMO master mix (red)
5	20 µl GMO positive control DNA	20 µl Plant master mix (green)
6	20 µl GMO positive control DNA	20 µl GMO master mix (red)



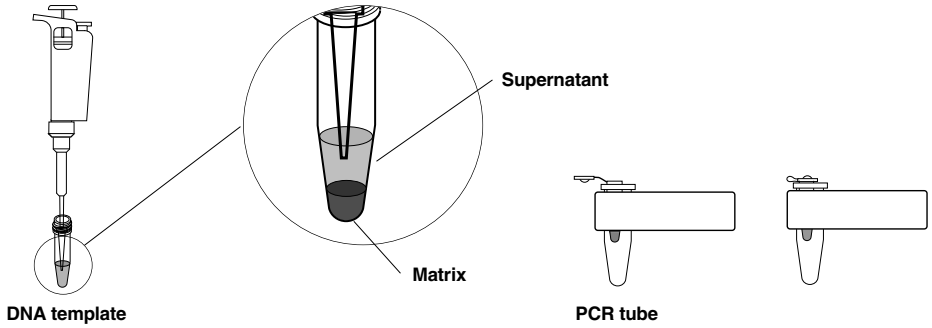
PCR tube Capless tube

2. Keep the tubes on ice for the remaining steps.
3. Using a fresh tip each time, add 20 µl of the indicated master mix to each tube. I.E. add 20 µl of green plant master mix (PMM) to tubes 1, 3, and 5. Then add 20 µl of red GMO master mix (GMM) to tubes 2, 4, and 6. Cap each tube.



Master mix

4. Using a fresh pipet tip for each tube, add 20 µl of the DNA to each tube as indicated in the table above. Take care not to transfer any of the InstaGene beads to your PCR reaction. If the beads are disrupted, recentrifuge your DNA samples to pellet the beads. Add 20 µl of non-GMO food control DNA to tube 1 and pipet up and down to mix. Discard your tip. Use a fresh tip to add 20 µl of non-GMO food control DNA to tube 2 and mix. Discard your tip. Similarly add 20 µl of test food DNA to tubes 3 & 4, and add 20 µl of GMO positive control DNA to tubes 5 & 6, changing your tip for every tube. Recap tubes.



5. When instructed to, place the PCR tubes in the thermal cycler.

Lesson 3 Electrophoresis of PCR Products

You have completed your PCR amplification. You cannot, however, at this point determine whether or not you have PCR products. To do this, you must visualize your products. You will do this using gel electrophoresis.

Your PCR product bands are very small compared to those in other DNA experiments you may have done. For example, fragments produced from a *Hind*III digest of lambda DNA are 23,130, 9,416, 6,557, 4,361, 2,322, 2,027, and 500 base pairs (bp). The product band sizes in this lab are 455 bp for the plant primers and 200 bp for the GMO primers, and a 1% gel would not separate these bands. Instead, a tighter gel matrix is needed to impede the movement of these bands so that they are separated more on the gel and can be seen. Therefore, if you are using agarose electrophoresis, you will use a 3% agarose gel. Alternatively, your teacher may elect to use a polyacrylamide gel, which has smaller pores, to separate your products. Polyacrylamide gel electrophoresis (PAGE) is used to separate smaller molecules for visualization.

Regardless of the gel type, you will load a molecular weight ruler (DNA standard) so that you have a reference to determine your product bands' sizes. The gel will then be stained with Fast Blast stain to make the bands visible.

Lesson 3

Focus Questions

1. Why did you resolve your PCR products by electrophoresis?

2. Explain why DNA fragments separate according to size in an electrophoresis gel.

3. Why do you need a molecular weight ruler alongside your samples?

4. What results do you expect in each lane? Fill in the chart below.

Lane	Sample	Expect band (Yes, No, Don't know)?
1	Sample 1: Non-GMO food control with plant primers	
2	Sample 2: Non-GMO food control with GMO primers	
3	Sample 3: Test food with plant primers	
4	Sample 4: Test food with GMO primers	
5	Sample 5: GMO positive control DNA with plant primers	
6	Sample 6: GMO positive control DNA with GMO primers	

Lesson 3

Student Workstation

Material	Quantity
Gel (3% agarose or 10% polyacrylamide)	1
Samples from previous lab period	6
Running buffer (1x TAE for agarose gels or 1x TBE for polyacrylamide gels)	300–350 ml
Orange G loading dye	1 vial
PCR molecular weight ruler	1 vial
2–20 µl adjustable-volume pipet or fixed-volume 20 µl micropipet	1
1–20 µl pipet tips, aerosol barrier	1 rack
Gel electrophoresis chamber (may be shared by 2 workstations)	1
Power supply (may be shared by multiple workstations)	1
Fast Blast DNA stain, 1x or 100x depending on protocol (at common workstation)	1
Gel staining tray	1

Protocol

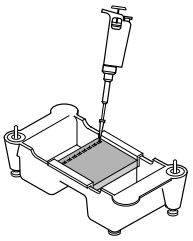
1. Set up your gel electrophoresis apparatus as instructed.

   Details on setting up electrophoresis equipment can be found in the Instructor's guide.

2. Using a fresh tip each time, add 10 µl of Orange G loading dye to each sample and mix well.

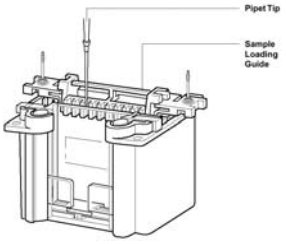
3. Load 20 µl of the PCR molecular mass ruler and 20 µl of each sample onto your gel in the order indicated below.

Lane	Sample	Load volume
1	Sample 1: Non-GMO food control with plant primers	20 µl
2	Sample 2: Non-GMO food control with GMO primers	20 µl
3	Sample 3: Test food with plant primers	20 µl
4	Sample 4: Test food with GMO primers	20 µl
5	Sample 5: GMO positive DNA with plant primers	20 µl
6	Sample 6: GMO positive DNA with GMO primers	20 µl
7	PCR molecular weight ruler	20 µl
8	Leave empty	



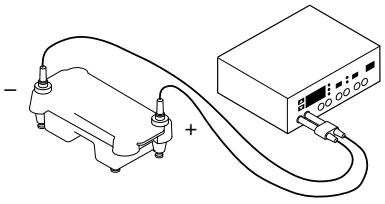
Agarose gel

or



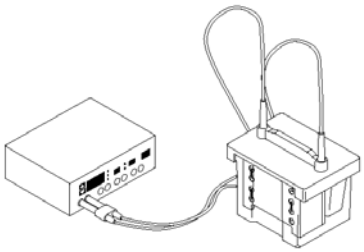
Polyacrylamide gel

4. The run time and voltage will depend on the type of gel you are running.
    - Run an agarose gel at 100 V for 30 minutes. Do not let the orange dye front migrate out of the agarose gel.
    - Run a polyacrylamide gel at 200 V for 30 minutes and do not let the red GMO primer dye front run out of the gel.



Agarose Gel Electrophoresis

or

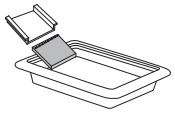


Polyacrylamide Gel Electrophoresis

5. Stain the gel in Fast Blast DNA stain. Refer to specific instructions below for your gel type.

Staining of Agarose Gels

1. When electrophoresis is complete, turn off the power and remove the lid from the gel box.
2. Carefully remove the gel tray and the gel from the gel box. Be careful, the gel is very slippery. Nudge the gel off the gel tray with your thumb and carefully slide it into your plastic staining tray.

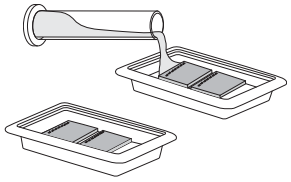


3. There are two protocols for staining your gel. Your instructor will inform you which one you will use.

    Protocol One: Overnight Staining

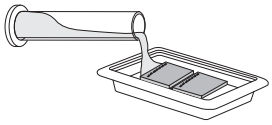
    a. Immerse your gel in 1x Fast Blast.

    b. Let the gels stain overnight, with gentle shaking for best results. No destaining is required.



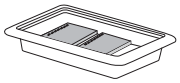
Protocol Two: Quick Staining (requires 20 minutes)–This method will allow you to see bands quickly (within 15 min) but may require extensive destaining to obtain optimal band-to-background intensity. Note: it is important to use **warm** water for destaining steps of this protocol.

a. Immerse your gel in 100x Fast Blast.



b. Stain the gel for 5 minutes with gentle agitation. Save the used stain for future use.

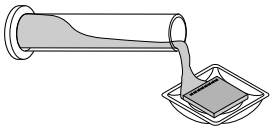
c. Transfer the gels into a large washing container and rinse with **warm** (40–55°C) tap water approximately 10 seconds.



d. Destain by washing three times in **warm** tap water for 5 minutes each with gentle shaking for best results. You should be able to visualize bands after 10 min if you view the gel with light coming through the bottom of the staining tray. If necessary continue destaining in **warm** water until the desired contrast is reached.

Staining of Polyacrylamide Gels

1. When gels are finished running, turn off the power supply and disconnect the leads. Remove the lid and lift out the electrode assembly and clamping frame.
2. Pour out the running buffer from the electrode assembly. Open the cams and remove the gel cassettes.
3. To keep the gel free of contamination from your fingertips, wear gloves to handle the gels from this point on. Lay a gel cassette flat on the bench with the short plate facing up. Cut the tape along the sides of the gel cassette. Carefully pry apart the gel plates, using a spatula or your fingertips. The gel will usually adhere to one of the plates. Transfer the plate with the gel adhering to it to a tray containing 1x Fast Blast stain (see below), allowing the liquid to detach the gel from the plate. The gel may also be lifted directly (and gently!) from the plate and placed into the stain.



4. Bands will start to appear after 10 minutes and staining will be complete in 1 hour. However, gels can be left in stain overnight. No destaining is required.

Lesson 4

Focus Questions

1. What was your test food?

2. Did your test food generate a 200 bp band with GMO primer (lane 4)?

3. What does this tell you about the GMO status of your food?

4. What other information do you need to confirm the GMO status of your sample?

5. How do the results of your other five PCR reactions help support or undermine your result for your test food?

6. If you were to repeat the procedure what laboratory practice might yield better results?