Module II: TESTING FOR GENETICALLY MODIFIED (GM) PLANTS

This module will teach you a way to test if a given plant has genetically modified (GM) content. This can be done by using a traditional PCR approach or Loop Mediated Isothermal Amplification (LAMP). Both ways are described below. The especially cool part about this module is that the experiments can be done in a lab setting, or in a home/field setting, depending on the available instruments.

You and your partner(s) will choose a vegetable or fruit available at the grocery store to test for GM content. Each group of four will test the same produce, but ideally from different stores/backgrounds (organic vs non-organic, locally grown, imported), etc, and you and your partner will present the results in form of a paper. You need to make a hypothesis about which plants you believe (based on literature, labels at the store, etc.) to be genetically-modified or not **before** doing the experiment. *Please **note: once you and your group have decided on a certain produce, please post your decision on Moodle Forum immediately. Each group should have a different fruit/vegetable/ plant.***

Upon completion of this lab, you will be able to:

- Analyze if a given plant has been genetically engineered
- Understand the difference between isothermal and traditional PCR amplification
- Use at least one type of indicator to test for the presence of amplified product
- Think about the use of LAMP vs. PCR for genetic testing

Week 1: PCR

A. *Background:* You hear about GMOs (genetically-modified organisms) all the time in the news. Genetically-modified foods are highly prevalent in the United States but which foods are really GM and which are really organic? To address this question, we have to first understand a little more about how GM-plants are made. Most GM-plants are made by inserting a piece of DNA into their genome that is meant to aid fruit develop, decrease reliance on insecticides in the environment, remove potentially harmful allergens, and much more. Genes are inserted into an organism's genome using plasmids (vectors), which are small, circular pieces of DNA, that are easily manipulated in the lab (Fig. 1). To express the inserted gene region in the plant genome, the inserted gene often is placed behind a promoter, usually the Cauliflower Mosaic Virus 35S promoter (a very strong constitutive promoter recognized by most plants for gene expression). The promoter is required to turn on gene expression and a terminator sequence is needed to end transcription of the inserted gene. A specific terminator sequence called the NOS terminator is most often used in vectors used to make GM-plants. So by targeting the CaMV 35S and/ or the NOS terminator sequence with specific primers, we can determine if a plant is

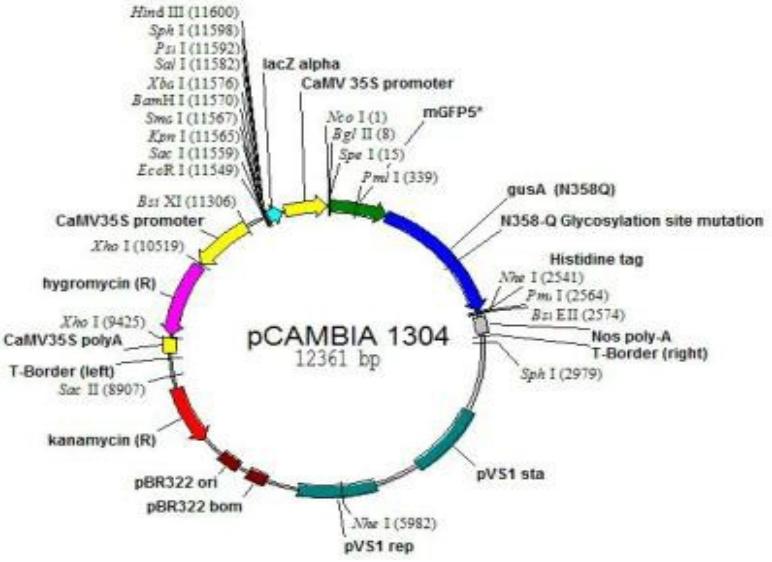

Figure 1.Typical GMO T-DNA vector

genetically modified. If it is a GM-plant, then primers specific to one or both of these gene regions will bind and amplify that region producing a product. If the plant is not a GM-plant, the primers won't bind and no product will be produced.

B. General Procedure

1. Traditional PCR

Step 1: DNA extraction using REDExtract-N-Amp™ Plant PCR Kit Protocol (Sigma-Aldrich)

1. Cut a small piece of tissue (about the size of a hole punch)
2. Place in 100 ul of 'E' Extraction solution.
3. Vortex and place in heating block at 95 oC for 10 min.
4. Add 100 ul of 'D' dilution solution and vortex. This will be your DNA for the PCR reaction.

Step 2: PCR reaction set up

1. Add 4 µl of lysate to 0.2 ml PCR tube.
2. Add 10 µl of PCR mix to same tube.
3. Add 2 µl of CaNos mixed primers.
4. Add sterile water to a total volume of 20 µl per a reaction.

Step 3:Set-up a positive and no template control (NTC) for your PCR reaction.

This is to check and make sure that a) the PCR itself is working, and b) products are not the result of contaminating DNA in the mix (not your template but something else).
To make the NTC, set-up the PCR reaction just like above but **using 4 µl of sterile water instead of lysate**. I will give you a positive control DNA sample instead of your own plant lysate. **Note: only one positive and NTC control reaction will need to be made per a lab.**

Step4:Place tubes in the PCR machine with the following program:

- 94 oC for 3 min initial denature
- 94 oC for 1 min denature double helix
- 54 oC for 1 min primers anneal
- 72 oC for 1 min extension

} repeat cycle 49 X

- 72 oC for 10 min clean-up of ends
- 12 oC hold temporary storage

The primers used for the PCR reaction were a 'mixed bag' of primers, targeting the CaMV 35S promoter and NOS (nopaline synthethase) terminator (Fig. 2). Most GM-plants contain at least one or both of these elements in the T-DNA construct. GM-plants may have up to three bands on a gel. Based on the placement of primers used to amplify the *CaMV* 35S or *Nos* terminator shown in Figure 2, **Determine the size of PCR products expected from GM-plants if they have the CaMV 35S or NOS terminator** based on the position of the forward and reverse primers for each region as shown in Fig. 2 **and fill out this information in your research notebook.**

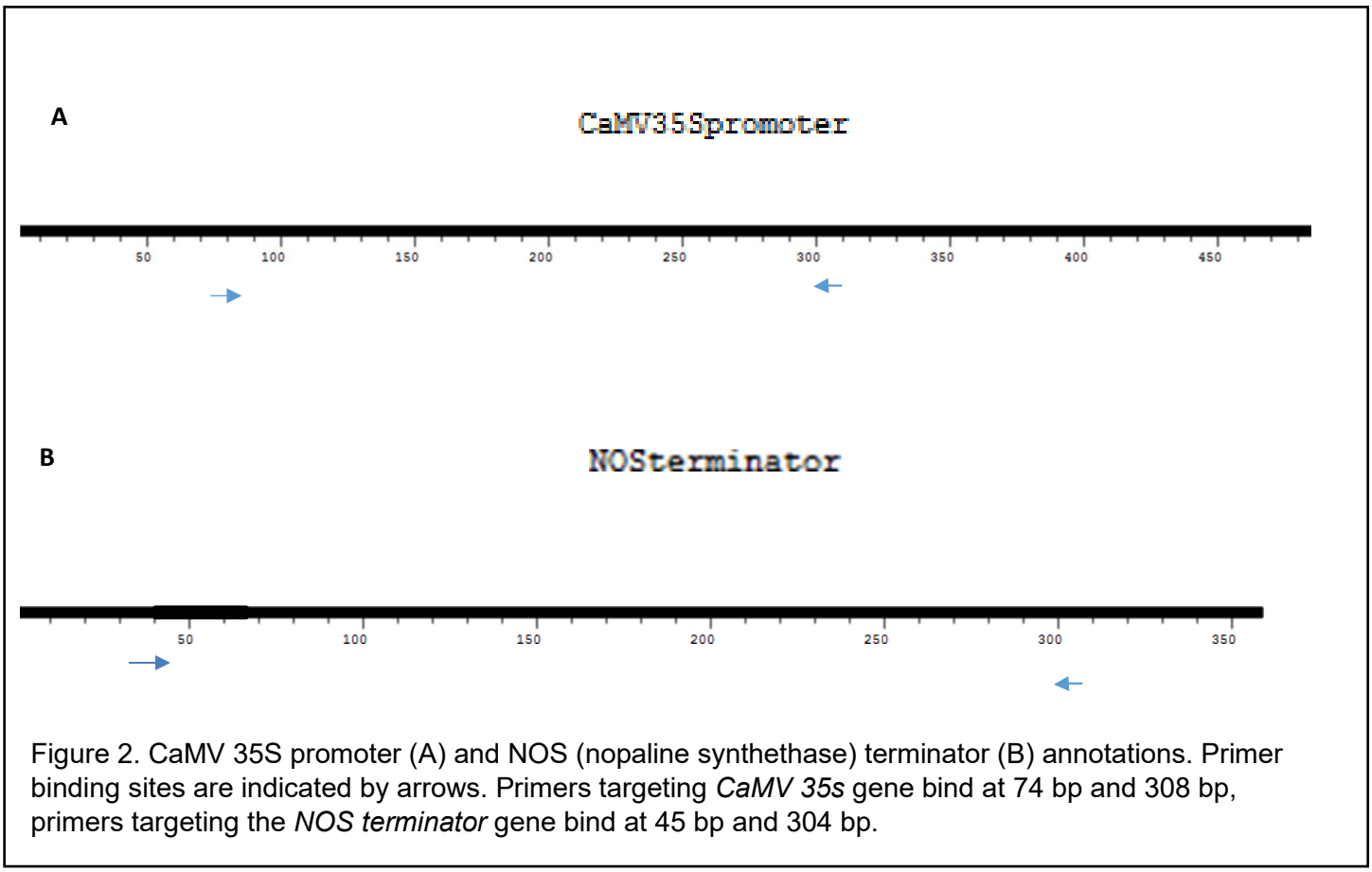

Figure 2. CaMV 35S promoter (A) and NOS (nopaline synthethase) terminator (B) annotations. Primer binding sites are indicated by arrows. Primers targeting *CaMV 35s* gene bind at 74 bp and 308 bp, primers targeting the *NOS terminator* gene bind at 45 bp and 304 bp.

2. LAMP assay

There is more than one way to amplify a region of DNA. Loop-mediated isothermal amplification (LAMP) is an amplification that requires different kinds of primers than PCR but can generate sensitive and specific reactions in a fraction of the time as traditional PCR without the need for expensive laboratory equipment. A slightly different DNA extraction method is needed since the chemicals in the extraction solution from the previous kit interfere with the LAMP reagents. Many countries or parts of countries do not have easily accessible lab equipment including PCR machines but still manage to amplify target regions of DNA. How can they do this? Loop-mediated isothermal amplification also known as LAMP is a process of DNA amplification using polymerases that are active at lower than normal temperatures and have strand-displacement ability to denature the template DNA. For example, the *Bst* DNA polymerase from *Bacillus stearothermophilus* which is part of DNA polymerase I, the polymerase responsible for removing RNA primers and replacing it with DNA nucleotides as part of DNA replication. Unlike Taq polymerase which is typically used in PCR and works at high temperatures, *Bst* DNA polymerase is most active between 60 and 65 ^{o}C. There are three main stages to PCR, stage 1: Denature DNA (requires 90-95 ^{o}C), stage 2: Anneal primers (typically 50-65 ^{o}C), and finally stage 3: Extension, the stage where the polymerase adds nucleotides to form the new complimentary DNA strands (with *Taq* polymerase this usually is run at 72 ^{o}C). The hardest part of amplifying regions of DNA template is how to get the DNA to denature. Harsh chemicals such as high levels of NaOH would inhibit or denature enzymes such as polymerases and prevent amplification, heating only works when you have a high-temperature stable polymerase such as *Taq* polymerase. So how can we denature the double strands of DNA in order to allow the primers to anneal and polymerase to make new copies of DNA?

Bst polymerase works by strand displacement meaning when this polymerase encounters double stranded DNA, it has its own built in helicase that open and unwinds the DNA. So no need for any additional denaturing of the DNA and the whole amplification reaction can be run at a single temperature for denaturing, annealing of primers, and extension.

Please review the following video to help you understand how primers are utilized in LAMP reactions. https://www.youtube.com/watch?v=GJkvQqDufh0. Although this seems difficult, most scientists use a program called Primer Explorer to help design LAMP primers. This program looks for the best regions for LAMP primers and essentially does the hard work for you. For practice, at the end of this lab, I will have you annotate the positions of the CaMV 35S LAMP primers we used for this lab.

Step 1: LAMP DNA extraction (slightly different from regular DNA extraction):

1. Cut a small 'bit' of plant leaf about the size of this circle. If you have a narrow-leafed plant, just accumulate enough material to fill the circle above.
2. Place in 1.5 ml tube that contains 100 µl of sterile water.
3. Use the sterile yellow tip provided to crush your plant material in the sterile water.
4. Keep the plant material in the sterile water after your complete crushing it.

Step 2: LAMP amplification:

1. Add 5 µl of your LAMP plant extract to a tube with pre-made LAMP mix already in it.
2. A positive control and NTC control for the LAMP reaction has already been set-up for you and has a '+' sign or NTC at the top of the tube. Take a photo of your LAMP tubes ***before*** you add the template to each tube.They should all be pink.
3. Place the strip of tubes at 65 ^{o}C for 20 minutes.
4. Take out your tubes and record (photograph and write) the color of each tube. If your positive control has not turned yellow yet, place back in the heating block for an additional 10 minutes.

Step 3: LAMP detection

How does LAMP work to indicate product? The LAMP color mix uses the release of a by-product after the addition of a monophosphate to the growing DNA chain. Now we can review a bit of DNA replication. Think back to learning DNA replication, when a new nucleotide is added to the growing DNA chain, its added in its mononucleotide form but starts as a trinucleotide (e.g. CTP is added as CMP). The extra two phosphates are released to help generate the energy needed to form the new phosphodiester bond in the growing DNA chain. These two phosphates are called pyrophosphate. A by-product of this reaction is the release of protons (dropping the pH of the solution to the acid range). Increased levels of acid cause phenol red to change color to yellow – this is how the color indication works with the color version of the LAMP kit. **You can only see color change if nucleotides have been added to the growing DNA chain.** So if you see a color change in the tube that contains your plant extract, your DNA template contains either CaMV or NOS sequence that the primers were able to bind. The amplification of the products caused a pH/ color change and you can draw your conclusion: Your sample is a GMO! The best part about LAMP is that it can be run even at your own home using an oven set at 150 oF (~65 oC)!

Make sure to place your LAMP reactions in the freezer to run on gels next week.

Week 2

Part A: Gel electrophoresis of traditional PCR reaction products

1. Set-up a 1.5 % agarose gel with a total volume of 50 mls. Loading dye is already in the PCR mix so does not have to be added. I will provide the DNA ladder.

1. Load 20µl of your PCR product from last week, along with positive control and NTC sample.
2. Run at 120V for ~ 1 hour.
3. Take gel photo. If your sample if a GMO, you will see PCR products (DNA bands) on your gel. You may see up to 3 bands (CAMV, NOS and CAMV-NOS).
4. LAMP reactions do not have a single specific product like PCR but instead look like multiple small products on a gel. Let's look at the difference between PCR products and LAMP products. Add 2 µl of loading dye to your LAMP reactions and load all 20 µl of your LAMP reactions on the gel.
5. Your traditional PCR gel electrophoresis result should be consistent with the color LAMP result from last week. **If the results are NOT consistent, discuss the possible reasons/errors in your lab notebook.**

Part B. Implications and Bioinformatics (Primer design) Answer in lab notebook.

1. Make a list of pros and cons for PCR vs. LAMP. Can you ever determine a specific product size using LAMP? Look up some LAMP gel images on Google. After reviewing those images, do you think LAMP would be appropriate for diagnostic genetic tests? If so, when?
2. Work such as this where you identify specific types of plants, etc. with a simple test has more than just a scientific impact. What are the implications of finding out non-GMO products actually contain GM plants to business and law? These same ideas impact conservation efforts (some companies lie about what ends up in the food, etc.), and medicine. Write at least one way a diagnostic test such as the GM test done in this lab could impact business, law, or policy.
3. Let's see how LAMP primers are really designed. Choose **any** gene of your choice from GenBank (go to www.pubmed.gov, click the NIH icon at the top of the blue screen, select nucleotide from the drop-down menu and then type in the gene of interest and genus species. Then select a file from the lists given. Make sure to click if you want DNA or cDNA (the RNA put together) and select 'FASTA' from the bottom of the screen. Save the FASTA file to your computer. Go to https://primerexplorer.jp/e/, click Primer Explorer v. 5 and select your FASTA file for input. You can also input a txt. File if the FASTA format does not work on your computer. A screen should appear with your sequence in it. Select 'Generate'. Then click 'Display'. You will now have the positions of the LAMP primers that were automatically generated annotated onto your sequence. You can use this information to see if 1. LAMP primers are possible for your sequence, and 2. Choose a set of primers suitable for the LAMP reaction you want to perform. The color coding matches the type of primer given on the previous web page. You can tie these primers to the design given in the video https://www.youtube.com/watch?v=GJkvQqDufh0.
   Write down your gene and the LAMP primer sequences you have designed in your

research notebook.