REGIONAL PROJECT FOR IMPLEMENTING NATIONAL BIOSAFETY FRAMEWORKS IN THE CARIBBEAN SUB-REGION

Detection of the CaMV 35S promoter and the NOS terminator using end-point polymerase chain reaction (PCR)

Laboratory Manual (3)

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Glossary of terms

CaMV 35S promoter	A strong constitutive promoter derived from the Cauliflower mosaic virus. It is one of the most widely used, general-purpose constitutive promoters
Cry1A	An insecticidal pore-forming toxin derived from *Bacillus thuringiensis*; can effectively control destructive lepidopteran corn pests
DNA	Deoxyribonucleic acid. The molecule found in chromosomes that contains the genetic code
DNA template	The sample DNA that contains the target sequence; the sequence of DNA that one wishes to amplify
dNTP	Deoxynucleotide-triphosphate; the base unit of DNA
ELISA	Enzyme-linked immunosorbent assay
Gel electrophoresis	A method used to separate DNA fragments on an agarose gel matrix. Fragments migrate on the basis of size and charge when an electric current is applied. The gel matrix acts as a sieve to separate the fragments based on size
Gene	An ordered series of nucleotide bases which code for a specific protein
GMO	Genetically modified organism; any organism whose genetic material has been altered using genetic engineering techniques
Lateral flow strip	An immunoassay performed on an easy to use strip format
LMO	Living modified organism; any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology
LMO-FFP	Living modified organism intended for direct use as food, feed or processing

NOS terminator	Nopaline synthase terminator (a DNA sequence that marks the end of a gene). It is frequently used in genetic engineering of plants to terminate the transcription of an inserted gene of interest
Nucleotide	The building block of DNA and consists of a nitrogenous base (adenine, thymine, guanine or cytosine), a five-carbon sugar (deoxyribose) and at least one phosphate group
PCR	Polymerase chain reaction; a method of amplifying a specific gene or region of DNA to produce millions of copies
Primer	A short oligonucleotide designed to anneal to specific regions of DNA in order to facilitate the PCR. Primers are designed to complement regions of DNA bounding the gene of interest
Real-time qPCR	Real-time quantitative PCR; based on PCR and is used to amplify and simultaneously detect or quantify a target DNA molecule
Taq polymerase	A specific, heat-stable DNA polymerase used to replicate DNA targets during PCR

Manual 3:

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End-point PCR analysis workflow

3.1 Introduction

Polymerase chain reaction (PCR) is a technology used to selectively amplify a specific DNA sequence from any source to generate billions of copies from a single or few copies. It is a relatively simple and inexpensive in vitro DNA amplification tool that allows one to multiply a particular segment of DNA billions of times in a few hours even in samples containing minute quantities of starting DNA [7]. In order for the PCR to work it must contain the DNA template, DNA polymerase, primers, nucleotides and buffer. The DNA template is the sample DNA that contains the target sequence (the sequence of DNA that one wishes to amplify) [8]. DNA polymerase is an enzyme that synthesizes new strands of DNA, while primers are short pieces of single-stranded DNA that bind to a specific region on either side of the target sequence. DNA polymerase begins synthesizing new DNA strands through the sequential addition of nucleotides to the end of the primer. A nucleotide is the building block of DNA and consists of a nitrogenous base (adenine, thymine, guanine or cytosine), a five-carbon sugar (deoxyribose) and at least one phosphate group. The buffer is a salt-solution that stabilizes the DNA and other components of the reaction and therefore provides the optimal condition for the PCR to work.

The three main stages in the PCR process are denaturation, annealing and extension. During the denaturation stage, DNA is heated to 94 to 95° C to break the weak hydrogen bonds in the double stranded DNA helix to produce single stranded DNA molecules. This is followed by the annealing stage where the mixture is usually cooled to 50 to 65° C. The lower temperature allows the primers to bind/ anneal to their complementary sequence in the template DNA. The third stage is extension, where the reaction is heated to 72° C, which allows DNA polymerase to extend the primers by adding nucleotides to their 3' ends to make a new copy of the DNA. The nucleotides are added in a sequential manner, using the target DNA as a template. The three stages are repeated for 20 to 50 times (cycles) during the PCR process. Also, DNA made in the previous cycles serve as template for the next cycle. For instance after one cycle, a single copy of double-stranded DNA template is amplified into two separate copies of double-stranded DNA. These two pieces are then available for amplification in the next cycle. After 2 cycles four DNA copies are produced, and after 3 cycles eight copies are produced. Therefore DNA is amplified exponentially and as the cycles are repeated, more and more copies are generated [7, 8]. The

reaction is performed in a thermocycler, which is an automated device that can rapidly heat and cool the reaction mixture.

In this protocol the Eurofins GeneScan GMO*Screen* 35S/NOS PCR kit would be utilized to screen samples for the presence of the CaMV 35S promoter from the Cauliflower mosaic virus and the NOS terminator from the nopaline synthase gene of *Agrobacterium tumefaciens* [9]. Detection of one or both genes strongly indicates the presence of a genetic modification. The initial steps in the protocol involve the processing of sample and the extraction of DNA which produces DNA that is purified from other cellular components that can degrade the molecule and inhibit the PCR reaction. This is followed by the quantification of the DNA, addition of the DNA to the PCR master mix, PCR amplification and post-PCR analysis using gel electrophoresis.

3.2 Precautions and notes

1. Use caution to prevent sample-to-sample cross-contamination with grain, fluids, or disposables. Thoroughly clean blender parts and containers to remove dust and residue prior to preparation of a second sample.
2. When using the Qiagen DNeasy Plant Mini kit, redissolve any precipitates (if necessary) in buffer AP1 and buffer AW1. Add ethanol to buffer AW1 and buffer AW2.
3. Safety goggles, lab coat, closed toe shoes and gloves must be worn; use UV goggles when working with the UV lightbox.
4. While most modern PCR machines use 0.2 mL tubes, some models may require reactions in 0.5 mL tubes. See the thermocycler's manual to determine the appropriate size tube.
5. Ethidium bromide is a mutagenic substance and should be treated before disposal and should be handled only with gloves. Ethidium bromide gels should be stored in a designated biohazard waste bin; used gels and gel waste should not be put down the sinks. Ethidium bromide spills should be cleaned up immediately, using proper precautions (wear gloves, put material used to clean up in biohazard waste bins, wipe contaminated area with ethanol or detergent).

6. Cover the buffer reservoirs in gel tanks during gel electrophoresis. Always turn off the power supply and unplug the leads before removing a gel.

3.3 Initial processing of sample and extraction of DNA

Procedure to extract DNA using the Qiagen DNeasy Plant Mini kit (catalog number 69104 and 69106) [10]

1. Determine the number and size of sub-samples (see Appendix 1 for additional guidelines).
2. Weigh out corn sample and grind with a Waring blender (or equivalent) on high speed for about 30 s or until all the grains are ground.
3. For every 100 mg of ground sample, add 400 µL buffer AP1 and 4 µL RNase A and vortex and incubate for 10 min at 65 °C (do not mix buffer AP1 and RNase A before use). Invert the tube 2 to 3 times during incubation.
4. Transfer 400 µL of the mixture to a 1.5 mL tube and add 130 µL buffer P3, mix and incubate on ice for 5 min.
5. Centrifuge the lysate for 5 min at 20,000 x g.
6. Pipet the lysate into a QIAshredder spin column in a 2 mL collection tube and centrifuge for 2 min at 20,000 x g.
7. Transfer the flow-through into a new tube without disturbing the pellet (if present) and add 1.5 volumes of buffer AW1 to the solution and mix by inverting the tube several times.
8. Transfer 650 µL of the mixture into a DNeasy Mini spin column in a 2 mL collection tube and centrifuge for 1 min at 6000 x g.
9. Discard the flow-through and repeat step 8 with the remaining sample.
10. Place the spin column into a new 2 mL collection tube and add 500 µL buffer AW2. Centrifuge for 1 min at 6000 x g and discard the flow-through.
11. Add another 500 µL buffer AW2 to the spin column and centrifuge for 2 min at 20,000 x g. Remove the spin column from the collection tube carefully so that the column does not come into contact with the flow-through and transfer it to a new 1.5 mL microcentrifuge tube.
12. Add 50 µL buffer AE to the column and incubate at room temperature for 5 min.

13. Centrifuge the tube for 1 min at 20,000 x g and store the eluate which contains the extracted DNA.
14. Quantify the DNA as described in Appendix IV and adjust the DNA concentration of the sample to between 10 to 20 ng/ µL using AE buffer.

3.4 PCR master mix preparation, addition of DNA and amplification

The following reagents are present in the Eurofins GeneScan GMO*Screen* 35S/NOS kit (catalog number 5221105010) [9]:

PREMaster CaMV 35S-Promoter (2 tubes)
PREMaster NOS-Terminator (2 tubes)
PREMaster CP/A (2 tubes)
Taq DNA polymerase (1 tube)
Control DNA Roundup Ready soy (2 tubes)
DNA length standard III (2 tubes)

PREMaster CaMV 35S-Promoter, PREMaster NOS-Terminator and PREMaster CP/A are master mix solutions. Each master mix contains primers, nucleotides [a mixture of deoxynucleotide triphosphates (dNTPs) with adenine, thymine, guanine and cytosine bases] and reaction buffer. However, the difference between the master mixes is that the PREMaster CaMV 35S-Promoter solution contains primers that would specifically amplify the CaMV 35S-Promoter, while the primers in PREMaster NOS-Terminator will amplify the NOS-Terminator and the primers in PREMaster CP/A would amplify chloroplast DNA. CaMV 35S-Promoter and NOS-Terminator are regulatory sequences and the detection of one or both genes in a sample would strongly indicate the presence of a genetic modification. The CP/A reaction is an internal amplification control reaction and is required to detect false negative results. Since chloroplast DNA is present in all plants, the detection of this gene would indicate that plant DNA was present in the original sample, DNA was extracted successfully, reagents in the kit worked and the PCR was not inhibited by components in the original DNA sample.

Example: Analyzing unknown sample (U1) for the presence of the 35S and NOS genes.

Procedure [9]

1. Pipette the appropriate volume of PREMaster CaMV 35S-Promoter and Taq DNA polymerase (in that order) into a 1.5 mL microfuge tube (volumes are shown in Table 3.1); assemble the reaction components on ice.
2. Vortex the mixture and spin the tube using a picofuge.
3. Aliquot the master mix into 0.2 mL PCR tubes (transfer 20 µL into each tube).
4. Repeat steps 1 to 3 using the PREMaster NOS-Terminator and PREMaster CP/A solutions; you should now have 12 tubes in total (label as shown in Table 3.2).
5. To tubes 1, 5 and 9 add 5 µL of U1 DNA (concentration should be 10 – 20 ng/ µL); tubes 2, 6 and 10 add 5 µL of Control DNA Roundup Ready soy (see kit), tubes 3, 7 and 11 add 5 µL of extraction control and tubes 4, 8 and 12 add 5 µL of nuclease free water. Seal the tubes (VERY IMPORTANT).
6. Vortex the mixtures, spin the tubes using a picofuge and quickly transfer the reactions to a thermocycler.
7. Perform the PCR with the temperature profile shown in Table 3.3

Table 3.1 Volumes of components in PCR master mix

Component	**1 PCR reaction (µL)**	**5 PCR reactions (µL)***
PREMaster	19.9	19.9 x 5 = 99.5
Taq DNA polymerase	0.16	0.16 x 5 = 0.8
Total	20.6	20.06 x 5 = 103

* 4 reactions are needed for the experiment, however an additional reaction is included to compensate for pipetting errors

Table 3.2 PCR tube set-up

	PCR tube number		
	35S	**NOS**	**CP/A**
Unknown sample (U1)	1	5	9
Control DNA Roundup Ready soy	2	6	10
Extraction control	3	7	11
PCR water control	4	8	12

Table 3.3 PCR temperature profile

Temperature	**Time**	
95 °C	10 min	
94 °C	25 s (denaturation)	50 cycles
62 °C	30 s (annealing)	
72 °C	45 s (extension)	
72 °C	10 min	
4 °C	∞	

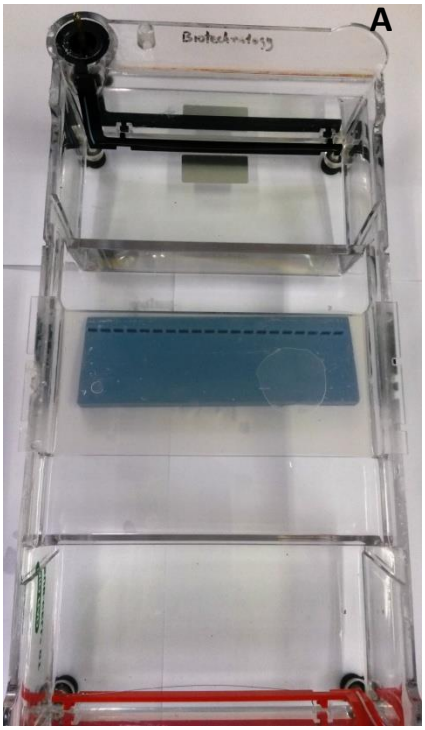
3.5 Post-PCR analysis: Visualization of DNA using agarose gel electrophoresis

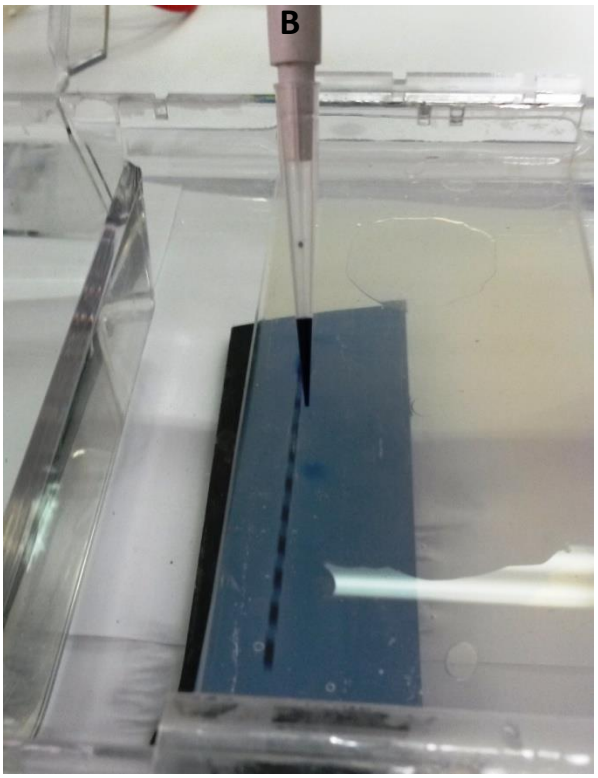
Procedure

1. To prepare a 2.5% agarose gel in 1X TAE buffer, weigh out 2.5 g agarose into a 250 mL flask and add 100 mL of 1X TAE buffer and heat the mixture in the microwave oven until it boils (~ 2 to 3 min; all the agarose must be dissolved but do not allow the mixture to boil over). Note: the total gel volume well vary depending on the size of the casting tray, therefore if the gel tray requires a different volume of agarose gel (ie not 100 mL) adjust

the amount of agarose and buffer accordingly so that the final concentration of the gel is 2.5%.

2. Add 2 µL of ethidium bromide solution (10 mg/ mL) to every 100 mL of gel mixture and let it cool to about 50 to 55 °C (swirling the flask occasionally to allow it to cool evenly).
3. Pour the mixture into the gel apparatus as described by the manufacturer's directions and allow the gel to solidify and remove the comb.
4. Place the gel in the electrophoresis chamber, ensuring that the wells are closest to the negative electrode (black) and add 1X TAE buffer so that there is at least 2 to 3 mm of buffer over the gel (Fig. 3.1).
5. Add 6 µL of 6X sample loading buffer to each 25 µL PCR reaction and mix.
6. Carefully pipette 12 to 20 µL of each PCR reaction/ sample loading buffer mixture into separate wells in the gel.
7. Add 5 to 10 µL of the DNA standard III included in the kit into at least one well.
8. Place the lid on the gel box and connect the electrode wires to the power supply; make sure the positive (red) and negative (black) are correctly connected. Note that DNA is negatively charged and would migrate towards the positive electrode. Turn on the power supply; the allowed voltage will vary depending on the size of the electrophoresis chamber however it should not exceed 5 volts/ cm from the shortest distance between the electrodes.
9. Check to make sure the current is running through the buffer by looking for bubbles forming on each electrode. Check to make sure that the current is running in the correct direction by observing the movement of the blue loading dye. This would take a couple of minutes and it would run in the same direction as the DNA.
10. Let the power run until the blue dye approaches midway of the gel. Turn off the power, disconnect the wires from the power supply, remove the lid of the electrophoresis chamber and carefully remove the tray and gel.
11. Visualize the DNA using a transilluminator according to the manufacturer's instructions.


A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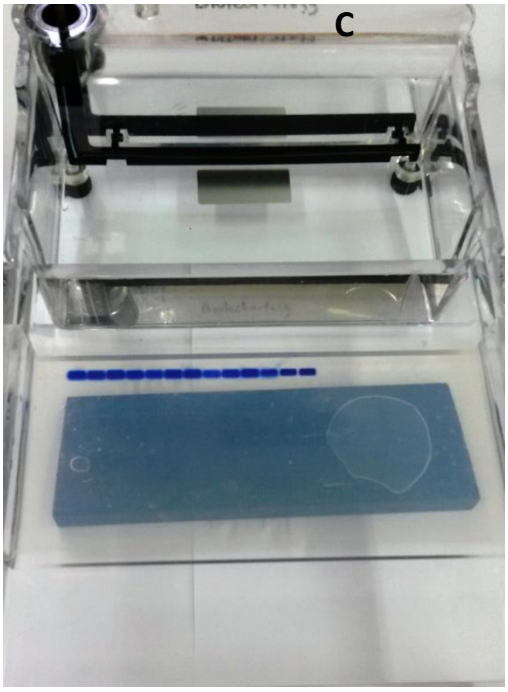
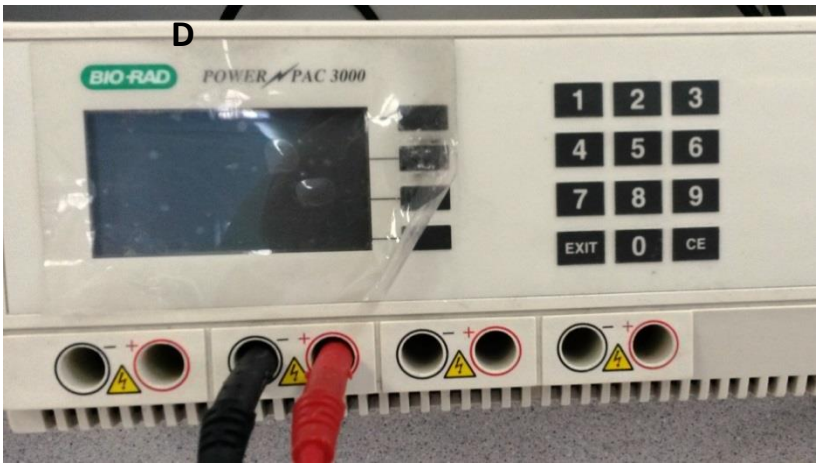
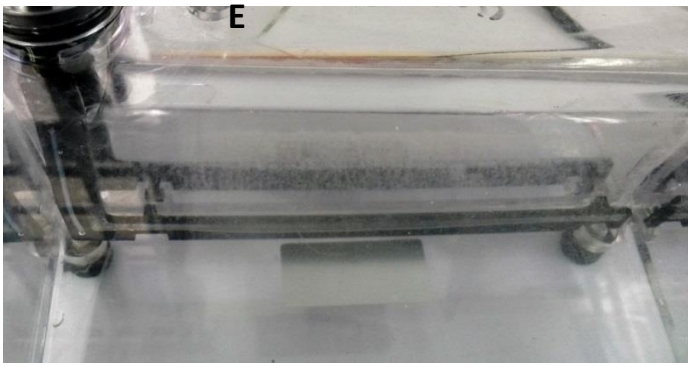


D





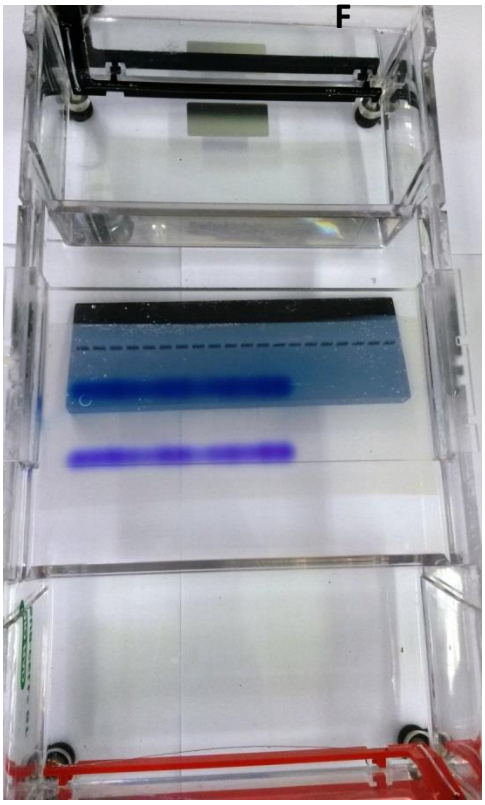


Fig. 3.1 Visualization of DNA using agarose gel electrophoresis. A, gel in the electrophoresis chamber; B and C, PCR reaction/ sample loading buffer mixture being pipetted into the wells of the gel; D, electrode wires connected to the power supply; E, bubbles forming on electrode; F, the loading buffer contained two dyes that ran at the same speed of DNA fragments of particular sizes and therefore the dyes can be used as a guide to estimate where the DNA is as it runs through the gel

3.6 Interpretation of results

Fig. 3.2 shows the PCR results of the 12 tubes. DNA length standard III (DNA ladder) is a set of DNA fragments of known molecular weight measured in base pairs (bp). After electrophoresis, the size of the PCR products can be estimated by comparing the size of the product to the DNA bands in the DNA ladder. A positive result for the 35S promoter and NOS terminator is the presence of a DNA band 123 bp in size, while a positive result for the CP/A gene is a DNA band 199 bp in size.

The results suggest that the unknown sample (U1) was positive for the 35S promoter and NOS terminator sequences indicating genetically modified DNA was present (Table 3.4). CP/A gene was also detected as expected. A similar result was also noted for the Roundup Ready soy control DNA (positive control) reactions. The extraction control was negative for all three genes indicating that during the course of DNA extraction and further handling, no contamination or impurities from the samples or foreign DNA were introduced. Likewise, the PCR water controls were negative indicating that no contamination of DNA occurred during pipetting of PCR reactions.

It should be noted that:

In the unknown, if the CP/A gene is detected but the 35S promoter and NOS terminator products are absent, then genetically modified DNA may be absent in the sample. If all three genes are undetected in the unknown sample, but are detected in the positive control, then extraction of DNA from the unknown may have failed or there may be inhibitors present in the DNA. If the positive control reactions lack PCR products, then the PCR reagents may not be working. If the DNA extraction control reveals a positive result, the extraction procedure and the PCR analysis must be repeated. If the water controls is positive, the PCR must be repeated.

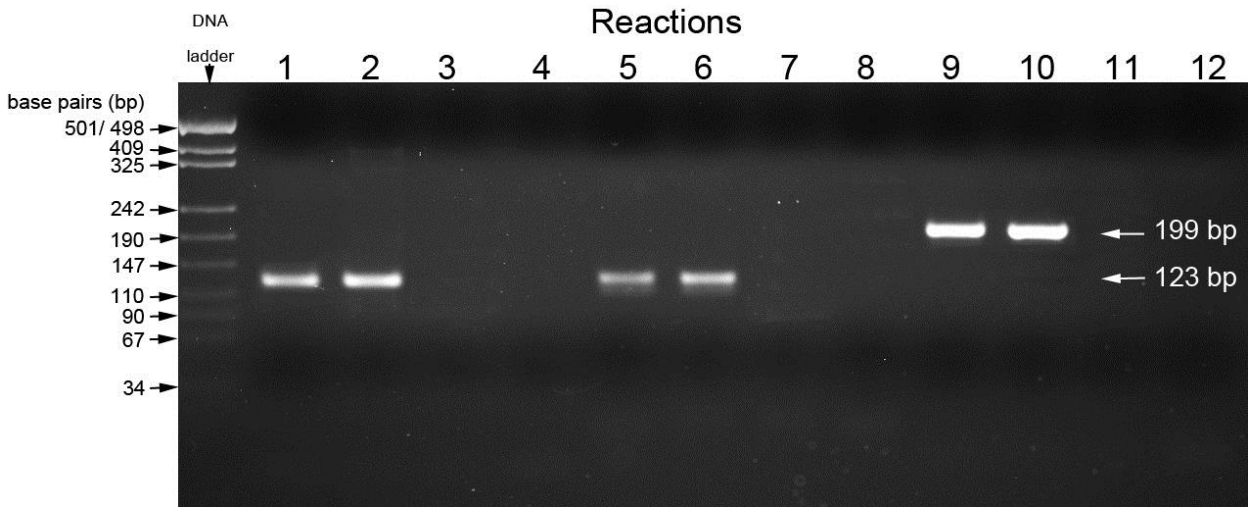


Fig 3.2 PCR results in 2.5% agarose

Table 3.4 Interpretation of the PCR gel

Reaction	**Gene assayed**	**Sample**	**Result**
1	35S promoter	Unknown sample (U1)	+
2		Control DNA Roundup Ready soy	+
3		Extraction control	-
4		PCR water control	-
5	NOS terminator	Unknown sample (U1)	+
6		Control DNA Roundup Ready soy	+
7		Extraction control	-
8		PCR water control	-
9	CP/A gene	Unknown sample (U1)	+
10		Control DNA Roundup Ready soy	+
11		Extraction control	-
12		PCR water control	-

Reference

1. Sajid, M., A.-N. Kawde, and M. Daud, *Designs, formats and applications of lateral flow assay: A literature review.* Journal of Saudi Chemical Society, 2014.

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15. U.S. Department of Agriculture Grain Inspections Packers and Stockyards Administration, *Practical application of sampling for the detection of biotech grains* http://www.gipsa.usda.gov/fgis/biotech/sample1.htm *(accessed September 18, 2015)*, 2000.

16. Barbas, C.F., et al., *Quantitation of DNA and RNA.* Cold Spring Harbor Protocols, 2007.

17. The University of the West Indies, *Molecular biology (BL38C) lab manual*, 2005.

Appendices

Appendix I Determination of number and size of sub-samples

Analyzing samples from a lot is an economical and practical means of estimating the characteristics of the entire lot [14]. The objective of sampling is to obtain a representative sample of an entire lot with a size suitable for the required test. Therefore, it is expected that the test results would reflect the average quality of the seed/ grain for the lot from which the sample was collected. The sampling and testing process consists of the following steps [14]:

1. Determine the type of lot (static or flowing) and thus the type of sampling strategy that is appropriate. The strategy may include representative or random approaches and the use of an appropriate probe or sampler.
2. Take multiple increment samples and combine the increments into one bulk sample.
3. Mix the bulk sample thoroughly and collect at least one sample for laboratory analysis and another (or more) for future reference (file sample).
4. Test the laboratory sample using the appropriate testing strategy.

Representative sampling (also known as practical sampling) procedures involve the collection of multiple small samples (increment samples) throughout a lot [14]. If the lot is heterogeneous, taking increment samples throughout the lot improves the chances of sampling areas with high and low concentrations. It should be noted that the equipment and techniques can differ depending on the nature of the lot. For instance, a flowing or static lot would require the use of a diverter sampler or probe sampler respectively [14]. On the other hand, random sampling (or simple random sampling) is a technique whereby samples are collected through a process in which every possible sample from the lot has an equal chance of being selected [14]. However, in both representative and random sampling, all the samples collected from the lot are combined to form the bulk sample.

Selection of the appropriate sample size is determined by the purity standard and the degree of confidence (statistical probability that the true GMO level in the seed lot is below the selected purity standard) required (Table A1).

Table A1 Samples sizes for selected lot concentrations and probability of rejecting the specified concentrations [15]

GMO concentration	99% Rejection		95% Rejection		90% Rejection	
	Kernels	Estimated weight (g)	Kernels	Estimated weight (g)	Kernels	Estimated weight (g)
0.05	9209	2709	5990	1762	4605	1355
0.10	4603	1354	2995	881	2302	678
0.20	2301	677	1497	441	1151	339
0.30	1533	451	998	294	767	226
0.40	1149	338	748	220	575	170
0.50	919	271	598	176	460	136

For further information on sampling protocols, please review the following literature:

1. U.S. Department of Agriculture, Grain Inspections, Packers and Stockyards Administrations. Grain Inspection Handbook-Book I, Grain Sampling. www.gipsa.usda.gov/fgis/handbook/gihbk1_inspec.aspx
2. Canadian Food Inspection Agency (CFIA) Grains and Oilseeds Sections, CFIA Seed Section, Canadian Seed Institute (CSI), and Canadian Grain Commission (CGC). 2015. Sampling Methods and Procedures Guide, version 3.0.1. http://www.grainscanada.gc.ca/pva-vpa/container-contenant/proc-301/proc3-0-1-en.pdf
3. Sampling for the Detection of Biotech Grains. www.gipsa.usda.gov/fgis/biotech/sample2.htm
4. Practical Application of Sampling for the Detection of Biotech Grains. www.gipsa.usda.gov/fgis/biotech/sample1.htm
5. The following is a helpful reference for use in designing a sampling plan: Remund, K.M., Dixon, D.A., Wright D.L., Holden, L.R. “Statistical considerations in seed purity testing for transgenic traits”, Seed Science Research, June 2001, Vol. 11 No.2, pp. 101-119.

Appendix II Safety in molecular biology laboratories

General laboratory safety

1. The work area should be cleaned and maintained in a sanitary condition. Surfaces and equipment should be routinely decontaminated.
2. Persons must wash their hands after working with hazardous materials and before leaving the laboratory.
3. Do not eat, drink, store food, smoke, handle contact lenses or apply cosmetics in laboratory areas. Do not store food in the laboratory.
4. Mouth pipetting is prohibited and mechanical pipetting devices must be used.
5. Wear laboratory coats, closed footwear, disposable gloves and safety glasses when working in the laboratory.
6. Use a fume hood to manipulate irritating, smelling, toxic, volatile, flammable and fine powder substances. Keep all noxious and volatile compounds in the fume hood.
7. Do not touch broken glassware with bare hands. Dispose of broken glassware in designated containers and not in general bins.
8. Dispose of all biological, toxic and nontoxic waste in designated labeled containers.
9. Do not dispose of hazardous or noxious chemicals in laboratory sinks or general bins.
10. Pregnant women must be cautious and not expose themselves to chemicals that may harm themselves and the fetus.
11. All reagents, solutions, biological materials and waste stored in the laboratory must be labeled.
12. Turn off all electrical devices before leaving the laboratory.
13. Dilute concentrated acids such as sulphuric acid, by adding acid to water.
14. Special care should be taken when using the following chemicals: phenol, is toxic and can cause severe burns; acrylamide is a potential neurotoxin; ethidium bromide is a carcinogen.
15. An emergency shower, eyewash station and first-aid kit must be readily available in the laboratory. There must be fire extinguishers and emergency exits at strategic locations. Know the location and proper use of safety devices.
16. Material safety data sheets for chemicals should be available.
17. Report all accidents immediately.
18. Emergency telephone numbers should be available.

Safety in an end-point PCR laboratory

1. Ethidium bromide is a mutagenic substance and should be treated before disposal and handled only with gloves. Ethidium bromide gels should be stored in a designated biohazard waste bin and not be thrown down the sink. Spills should be cleaned up immediately.
2. Gloves contaminated with ethidium bromide should not be worn to answer the telephone or be use with other equipment.
3. Always cover running buffer reservoirs during electrophoresis and turn off the power supply and unplug the leads before removing a gel.
4. Always wear UV safety goggles when using UV lamps.

Appendix III Preparation of solutions

100 mL 0.5 M EDTA (pH 8.0)

Add 18.6 g EDTA to 40 mL distilled water and adjust the pH to 8.0 with NaOH pellets (EDTA will not go into solution until pH is close to 8.0). When dissolved, adjust the volume to 100 mL with distilled water and autoclave for 20 min.

1 L 10X TAE

Add 48.4 g Tris base, 11.42 mL glacial acetic acid and 20 mL 0.5 M EDTA (pH 8.0) to 400 mL distilled water. Dissolve and bring the total volume up to 1 L with distilled water.

1 L 5X TBE

Dissolve 54 g Tris base, 27.5 g boric acid and 20 mL 0.5 M EDTA (pH 8.0) in 500 mL distilled water and adjust the volume to 1 L.

100 mL 6X loading buffer [0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol]

Add 0.25 g bromophenol blue, 0.25 g xylene cyanol FF and 30 mL glycerol to 50 mL distilled water. Adjust the volume to 100 mL and autoclave for 20 min.

1 mL ethidium bromide (10 mg/ mL)

Prepare 10 mg of ethidium bromide in 1 mL of distilled water.

100 mL 1X TE (10 mM Tris and 1 mM EDTA)

Add 1 mL of 1 M Tris buffer (pH 8.0) and 0.2 mL of 0.5 M EDTA solution (pH 8.0) to 80 mL distilled water. Adjust the pH to 8.0, make-up to 100 mL and autoclave for 20 min.

Note:

1. A molar solution is one in which 1 liter of solution contains the number of grams equal to its molecular weight.

2. Percentage (w/v) = weight (g) in 100 mL of solution; percentage (v/v) = volume (mL) in 100 mL of solution.
3. Many buffers in molecular biology are prepared as concentrated solutions for instance 5X or 10X (five or ten times the concentration of the working solution). The solutions are subsequently diluted to 1X working solutions when needed.
4. The following formula is useful when preparing dilutions of solutions:

$C_1V_1 = C_2V_2$

Where:

C_1 = initial concentration of solution

V_1 = initial volume

C_2 = final concentration of solution

V_2 = final volume

Appendix IV DNA quantification

There are a number of methods that can be used to determine DNA concentrations; however, a method typically used involves measuring the absorbance of the sample at 260 nm on a spectrophotometer (Fig. A4). This method is simple to perform and shows little sample to sample variation, making it a desirable technique.

Procedure [16]

1. Turn on the spectrophotometer at least 15 min before use and adjust the wavelength to 260 nm.
2. To a 100 µL quartz cuvette, add 100 µL of DNase free water and zero the spectrophotometer. Discard the liquid.
3. Make a 1:20 dilution of the unknown DNA sample in a microfuge tube (add 5 µL of sample to 95 µL of DNase free water).
4. Add the 1:20 diluted DNA to the cuvette, pipette up and down several times to mix and read the absorbance at 260 nm; ensure that there are no bubbles in the cuvette.
5. Calculate the concentration of the DNA (take into account the 1:20 dilution that was made). Note that for a 1 cm light path length, the optical density at 260 nm (OD_{260}) equals 1.0 for 50 ng/ µL solution of double stranded DNA.

<u>Example of calculation</u>

A sample of double stranded DNA was diluted 20 times (1:20 dilution) and measured on a spectrophotometer at 260 nm. The diluted sample gave an OD_{260} reading of 0.65. To determine the concentration of DNA in the original sample, perform the following calculation:

DNA concentration = 50 ng/ µL × OD_{260} × dilution factor
DNA concentration = 50 ng/ µL × 0.65 × 20
DNA concentration = 650 ng/ µL

For accurate readings, dilute the sample to give readings between 0.1 and 1.0. Contamination of DNA solutions makes spectrophotometric quantification inaccurate. However, an indication of DNA purity can be determined from the absorbance ratios of 260/ 280 nm. Pure DNA has an

OD_{260}/ OD_{280} ratio of approximately 1.8. A lower ratio could be caused by protein or phenol contamination, while a higher ratio may be due to RNA contamination.

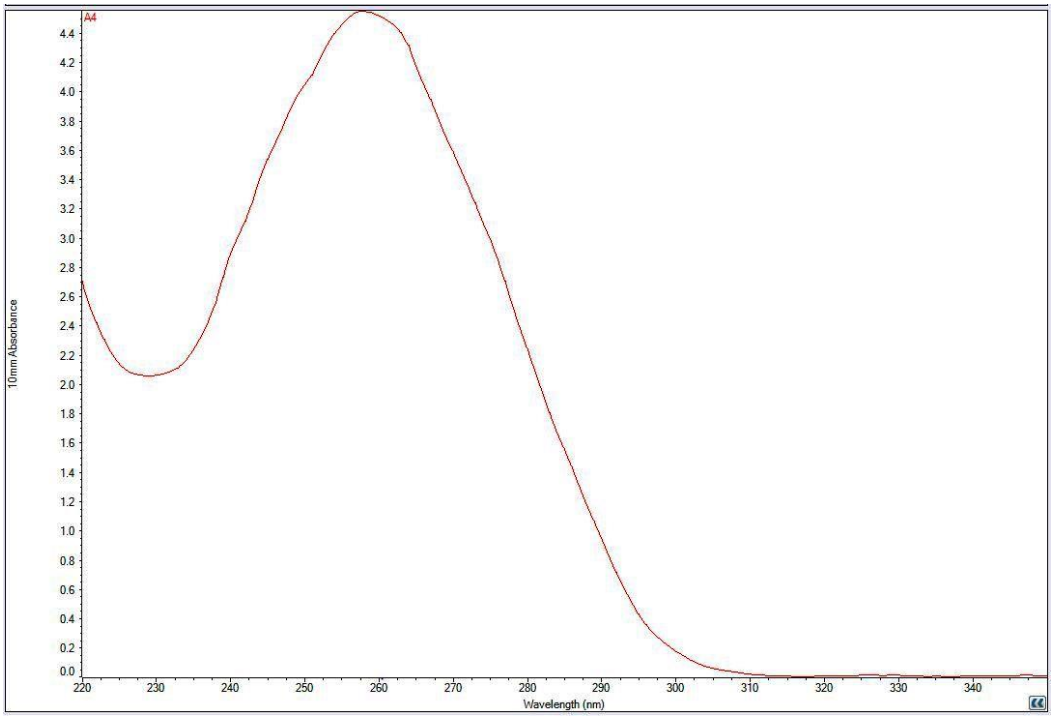

Fig. A4 The wavelength of maximum absorption for DNA is 260 nm. Graph obtained using a NanoDrop 2000 UV-Vis spectrophotometer

Appendix V Agarose gel electrophoresis

Agarose gel electrophoresis is a widely used technique for analyzing DNA. It allows one to determine the presence or absence of DNA molecules and estimate their size. To prepare an agarose gel, agarose powder is boiled in a buffer solution (usually TBE or TAE) then poured into a casting tray and allowed to cool forming a flexible gelatin-like slab. Subsequently, the gel is submersed in a chamber containing a **running buffer** (TBE or TAE solution) that provides ions that carry the current and sets up an electric field across the gel between the positive and negative electrodes. When exposed to an electric field, DNA migrates through the pores of the agarose towards the positive electrode (red) and away from the negative electrode (black). The strength of the electrical field, the concentration of agarose in the gel and the size of the DNA fragments (smaller DNA molecules travel faster than larger molecules) can determine the speed at which the DNA migrates through the gel.

Since DNA samples are usually colorless and have a density similar to the running buffer, it can be difficult to add samples into the wells of agarose gels as the solutions cannot be visualize and tend to float away. These problems can be overcome by adding **loading buffer** to the DNA samples. Loading buffer contains at least one dye and when mixed with the sample, it allows one to visualize the sample when being added to the wells of the gel. Additionally, it usually contains glycerol or sucrose and when mixed with the DNA sample, it causes it to become denser than the running buffer and therefore the sample to sink to the bottom of the well. Also, the dye usually runs at a speed that is similar to a particular size of DNA. Therefore the dye can be used as a guide to estimate where the DNA sample is as it runs through the gel.

DNA is not visible to the naked eye within the agarose gel, however, dyes such as **ethidium bromide** can be used to detect the molecule. Ethidium bromide is an intercalating agent and can bind to double stranded DNA molecules. When ethidium bromide intercalates with DNA and it is exposed to ultraviolet light it fluoresces; hence it provides a means of tagging and visualizing DNA. It should be noted that ethidium bromide is very toxic if swallowed or inhaled, can be irritating to eyes, respiratory system and skin and it is believed to be a mutagen, carcinogen and teratogen.

The size of the DNA molecule can be estimated by comparing it to the size of DNA fragments in a **DNA ladder**. A DNA ladder usually contains a set of known DNA fragments

with different sizes in base pairs (bp) or kilo bases (kb). These DNA fragments are separated and visualized as DNA bands on a gel in parallel to the DNA from the samples.

Appendix VI How to use a micropipette

Most molecular biology reactions are based on micro-chemical protocols that use very small volumes of reagents. They require the use of an adjustable micropipette that can measure as little as 1 microliter (µL) [17]. There are three micropipettes that are commonly used for different volume ranges: P20, P200 and P1000 that measures 2 to 20 µL, 20 to 200 µL and 200 to 1000 µL respectively. These instruments must be treated with care to ensure their reliability. The following rules should adhere to:

1. Never drop the micropipette.
2. Never rotate the volume adjustor beyond the upper or lower range as stated by the manufacturer.
3. Never use the micropipette without a tip as this could ruin the precision piston that measures the volume of fluid.
4. Never lay down the micropipette with a filled tip as fluid could run into the piston.
5. Never let the plunger snap back after withdrawing or ejecting fluid.
6. Never immerse the barrel of the micropipette in fluid.
7. Never flame the micropipette tip.

<u>Micropipetting instructions [17]</u>

Rotate the volume adjustor to the desired setting; be sure to locate the decimal point properly when reading the volume setting. Firmly seat a proper-sized tip on the end of the micropipette. To withdraw or expel fluid into a tube, hold the tube at eye level (firmly between the thumb and forefinger) to observe the change in fluid level. Do not pipette with the tube in a rack or have another person hold the tube while pipetting. Hold the micropipette almost vertical when filling; the result of holding a pipette at an angle can create inaccuracy. Usually, micropipettes have a two-position plunger with friction stops. Depressing to the first stop measures the desired volume, while depressing to the second stop introduces an additional volume of air to blow out any solution remaining in the tip. Pay attention to these friction stops, which can be felt with the thumb.

Withdraw sample from reagent tube:

1. Depress the plunger to the first stop and hold in this position. Dip into the solution to be pipetted so that the tip is just below the surface of the liquid and draw the fluid into the tip by gradually releasing the plunger. Move the tip down the tube to ensure the opening is always below the liquid surface.
2. Slide the tip out along the inside wall of the reagent tube to dislodge excess droplets adhering to the outside of the tip.
3. Check that there is no air space at the very end of the tip. To avoid future pipetting errors learn to recognize the approximate level particular volumes reach in the tip.

Expel sample into reaction tube:

1. Touch the tip to the inside wall of the reaction tube into which the sample will be emptied. This creates a capillary effect that helps draw fluid from the tip.
2. Slowly depress the plunger to the first stop to expel sample. Depress to the second stop to blow out the last bit of fluid. Hold plunger in depressed position.
3. Slide the pipette out of the reagent tube with the plunger depressed to avoid sucking any liquid back into the tip.
4. Manually remove or eject the tip by depressing the measurement plunger beyond the second stop or by depressing a separate tip-ejector button, depending on the micropipette being used.

Preventing cross-contamination of reagents:

1. Always add appropriate amounts of a single reagent sequentially to all reaction tubes.
2. Use a fresh tip for each new reagent to be pipetted.
3. If the tip touches one of the other reagents in the tube, change to a new tip.