# Using Multiplex PCR to Determine Genetic Insertion in Genetically Modified Soybeans

Bella Liu, Katie Freeman
Section: MF
Dec. 15, 2022

# I. Introduction

The goal of this project is to design a set of optimal conditions to detect the presence of a genetic insertion in GMO soybeans using multiplex PCR. The project will isolate the genomic DNA from ground soy and soy food products, and determine the optimal multiplex PCR conditions by examining simplex PCR reactions for genetic modification detection.

GMO and GMO soyplants

GMO (genetically modified organism) refers to a plant, animal, or microorganism that has had its genetic material (DNA) altered through the process of genetic engineering.[8] Most of the GMO crops grown today were developed to help farmers prevent crop loss. The three most common traits found in GMO crops are resistance to insect damage, tolerance to herbicides, and resistance to plant diseases.[8] The most common GMO crops were developed to address the needs of farmers, but in turn they can help foods become more accessible and affordable for consumers. Non-GMO food products are labeled with the "USDA organic" symbol, which mostly guarantees that the food product is certified organic and does not contain any genetically modified sources. All other organic labels are not recognized by the government as true GM free food products.

In 2020, genetically modified soybeans made up 94% of all soybeans planted.[8] One of the most common causes of death for soy plants is weeds. While normally the use of herbicide can effectively control the growth of weeds, it also damages soy plants and therefore decreases soy production.

Genetically modifying soy plants can effectively increase soy plants yield and quality. Roundup Ready soybeans, for example, are genetically engineered soybeans that are glyphosate-resistant.[8] The modification makes the soybeans resistant to the herbicide Roundup, which allows the farmers to use the herbicide to keep weeds from growing without killing the soy plants.

The most common gene inserted into soy plants is the EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) gene, which is from the soil bacterium Agrobacterium tumefaciens.[9] The expression of this protein gives the soy plants the Roundup herbicide immunity. Expression of the EPSPS gene in the plasmid used for transformation was regulated by promoter (E35S) from cauliflower mosaic virus (CaMV), and a nopaline synthase (nos 3') transcriptional termination element from Agrobacterium tumefaciens.[9] Genes are inserted in a cassette, consisting of:[1]

| Promoter | EPSPS gene | Terminator |
|---|---|---|

Simplex and multiplex PCR

Polymerase chain reaction (PCR) is a laboratory technique for rapidly producing millions to billions of copies of a specific segment of DNA by amplifying a sequence of DNA *in vitro*. [6] The components of a PCR reaction includes:

- **Template DNA** to be copied and amplified during PCR
- **dNTPs** (deoxynucleotide triphosphates) that serve as substrates for DNA synthesis
- **DNA polymerase** that synthesizes new strands of DNA complementary to the target sequence. For this project, Taq DNA polymerase is used, which is an thermostable enzyme which is not irreversibly denatured by the high temperature used during the melting period of the cycle. The enzyme is enzymatically active at synthesis period temperature of 50-85 °C, with an optimal temperature of elongation at 74 °C. [4]
- **Buffer** that maintains optimal pH and contains $Mg^{2+}$, which is a cofactor for polymerase.
- **Primers** that initiate DNA synthesis at the location of interest. Internal (self) complementarity or complementarity among primers should be avoided to help reduce the formation of primer dimers in the PCR reaction. The difference in melting temperature between two primers should not exceed 5 °C.

Simplex PCR uses one pair of primers to amplify a single target, while multiplex PCR uses several pairs of primers to amplify more than one target sequence. In this project, simplex PCRs are performed and analyzed first to obtain optimal conditions for multiplex PCR.

In this project, three sets of primers are selected for detecting genetic modification in food products that contain soy. The forward and reverse primers CP4EPSPSF/CP4EPSPSR targets EPSP genetic segment with expected size of 356 bp; CAMV35SF158/CAMV35SR158 targets CaMV35S genetic segment with expected size of 150 bps; NPTIIF/NPTIIR targets NPT II (Kanr), which is a selectable marker sequence for kanamycin resistance in GMO soy plants[9], with expected size of 794 bp. The three sets of primers target different genetic sequences expected to be seen in GMO soys, have melting temperature difference within 5 °C, and have products with differentiable expected sizes.

Isolation of genomic DNA using NucleoSpin Prep

NucleoSpin Food is designed for isolation of genomic DNA from food samples. NucleoSpin food ensures good recovery for small genomic DNA fragments (< 1kbp) out of processed, complex food matrices. [5]

With food samples homogenized, the DNA can be extracted with lysis buffers containing chaotropic salts, denaturing agents and detergents. The standard isolation ensures lysis using Lysis Buffer CF. Contaminants and residual cellular debris can be removed by centrifugation or filtration. The clear supernatant is then mixed with binding buffer and ethanol for optimal

binding to NucleoSpin Silica Membrane. Potential PCR inhibitors can be removed by washing with two different buffers. DNA can then be eluted in a low salt buffer. [5]

DNA scans

DNA Spectrophotometers use detectors that record the intensity of a light beam at a range of wavelengths. [7] Scanning DNA in the spectrophotometers can be used to determine the purity and concentration of acquired plasmid DNA. In a solution, DNA spectrophotometers can measure the levels of ultraviolet light absorbed by the bases. DNA and other nucleic acids absorb light at a peak wavelength of 260 nm. The amount of light absorbed is proportional to the concentration of DNA in the sample. The concentration is calculated from the amount of transmitted light using the Beer-Lambert equation. [7]

In this project, Thermo Scientific Nanodrop One is used for the analysis of DNA concentration and purity. The samples are scanned for absorbance from 200 to 400 nm, which will indicate whether other factors are interfering with the absorbance at 260 and 280 nm. DNA and RNA maximally absorb at a wavelength of 260 nm. [3]

The absorbance values at 230, 260, and 280 nm are recorded, which should be between 0.1 and 1.0. An absorbance of 1.0 at 260 nm corresponds to 50 ug of DNA per mL, which can be used to determine concentration. The purity is determined by the Abs260/Abs280 ratio. Pure DNA has a ratio of 1.7-1.9. [3]

Agarose gel electrophoresis

Agarose gel electrophoresis is a technique used to analyze the size and purity of the DNA fragments.

Melted agarose can be poured and cooled to form a molded gel as a horizontal slab to prepare for agarose gel. To run electrophoresis, DNA fragments are loaded into the three-dimensional matrix composed of agarose. Ethidium bromide (EtBr) is an intercalating dye added to the gel as it is poured to allow us to visualize the DNA when the gel is finished running. An electric current is applied to the matrix and the DNA would migrate through the agarose as a result. [3]

The purity can be determined by agarose gel electrophoresis. All nucleic acids, including RNA and genomic DNA, absorb at 260 nm. [3] If RNA is present, it will appear as a large, intense band at low molecular weight. If genomic DNA is present, it will appear as a smear throughout the lane. [3] The integrity of the plasmid DNA can also be seen. Nicked dsDNA will relax and consequently travel differently through the gel than pristine plasmid DNA. [3] Plasmid DNA preparations contain three types of DNA conformations: linear, relaxed circular (or nicked) and supercoiled. Usually, but not always, the supercoiled runs fastest, linear next, then the relaxed circular.

The agarose gel electrophoresis can also determine the size of DNA fragments by running a DNA ladder on the gel. A DNA ladder is a solution of known sizes of DNA. [3] By comparing the distance traveled of the fragments in the ladder, the size of the fragment can be determined. Smaller fragments can travel faster through the gel than larger fragments because they encounter less resistance, and will appear at the bottom of the gel compared to the larger fragments. [3]

**References:**


(1) Dr. Doonan, etc. - Final Project Documentation, 2022
(2) Dr. Doonan - Prelab Talk on Final Project, 11/4/2022
(3) Dr. Kauffman, Dr. Doonan, Dr. McGuier, and Dr. Drill---Experimental Genetics Lab Manual, Experiment 7, 2022
(4) Dr. Kauffman, Dr. Doonan, Dr. McGuier, and Dr. Drill---Experimental Genetics Lab Manual, Experiment 10, 2022
(5) Genomic DNA from Food, User Manual, NucleoSpin Food, February 2021/Rev. 10
(6) Polymerase Chain Reaction, National Library of Sciance, https://www.ncbi.nlm.nih.gov/probe/docs/techpcr/
(7) National Institute of Standards and Technology, Spectrophotometry, 2009, https://www.nist.gov/programs-projects/spectrophotometry
(8) GMO Crops, Animal Food, and Beyond, U.S. Food and Drug Administration, https://www.fda.gov/food/agricultural-biotechnology/gmo-crops-animal-food-and-beyond
(9) Roundup Ready™ Soybean, GM Approval Database, International Service for the Acquisition of Agri-biotech Applications, https://web.archive.org/web/20110930182016/http://www.isaaa.org/gmapprovaldatabase/events/default.asp?EventID=94

## Project Planning

| Day | Step | Materials Needed |
|---|---|---|
| Day 1<br><br>Date: 11.14 Mon | Pour mini gel | 2x<br>0.35 g agarose in 45 mL distilled water<br>5 mL 10x TBE buffer<br>50 µL Ethidium Bromide<br>50 mL10x TBE buffer (to cover)<br>450 mL DI water |
| | NucleoSpin Prep | 8 X each<br>550 µL Buffer CF (65℃)<br>10 µLProteinase K<br>10 µL RNase A<br>1 vol Buffer C4<br>1 vol ethanol<br>400 µL Buffer CQW<br>900 µL Buffer C5<br>100 µL Elution Buffer CE (70℃) |
| | Load gel | 12 µL 25 ng/µL mass marker<br>4 x 1 kb ladder<br>16 µL STOP ‘n’ Dye |
| | NanoDrop if time | |
| Day 2<br><br>Date: 11.18 Fri | Pour mini gel | 2x<br>0.35 g agarose in 45 mL distilled water<br>5 mL 10x TBE buffer<br>50 µL Ethidium Bromide<br>50 mL10x TBE buffer (to cover)<br>450 mL DI water |
| | Simplex PCR | 8 X each<br>Sterile distilled water<br>2.5 µL (each) Forward and reverse primers (CAMV35SF158, |

| | | |
|---|---|---|
| | | CAMV35SR158)<br>Template DNA<br>PCR Beads |
| | Load gel | 12 µL 25 ng/µL mass marker<br>4 x 100 bp ladder<br>16 µL STOP ‘n’ Dye |
| Day 3<br><br>Date: 11.21 Mon | Simplex PCR | 48 X each<br>Sterile distilled water<br>2.5 µL (each) Forward and reverse primers (CP4EPSPSF, CP4EPSPSR, CAMV35SF158, CAMV35SR158, NPTIIF, NPTIIR)<br>Template DNA<br>PCR Beads |
| | Pour 2 X midi gel | 1.5 g agarose in 90 mL distilled water<br>10 mL 10x TBE buffer<br>100 µL Ethidium Bromide<br>100 mL10x TBE buffer (to cover)<br>900 mL DI water |
| Date: THANKSGIVING | | |
| Day 4<br><br>Date: 11.28 Mon | Load Midi Gel | 6 µL 25 ng/µL mass marker<br>4 x 100 bp ladder<br>48 µL STOP ‘n’ Dye |
| Day 5<br><br>Date: 12.2 Fri | Multiplex PCR | Sterile distilled water<br>2.5 µL (each) Forward and reverse primers (CP4EPSPSF, CP4EPSPSR, CAMV35SF158, CAMV35SR158, NPTIIF, NPTIIR)<br>Template DNA<br>PCR Beads |

| | | |
|---|---|---|
| | Load Midi Gel | 12 µL 25 ng/µL mass marker<br>8 x 100 bp ladder<br>96 µL STOP ‘n’ Dye |

# II. Methods

| Date | Method | Purpose |
|---|---|---|
| 11/14/22 | NucleoSpin Prep | Isolate genomic DNA from food products |
| 11/18/22 | NanoDrop | Assess the concentration and purity of the genomic DNA with respect to protein |
| | Gel Electrophoresis | Assess the purity and quality of the DNA |
| | NucleoSpin Prep (1st half) | Isolate genomic DNA from food products for a second time to obtain higher quality DNA |
| 11/21/22 | NucleoSpin Prep (2nd half) | Isolate genomic DNA from food products for a second time to obtain higher quality DNA |
| | NanoDrop | Assess the concentration and purity of the genomic DNA with respect to protein |
| | Gel Electrophoresis | Assess the purity and quality of the DNA |
| | Simplex PCR | Test primary PCR conditions for the promoter (57℃ annealing temperature, 40x cycles) |
| 11/28/22 | Gel Electrophoresis | Assess the quality of the promoter PCR product |
| | Simpex PCR | Test new conditions for PCR on the promoter (59℃ annealing temperature, 40x cycles) |
| 11/30/22 | Gel Electrophoresis | Assess the quality of the promoter PCR product |
| | Simpex PCR | Test primary PCR conditions for EPSPS and soy lectin (59℃ annealing temperature, 40x cycles) |
| 12/2/22 | Gel Electrophoresis | Assess the quality of the EPSPS and soy lectin PCR products |
| | Simpex PCR | PCR for promoter, EPSPS, and soy lectin (59℃ annealing temperature, 35x cycles) |
| 12/5/22 | Gel Electrophoresis | Assess the quality of the promoter, EPSPS, and soy lectin PCR products |
| | Multiplex PCR | PCR for promoter, EPSPS, and soy lectin (59℃ annealing temperature, 35x cycles) |
| 12/9/22 | Gel Electrophoresis | Assess the quality of the promoter, EPSPS, and soy lectin multiplex PCR products |

| **Step** | **Purpose** |
|---|---|
| *NucleoSpin prep* | Isolate genomic DNA from the food product. |
| Homogenize 200 mg of sample | Grinding up the sample allows more surface area for the extraction buffers to act on. |
| Transfer powder to into a 2mL collection tube and add 550 µL Buffer CF (preheated to 65℃)<br><br>Mix carefully | Buffer CF likely contains:<br><br>NaCl to maintain osmotic pressure inside the cells. If there is a hypotonic environment outside of the cells, water will rush in and the cells will burst. If it is not NaCl, it is likely another ionic salt or a sugar such as glucose. [1]<br><br>Tris Buffer to maintain an ideal pH for DNA. If it is not Tris, it is likely a buffer that maintains a pH from 7-9. [6]<br><br>EDTA to bind divalent cations to prevent the activation of nucleases, which will degrade DNA. [6]<br><br>Cetrimonium Bromide (CTAB), which is a quaternary ammonium surfactant, to remove membrane lipids and to promote cell lysis. CTAB forms an insoluble complex with nucleic acids at a specific NaCl concentration. Polysaccharides, phenolic compounds, and other enzyme-inhibiting contaminants are removed when centrifuged because they will not precipitate [2]. If it is not this specific surfactant, other possibilities are SDS or N-lauroylsarcosine. The incubation temperature, however, points to CTAB, with SDS not requiring an incubation and N-lauroylsarcosine operating best with a 55℃ incubation. |
| Add 10 µL proteinase K<br><br>Mix gently | Proteinase K is a broad spectrum serine protease that hydrolyzes peptide bonds and esters. This is needed for food products as there are many more protein contaminants than in a single celled organism. This will digest cell contaminants as well as any other proteins in the food sample.[3][5] |
| Incubate at 65℃ for 30 minutes (minimum) | The heat promotes detachment of genomic DNA from the food sample. Additionally, this provides optimal conditions for the components of Buffer CF to fully act on the sample. [2] |

| | |
|---|---|
| Add 10 µL RNase A (20 mg/mL) per 550 µL lysis buffer<br><br>Incubate at RT for 30 minutes | * Step was performed the first round to remove RNA from the genomic DNA, however it was omitted the second time.<br><br>RNase A removes RNA from the sample.<br><br>The incubation is performed to allow the enzyme to fully digest RNA in the sample. [6] |
| Centrifuge at >10,000 rpm for 10 minutes | Centrifugation takes advantage of the differential densities of the components within the sample.<br><br>Pellet: food debris, cell debris, some proteins, carbohydrates, lipids<br><br>Supernatant: gDNA, proteins, carbohydrates, lipids<br><br>Supernatant contains the desired gDNA, so this will be transferred and the pellet disposed of. [1] |
| Transfer supernatant to a microfuge tube.<br><br>Add 1 vol Buffer C4<br><br>Add 1 vol ethanol<br><br>Vortex for 30 seconds | Buffer C4 contains:<br><br>Guanidine hydrochloride (noted as a component in the safety data sheets[8]) which decreases enzyme activity and increases the solubility of hydrophobic molecules. It also causes proteins to lose their ordered structure. Additionally, it is a chaotropic salt, which is needed for DNA to bind to the silica membrane. [4]<br><br>Ethanol adjusts the polarity. Because it is less polar than water, it allows ions to interact with the gDNA. It also allows the primary structured proteins and the hydrophobic molecules to dissolve. [6] Vortexing promotes dissolution.<br><br>Buffer C4 and ethanol adjusts DNA binding conditions, which promotes gDNA binding to the silica membrane. |
| Pipette 700 µL into the NucleoSpin® food column.<br><br>Spin for 1 minute at 11,000 rpm.<br><br>Discard flowthrough<br><br>Repeat for the rest of the sample. | The silica membrane is designed to bind the large and charged gDNA.<br><br>Spinning down the sample allows a majority of the proteins, carbohydrates, and lipids to flow through with the binding buffer C4. [4]<br><br>Repeating for the rest of the sample allows a maximum amount of genomic DNA to bind to the membrane. |

| | |
|---|---|
| Pipette 400 µL Buffer CQW into the NucleoSpin® food column.<br><br>Spin for 1 minute at 11,000 rpm.<br><br>Discard flowthrough | Buffer CQW contains:<br><br>Guanidine hydrochloride and ethanol (which are both outlined in the safety data sheets) both reduce polarity and aid in the removal of organic components. [4]<br><br>It also likely contains detergents to remove lipids from the sample.<br><br>The DNA will remain bound to the silica membrane and organic components will flow through the membrane. |
| Pipette 700 µL Buffer C5 into the NucleoSpin® food column.<br><br>Spin for 1 minute at 11,000 rpm.<br><br>Discard flowthrough | Buffer C5 contains:<br><br>At least 96% ethanol which will wash the DNA and remove salts and solvents. [6]<br><br>The flowthrough will be mostly salts, solvents, and ethanol. |
| Pipette another 200 µL Buffer C5 into the NucleoSpin® food column.<br><br>Spin for 2 minutes at 11,000 rpm.<br><br>Discard flowthrough | This will ensure that the DNA is completely washed of salts and solvents.<br><br>Spinning for 2 minutes ensures that the membrane is completely dry. |
| Place the NucleoSpin® food column into a new microfuge tube.<br><br>Pipette 100 µL Elution Buffer CE (preheated to 70℃) onto the membrane.<br><br>Incubate for 5 minutes at RT. | Elution Buffer CE likely contains:<br><br>The EDTA will bind divalent cations to prevent nuclease activities.<br><br>Tris will bring the mixture to the correct pH and solubilize the DNA.<br><br>Buffer CE will stabilize and suspend the DNA. [6]<br><br>The preheated buffer and the 5 minute incubation allows the extraction buffer to work on the very large and charged genomic DNA to allow it to detach from the membrane. |

| | |
|---|---|
| Spin for 1 minute at 11,000 rpm | The aqueous buffer allows the DNA to detach from the silica membrane and elute with the centrifugal force. |
| *NanoDrop* | The NanoDrop measures the absorbance at 230, 260, and 280 nm. Because different components absorb at different wavelengths (peptide bonds at 230 nm, nucleic acids at 260 nm, and aromatic rings at 280 nm), the ratios between the values can indicate whether the sample is pure with respect to protein. There will also be a graph output which will give more insight into potential contaminants. The NanoDrop will also give a concentration, however it is not as accurate as comparing bands on a gel to known mass markers. [7] |
| *Gel Electrophoresis* | Gel Electrophoresis allows for the visualization of the purity, concentration, and quality of the genomic DNA. Additionally, it will show if the PCR was successful by the position of the band when compared to a ladder of known sizes. [7] |
| *Simplex and Multiplex PCR* | PCR for promoter, EPSPS, and soy lectin (59℃ annealing temperature, 35x cycles). |
| For *simplex* PCR:<br>Add 2.5 µL forward and 2.5 µL reverse primer for one set of chosen primers to PCR bead.<br><br>For *multiplex* PCR:<br>Add 2.5 µL forward and 2.5 µL reverse primer for all three sets of chosen primers to the PCR bead. | The forward and reverse primer initiate DNA synthesis at the location of interest.<br>The PCR beads contain 2.5 units of Taq DNA polymerase, 0.2mM of each of the dNTP, and buffer. [10]<br>DNA polymerase is a type of enzyme that synthesizes new strands of DNA complementary to the target sequence. Taq DNA polymerase is an thermostable enzyme which is not irreversibly denatured by high temperature used during the melting period of the cycle. The enzyme is also enzymatically active at the synthesis period temperatures (50-80°C), and has an optimal temperature of elongation at 74℃. [9]<br>dNTPs serve as substrates for DNA synthesis, and are often added at a concentration of 0.2 mM to ensure continuous synthesis of the DNA products. [9]<br>The buffer ensures that the DNA is kept at optimal pH. |
| Add 2-5µL template DNA depending on the concentration of the sample | The template DNA is the DNA that will be copied and amplified during PCR. In this project, the template DNAs are the purified genomic DNA of soy-containing products. [9]<br>Samples with higher concentration are added with a lower volume, and samples with higher concentration are added with a higher volume.<br><br>* In the initial round of PCR, 5 µL was added for each sample. The sample volumes were reduced correspondingly based on degree of non-specific binding products band. |

| | |
|---|---|
| | In this project, the following volume was used for each sample in the final round: 2 µL for positive control (certified GMO soy powder), 3 µL for organic soy flour, 4 µL for Silk soy milk, 4 µL for Silk oat/soy milk, 4 µL for stroopwafel, 4 µL for protein bar, 5 µL for organic tofu. |
| Add 15-20 µL sterile distilled water | The sterile water brings the total volume of each PCR tube to 25 µL, which is the volume to dissolve the PCR bead. |
| Mix gently; short spin in the centrifugation machine | Uniformly mix the PCR tubes and bring all the liquid to the bottom of the tube. |
| Denature at 95℃ for 5 minutes | The initial denaturation ensures that all the template DNA has been completely melted into ssDNA. [9] |
| 35 cycles<br><br>Denaturation at 95℃ for 20 seconds,<br><br>Annealing at 59℃ for 40 seconds,<br><br>Extension at 72℃ for 1 minute. | * Initial conditions for the simplex PCR were 40 cycles and 57 ℃. The cycles were decreased to 35 because there was non-specific binding shown on the gel. The temperature was increased to 59 ℃ because very little PCR products showed up at 57 ℃.<br><br>During denaturation, DNA is melted and strands are separated to make the template accessible to the primers. [9]<br><br>During annealing, temperature is cooled down and primers are annealed to their complementary sequence on the template by sequence specific pairing. [9]<br><br>During extension, the polymerase synthesizes a complementary copy of DNA by reading the opposite strand and extending the primer using the appropriate dNTP substrates. [9]<br><br>The cycle is repeated for an exponential increase in the desired product. [9] |
| Final extension at 72℃ for 10 minutes | The final extension allows for a final round of increase in the desired product. [9] |
| Hold at 4℃ | The cold temperature keeps the DNA stable. |

**References**

[1] Richards, E., Reichardt, M. and Rogers, S. (1994), Preparation of Genomic DNA from Plant Tissue. Current Protocols in Molecular Biology, 27: 2.3.1-2.3.7. https://doi.org/10.1002/0471142727.mb0203s27

[2] Aboul-Maaty, N.AF., Oraby, H.AS. Extraction of high-quality genomic DNA from different plant orders applying a modified CTAB-based method. Bull Natl Res Cent 43, 25 (2019). https://doi.org/10.1186/s42269-019-0066-1

[3] Saenger, W. (2013). Proteinase K. Handbook of Proteolytic Enzymes, 3240-3242. https://doi.org/10.1016/B978-0-12-382219-2.00714-6

[4] "DNA Purification." Promega, https://www.promega.com/resources/guides/nucleic-acid-analysis/dna-purification/#:~:text=DNA%20is%20soluble%20in%20low,and%20the%20eluate%20is%20collected.

[5] "10 Questions You Want to Ask about Proteinase K." Astral Scientific, https://astralscientific.com.au/blogs/astral-scientific/10-questions-you-want-to-ask-about-proteinase-k#:~:text=During%20the%20extraction%20of%20DNA,to%20digest%20these%20contaminating%20proteins.

[6] Dr. McGuier, 03-343, experimental genetics, Pre-Lab Lecture, September 23, 2022.

[7] Dr. McGuier, 03-343, experimental genetics, Pre-Lab Lecture, October 3, 2022.

[8] "Genomic DNA from Food ." MACHEREY-NAGEL, https://www.takarabio.com/documents/User%20Manual/NucleoSpin%20Food%20Genomic%20DNA%20Isolation%20User%20Manual%20%28PT4015/NucleoSpin%20Food%20Genomic%20DNA%20Isolation%20User%20Manual%20%28PT4015-1%29_Rev_11.pdf.

[9] Dr. Doonan, Dr. Drill, Dr. McGuier, and Dr. Kaufmann, Experimental Genetics and Molecular Biology Lab Manual, Experiment 9, pages 117-125, 2022.

[10] Dr Doonan, Promoter Bead PCR Worksheet, Experimental Genetics and Molecular Biology.

# III. Data and Analysis

## Abstract

The goal of this project was to determine if certain food products are formulated with genetically modified soybeans. The food products were organic tofu, organic soy flour, original Silk milk, Silk next milk, stroopwafel, and chocolate chip Clif Bar. These products had a variety of labels with the organic tofu and organic soy flour having a USDA certified organic label, the two Silk products having a GMO Project label, the Clif Bar an internal label, and the stroopwafel no label. To determine the GM content of these products, DNA extraction, spectrophotometry, gel electrophoresis, simplex PCR, and multiplex PCR were utilized. The genomic DNA extraction was performed with the NucleoSpin prep. The NanoDrop spectrophotometer was used to assess the quality and purity of the genomic DNA. Simplex PCR was performed to determine optimal conditions for the primers separately before they were combined in multiplex PCR. These procedures amplified the GM gene sequences of a promoter and a promoter/roundup resistance junction to determine if the food products contain inserted sequences. The gel electrophoresis allows the products to be assessed and conditions adjusted. The results of the simplex PCR showed that the positive control and the organic tofu showed definitive signs of genetic modification by the roundup resistance gene.

## Calendar

| Date | Method | Purpose |
|---|---|---|
| 11/14/22 | NucleoSpin Prep | Isolate genomic DNA from food products |
| 11/18/22 | NanoDrop | Assess the concentration and purity of the genomic DNA with respect to protein |
| | Gel Electrophoresis | Assess the purity and quality of the DNA |
| | NucleoSpin Prep (1st half) | Isolate genomic DNA from food products for a second time to obtain higher quality DNA |
| 11/21/22 | NucleoSpin Prep (2nd half) | Isolate genomic DNA from food products for a second time to obtain higher quality DNA |
| | NanoDrop | Assess the concentration and purity of the genomic DNA with respect to protein |
| | Gel Electrophoresis | Assess the purity and quality of the DNA |
| | Simplex PCR | Test primary PCR conditions for the promoter (57℃ annealing temperature, 40x cycles) |
| 11/28/22 | Gel Electrophoresis | Assess the quality of the promoter PCR product |
| | Simpex PCR | Test new conditions for PCR on the promoter (59℃ annealing temperature, 40x cycles) |
| 11/30/22 | Gel Electrophoresis | Assess the quality of the promoter PCR product |
| | Simpex PCR | Test primary PCR conditions for EPSPS and soy lectin (59℃ annealing temperature, 40x cycles) |
| 12/2/22 | Gel Electrophoresis | Assess the quality of the EPSPS and soy lectin PCR products |
| | Simpex PCR | PCR for promoter, EPSPS, and soy lectin (59℃ annealing temperature, 35x cycles) |
| 12/5/22 | Gel Electrophoresis | Assess the quality of the promoter, EPSPS, and soy lectin PCR products |
| | Multiplex PCR | PCR for promoter, EPSPS, and soy lectin (59℃ annealing temperature, 35x cycles) |
| 12/9/22 | Gel Electrophoresis | Assess the quality of the promoter, EPSPS, and soy lectin multiplex PCR products |

**Day 1: 11/14/22**

**Goal**

Isolate genomic DNA from soy food products with the NucleoSpin prep kit. To isolate the genomic DNA from, we each picked three products in addition to the given positive control. These products included organic tofu, stroopwafel, and original Silk milk (KF) as well as organic soy flour, Clif bar, and oat/silk Silk milk (BL). The positive control was a certified GM soy powder.

**Data**

No data was collected or analyzed this day, however the ingredients of the products were noted.


Ingredients: Water, Organic Soybeans, Less than 2% of Magnesium Chloride, Calcium Sulfate.

Contains: Soy

Label: USDA Organic


Ingredients: Glucose syrup, Wheat Flour, Sugar, Palm Oil, Butter, Molasses, Soy Flour, Canola Oil, Salt, Wheat Dextrin, Soy Lectin, Baking Soda, Cinnamon, Citric Acid, Vanilla Bean, Mono-and Diglycerides of Fatty Acids.

Contains : Milk, Wheat, and Soy

Label: n/a


Ingredients: Soymilk (Filtered Water, Soybeans), Cane Sugar, Vitamin and Mineral Blend (Tricalcium Phosphate, Calcium Carbonate, Vitamin A Palmitate, Vitamin D2, Riboflavin B2, Vitamin B12), Sea Salt, Natural Flavor, Gellan Gum.

Contains: Soy

Label: Non-GMO Project Verified


Ingredients: 100% Organic Soy Flour.

Contains: Soy

Label: USDA Organic


Ingredients: Organic Rolled Oats, Organic Brown Rice Syrup, Soy Rice Crisps (Soy Protein Isolate, Rice Flour, Barley Malt Extract), Organic Roasted Soybeans, Organic Tapioca Syrup, Organic Cane Syrup, Unsweetened Chocolate, Chicory Fiber, Organic Soy Flour, Organic High Oleic Sunflower Oil, Natural Flavors, Sea Salt, Organic Cinnamon.

Contains: Soy

Label: Non-GMO


Ingredients: Oatmilk (Filtered Water, Oat Concentrate), Coconut Milk (Filtered Water, Coconut Cream), Soy Protein Isolate, Coconut Oil, Chicory Root Extract, Cane Sugar, Sunflower Oil, Vitamin and Mineral Blend (Calcium Carbonate, Vitamin A Palmitate, Vitamin D2, Riboflavin B2, Vitamin B12), Sea Salt, Locust Bean Gum, Gellan Gum, Sunflower Lecithin, Soy Lecithin, Natural Flavor.

Contains: Soy

Label: Non-GMO Project Verified

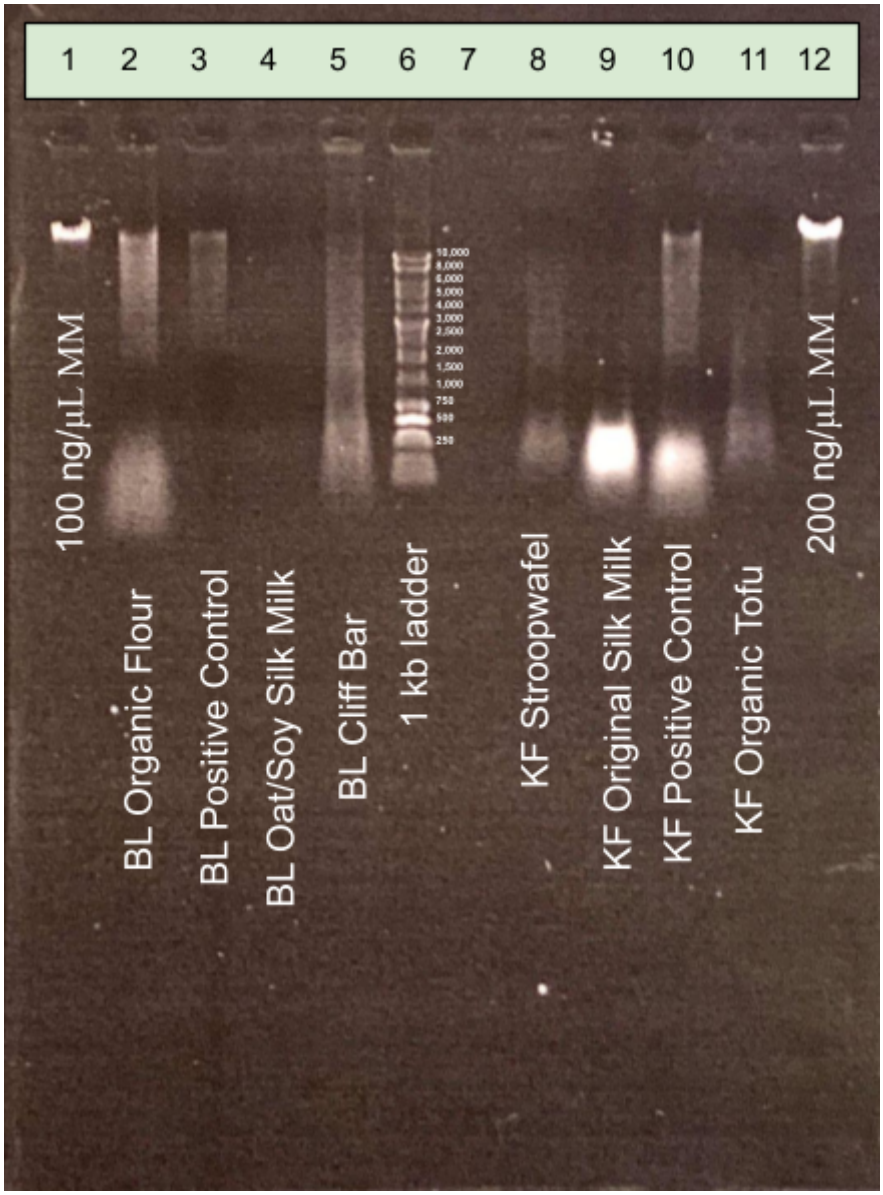
**Day 2: 11/18/22**

**Goal**

Assess the quality and purity of the genomic DNA and isolate better quality genomic DNA from soy food products.

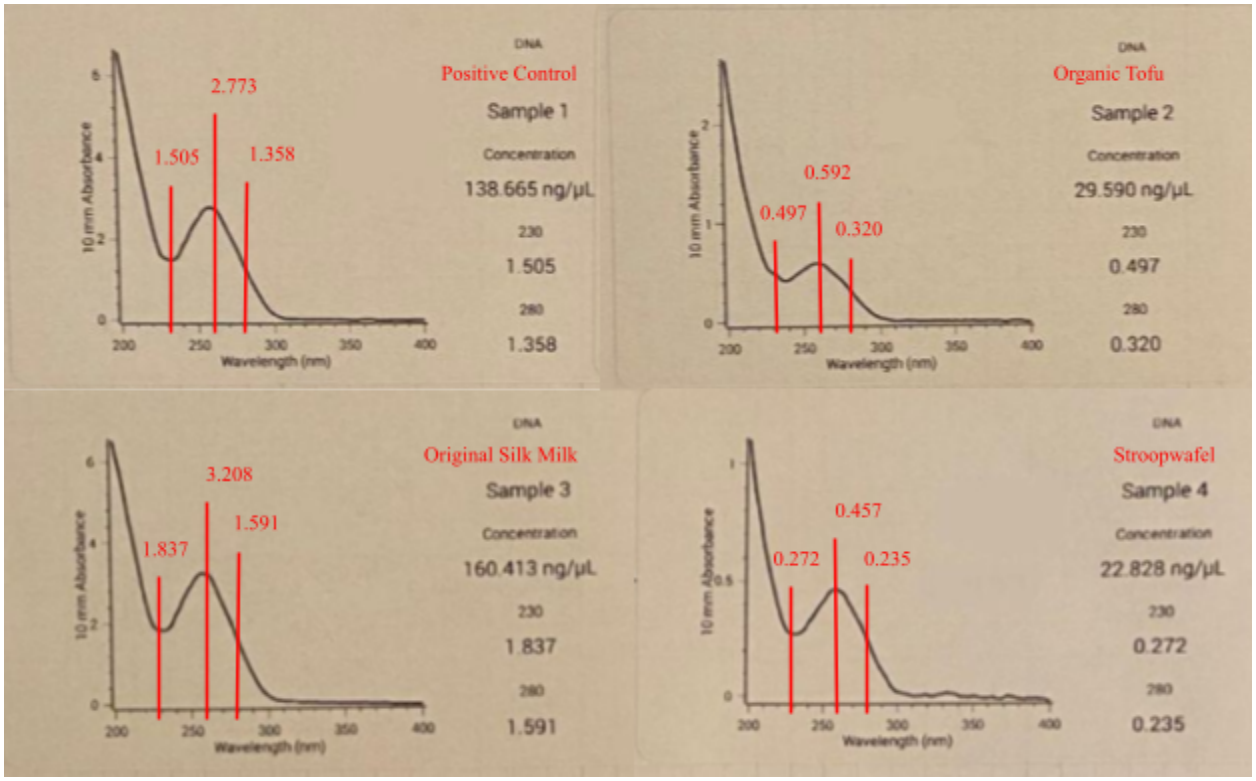
**Data**

**Figure 2.1** | Soy food product genomic DNA run on a 1.5% mini gel




[a] 8 µL of genomic DNA loaded into each well

**Figure 2.2** | NanoDrop scans of genomic DNA samples (KF)




[a] Sample 1: Positive Control
[b] Sample 2: Organic Tofu
[c] Sample 3: Original Silk Milk
[d] Sample 4: Stroopwafel
[e] Absorbances recorded at 230, 260, and 280 nm

**Table 2.1** | Absorbance, purity, and concentration of genomic DNA samples measured on the NanoDrop (KF)

| **Genomic DNA Sample**[a] | **Absorbance**[b] | | | **Purity**[c] **($Abs_{260}/Abs_{280}$)**[d] | **Concentration NanoDrop (ng/µL)** | **Concentration from Gel**[e] **(ng/µL)** | **Total Yield from Gel**[f] **(µg)** |
|---|---|---|---|---|---|---|---|
| | **$Abs_{230}$** | **$Abs_{260}$** | **$Abs_{280}$** | | | | |
| Positive Control | 1.505 | 2.773 | 1.358 | 2.042 | 138.665 | 100 | 10 |
| Organic Tofu | 0.497 | 0.592 | 0.320 | 1.850 | 29.590 | 25 | 2.5 |
| Original Silk Milk | 1.837 | 3.208 | 1.591 | 2.016 | 160.413 | 500 | 50 |
| Stroopwafel | 0.272 | 0.457 | 0.235 | 1.945 | 22.828 | 25 | 2.5 |

[a] 2 µL of each sample loaded on the NanoDrop

[b] Absorbances measured at 230, 260, and 280 nm

[c] Considered "pure" with respect to protein if the $Abs_{260}/Abs_{280}$ is between 1.7-1.9

[d] Example calculation - Purity:

$Abs_{260}/Abs_{280}$ = purity

For the positive control,

2.773/1.358 = 2.042

[e] Estimated based on the size of the known mass marker band on the gel

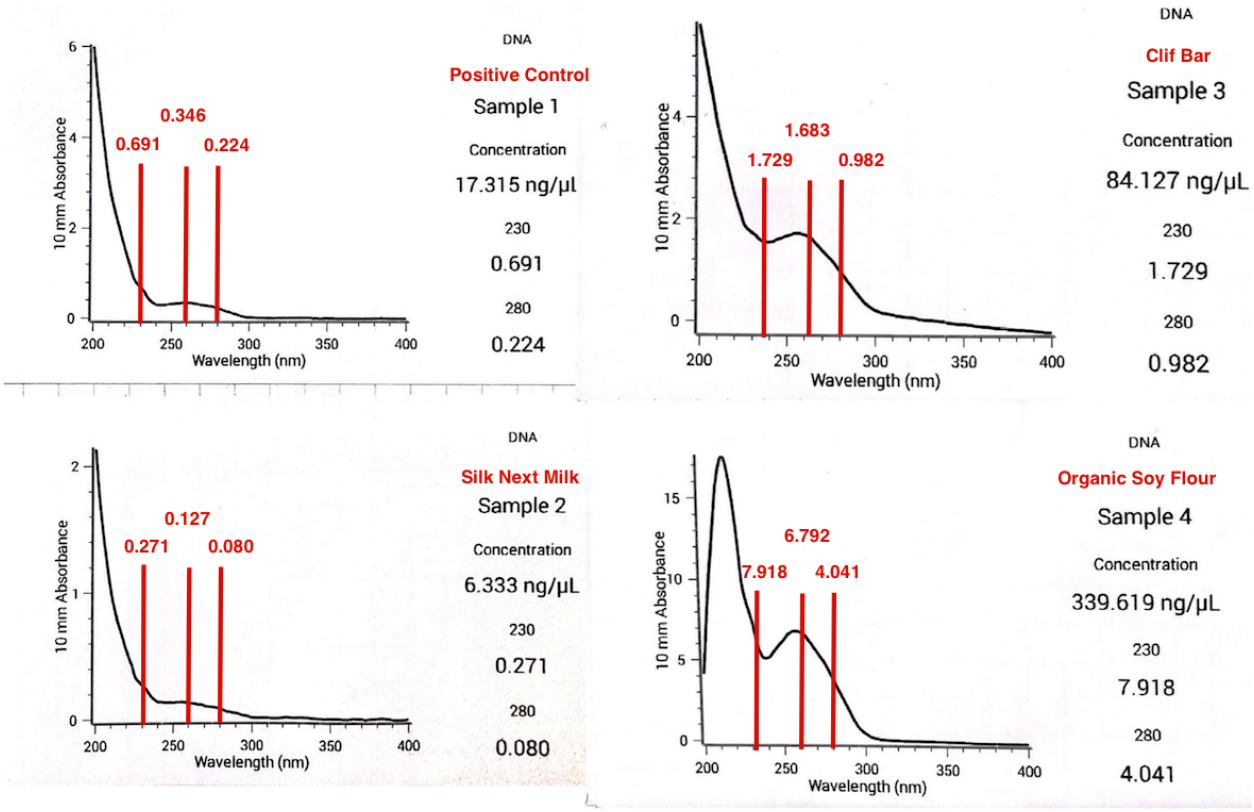
[f] Example calculation - Total Yield:

Concentration (ng/µL) * Volume (µL) * (1 µg/1000 ng)

For the positive control,

100 ng/µL * 100 µL (eluted from NucleoSpin) * (1 µg/1000 ng) = 10 µg

**Figure 2.3** | NanoDrop scans of genomic DNA samples (BL)




[a] Sample 1: Positive Control
[b] Sample 2: Oat/Soy Silk Next Milk
[c] Sample 3: Clif Bar
[d] Sample 4: Organic Soy Flour
[e] Absorbances were recorded at 230, 260, and 280 nm

**Table 2.2** | Absorbance, purity, and concentration of genomic DNA samples measured on the NanoDrop (BL)

| **Genomic DNA Sample**[a] | **Absorbance**[b] | | | **Purity**[c] **($Abs_{260}/Abs_{280}$)**[d] | **Concentration NanoDrop (ng/µL)** | **Concentration from Gel**[e] **(ng/µL)** | **Total Yield from Gel**[f] **(µg)** |
|---|---|---|---|---|---|---|---|
| | **$Abs_{230}$** | **$Abs_{260}$** | **$Abs_{280}$** | | | | |
| Positive Control | 0.691 | 0.346 | 0.224 | 1.545 | 17.315 | 10 | 1 |
| Oat/Soy Silk Next Milk | 0.271 | 0.127 | 0.080 | 1.587 | 6.333 | 5 | 0.5 |
| Clif Bar | 1.729 | 1.683 | 0.982 | 1.714 | 84.127 | 50 | 5 |
| Orgnanic Soy Flour | 7.918 | 6.792 | 4.041 | 1.681 | 339.619 | 50 | 5 |

[a] 2 µL of each sample loaded on the NanoDrop

[b] Absorbances were measured at 230, 260, and 280 nm

[c] DNA samples are considered to be "pure" with respect to protein if the $Abs_{260}/Abs_{280}$ is between 1.7-1.9

[d] Example calculation for purity:

Purity Ratio = $Abs_{260}/Abs_{280}$

For the positive control,

Purity Ratio = 0.346/0.224 = 1.545

[e] Concentration from gel is estimated based on the size of the known mass marker band on the gel

[f] Example calculation for total yield from gel:

Yield = Concentration (ng/µL) * Volume (µL) * (1 µg/1000 ng)

For the positive control,

Yield = 10 ng/µL * 100 µL (eluted from NucleoSpin) * (1 µg/1000 ng) = 1 µg

**Review**

*Purity and quality of gDNA samples*

The genomic DNA that was isolated from the NucleoSpin food prep kit in Day 1 was analyzed for purity, quality, and concentration in Day 2. This was done by using gel electrophoresis and a NanoDrop scan to collect absorbances and visualize the quality of the genomic DNA. The NanoDrop measures the absorbance at 230, 260, and 280 nm. Because different components absorb at different wavelengths (peptide bonds at 230 nm, nucleic acids at 260 nm, and aromatic rings at 280 nm), the ratios between the values can indicate whether the sample is pure with respect to protein [1]. Additionally, there will also be a graph output which will give more insight into potential contaminants. The ideal graph output is a large peak at 260 nm with no observable peaks at 230 or 280 nm [1]. The NanoDrop will also give a concentration, however it is not as accurate as comparing bands on a gel to known mass markers. This is because the concentration from the NanoDrop is determined based on the absorbance at 260 nm.

*(KF) Description of Scans and Gels*

Looking at Figure 2.1, lanes 8, 9, 10, and 11, the quality of the DNA can be assessed. Starting at lane 8 with the stroopwafel, there is very little DNA on the gel which can be visualized by the very faint band. From the gel, it can also be seen that there is a significant amount of genomic DNA smearing present above the band. This is due to the shearing of gDNA which causes the many different sized fragments to move down the gel at different rates [1]. In Table 2.1, the purity of the sample is also not ideal with a $Abs_{260}/Abs_{280}$ ratio of 1.945. Because this value is above the 1.7-1.9 range, we can conclude that this sample is not pure with respect to protein. Because it is >1.9, it cannot be definitively stated what is causing the contamination. The shape of the curve in Figure 2.2 is consistent with the expected shape, with one large peak at 260 nm. The concentration of 22.828 ng/μL from the NanoDrop corroborates the low concentration on the gel that was determined to be 25 ng/μL based on the known mass markers.

Looking next at lane 9 of Figure 2.1, the Silk milk band seems to be free from the gDNA smear and is well concentrated. There is a large white band without any extraneous DNA. In Table 2.1, there is less positive data with a $Abs_{260}/Abs_{280}$ ratio of 2.016 indicating that the gDNA sample is not pure with respect to protein. Because it is >1.9, it cannot be definitively stated what is causing the contamination. The shape of the curve in Figure 2.2 is consistent with the expected shape with a singular large peak at 260 nm. The concentration of 160.413 ng/μL from the NanoDrop is significantly lower than the estimated concentration of 500 ng/μL from the known mass markers. Because the concentration was sufficiently high and the purity and quality of the gDNA was up to the standard required for PCR samples, the Silk milk DNA was not re-extracted on the third day with the other samples.

Lane 10 of Figure 2.1, the positive control band seems to have relatively high concentration, however, it shows signs of genomic DNA smearing. This can be visualized by the streaking above the solid band. This is due to the shearing of gDNA which causes the many different sized fragments to move down the gel at different rates. Additionally, the figure shows that there was not an incredibly high yield like the band for Silk milk. This can be seen by the diffuse and not extremely bright band. In Table 2.1, there is less positive data with a $Abs_{260}/Abs_{280}$ ratio of 2.042 indicating that the gDNA sample is not pure with respect to protein. Because it is >1.9, it cannot be definitively stated what is causing the contamination. The shape of the curve in Figure 2.2 is consistent with the expected shape, with one large peak at 260 nm. The concentration of 138.665 ng/μL from the NanoDrop is larger than the concentration on the gel that was determined to be 100 ng/μL based on the known mass markers.

Ending with Figure 2.1 lane 11 with the organic tofu, there is very little DNA on the gel which can be visualized by the very faint band. From the gel, it can also be seen that there is a significant amount of genomic DNA smearing present above the band. This is due to the shearing of gDNA which causes the many different sized fragments to move down the gel at different rates. In Table 2.1, the purity of the sample is ideal with a $Abs_{260}/Abs_{280}$ ratio of 1.850. Because this value is within the 1.7-1.9 range, we can conclude that this sample is pure with respect to protein. The shape of the curve in Figure 2.2 is consistent with the expected shape, with one large peak at 260 nm. The concentration of 29.590 ng/μL from the NanoDrop corroborates the low concentration on the gel that was determined to be 25 ng/μL based on the known mass markers.

*(BL) Description of Scans and Gels*

The concentration and quality of the four gDNA samples can be assessed from the gel photo from Figure 2.1, NanoDrop information from Figure 2.3 and the conclusion in Table 2.2.

Positive control has very low concentration and is impure. From Figure 2.1 lane 3, it can be seen that the gDNA band is very faint, indicating extremely low concentration. Smearing can also be seen on the lane, indicating gDNA shearing, which causes damage to the DNA sample and different-sized fragments travel at different speeds. From Figure 2.3, no significant peak occurs at 260 nm, which indicates low concentration. This is consistent with the estimated concentration of 10 ng/μL from gel photo in Figure 2.1, and is mostly consistent from the NanoDrop detected concentration of 17.315 ng/μL. The sample is impure, since it has an Abs260/Abs280 ratio of 1.545, which is lower than the 1.7-1.9 range, indicating protein contamination.

Organic soy flour has low concentration and is impure. From Figure 2.1 lane 2, it can be seen that the gDNA band exists but is very dim, indicating relatively lower concentration.

Significant smearing can be seen on the lane, indicating gDNA shearing. From Figure 2.3, peak can be detected at 260 nm, which is consistent with the expectation. However, the NanoDrop detected concentration of 339.619 ng/μL is significantly higher than the estimated concentration of 50 ng/μL from mass marker on gel photo in Figure 2.1. The sample is impure, since it has an Abs260/Abs280 ratio of 1.681, which is slightly lower than the 1.7-1.9 range, indicating protein contamination.

Oat/Soy Silk Next Milk has extremely low concentration and is impure. From Figure 2.1 lane 4, almost no gDNA band can be seen, indicating that the concentration is extremely low. This is consistent with Figure 2.3, where no peak can be seen at 260 nm. The NanoDrop detected concentration of 6.333 ng/μL from Table 2.2 is mostly consistent with the estimated 5 ng/μL from gel mass marker from Figure 2.1, and both are extremely low. The sample is impure, since it has an Abs260/Abs280 ratio of 1.587, which is slightly lower than the 1.7-1.9 range, indicating protein contamination.

Clif Bar has very low concentration but is pure. From Figure 2.1 lane 5, almost no gDNA band can be seen, indicating very low concentration. Significant smearing can be seen on the entire lane, indicating gDNA shearing and significant DNA damages. From Figure 2.3, slight peak can be detected at 260 nm, which is mostly consistent with the expected curve shape. The NanoDrop detected concentration of 84.127 ng/μL is slightly higher than the estimated 50 ng/μL from mass marker on gel photo from Figure 2.1. The sample is pure, since it has an Abs260/Abs280 ratio of 1.714, which is within the 1.7-1.9 range.

*Re-extraction of gDNA with the NucleoSpin Prep*

Because seven out of the eight samples were not up to PCR standards, due to the extreme amount of gDNA shearing and low concentration, we chose to repeat the NucleoSpin preparation to extract better quality DNA. To do this, we ensured that the DNA was not vortexed or centrifuged for longer than the prep required. Additionally, forcefully pipetting the samples can lead to shearing as the large gDNA moves through the small tip [3]. So, in the second prep, we made sure to pipette as gently as possible to mitigate this risk

**References**

[1] Dr. McGuier, 03-343, experimental genetics, Pre-Lab Lecture, October 3, 2022.
[2] Dr. Doonan, Dr. Drill, Dr. McGuier, and Dr. Kaufmann, Experimental Genetics and Molecular Biology Lab Manual, Experiment 8, 2022.
[3] Dr. Doonan, 03-343, Experimental Genetics, Pre-Lab Lecture, November 18, 2022.

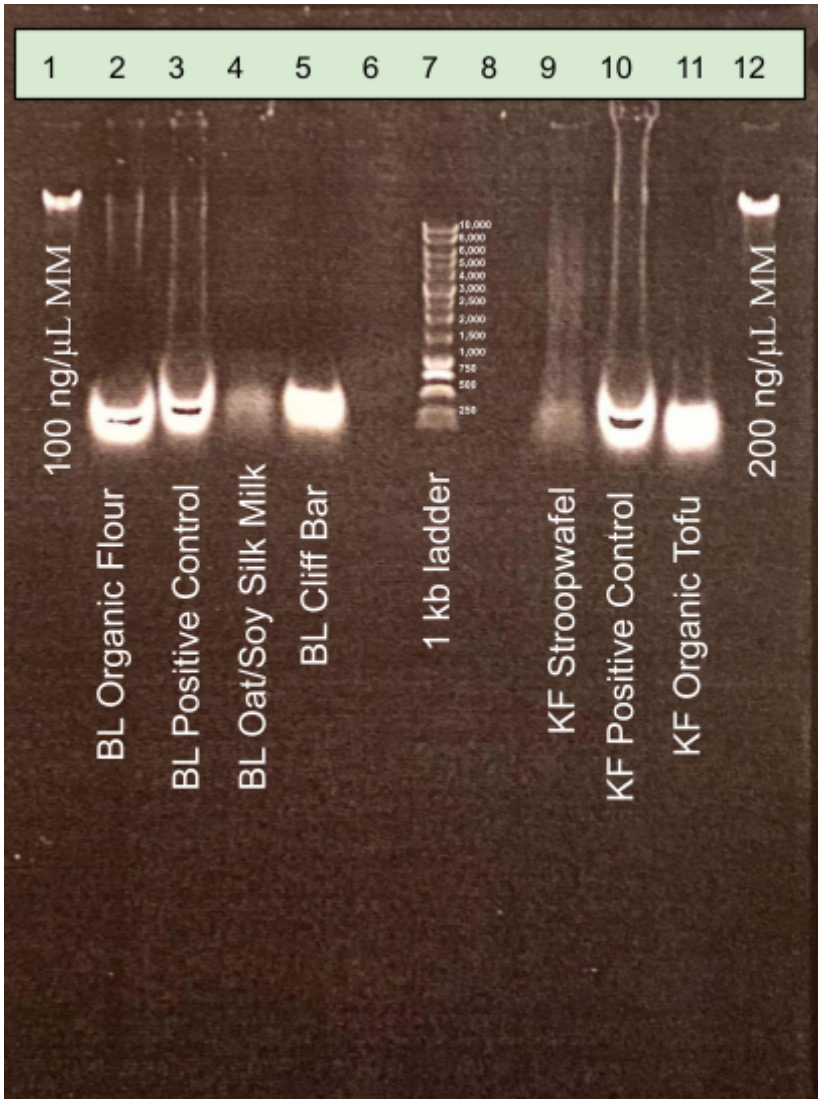
**Day 3: 11/21/22**

**Goal**

Isolate better quality genomic DNA from soy food products, assess the quality and purity of the DNA, and test primary simplex PCR conditions for the CaMV35S promoter.

**Data**

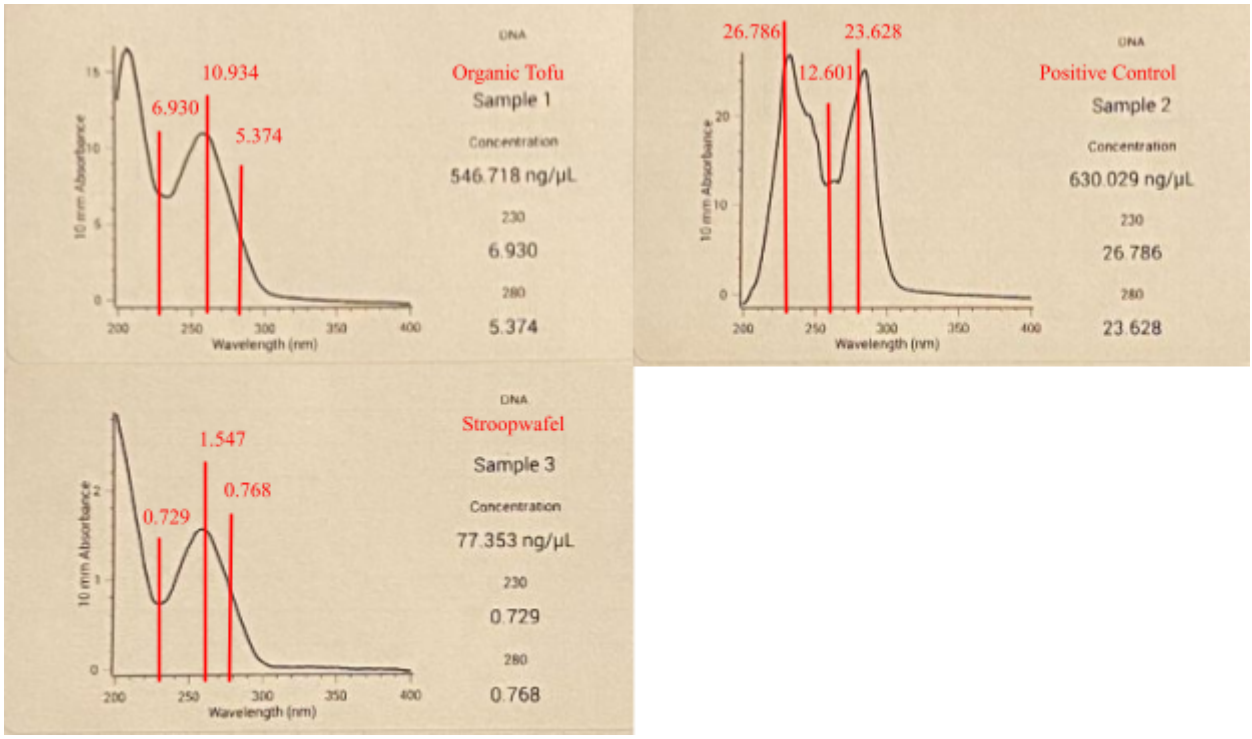
**Figure 3.1** | Soy food product second extraction of genomic DNA run on a 1.5% mini gel




[a] 8 µL of genomic DNA loaded into each well

[a] (KF) gDNA was not loaded for the Original Silk Milk sample as it did not need to be repeated and was previously visualized in Figure 2.1 and Figure 2.2.

**Figure 3.2** | NanoDrop scans of genomic DNA samples (KF)




[a] Sample 1: Organic Tofu
[b] Sample 2: Positive Control
[c] Sample 3: Stroopwafel
[d] Absorbances recorded at 230, 260, and 280 nm

**Table 3.1** | Absorbance, purity, and concentration of genomic DNA samples measured on the NanoDrop (KF)

| **Genomic DNA Sample**[a] | **Absorbance**[b] | | | **Purity**[c] **($Abs_{260}/Abs_{280}$)**[d] | **Concentration NanoDrop (ng/µL)** | **Concentration from Gel**[e] **(ng/µL)** | **Total Yield from Gel**[f] **(µg)** |
|---|---|---|---|---|---|---|---|
| | **$Abs_{230}$** | **$Abs_{260}$** | **$Abs_{280}$** | | | | |
| Organic Tofu | 6.930 | 10.934 | 5.374 | 2.035 | 546.718 | 500 | 50 |
| Positive Control | 26.786 | 12.601 | 23.628 | 0.533 | 630.029 | 600 | 60 |
| Stroopwafel | 0.729 | 1.547 | 0.768 | 2.014 | 77.353 | 50 | 5 |

[a] 2 µL of each sample loaded on the NanoDrop

[b] Absorbances measured at 230, 260, and 280 nm

[c] Considered "pure" with respect to protein if the $Abs_{260}/Abs_{280}$ is between 1.7-1.9

[d] Example calculation:

$Abs_{260}/Abs_{280}$ = purity

For the organic tofu,

10.934/5.374 = 2.035

[e] Estimated based on the size of the known mass marker band on the gel

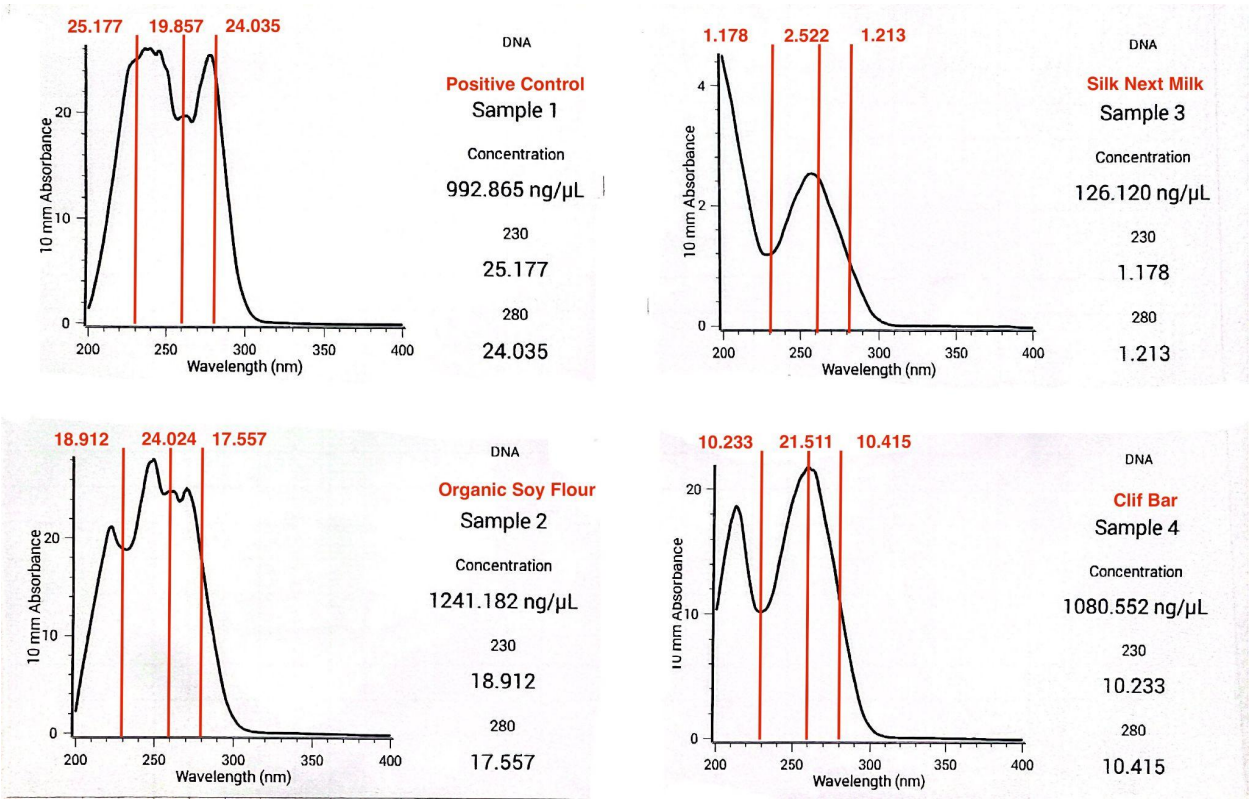
[f] Example calculation:

Concentration (ng/µL) * Volume (µL) * (1 µg/1000 ng)

For the organic tofu,

500 ng/µL * 100 µL (eluted from NucleoSpin) * (1 µg/1000 ng) = 50 µg

**Figure 3.3** | NanoDrop scans of genomic DNA samples (BL)




[a] Sample 1: Positive Control
[b] Sample 2: Organic Soy Flour
[c] Sample 3: Oat/Soy Silk Next Milk
[d] Sample 4: Clif Bar
[e] Absorbances were recorded at 230, 260, and 280 nm

**Table 3.2** | Absorbance, purity, and concentration of genomic DNA samples measured on the NanoDrop (BL)

| **Genomic DNA Sample**[a] | **Absorbance**[b] | | | **Purity**[c] **($Abs_{260}/Abs_{280}$)**[d] | **Concentration NanoDrop (ng/µL)** | **Concentration from Gel**[e] **(ng/µL)** | **Total Yield from Gel**[f] **(µg)** |
|---|---|---|---|---|---|---|---|
| | **$Abs_{230}$** | **$Abs_{260}$** | **$Abs_{280}$** | | | | |
| Positive Control | 25.177 | 19.857 | 24.035 | 0.826 | 992.865 | 600 | 60 |
| Organic Soy Flour | 18.912 | 24.024 | 17.557 | 1.368 | 1241.182 | 600 | 60 |
| Silk Next Milk | 1.178 | 2.522 | 1.213 | 2.079 | 126.120 | 50 | 5 |
| Clif Bar | 10.233 | 21.511 | 10.415 | 2.065 | 1080.552 | 500 | 50 |

[a] 2 µL of each sample loaded on the NanoDrop

[b] Absorbances were measured at 230, 260, and 280 nm

[c] DNA samples are considered to be "pure" with respect to protein if the $Abs_{260}/Abs_{280}$ is between 1.7-1.9

[d] Example calculation for purity:

Purity Ratio = $Abs_{260}/Abs_{280}$

For the positive control,

Purity Ratio = 19.857/24.035 = 0.826

[e] Concentration from gel is estimated based on the size of the known mass marker band on the gel

[f] Example calculation for total yield from gel:

Yield = Concentration (ng/µL) * Volume (µL) * (1 µg/1000 ng)

For the positive control,

Yield = 600 ng/µL * 100 µL (eluted from NucleoSpin) * (1 µg/1000 ng) = 60 µg

**Review**

*(KF) Description of Scans and Gels*

Looking at Figure 3.1, lanes 9, 10, and 11, the quality of the DNA can be assessed. Starting at lane 9 with the stroopwafel, there is slightly more DNA on the gel which can be visualized by the stronger band. While there is still not a significant amount of DNA on the gel, the stroopwafel is highly processed so we have lower expectations for the concentration of DNA. In Table 3.1, the purity of the sample is also not ideal with a $Abs_{260}/Abs_{280}$ ratio of 2.014. Because this value is above the 1.7-1.9 range, we can conclude that this sample is not pure with respect to protein. Because it is >1.9, it cannot be definitively stated what is causing the contamination. The shape of the curve in Figure 2.2 is consistent with the expected shape, with one large peak at 260 nm. The concentration of 77.353 ng/μL from the NanoDrop as well as the estimation from the gel that was determined to be 50 ng/μL based on the known mass markers corroborates that the concentration is higher than the first extraction.

Lane 10 of Figure 3.1, the positive control band seems to have very high concentration, with no signs of genomic DNA smearing. This shows a very high yield, which is already improved from the primary extraction. This can be seen by the extremely bright and condensed band. In Table 3.1, there is less positive data with a $Abs_{260}/Abs_{280}$ ratio of 0.533 indicating that the gDNA sample is not pure with respect to protein. Because it is <1.7, it can be determined that protein is causing the contamination. The gel in Figure 3.1 corroborates this protein contamination as seen by the trapped DNA in the well. Proteins bind up DNA and cause the sample to be trapped in the well due to its large size. The shape of the curve in Figure 2.2 is consistent with the protein contamination seen in the absorbance ratio, with one large peak at 260 nm as well as another large peak at 230 nm. Because the PCR process is not greatly affected by protein contamination, another extraction was not performed. The concentration of 630.029 ng/μL from the NanoDrop corroborated the high concentration on the gel that was determined to be 600 ng/μL based on the known mass markers.

Ending with Figure 3.1 lane 11 with the organic tofu, the band shows very high concentration, with no signs of genomic DNA smearing. The figure shows a very high yield, which is already improved from the primary extraction, which can be seen by the extremely bright and condensed band. Table 3.1 shows an impure sample with a $Abs_{260}/Abs_{280}$ ratio of 2.035. Because this value is above the 1.7-1.9 range, we can conclude that this sample is not pure with respect to protein. Because it is >1.9, it cannot be definitively stated what is causing the contamination. The shape of the curve in Figure 2.2 is consistent with the expected shape, with one large peak at 260 nm. The concentration of 546.718 ng/μL from the NanoDrop corroborated the high concentration on the gel that was determined to be 500 ng/μL based on the known mass markers.

*(BL) Description of Scans and Gels*

The concentration and quality of the four gDNA samples can be assessed from the gel photo from Figure 3.1, NanoDrop information from Figure 3.3 and the conclusion in Table 3.2.

Positive control has high concentration but is impure. From Figure 3.1 lane 3, it can be seen that the gDNA band is large, bright and condensed, with no sign of smearing. This indicates high concentration and very little DNA damage. This is supported by the estimated concentration of 600 ng/μL from gel, and the NanoDrop concentration 992.865 ng/μL from Table 3.2. The NanoDrop concentration is significantly higher than the estimated concentration from gel. From Figure 3.3, a peak occurs at 260 nm, which is consistent with expectation. However, there are significant peaks at 230 nm and 280 nm as well as multiple random peaks, which indicates contamination. This is consistent with Abs260/Abs280 ratio of 0.826, which is significantly lower than the 1.7-1.9 range, indicating a large amount of protein contamination.

Organic soy flour has high concentration but is impure. From Figure 3.1 lane 2, it can be seen that the gDNA band is large, bright and condensed, with no sign of smearing. This indicates high concentration and very little DNA damage. This is supported by the estimated concentration of 600 ng/μL from gel, and the NanoDrop concentration 1241.182 ng/μL from Table 3.2. The NanoDrop concentration is significantly higher than the estimated concentration from gel. From Figure 3.3, a peak occurs at 260 nm, which is consistent with expectation. However, there are multiple peaks from 230 - 280 nm rangel, which indicates contamination. This is consistent with Abs260/Abs280 ratio of 1.368, which is lower than the 1.7-1.9 range, indicating existence of protein contamination.

Oat/Soy Silk Next Milk has very low concentration and is impure. From Figure 3.1 lane 4, it can be seen that gDNA band is very faint, indicating low concentration. This is supported by the Nanodrop concentration of 126.120 ng/μL and the estimated concentration ot 50 ng/μL from gel in Table 3.2. From Figure 3.3, a peak occurs at 260 nm, which is consistent with the expectation. The sample is impure because it has a Abs260/Abs280 ratio of 2.079, which is higher than the 1.7-1.9 range.

Clif bar has high concentration but is impure. From Figure 3.1 lane 5, it can be seen that the gDNA band is large, bright and condensed, with no sign of smearing. This indicates high concentration and very little DNA damage. This is supported by the estimated concentration of 500 ng/μL from gel, and the NanoDrop concentration 1080.552 ng/μL from Table 3.2. The NanoDrop concentration is significantly higher than the estimated concentration from gel. From Figure 3.3, a peak occurs at 260 nm, which is consistent with expectation. The product is impure because it has Abs260/Abs280 ratio of 2.065, which is higher than the 1.7-1.9 range.

**Table 3.3** | PCR primers selected for the amplification of the EPSPS, CaMV35S, and kanamycin resistance genes

| Gene of Interest | Primer | Primer Sequence | $T_m$[a] (℃) | Expected Size[b] |
|---|---|---|---|---|
| EPSPS[d] | CP4EPSPSF | TGG CGC CCA AAG CTT GCA TG | 59.35 | 356 |
| | CP4EPSPSR | CC CAA GTT CCT AAA TCT TCA AGT | 59.82 | |
| CaMV35S | CAMV35SF158[c] | CCG ACA GTG GTC CCA AAG ATG | 61.78 | 158[2] |
| | CAMV35SR158 | AGA GGA AGG GTC TTG CGA AGG | 61.78 | |
| NPT II (Kanr)[d] | NPTIIF | ACA AGA TGG ATT GCA CGC AGG | 59.82 | 794 |
| | NPTIIR | AAC TCG TCA AGA AGC CGA TAG | 57.87 | |

[a] Example calculation

$L > 18$, $69.3 + 0.41(GC\%) - 650/L = T_m$

For the CaMV35S forward primer,

$69.3 + 0.41(57.14\%) - 650/21 = 61.78$℃

[b] Based on literature values

[c] Also called 35SFZMP1, 35SFZMP2

[d] Later changed due to the low melting temperatures, however the initial simplex PCR conditions were designed with these primers in mind.

**Table 3.4** | Simplex PCR conditions for the amplification of the CaMV35S gene

| PCR Step | | Temperature (℃) | Time |
|---|---|---|---|
| Initial Denaturation | | 95 | 5 min |
| 40x Cycles | Denaturation | 95 | 20 sec |
| | Annealing | 57 | 40 sec |
| | Extension | 72 | 1 min |
| Final Extension | | 72 | 10 min |
| Holding | | 4 | Overnight |

**Table 3.4** | Simplex PCR design for the amplification of the CaMV35S gene using the *Taq* PCR beads

| **Sample (Initials)** | **gDNA** | **Forward Primer** | **Reverse Primer** | **dd$H_2O$** | **Total Volume** |
|---|---|---|---|---|---|
| Positive Control (KF) | 3 µL | 2.5 µL | 2.5 µL | 17 µL | 25 µL |
| Stroopwafel (KF) | 5 µL | 2.5 µL | 2.5 µL | 15 µL | 25 µL |
| Silk Milk (KF) | 5 µL | 2.5 µL | 2.5 µL | 15 µL | 25 µL |
| Organic Tofu (KF) | 3 µL | 2.5 µL | 2.5 µL | 17 µL | 25 µL |
| Negative Control (KF) | 0 µL | 2.5 µL | 2.5 µL | 20 µL | 25 µL |
| Positive Control (BL) | 3 µL | 2.5 µL | 2.5 µL | 17 µL | 25 µL |
| Clif Bar (BL) | 3 µL | 2.5 µL | 2.5 µL | 17 µL | 25 µL |
| Silk Next Milk (BL) | 5 µL | 2.5 µL | 2.5 µL | 15 µL | 25 µL |
| Organic Soy Flour (BL) | 3 µL | 2.5 µL | 2.5 µL | 17 µL | 25 µL |
| Negative Control (BL) | 0 µL | 2.5 µL | 2.5 µL | 20 µL | 25 µL |

**Review**

*Primer Rationale*

The primers in Table 3.3 were selected due to their similar melting temperatures and different product sizes. The product sizes must be different so that they can easily be distinguished on a multiplex PCR gel. The melting temperatures must be within 5℃ of each other so that the annealing temperatures can all be within the 2-5℃ below the melting temperature range required for PCR [3]. In a multiplex PCR cycler, all of the primers must be run at the same temperatures and they must all work optimally at the same annealing temperature.

*PCR Conditions Rationale*

The initial denaturation is at 95℃, which denatures the DNA and separates the two strands. Once the cycles begin, the denaturation at 95℃ ensures that all of the double stranded DNA has separated to allow the DNA polymerase to bind. The annealing at 57℃ falls within the recommended annealing temperatures for all except the kanamycin resistance reverse primer. This temperature was chosen to ensure that this primer would not melt, but would produce PCR products from all other primer pairs. The annealing step is where the primers bind to their respective sequences on the template strand and indicate the start of replication. The extension at 72℃ is the optimal temperature for Taq polymerase to replicate the DNA. These three steps are repeated for 40 cycles to ensure that enough product is produced to be clearly visible on an agarose gel. The final extension is to ensure all products have been completely formed. The holding temperature is 4℃ to keep the DNA in optimal conditions and ensure that there is no enzyme activity to damage the sample [3].

*PCR Design*

The PCR for this experiment was performed in tubes with lyophilized beads containing Taq polymerase, dNTPs, and buffer. The tubes hold a 25 µL total reaction volume. The primer volume was based off of the concentration of the stock solutions in units of enzyme. The amount of template DNA added was determined by the concentration of the gDNA determined by the purity and quality gel electrophoresis performed earlier. The ideal concentration of genomic DNA for PCR is between 50-100 ng [1]. This is higher than the amount required for plasmid PCR because genomic DNA is much larger and the primers have a much greater surface to scan. Increasing the amount of DNA ensures that the primers will all find a site to bind to and amplify. The deionized water brings the reaction up to volume. There was a negative control sample that contained all of the same components except for the template DNA that will serve as a measure for contamination and the positive control was our certified GM soy powder that we know should contain these genes of interest.

**References**

[1] Dr. Doonan, 03-343, Experimental Genetics, Pre-Lab Lecture, November 7, 2022.
[2] James, Delano, et al. “Reliable Detection and Identification of Genetically Modified Maize, Soybean, and Canola by Multiplex PCR Analysis.” Journal of Agricultural and Food Chemistry, U.S. National Library of Medicine, 19 Aug. 2003, https://pubmed.ncbi.nlm.nih.gov/13129280/.
[3] Dr. McGuire, 03-343, Experimental Genetics, Pre-Lab Lecture, October 24, 2022.

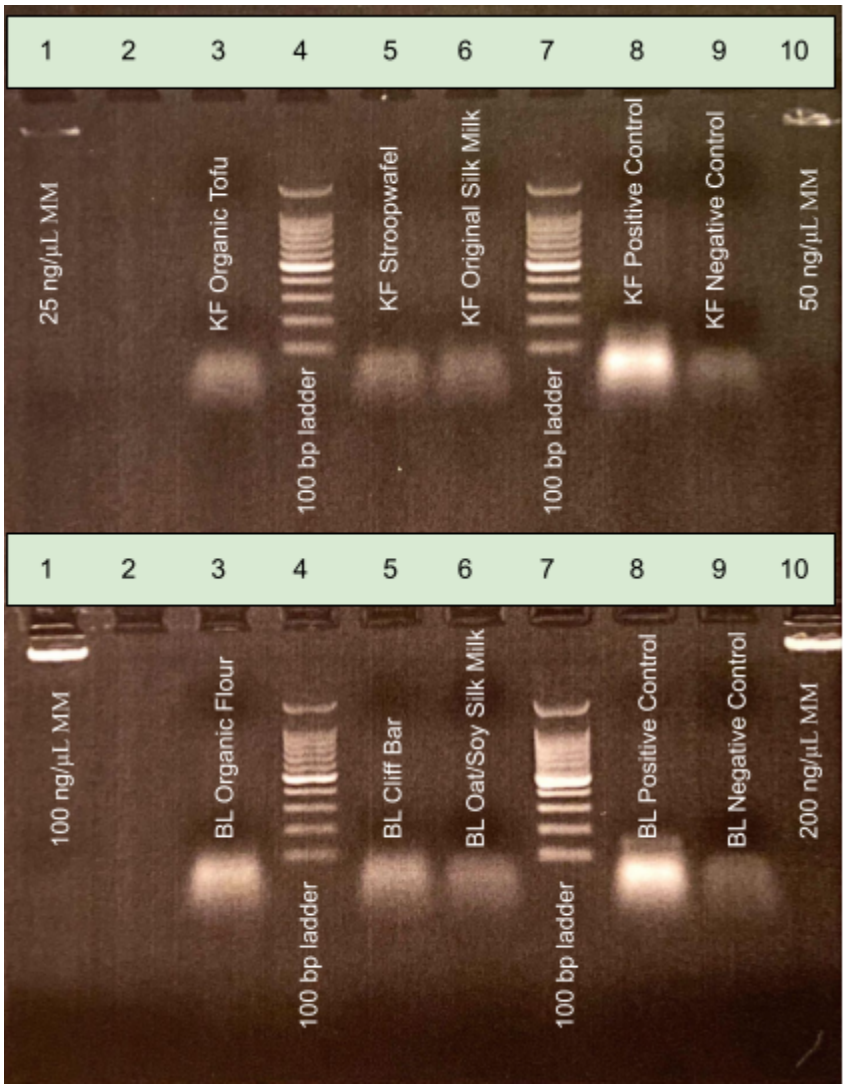
**Day 4: 11/28/22**

**Goal**

Analyze the CaMV35S promoter simplex PCR products and change conditions to optimize PCR for the promoter.

**Data**

**Figure 4.1** | Primary simplex PCR (57 °C annealing temp, 40 cycles) for the CaMV35S promoter products on double lane 1.5% midi gel




[a] 9 µL of genomic DNA loaded into each well because mineral oil was used during PCR process. The mineral oil was used to prevent evaporation and conduct heat, however it could be mixed with the samples slightly when drawing the sample for gel loading. Therefore, extra 1µL was drawn to compensate for that.

[b] Ladder did not fully separate

**Table 4.1** | PCR primers selected for the amplification of the CaMV35S/EPSPS Junction, CaMV35S, and Soy Lectin genes

| Gene of Interest | Primer | Primer Sequence | $T_m$[a] (℃) | Expected Size[b] |
|---|---|---|---|---|
| CaMV35S/ EPSPS Junction[c] | F35S-EPSPS 447KA | CCA CTG ACG TAA GGG ATG ACG | 61.8 | 447[2] |
| | R35S-EPSPS 447KA | CAT GAA GGA CCG GTG GGA GAT | 61.8 | |
| CaMV35S | CAMV35SF158[e] | CCG ACA GTG GTC CCA AAG ATG | 61.78 | 158[3] |
| | CAMV35SR158 | AGA GGA AGG GTC TTG CGA AGG | 61.78 | |
| Soy Lectin[c] | SOYLECTF210[f] | GGG TGA GGA TAG GGT TCT CTG | 61.7 | 210[3] |
| | SOYLECTR210 | GCG ATC GAG TAG TGA GAG TCG | 61.7 | |

[a] Example calculation
$L > 18$, $69.3 + 0.41(GC\%) - 650/L = T_m$
For the CaMV35S forward primer,
$69.3 + 0.41(57.14\%) - 650/21 = 61.78$
[b] Based on literature values
[c] Changed primers due to low melting point.
[d] Increased melting point allowed the annealing temperature to be increased in the simplex PCR
[e] Also called 35SFZMP1, 35SFZMP2
[f] Also called lecMP1, lecMP2

**Table 4.2** | Simplex PCR conditions for the amplification of the CaMV35S/EPSPS junction and soy lectin genes

| PCR Step | | Temperature (℃) | Time |
|---|---|---|---|
| Initial Denaturation | | 95 | 5 min |
| 40x Cycles | Denaturation | 95 | 20 sec |
| | Annealing | 59 | 40 sec |
| | Extension | 72 | 1 min |
| Final Extension | | 72 | 10 min |
| Holding | | 4 | Overnight |

**Table 4.3** | Simplex PCR design for the amplification of the CaMV35S gene using the *Taq* PCR beads

| **Sample (Initials)** | **gDNA** | **Forward Primer** | **Reverse Primer** | **dd$H_2O$** | **Total Volume** |
|---|---|---|---|---|---|
| Positive Control (KF) | 5 µL | 2.5 µL | 2.5 µL | 15 µL | 25 µL |
| Stroopwafel (KF) | 5 µL | 2.5 µL | 2.5 µL | 15 µL | 25 µL |
| Silk Milk (KF) | 5 µL | 2.5 µL | 2.5 µL | 15 µL | 25 µL |
| Organic Tofu (KF) | 5 µL | 2.5 µL | 2.5 µL | 15 µL | 25 µL |
| Negative Control (KF) | 0 µL | 2.5 µL | 2.5 µL | 20 µL | 25 µL |
| Positive Control (BL) | 5 µL | 2.5 µL | 2.5 µL | 15 µL | 25 µL |
| Clif Bar (BL) | 5 µL | 2.5 µL | 2.5 µL | 15 µL | 25 µL |
| Silk Next Milk (BL) | 5 µL | 2.5 µL | 2.5 µL | 15 µL | 25 µL |
| Organic Soy Flour (BL) | 5 µL | 2.5 µL | 2.5 µL | 15 µL | 25 µL |
| Negative Control (BL) | 0 µL | 2.5 µL | 2.5 µL | 20 µL | 25 µL |

**Review**

*Simplex PCR Results*

Because we decided to analyze our CaMV35S promoter simplex PCR products on a double lane midi gel, we did not allow enough space for our ladders to completely separate and the primer dimers to migrate below the PCR products. From this gel in Figure 4.1, we were not able to make any conclusive measurements to calculate the size of the products, however we were able to see which samples had potentially produced products. The positive controls in lane 8 of the top and bottom shows the potential for having a product based on the brightness of the band present. The other lanes, however, do not show as much promise. We determined that this could have been due to a low annealing temperature and possibly not enough DNA added to the PCR reaction.

*Changes in Primers and PCR Conditions Rationale*

Because the EPSPS and NPT II primers had melting temperatures that prevented the increase of the PCR annealing temperature, we made the decision to change these primers. Looking at Table 4.1, we changed the EPSPS primer to an CaMV35S/EPSPS junction primer and the NPT II primer to a soy lectin primer. In doing so, we were able to find primers that all had melting temperatures greater than 61℃ at 61.8℃ and 61.7 respectively. Because the melting temperatures were all similar, we were able to raise the annealing temperatures from 57℃ to 59℃ to increase our PCR products. As seen in Table 4.2, all other conditions have remained the same.

*Changes in PCR Design Rationale*

In Table 4.3, the template DNA amount was increased to account for the low yield in Figure 4.1. By increasing the amount of template DNA in the reaction, there is a greater chance primers will find a binding site and be able to amplify the correct sequence.

**References**

[1] Dr. Doonan, 03-343, Experimental Genetics, Pre-Lab Lecture, November 7, 2022.

[2] Cardarellia, Paola, et al. "Detection of GMO in Food Products in Brazil: The INCQS Experience." Food Control, Elsevier, 28 Sept. 2004, https://www.sciencedirect.com/science/article/pii/S095671350400177X?casa_token=vTtvWff5uwsAAAAA%3Ajq6YVQVsoYcDxQzFvsDQC829RNtkeUEX0ASNIcN9b5FoB7GAN5pWMDoonNYq8C19Zi9uIUzdL8s.

[3] James, Delano, et al. "Reliable Detection and Identification of Genetically Modified Maize, Soybean, and Canola by Multiplex PCR Analysis." Journal of Agricultural and Food Chemistry, U.S. National Library of Medicine, 19 Aug. 2003, https://pubmed.ncbi.nlm.nih.gov/13129280/.

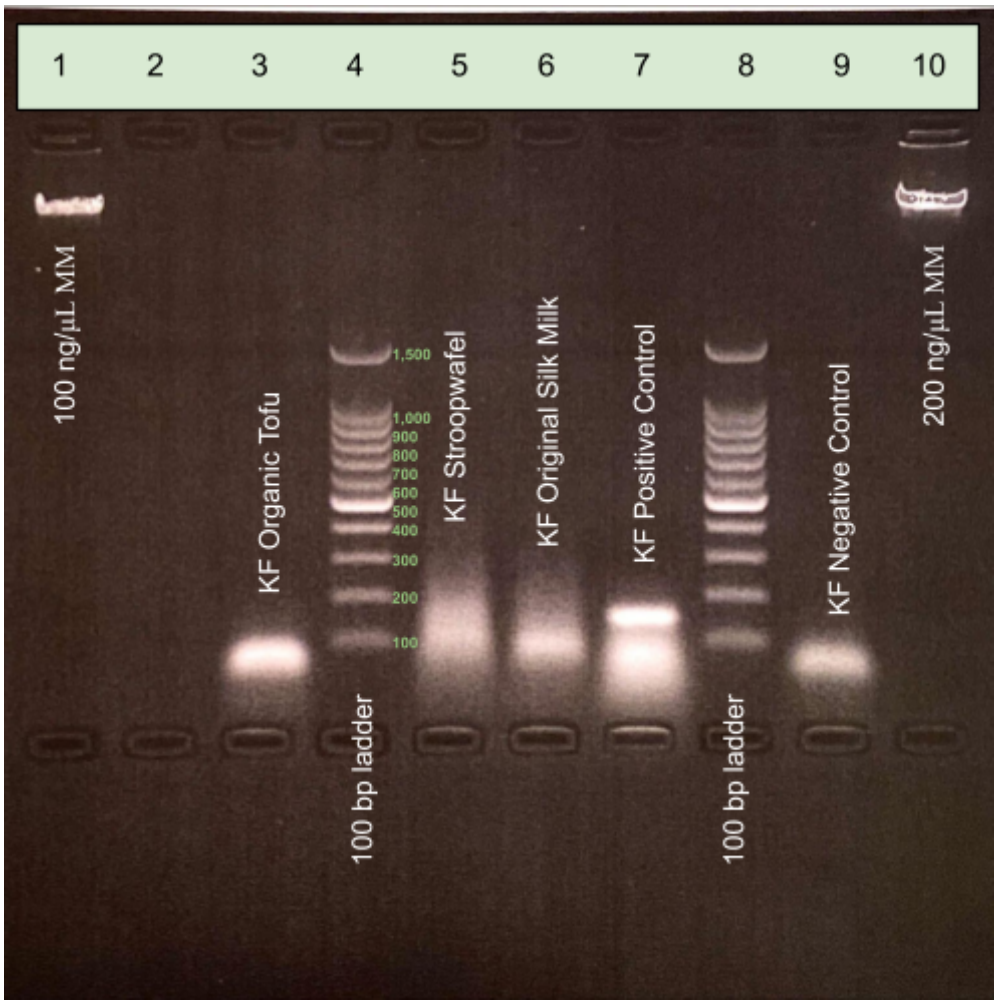
**Day 5: 11/30/22**

**Goal**

Analyze the CaMV35S promoter simplex PCR products and perform simplex PCR for the soy lectin and CaMV35S/EPSPS junction.

**Data**

**Figure 5.1** | Primary simplex PCR for the CaMV35S promoter products on single lane 1.5% midi gel (KF)




[a] 8 µL of genomic DNA loaded into each well
[b] The CAMV35SR158 primer forms a primer dimer which can be seen at <100 bp

**Figure 5.2** | Relationship of distance migrated vs. log molecular weight of 100 kb DNA ladder (KF)

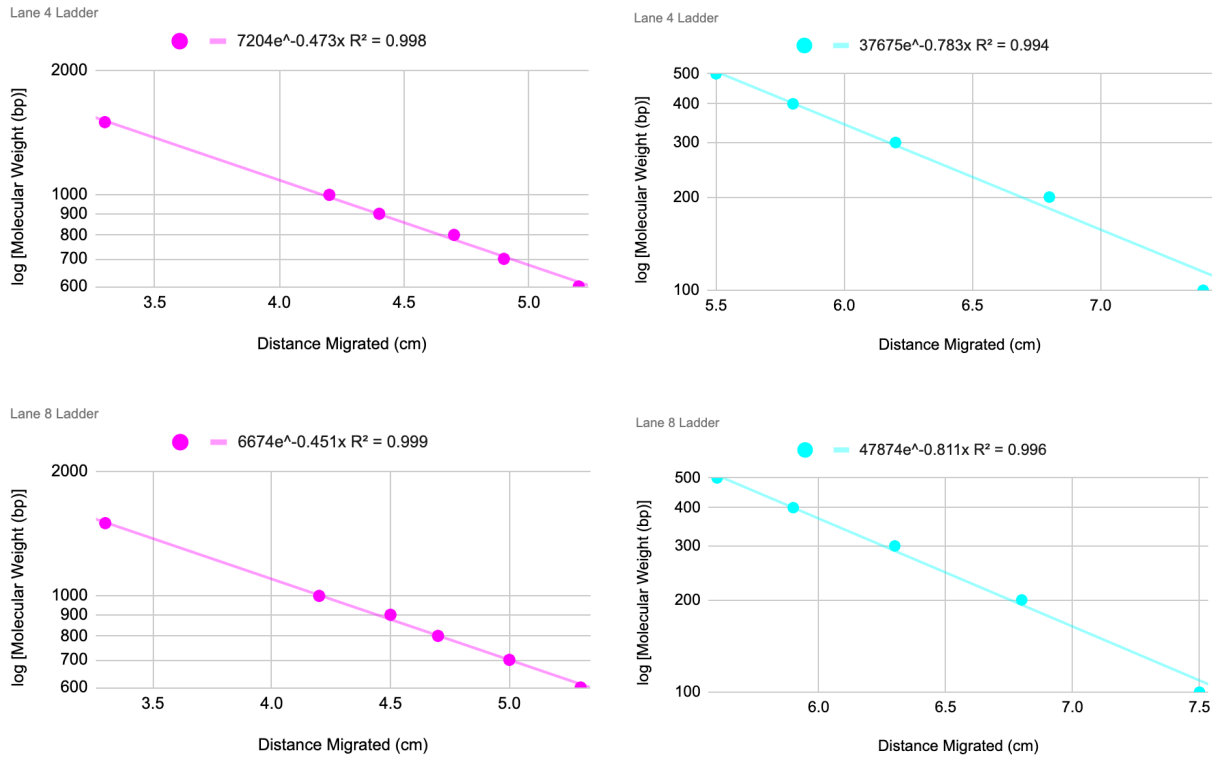



**Table 5.1** | Distance traveled and calculated DNA size for simplex PCR for the CaMV35S promoter products (KF)

| Lane | Genomic DNA Sample | Expected DNA sizes (kb) | Band Present (Yes/No) | Distance Traveled (cm) | Calculated DNA sizes (bp)[b] | Which Ladder Lane? | Estimated Concentration (ng/µL) |
|---|---|---|---|---|---|---|---|
| 3 | Organic Tofu | 158 | N | n/a | n/a | n/a | n/a |
| 5 | Stroopwafel | 158 | Y | 7.0 | 157 | 4 | 50 |
| 6 | Original Silk | 158 | N | n/a | n/a | n/a | n/a |
| 7 | Positive Control | 158 | Y | 7.15 | 145 | 8 | 200 |
| 9 | Negative Control | n/a | N | n/a | n/a | n/a | n/a |

[a] Determined by estimating size based on the closest ladder

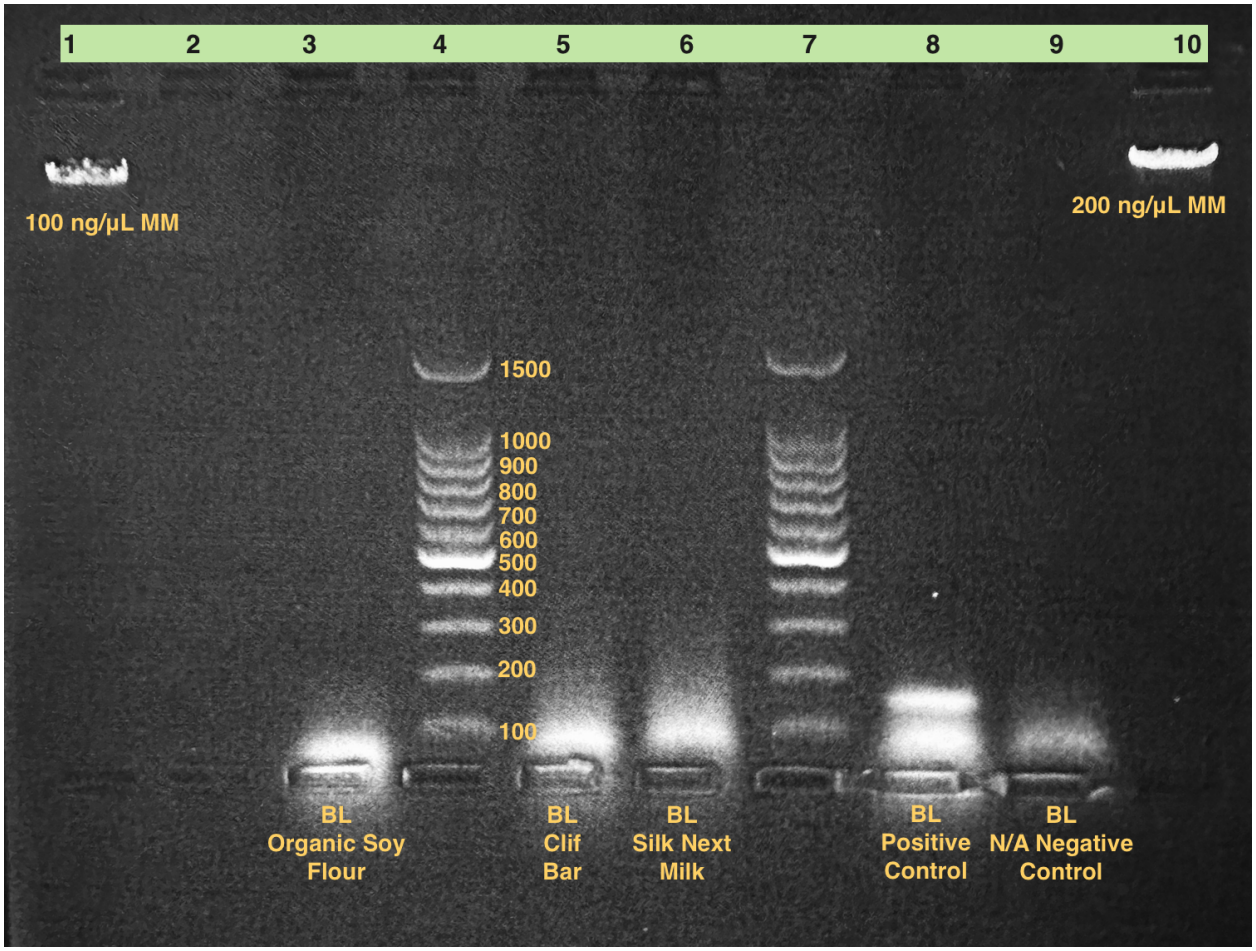
[b] Sample Calculation:

$47874e^{-0.811(\text{Distance Traveled})} = \text{Molecular Weight}$

For Positive Control,

$47874e^{-0.811(7.15)} = 145$ bp

**Figure 5.3** | Primary simplex PCR (59 °C annealing temp, 40 cycles) for the CaMV35S promoter products on single lane 1.5% agarose midi gel (BL)




[a] 8 µL of genomic DNA loaded into each well

[b] The CAMV35SR158 primer forms a primer dimer which can be seen at <100 bp

**Figure 5.4** | Relationship of distance migrated vs. log molecular weight of 100 kb DNA ladder for ladders on lane 4 and 7

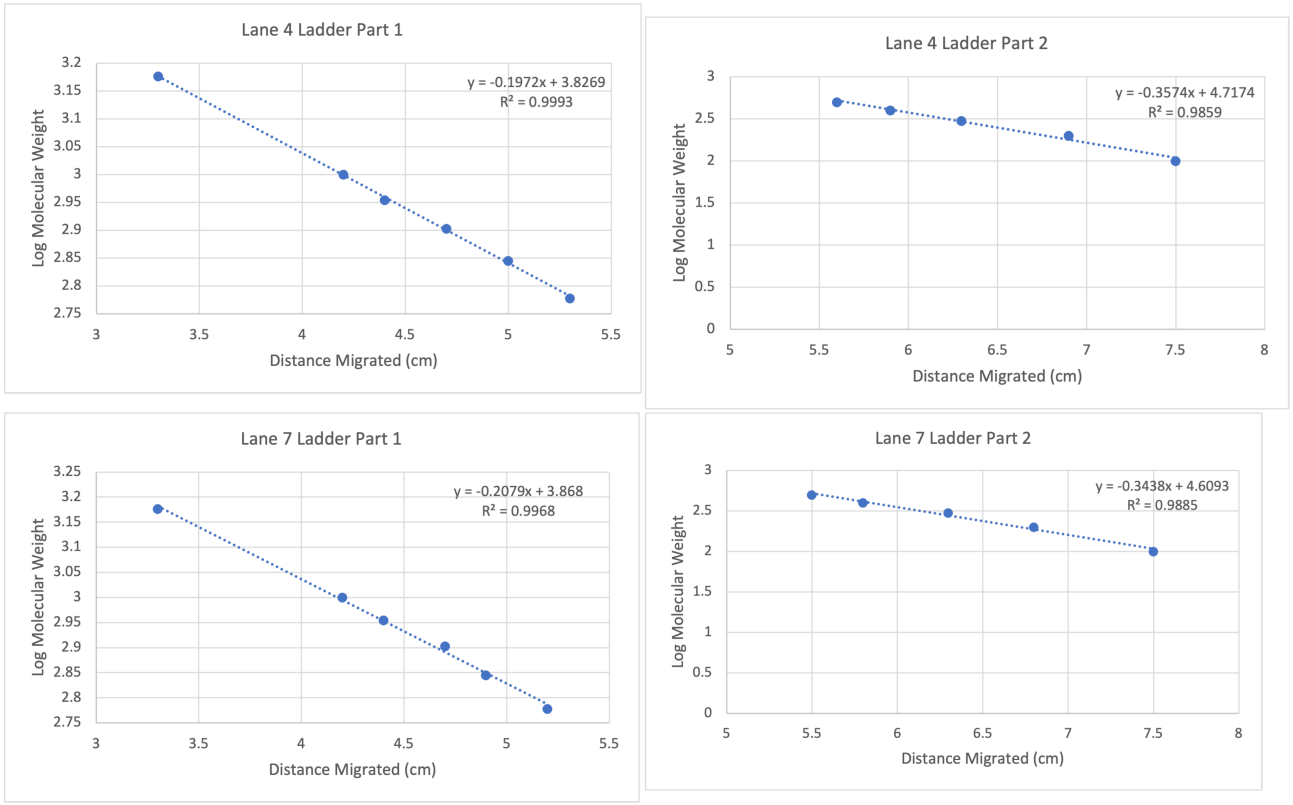



**Table 5.2** | Distance traveled and calculated DNA size for simplex PCR (59 °C annealing temp, 40 cycles) for the CaMV35S promoter products

| Lane | Genomic DNA Sample | Expected DNA sizes (kb) | Band Present (Yes/No) | Distance Traveled (cm) | Calculated DNA sizes (bp)[b] | Which Ladder Lane? | Estimated Concentration (ng/µL) |
|---|---|---|---|---|---|---|---|
| 3 | Organic Soy Flour | 158 | N | / | / | / | / |
| 5 | Clif Bar | 158 | N | / | / | / | / |
| 6 | Silk Next Milk | 158 | N | / | / | / | / |
| 8 | Positive Control | 158 | Y | 7.0 | 159.5 | 7 | 100 |
| 9 | Negative Control | n/a | N | / | / | / | / |

[a] Determined by estimating size based on the closest ladder

[b] Sample Calculation for molecular weight:

$y = m * x + b$ represents the best fitted line, Molecular Weight = $10^{(m * \text{distance migrated} + b)}$

For positive control, molecular weight = $10^{(-0.3438 * 7 + 4.6093)}$ = 159.5 bp

**Review**

*(KF) CaMV35S Simplex PCR product analysis*

Because of the sequence of the CaMV35S promoter reverse primer, it forms what is known as a primer dimer or a self dimer. The primer dimers showed up on the gel as a band that runs past the 100 bp ladder. Below is an example of how a self dimer forms between the promoter reverse primer [1].


In Figure 5.1, only two samples showed the presence of a PCR product for the CaMV35S promoter. The positive control showed a very bright and distinct band and the stroopwafel had a slightly less bright band that was slightly obscured by the primer dimer. Looking closely at the organic tofu and negative control lanes, the primer dimers are only present below the 100 bp marker on the ladder. For the stroopwafel, there is a faint band between the 100 and 200 bp marker. In this lane, there is also evidence of non-specific binding with the bright smear around the band. This is also present in the silk milk sample and the positive control. As stated before, the organic tofu and negative control show no sign of a PCR product.

In Figure 5.2, two regression lines were determined to be best fitting because there was a significant difference in slope between the upper and lower weights in the 100 bp ladder. Both ladders were graphed, and the closest ladder to the product was used to calculate the size. The stroopwafel product was calculated off of ladder 4 and the positive control off of ladder 8. Choosing a ladder in the same environment as the DNA is essential in calculating the correct mass as the high heat causes the gel to run unevenly. For all four ladder regressions, the $R^2$ values are greater than 0.99, confirming that two best fit lines was the correct decision.

Starting with the stroopwafel in Table 5.1, the expected size for the promoter PCR product was 158 bp and the calculated size was 157 bp. This is extremely close to the expected size, so it is highly unlikely that this is due to anything other than the promoter primer. The non-specific binding present in this sample could be due to a high concentration of DNA, too high of an annealing temperature, or too many PCR cycles. So, this does agree with the expected size.

For the positive control, the expected size of 158 bp is slightly higher than the calculated size of 145 bp from ladder 8. Because this particular sample was highly contaminated, that could have caused the DNA to move unpredictably through the gel. There was also non-specific binding present in this sample which could be due to any of the reasons stated above. Even though it was slightly lower than expected, it still does agree with the expected size.

*(BL) CaMV35S simplex PCR product analysis*

From Figure 5.3, we can see that only positive control sample on lane 8 shows a significant bright band in between 200 and 100 bp according to the ladder, indicating the presence of a PCR product for CaMV35S promoter. Organic soy flour on lane 3, Clif bar on lane 5, Silk Next Milk on lane 6, and negative control on lane 9 do not have indicative evidence that suggests the existence of PCR product for the promoter. All lanes show the presence of primer dimer because of the band shown at around 100 bp according o the ladder. There is also the presence of non-specific binding indicated by the smearing on each lane.

Both ladders from lane 4 and lane 7 from Figure 5.3 are graphed in Figure 5.4. Two regression lines were used to for each ladder to achieve best fitting result. For both parts on both ladders, the $R^2$ are either above 0.99 or close to 0.99, which indicates good fitting for the regression line. The ladder on lane 7 was used to calculate the size for the band for the positive control sample on lane 8 because it was the closest.

Using the regression lines from Figure 5.4 and the band travel distance from Figure 5.3, we can estimate the DNA sizes. From Table 5.2, it is calculated that the estimated DNA size for positive control PCR product band is around 159.5 bp, which is very close to the expected band size of 158 bp for CaMV35S promoter products. This is indicative evidence for the existence of GM soy in the positive control gDNA sample.

**References**

[1] Dr. Doonan, 03-343, Experimental Genetics, Pre-Lab Lecture, November 7, 2022.

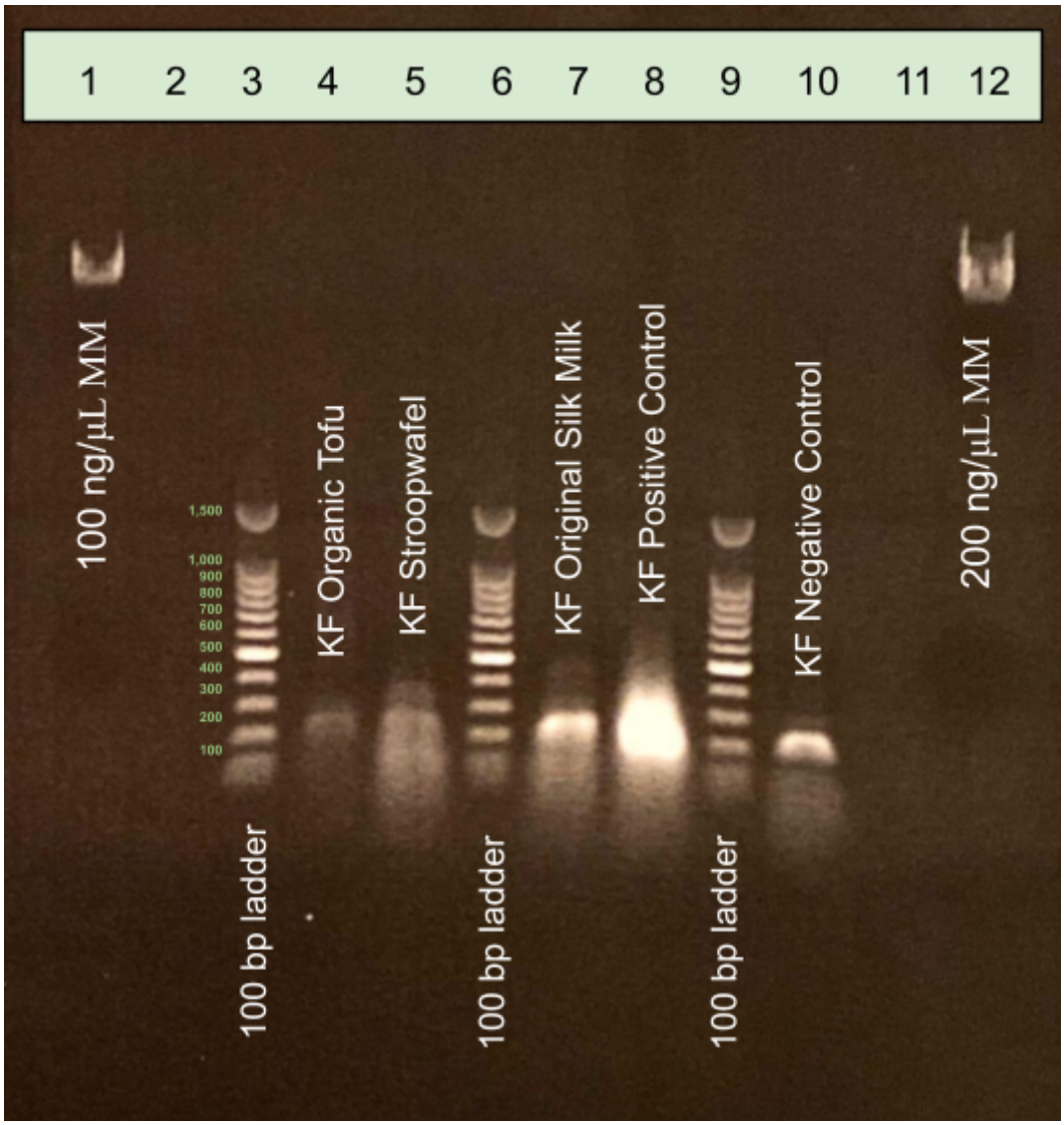
**Day 6: 12/2/22**

**Goal**

Analyze simplex PCR products for the soy lectin and CaMV35S/EPSPS junction genes as well as perform simplex PCR under the same conditions for the CaMV35S, soy lectin, and CaMV35S/EPSPS junction genes.

**Data**

**Figure 6.1** | Simplex PCR for the soy lectin primers on 1.5% agarose mini gel (KF)




[a] 8 µL of genomic DNA loaded into each well

**Figure 6.2** | Relationship of distance migrated vs. log molecular weight of 100 kb DNA ladder (KF)

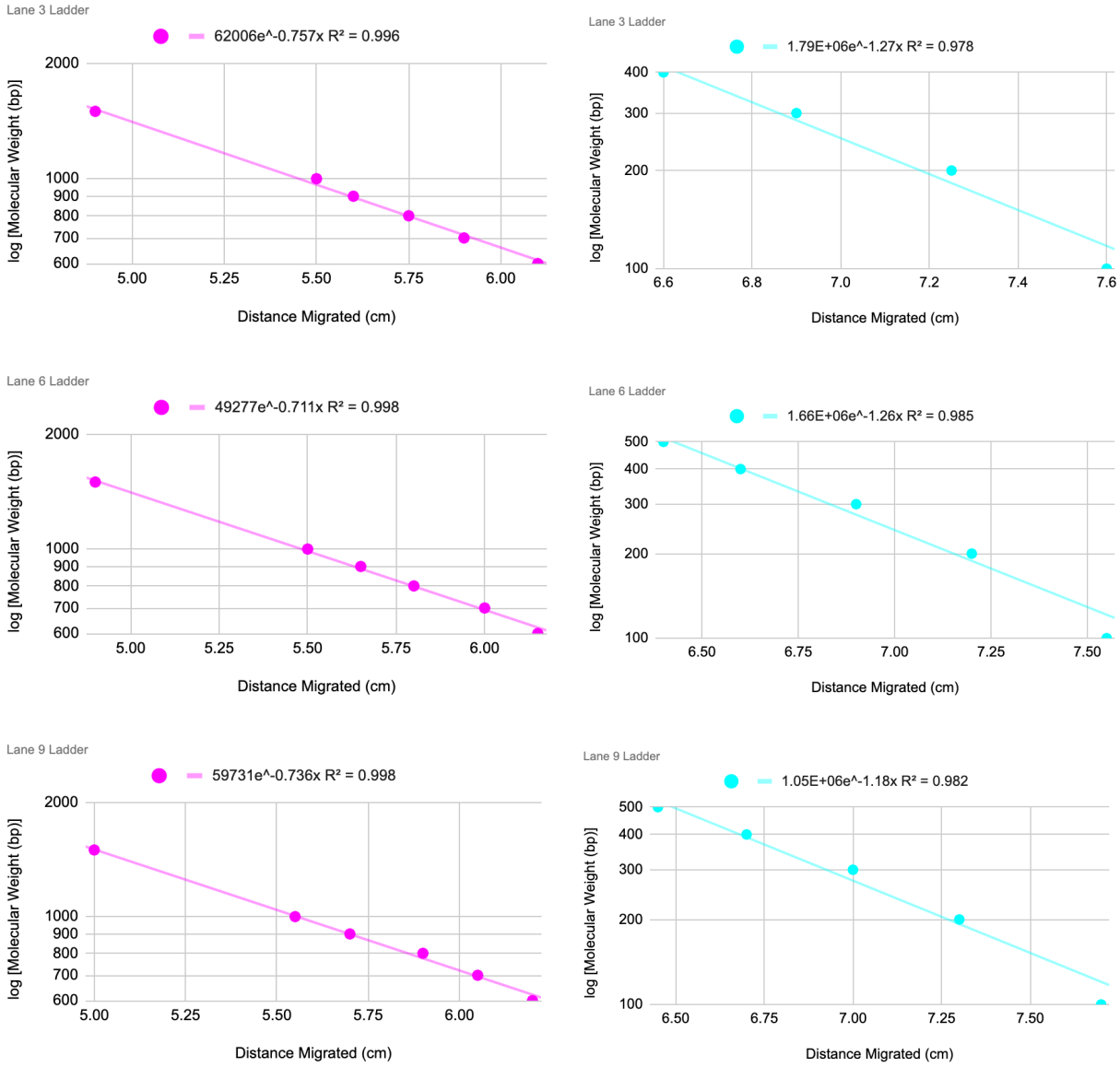


**Table 6.1** | Distance traveled and calculated DNA size for simplex PCR for the soy lectin products (KF)

| Lane | Genomic DNA Sample | Expected DNA sizes (bp) | Band Present (Yes/No) | Distance Traveled (cm) | Calculated DNA sizes (bp)[b] | Which Ladder Lane? | Estimated Concentration (ng/µL) |
|---|---|---|---|---|---|---|---|
| 3 | Organic Tofu | 210 | Y | 7.05 | 231 | 3 | 25 |
| 5 | Stroopwafel | 210 | Y | 7.05 | 230 | 6 | 50 |
| 6 | Original Silk | 210 | Y | 7.1 | 216 | 6 | 200 |
| 7 | Positive Control | 210 | Y | 7.1 | 241 | 9 | 800 |
| 9 | Negative Control | n/a | Y | 7.2 | 214 | 9 | 100 |

[a] Determined by estimating size based on the closes ladder

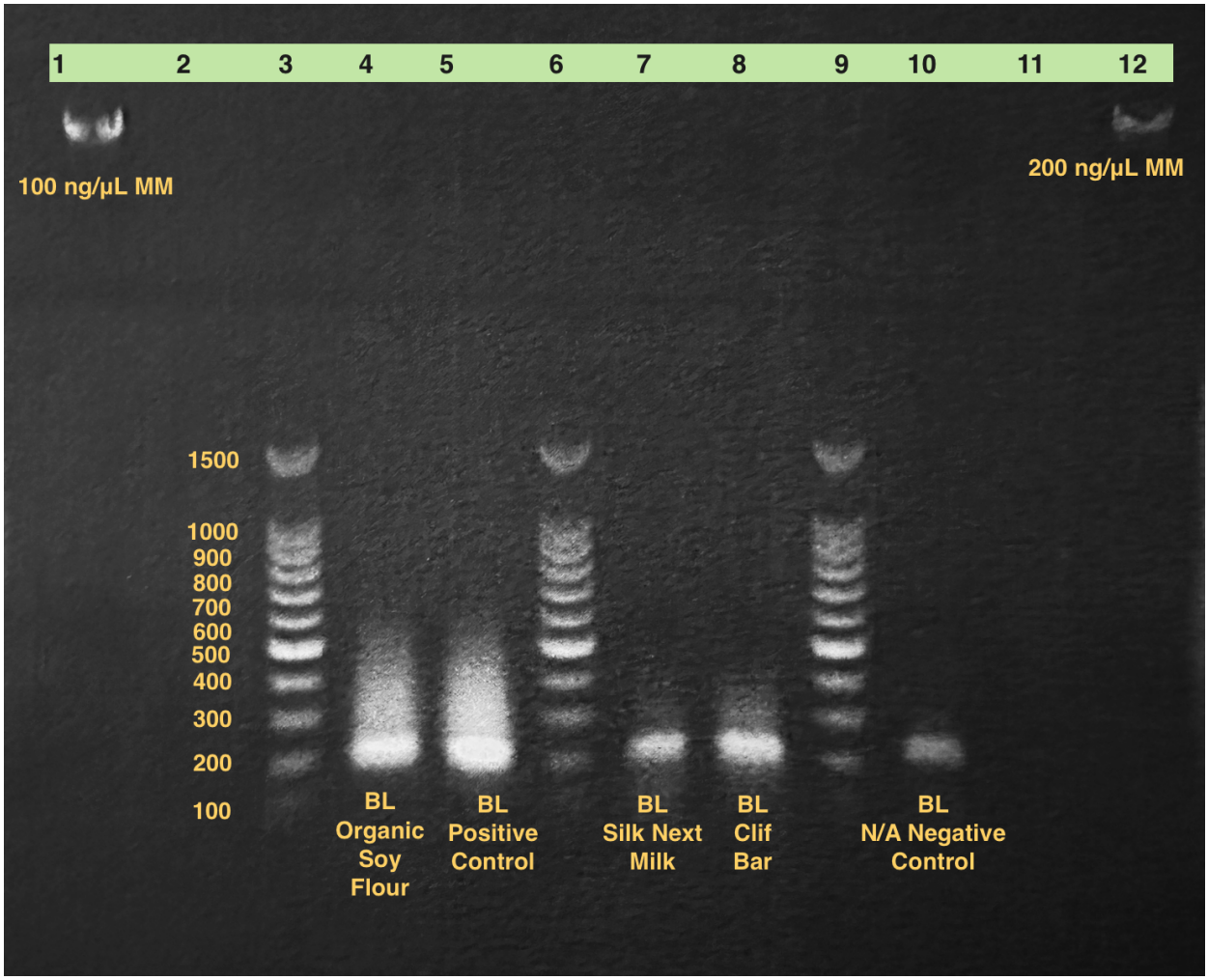
[b] Sample Calculation:

$1.05E06e^{-1.18(\text{Distance Traveled})} = \text{Molecular Weight}$

For the positive control,

$1.05E06e^{-1.18(7.1)} = 241$ bp

**Figure 6.3** | Simplex PCR (59 °C annealing temp, 40 cycles) for the soy lectin products on 1.5% agarose mini gel (BL)




[a] 8 µL of genomic DNA loaded into each well

**Figure 6.4** | Relationship of distance migrated vs. log molecular weight of 100 kb DNA ladder for ladders on lane 3, 6, 9 for Figure 6.3

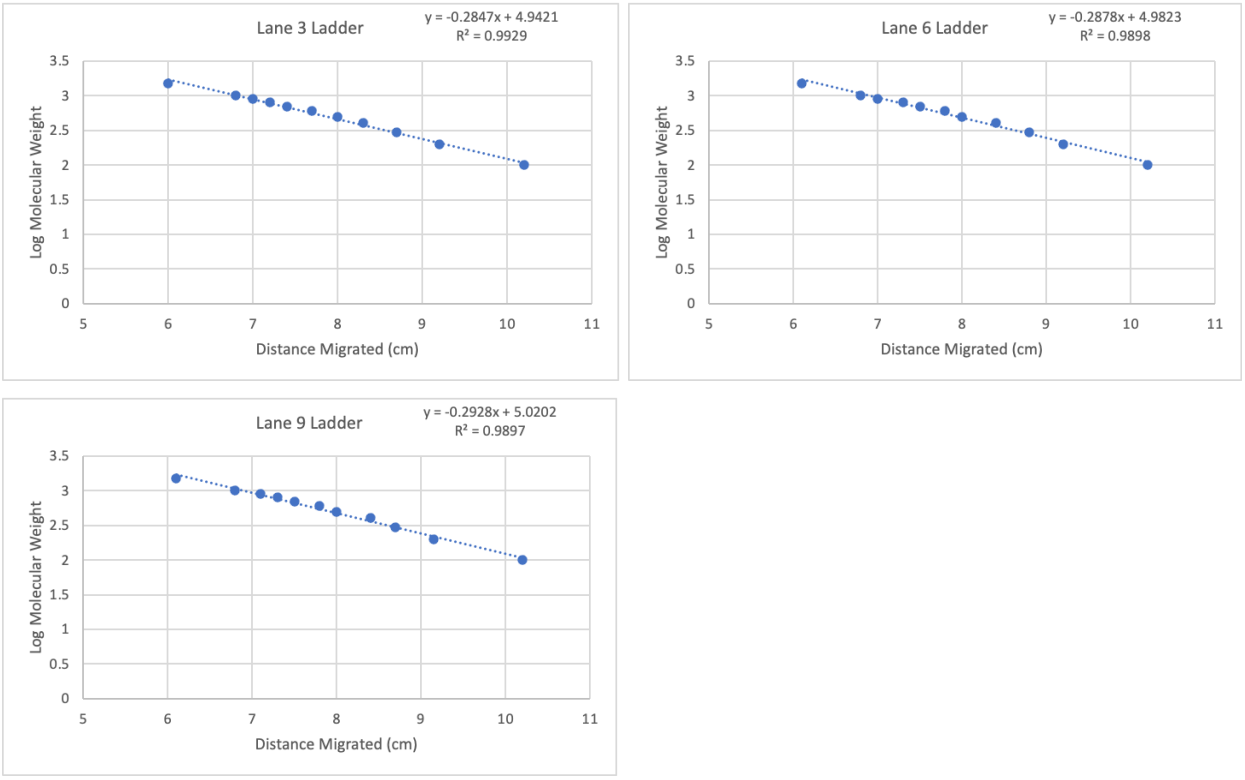



**Table 6.2** | Distance taveled and calculated DNA size for simplex PCR (59 °C annealing temp, 40 cycles) for the soy lectin primer products

| Lane | Genomic DNA Sample | Expected DNA sizes (kb) | Band Present (Yes/No) | Distance Traveled (cm) | Calculated DNA sizes (bp)[b] | Which Ladder Lane? | Estimated Concentration (ng/µL) |
|---|---|---|---|---|---|---|---|
| 4 | Organic Soy Flour | 210 | Y | 9.1 | 224.5 | 3 | 400 |
| 5 | Positive Control | 210 | Y | 9.1 | 230.8 | 6 | 400 |
| 7 | Silk Next Milk | 210 | Y | 9.1 | 230.8 | 6 | 100 |
| 8 | Clif Bar | 210 | Y | 9.1 | 226.8 | 9 | 200 |
| 10 | Negative Control | n/a | Y | 9.1 | 226.8 | 9 | 50 |

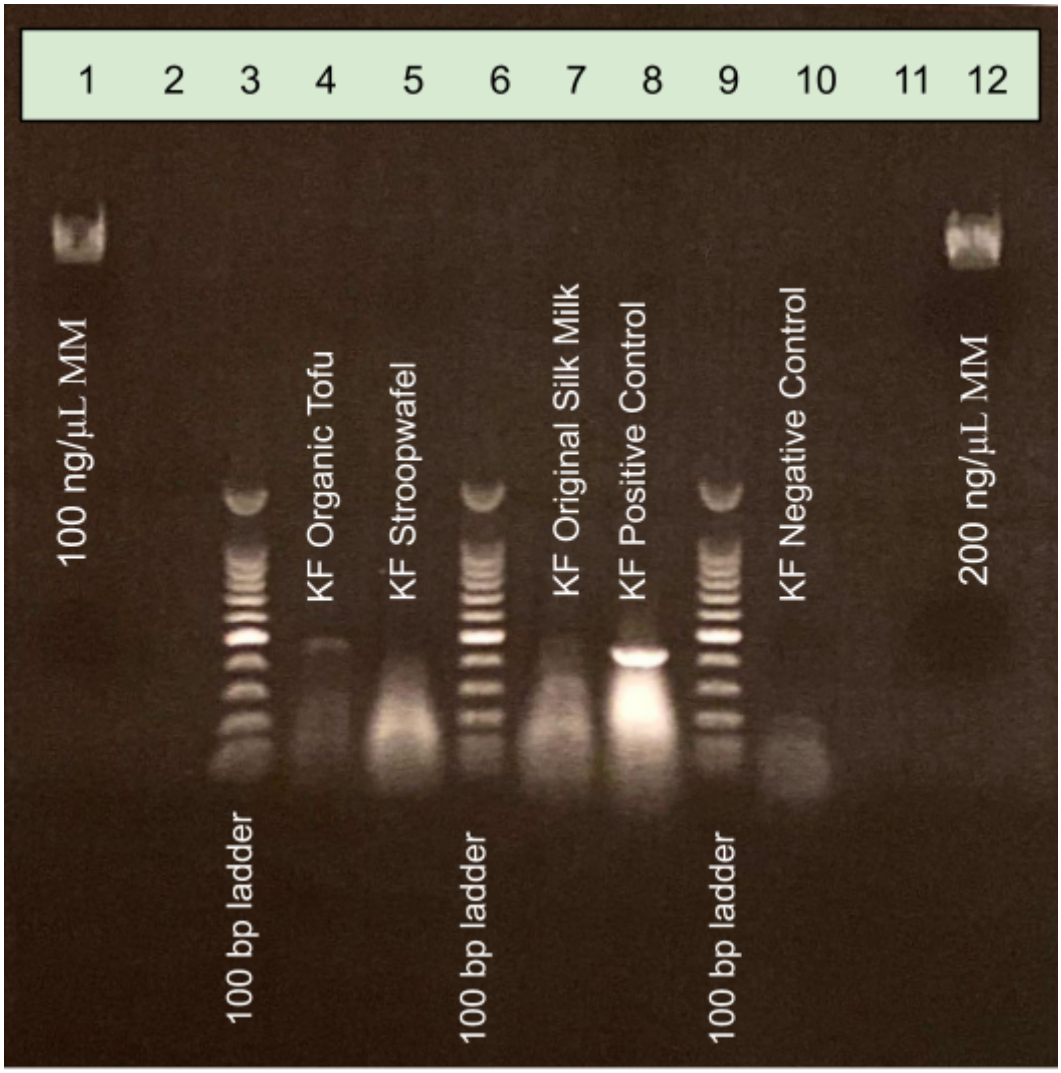
[a] Determined by estimating size based on the closest ladder

[b] Sample Calculation for molecular weight:

y = m * x + b represents the best fitted line, Molecular Weight = $10^{(m * \text{distance migrated} + b)}$

For organic soy flour, molecular weight = $10^{(-0.2847 * 9.1 + 4.9421)}$ = 224.5 bp

**Figure 6.5** | Simplex PCR from the CaMV35S/EPSPS junction run on a 1.5% agarose mini gel (KF)




[a] 8 µL of genomic DNA loaded into each well

**Figure 6.6** | Relationship of distance migrated vs. log molecular weight of 100 kb DNA ladder (KF)

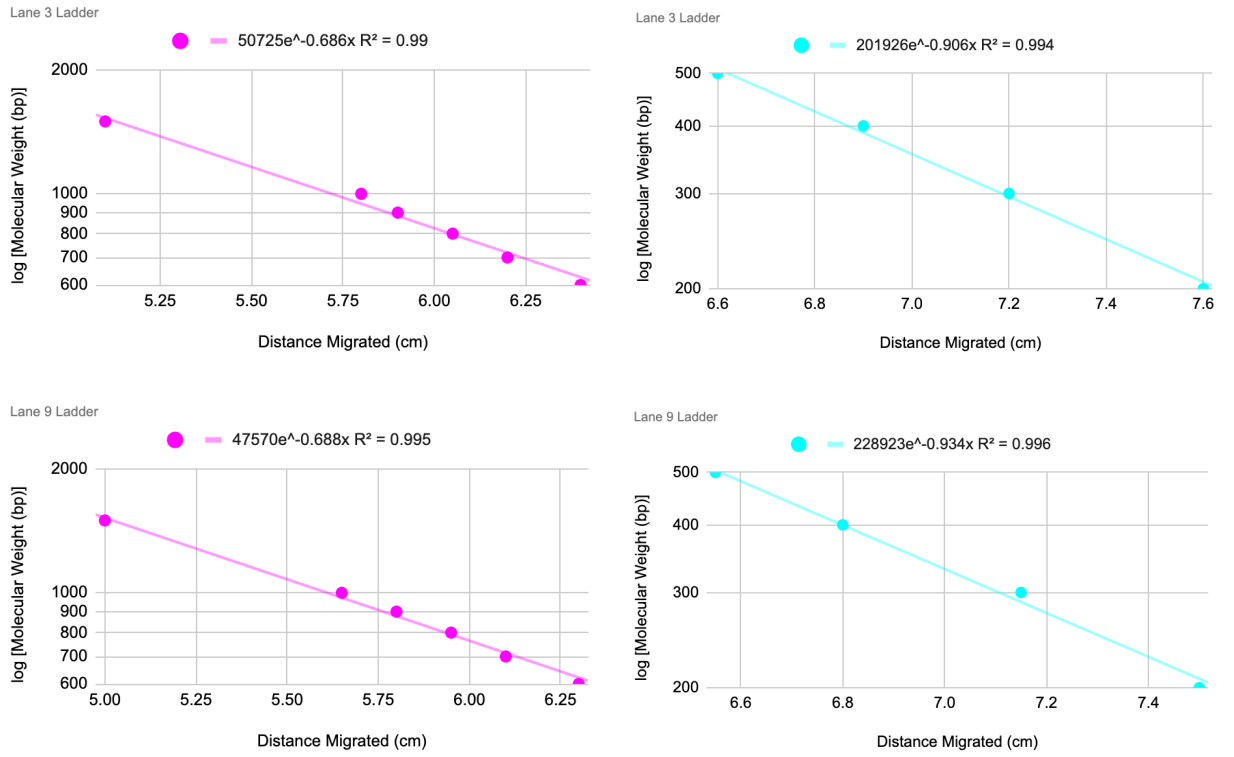



**Table 6.3** | Distance traveled and calculated DNA size for simplex PCR for the CaMV35S/EPSPS junction products (KF)

| Lane | Genomic DNA Sample | Expected DNA sizes (bp) | Band Present (Yes/No) | Distance Traveled (cm) | Calculated DNA sizes (bp)[b] | Which Ladder Lane? | Estimated Concentration (ng/µL) |
|---|---|---|---|---|---|---|---|
| 3 | Organic Tofu | 447 | Y | 6.65 | 488 | 3 | 25 |
| 5 | Stroopwafel | 447 | N | n/a | n/a | n/a | n/a |
| 6 | Original Silk | 447 | N | n/a | n/a | n/a | n/a |
| 7 | Positive Control | 447 | Y | 6.8 | 400 | 9 | 200 |
| 9 | Negative Control | n/a | N | n/a | n/a | n/a | n/a |

[a] Determined by estimating size based on the closes ladder

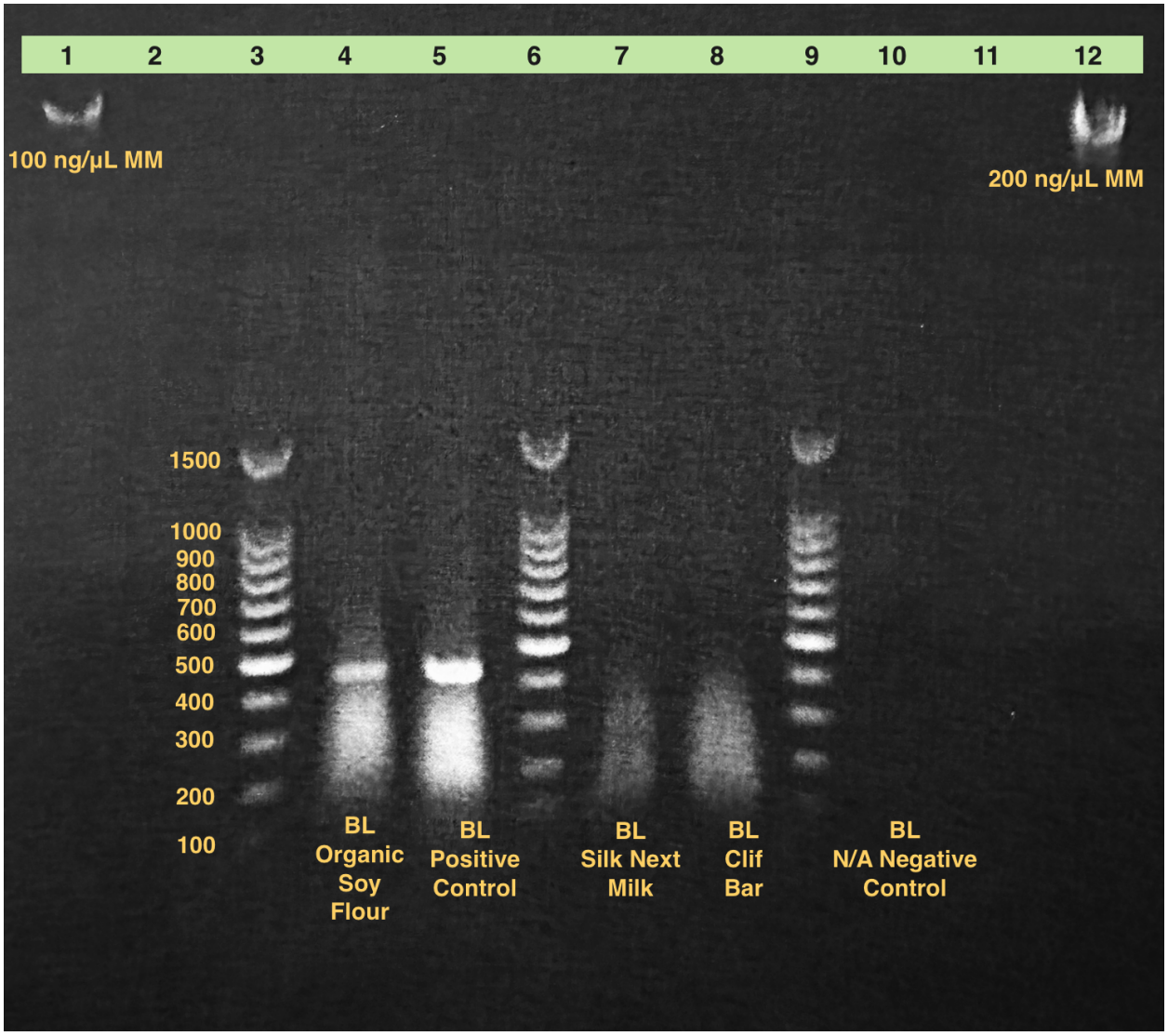
[b] Sample Calculation:

$228923e^{-0.934(\text{Distance Traveled})} = \text{Molecular Weight}$

For the positive control,

$228923e^{-0.934(6.8)} = 400$ bp

**Figure 6.7** | Simplex PCR (59 °C annealing temp, 40 cycles) for CaMV35S/EPSPS junction products on 1.5% agarose mini gel (BL)




[a] 8 µL of genomic DNA loaded into each well

**Figure 6.8** | Relationship of distance migrated vs. log molecular weight of 100 kb DNA ladder for ladders on lane 3, 6, 9 for Figure 6.7

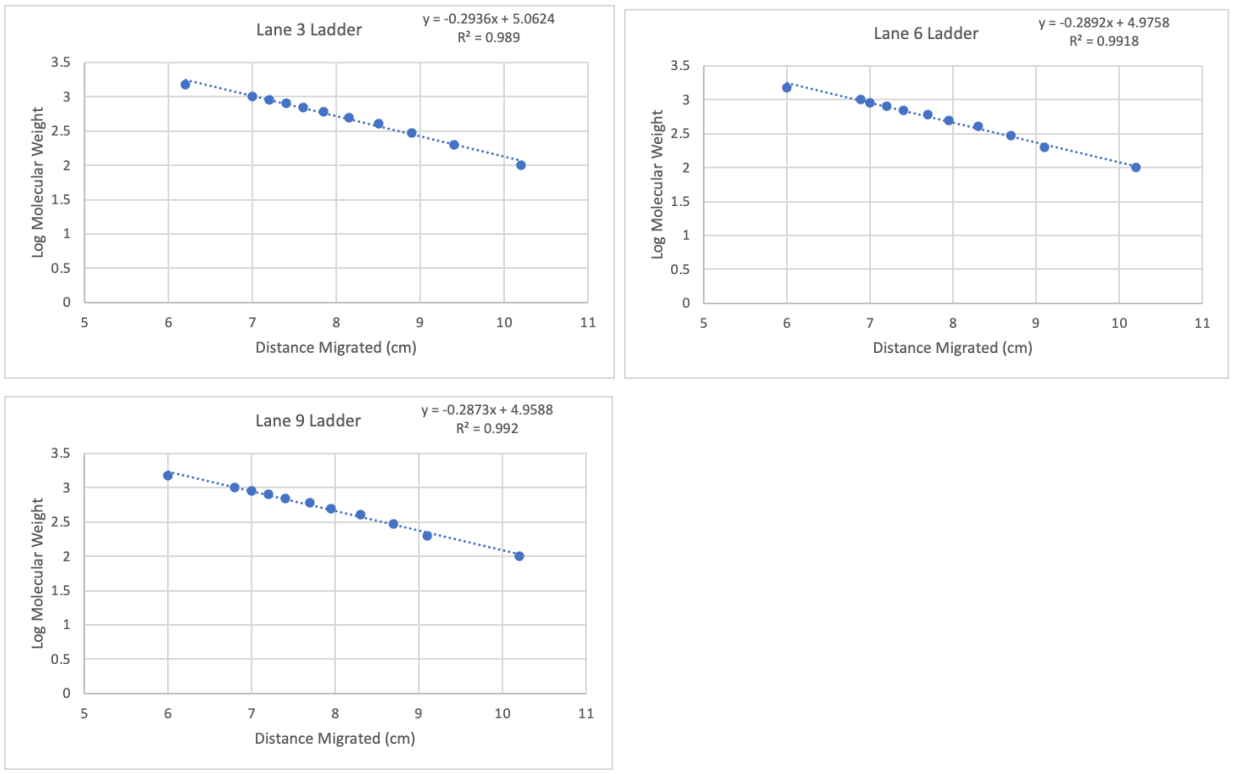



**Table 6.4** | Distance taveled and calculated DNA size for simplex PCR (59 °C annealing temp, 40 cycles) for the CaMV35S/EPSPS junction primer products

| Lane | Genomic DNA Sample | Expected DNA sizes (kb) | Band Present (Yes/No) | Distance Traveled (cm) | Calculated DNA sizes (bp)[b] | Which Ladder Lane? | Estimated Concentration (ng/µL) |
|---|---|---|---|---|---|---|---|
| 4 | Organic Soy Flour | 447 | Y | 8.2 | 451.7 | 3 | 100 |
| 5 | Positive Control | 447 | Y | 8.2 | 402.1 | 6 | 300 |
| 7 | Silk Next Milk | 447 | N | / | / | / | / |
| 8 | Clif Bar | 447 | N | / | / | / | / |
| 10 | Negative Control | n/a | N | / | / | / | / |

[a] Determined by estimating size based on the closest ladder

[b] Sample Calculation for molecular weight:

y = m * x + b represents the best fitted line, Molecular Weight = $10^{(m * \text{distance migrated} + b)}$

For organic soy flour, molecular weight = $10^{(-0.2936 * 8.2 + 5.0624)}$ = 451.7 bp

**Review**

*(KF) Soy Lectin Simplex PCR Product Analysis*

In Figure 6.1, there is evidence of the soy lectin simplex PCR product for all samples including the negative control. The soy lectin gene acts as an internal positive control because every product containing soy, genetically modified or not, should produce a product with this primer pair. The negative control was not expected to produce a PCR product as it does not contain any template DNA. This would mean that the tube was likely contaminated with the template DNA of one of the other samples. The expected band size for this primer pair is 210 bp.

In Figure 6.2, two regression lines were determined to be best fitting because there was a significant difference in slope between the upper and lower weights in the 100 bp ladder. Both ladders were graphed, and the closest ladder to the product was used to calculate the size. Choosing a ladder in the same environment as the DNA is essential in calculating the correct mass as the high heat causes the gel to run unevenly. For all four ladder regressions, the $R^2$ values are greater than 0.97, confirming that two best fit lines was the correct decision.

In Table 6.1, the organic tofu sample has a calculated size of 231 bp based on ladder 3. This is slightly higher than the expected size of 210 bp. This could be because the ladder is closer to the outside of the gel, meaning that it would experience greater heat than the sample. If the ladder runs faster than the sample, the sample will have a higher calculated size than expected. In terms of concentration, the band is not the brightest, however, there is no non-specific binding present.

The stroopwafel sample has a calculated size of 230 bp based on ladder 6. This is slightly higher than the expected size of 210 bp. The sample contains a significant amount of non-specific binding. Because of this, it is difficult to pinpoint exactly where the PCR product band is located. This makes it difficult to make an accurate measurement, causing the calculation size to be less than ideal. Despite the slight deviations, the band agrees with the expected size for the soy lectin primers.

The original Silk milk sample has a calculated size of 216 bp based on ladder 6. This is nearly the same as the expected size of 210 bp. It most definitely agrees with the expected size for the primer pair. Even though the concentration is optimal, there is a fair amount of non-specific binding present, so the parameters need to be adjusted accordingly.

The positive control sample has a calculated size of 241 bp based on ladder 9. This is higher than the expected size of 210 bp. The sample is at a very high concentration and contains a significant amount of non-specific binding. Because of this, it is difficult to pinpoint exactly where the PCR product band is located. This makes it difficult to make an accurate measurement, causing the

calculation size to be less than ideal. Despite the slight deviations, the band agrees with the expected size for the soy lectin primers.

The negative control sample has a calculated size of 214 bp based on ladder 9. Because this is the negative control and should not have produced a product, the size does not indicate anything significant.

*(KF) CaMV35S/EPSPS Simplex PCR Product Analysis*

In Figure 6.5, there is evidence of the CaMV35S/EPSPS junction simplex PCR in the organic tofu and positive control samples. The organic tofu is USDA certified organic, so we would not expect a band to be present, but we cannot write it off as contamination. Repeat simplex and multiplex PCR gels will confirm if there is truly GM soy present in this food sample. The expected band size for this primer pair is 447 bp.

In Figure 6.6, two regression lines were determined to be best fitting because there was a significant difference in slope between the upper and lower weights in the 100 bp ladder. Both ladders were graphed, and the closest ladder to the product was used to calculate the size. Choosing a ladder in the same environment as the DNA is essential in calculating the correct mass as the high heat causes the gel to run unevenly. For all four ladder regressions, the $R^2$ values are greater than or equal to 0.99, confirming that two best fit lines was the correct decision.

In Table 6.3, the organic tofu sample has a calculated size of 488 bp based on ladder 3. This is slightly higher than the expected size of 447 bp. This could be because the ladder is closer to the outside of the gel, meaning that it would experience greater heat than the sample. If the ladder runs faster than the sample, the sample will have a higher calculated size than expected. Even though the calculated band is not exact, it still agrees with the expected size. The concentration seems low for this sample, which will be adjusted in subsequent PCR reactions.

The positive control sample has a calculated size of 400 bp based on ladder 9. This is lower than the expected size of 447 bp. This could be due to the high protein contamination in this sample making the DNA move through the gel unpredictably. Despite the deviations, the band agrees with the expected size for the junction primers. The sample is at a very high concentration and contains a significant amount of non-specific binding.

*(BL) Soy Lectin Simplex PCR Product Analysis*

Soy lectin is an internal positive control and should be contained in all soy products. From Figure 6.3, it can be seen that all four samples with gDNA shows band for soy lectin PCR product, which is consistent with the expectation.

Regression lines were determined for each of the ladder on lane 3, 6, and 9 as shown in Figure 6.4. The $R^2$ values are above or very close to 0.99, so the regression lines fit the data well and can relatively estimate the band size accurately.

The expected band size is 210 kb. Positive control has an estimated band size of 230.8 bp based on ladder 6; organic soy flour has an estimated band size of 224.5 bp based on ladder 3; Silk Next Milk has an estimated band size of 230.8 bp based on ladder 6; Clif Bar has an estimated band size of 226.8 based on ladder 9. The sizes are within reasonable range with the expected size. All lanes show some amount of smearing around the band, which is a sign of non-specific binding. The negative control with no gDNA also shows a faint band, which is inconsistent with expectation. This indcicates that the primers for soy lectin were probably contaminated with gDNA from the beginning.

*(BL) CaMV35S/EPSPS Simplex PCR Product Analysis*

CaMV35S/EPSPS junction should appear in GM soy products. From Figure 6.7, it can be seen that positive control and organic soy flour show band for CaMV35S/EPSPS junction PCR product. This is inconsistent with the expectation because CaMV35S and EPSPS should only be detected in GM soy products. Organic soy flour should not contain any genetically modified soy and therefore should not show band for the junction gene.

Regression lines were determined for each of the ladder on lane 3, 6, and 9 as shown in Figure 6.4. The $R^2$ values are above or very close to 0.99, so the regression lines fit the data well and can relatively estimate the band size accurately.

The expected band size is 447 kb. Positive control has a calculated band size of 402.1 bp based on ladder 6, which is lower than the expected size but still within relatively normal range. Organic soy flour has a calculated band size of 402.1 bp based on ladder 3, which is very close to the expected size. All lanes show some amount of smearing around the band, which is a sign of non-specific binding. The negative control with no gDNA does not have any product, which is as expected and indicates that the primers are not contaminated.

**Table 6.5** | Simplex PCR conditions for the amplification of the CaMV35S, CaMV35S/EPSPS junction, and soy lectin genes

| PCR Step | | Temperature (℃) | Time |
|---|---|---|---|
| Initial Denaturation | | 95 | 5 min |
| 35x Cycles | Denaturation | 95 | 20 sec |
| | Annealing | 59 | 40 sec |
| | Extension | 72 | 1 min |
| Final Extension | | 72 | 10 min |
| Holding | | 4 | Overnight |

**Table 6.6** | Simplex PCR design for the amplification of the CaMV35S, CaMV35S/EPSPS junction, and soy lectin genes using the *Taq* PCR beads

| Sample (Initials) | gDNA | Forward Primer | Reverse Primer | $ddH_2O$ | Total Volume |
|---|---|---|---|---|---|
| Positive Control (KF) | 3 µL | 2.5 µL | 2.5 µL | 17 µL | 25 µL |
| Stroopwafel (KF) | 4 µL | 2.5 µL | 2.5 µL | 16 µL | 25 µL |
| Silk Milk (KF) | 4 µL | 2.5 µL | 2.5 µL | 16 µL | 25 µL |
| Organic Tofu (KF) | 5 µL | 2.5 µL | 2.5 µL | 15 µL | 25 µL |
| Negative Control (KF) | 0 µL | 2.5 µL | 2.5 µL | 20 µL | 25 µL |
| Positive Control (BL) | 3 µL | 2.5 µL | 2.5 µL | 17 µL | 25 µL |
| Organic Soy Flour (BL) | 3 µL | 2.5 µL | 2.5 µL | 17 µL | 25 µL |
| Silk Next Milk (BL) | 4 µL | 2.5 µL | 2.5 µL | 16 µL | 25 µL |
| Clif Bar (BL) | 4 µL | 2.5 µL | 2.5 µL | 16 µL | 25 µL |
| Negative Control (BL) | 0 µL | 2.5 µL | 2.5 µL | 20 µL | 25 µL |

**Review**

*Changes in PCR Conditions Rationale*

For the final round of simplex PCR, where all three sets of primers are individually tested with each of the samples, the cycle number of the PCR was reduced from 40 to 35 as seen in Table 6.5. This is due to the existence of heavy non-specific binding, which was seen by the smearing on the lanes in Figure 6.1, 6.3, 6.5, 6.7. Such heavy and bright smearing on the lane could obscure the existence of bands. Reducing cycles would decrease the amount of PCR products, which in turn would decrease the amount of non-specific binding.

*Changes in PCR Design Rationale*

In Table 6.6, it can be seen that the volume of gDNA added for each sample is reduced accordingly to reduce the amount of non-specific binding smear so that the product band can be seen more clearly.

From Figure 6.3 (BL), organic soy flour on lane 4 and positive control on lane 5 show significant non-specific binding smear, so the volumes were both reduced from 5 µL to 3 µL. Silk Next milk on lane 7 and Clif bar on lane 8 show some degree of non-specific binding smear, so the volumes were reduced form 5 µL to 4 µL.

From Figure 6.1 (KF), positive control on lane 8 show significant non-specific binding smear, so the volume was reduced from 5 µL to 3 µL. Organic silk milk on lane 7 and stroopwafel on lane 5 show some degree of non-specific binding smear, so the volumes were reduced from 5 µL to 4 µL. Organic tofu on lane 4 did not show much non-specific binding, so the 5 µL was kept.

**Day 7: 12/5/22**

**Goal**

Analyze simplex PCR results for the CaMV35S, soy lectin, and CaMV35S/EPSPS junction genes and perform multiplex PCR under identical conditions.

**Data**

**Figure 7.1** | 200 mL gel electrophoresis apparatus

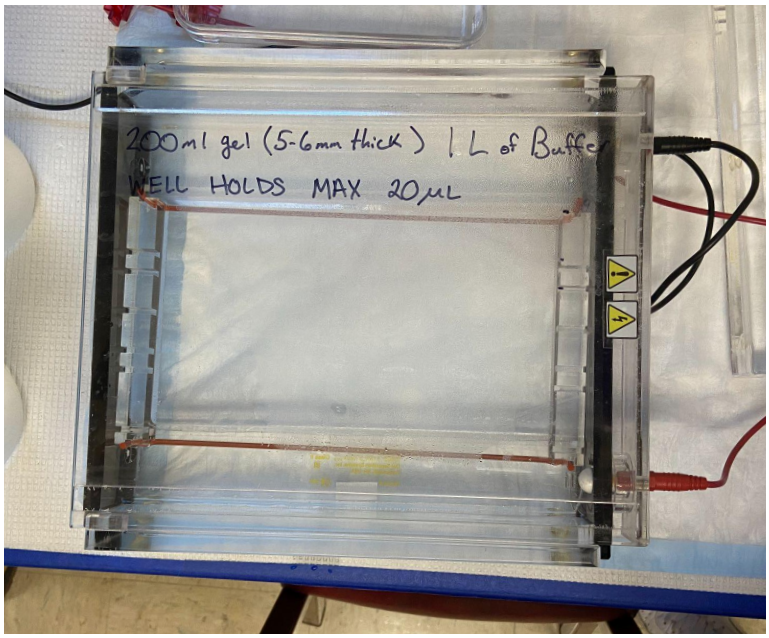



**Figure 7.2** | Simplex PCR (35 cycles, 59 ℃) samples from CaMV35s, CaMV35s/EPSPS, and Soy Lectin primers run on a 1.5% agarose 200 mL gel

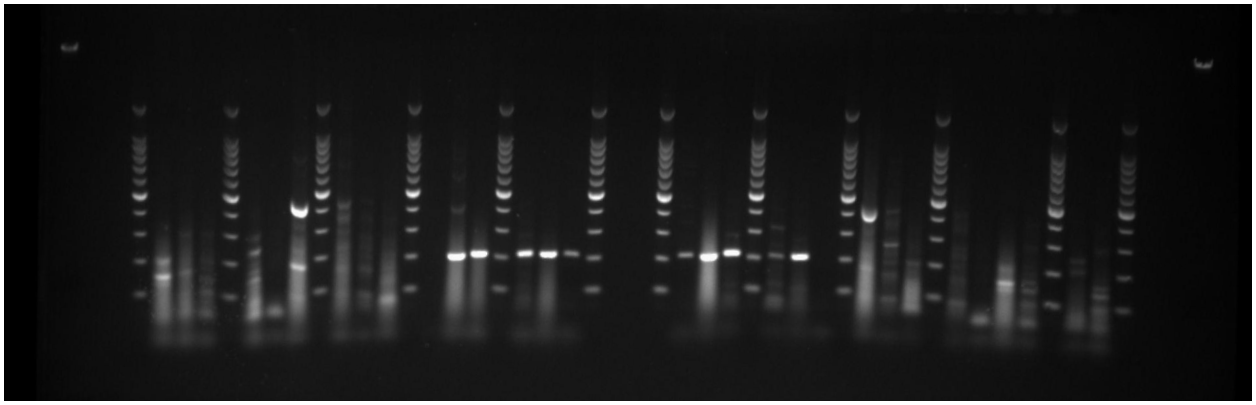
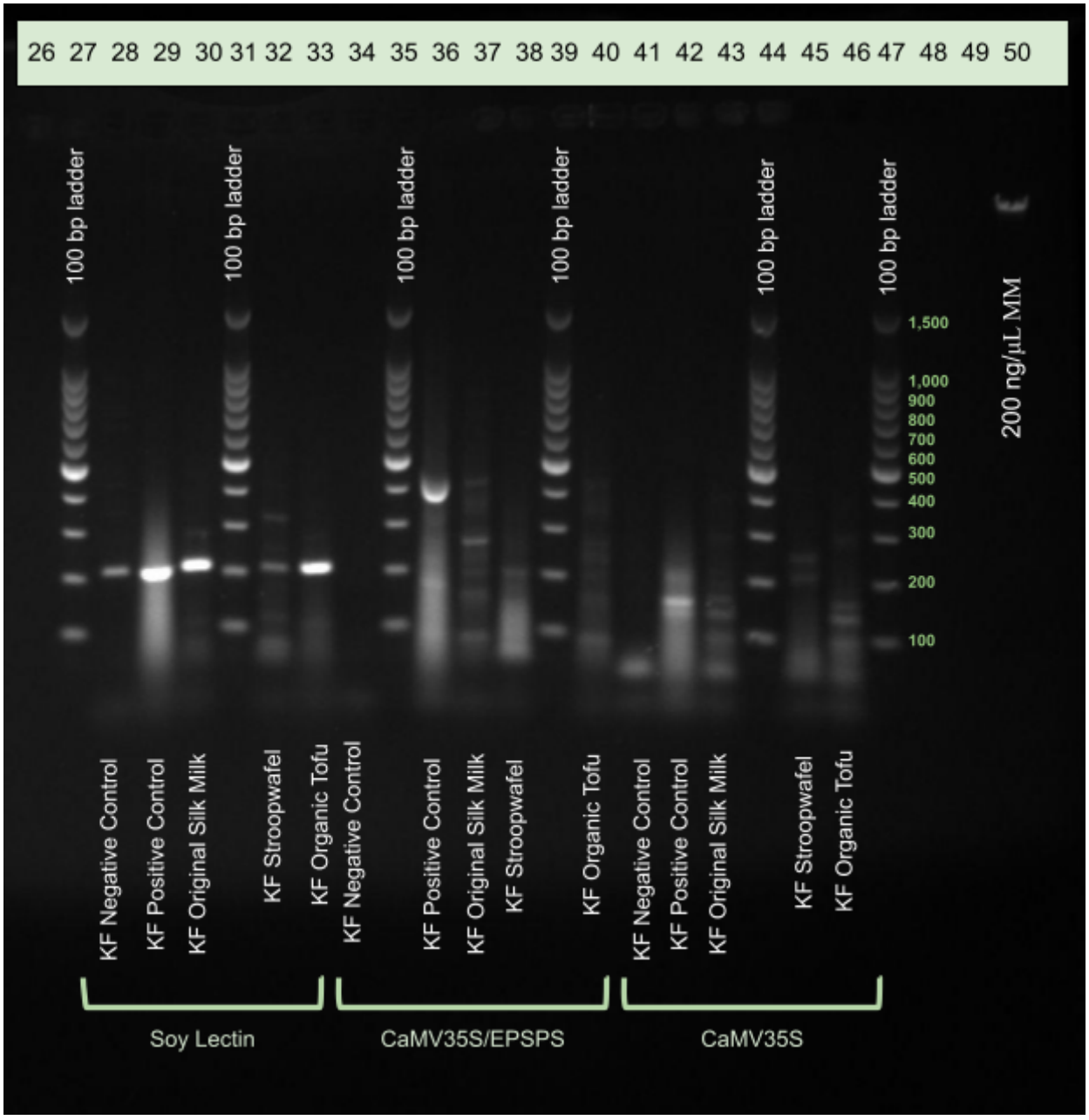


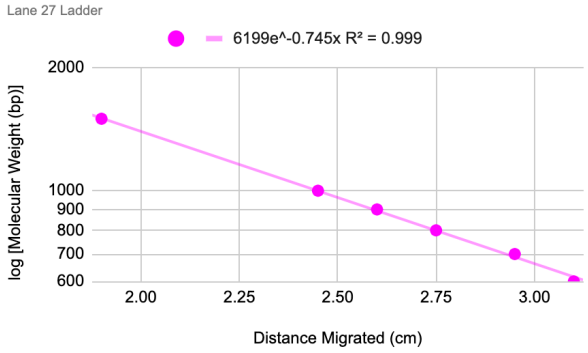
**Figure 7.3** | Right half of Figure 7.2 gel enlarged and labeled (KF)

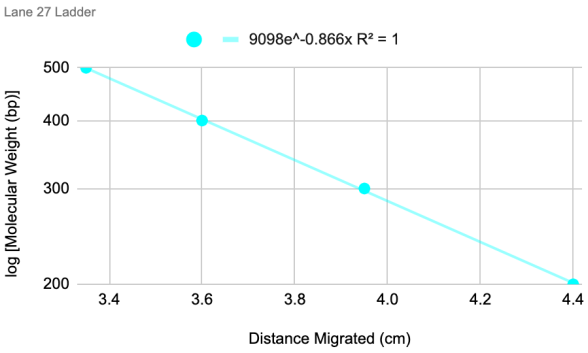


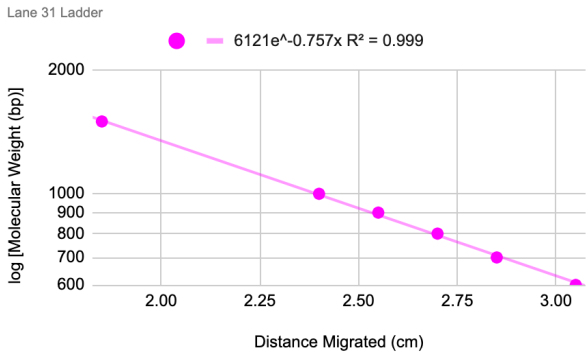

[a] 8 µL of genomic DNA loaded into each well

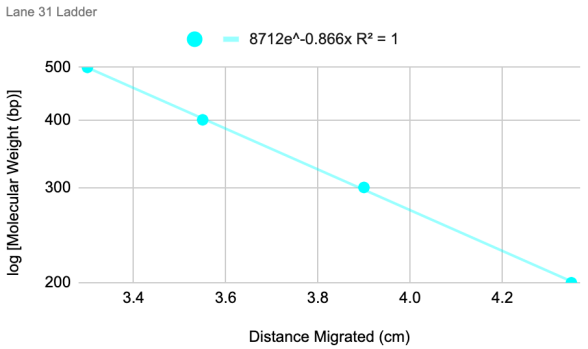
[b] The bands throughout at unexpected sizes are caused by primers binding to other sequences as well as potentially contamination from other primers when the band sizes match another gene of interest

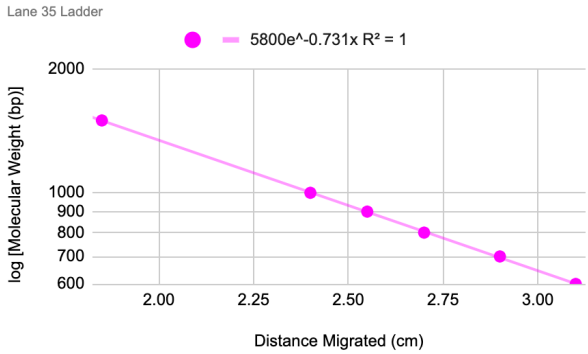
**Figure 7.4** | Relationship of distance migrated vs. log molecular weight of 100 kb DNA ladder (KF)

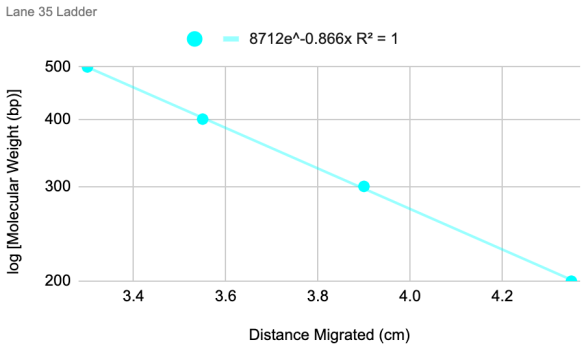


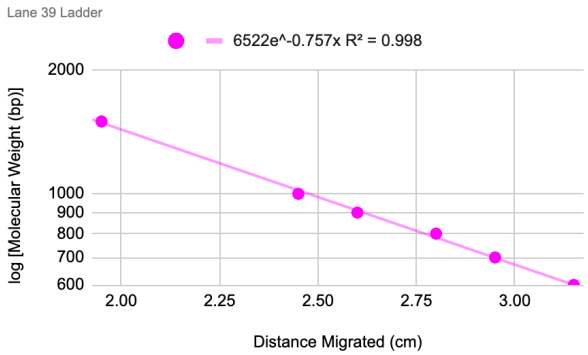



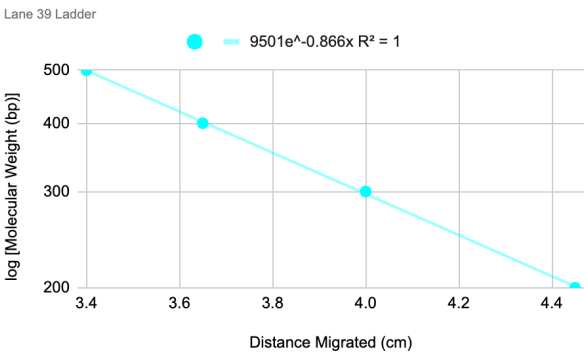



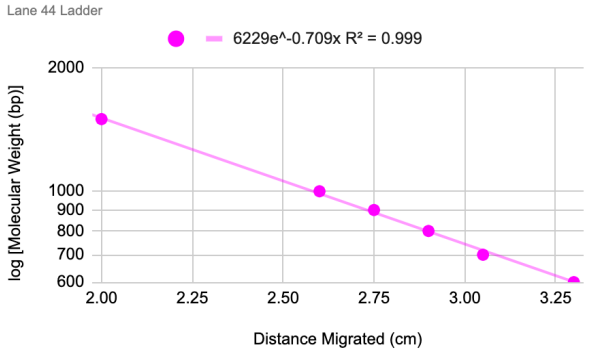



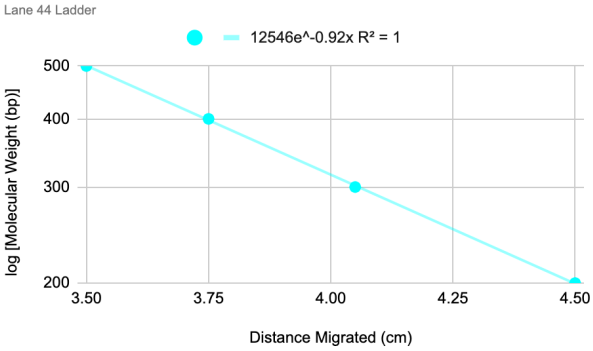



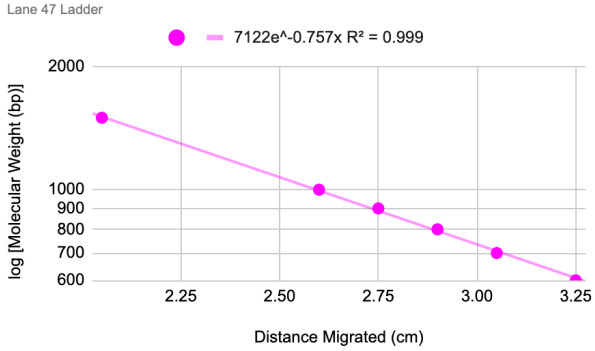




Lane 44 Ladder
6229e^-0.709x R² = 0.999
log [Molecular Weight (bp)]
2000
1000
900
800
700
600
2.00
2.25
2.50
2.75
3.00
3.25
Distance Migrated (cm)


Lane 44 Ladder
12546e^-0.92x R² = 1
log [Molecular Weight (bp)]
500
400
300
200
3.50
3.75
4.00
4.25
4.50
Distance Migrated (cm)


Lane 47 Ladder
7122e^-0.757x R² = 0.999
log [Molecular Weight (bp)]
2000
1000
900
800
700
600
2.25
2.50
2.75
3.00
3.25
Distance Migrated (cm)

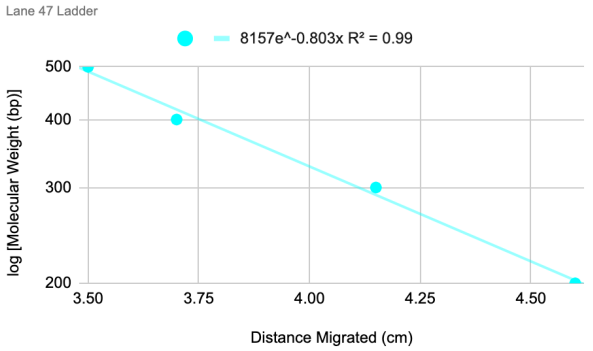

Lane 47 Ladder
8157e^-0.803x R² = 0.99
log [Molecular Weight (bp)]
500
400
300
200
3.50
3.75
4.00
4.25
4.50
Distance Migrated (cm)

**Table 7.1** | Distance traveled and calculated DNA size for simplex PCR for the soy lectin, CaMV53S/EPSPS, and CaMV53S products (KF)

| Lane | Genomic DNA Sample | Primers Used | Expected DNA sizes (bp) | Band Present (Yes/No) | Distance Traveled (cm) | Calculated DNA sizes (bp)[b] | Which Ladder Lane? | Estimated Concentration (ng/µL) |
|---|---|---|---|---|---|---|---|---|
| 28 | Negative Control | Soy Lectin | n/a | Y | 4.3 | 219 | 27 | 200 |
| 29 | Positive Control | Soy Lectin | 210 | Y | 4.35 | 210 | 27 | 400 |
| 30 | Silk Milk | Soy Lectin | 210 | Y | 4.25 | 220 | 31 | 300 |
| 32 | Stroopwafel | Soy Lectin | 210 | Y | 4.3 | 210 | 31 | 100 |
| 33 | Organic Tofu | Soy Lectin | 210 | Y | 4.3 | 210 | 31 | 300 |
| 34 | Negative Control | CaMV53S/ EPSPS | n/a | N | n/a | n/a | n/a | n/a |
| 36 | Positive Control | CaMV53S/ EPSPS | 447 | Y | 3.6 | 386 | 35 | 400 |
| 37 | Silk Milk | CaMV53S/ EPSPS | 447 | Y | 3.5 | 459 | 39 | 100 |
| 38 | Stroopwafel | CaMV53S/ EPSPS | 447 | N | n/a | n/a | n/a | n/a |
| 40 | Organic Tofu | CaMV53S/ EPSPS | 447 | Y | 3.5 | 459 | 39 | 50 |
| 41 | Negative Control | CaMV53S | n/a | N | n/a | n/a | n/a | n/a |
| 42 | Positive Control | CaMV53S | 158 | Y | 4.7 | 162 | 44 | 300 |
| 43 | Silk Milk | CaMV53S | 158 | Y | 4.7 | 162 | 44 | 200 |
| 45 | Stroopwafel | CaMV53S | 158 | N | n/a | n/a | n/a | n/a |
| 46 | Organic Tofu | CaMV53S | 158 | Y | 4.75 | 159 | 47 | 200 |

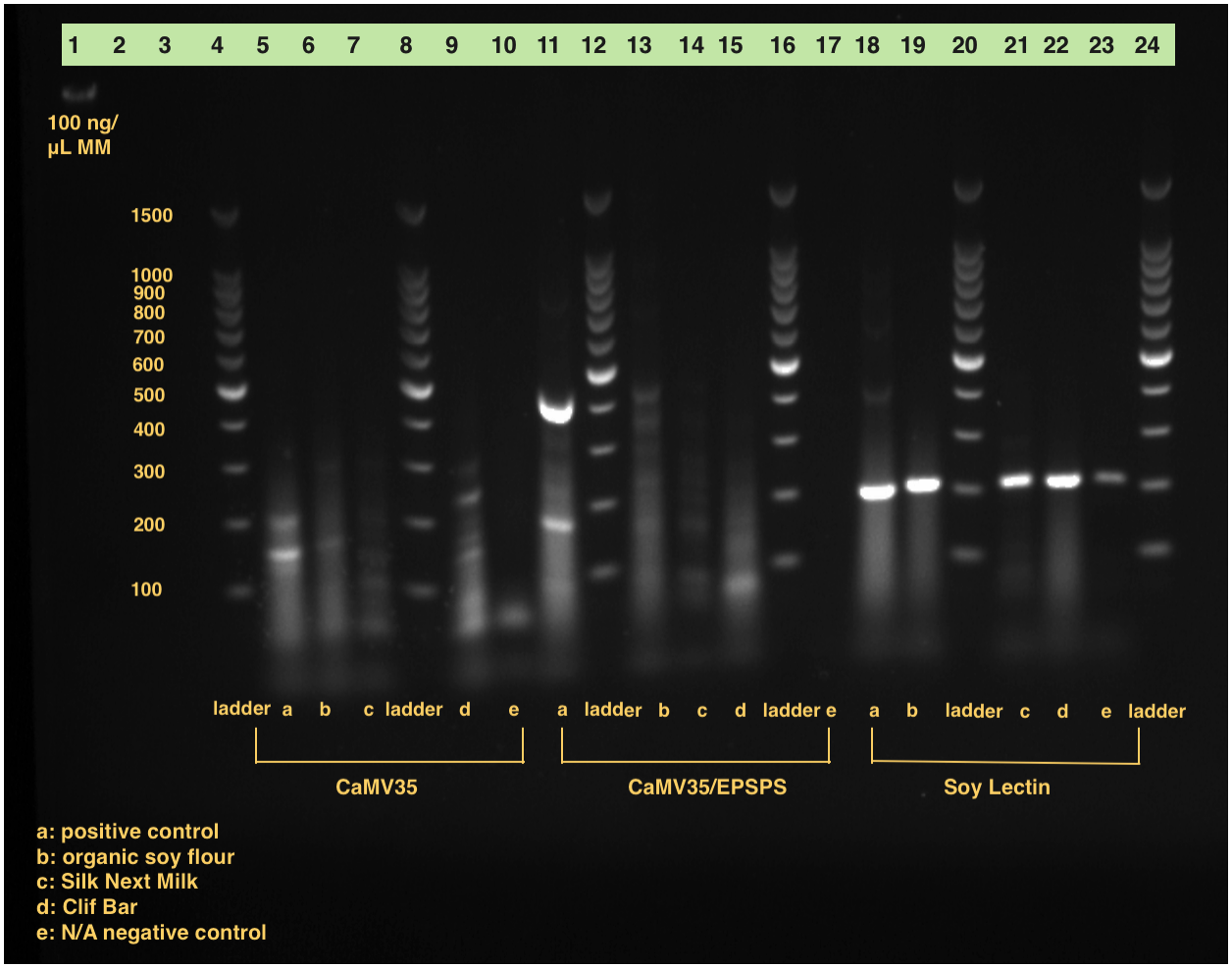
[a] Determined by estimating size based on the closes ladder

[b] Sample Calculation:

$8157e^{-0.803(\text{Distance Traveled})} = \text{Molecular Weight}$

For the organic tofu CaMV35S, $8157e^{-0.803(4.75)} = 159$ bp

**Figure 7.5** | Left half of Figure 7.2 gel enlarged and labeled (BL)




[a] 8 µL of genomic DNA loaded into each well

[b] The bands throughout at unexpected sizes are caused by primers binding to other sequences as well as potentially contamination from other primers when the band sizes match another gene of interest

**Figure 7.6** | Relationship of distance migrated vs. log molecular weight of 100 kb DNA ladder for ladders on lane 4, 8, 12, 16, 20, 24 for Figure 7.5

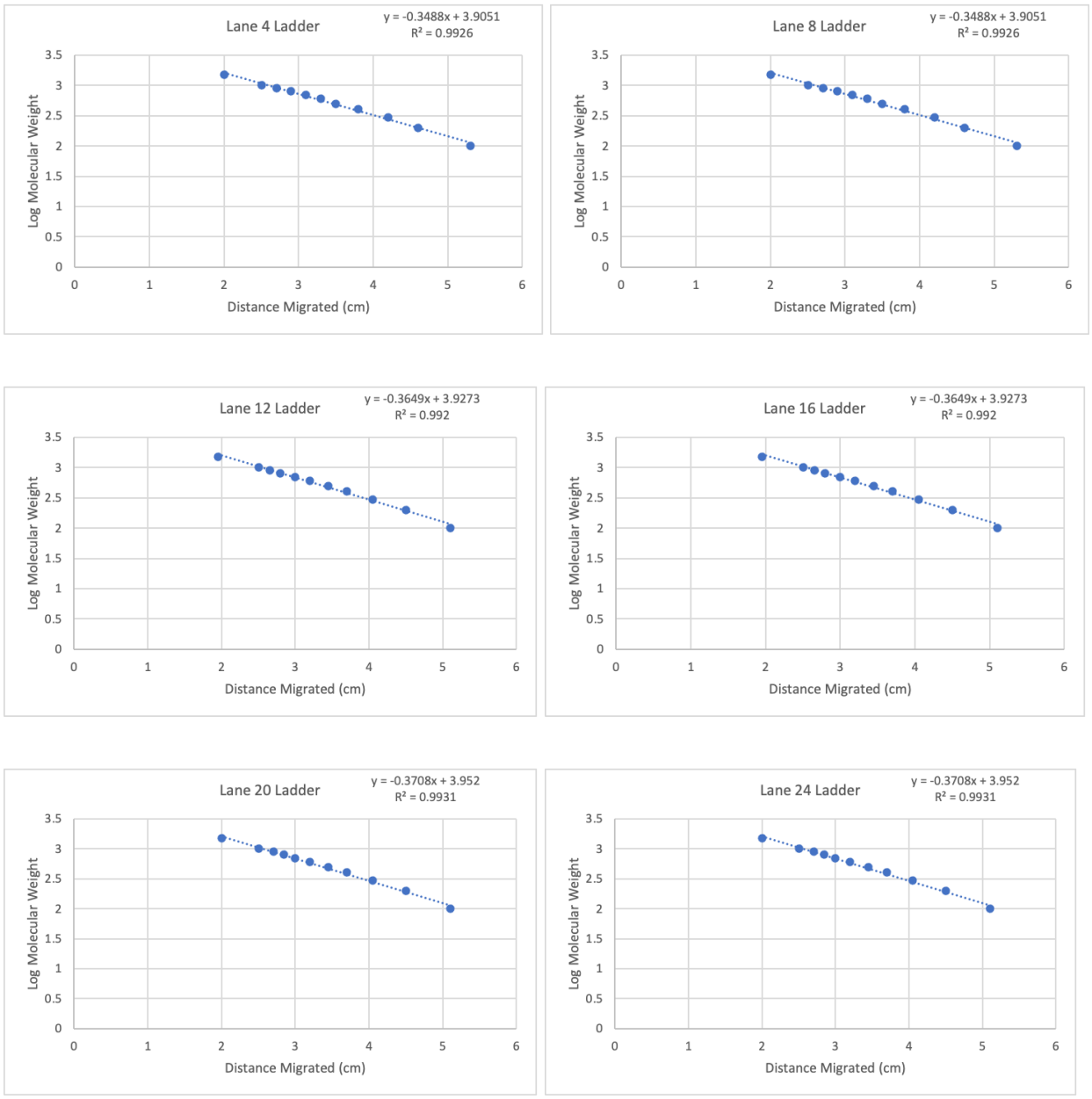


**Table 7.2** | Distance traveled and calculated DNA size for simplex PCR for the soy lectin, CaMV53S/EPSPS, and CaMV53S products (BL)

| Lane | Genomic DNA Sample | Primers Used | Expected DNA sizes (bp) | Band Present (Yes/No) | Distance Traveled (cm) | Calculated DNA sizes (bp)[b] | Which Ladder Lane? | Estimated Concentration (ng/µL) |
|---|---|---|---|---|---|---|---|---|
| 5 | Positive ctrl | CaMV53S | 158 | Y | 4.9 | 157 | 4 | 100 |
| 6 | Organic Soy Flour | CaMV53S | 158 | N | / | / | / | / |
| 7 | Silk Next Milk | CaMV53S | 158 | N | / | / | / | / |
| 9 | Clif Bar | CaMV53S | 158 | N | / | / | / | / |
| 10 | Negative ctrl | CaMV53S | / | N | / | / | / | / |
| 11 | Positive ctrl | CaMV53S/ EPSPS | 447 | Y | 3.7 | 377.7 | 12 | 400 |
| 13 | Organic Soy Flour | CaMV53S/ EPSPS | 447 | N | / | / | / | / |
| 14 | Silk Next Milk | CaMV53S/ EPSPS | 447 | N | / | / | / | / |
| 15 | Clif Bar | CaMV53S/ EPSPS | 447 | N | / | / | / | / |
| 17 | Negative ctrl | CaMV53S/ EPSPS | / | N | / | / | / | / |
| 18 | Positive ctrl | Soy Lectin | 210 | Y | 4.35 | 218.3 | 20 | 400 |
| 19 | Organic Soy Flour | Soy Lectin | 210 | Y | 4.4 | 209.1 | 20 | 400 |
| 21 | Silk Next Milk | Soy Lectin | 210 | Y | 4.4 | 209.1 | 20 | 200 |
| 22 | Clif Bar | Soy Lectin | 210 | Y | 4.4 | 209.1 | 20 | 400 |
| 23 | Negative ctrl | Soy Lectin | / | Y | 4.4 | 209.1 | 24 | 100 |

[a] Determined by estimating size based on the closest ladder

[b] Sample Calculation for molecular weight:

$y = m * x + b$ represents the best fitted line, Molecular Weight = $10^{(m * \text{distance migrated} + b)}$

For lane 5, molecular weight = $10^{(-0.3488 * 4.9 + 3.9051)} = 157$ bp

**Review**

*(KF) Soy Lectin Simplex PCR Results*

In Figure 7.3, there is evidence of the soy lectin simplex PCR product for all samples including the negative control. The soy lectin gene acts as an internal positive control because every product containing soy, genetically modified or not, should produce a product with this primer pair. The negative control was not expected to produce a PCR product as it does not contain any template DNA. This would mean that the tube was likely contaminated with the template DNA of one of the other samples. The expected band size for this primer pair is 210 bp.

In Table 7.1, the positive control, stroopwafel, and the organic tofu all have a calculated size of 210 bp, which is the same as the expected. The positive control and the tofu sample have an ideal concentration, with the stroopwafel being slightly lower than ideal. This could be due to the fact that the sample is much more processed, making the DNA extraction less pure and concentrated. The positive control sample did experience some non-specific binding, so the volume should be decreased for all subsequent PCR reactions.

The negative control and the original Silk milk samples have calculated sizes of 219 bp and 220 bp respectively. This still agrees with the expected size of 210 bp. Because the gel was so wide, the resolution of the ladders was not ideal and very minute measurement inaccuracies could cause the calculated sizes to change. So, with this considered, these calculations are close to ideal. The Silk milk sample is at an ideal concentration and there is not much evidence of non-specific binding. The negative control should not have a band present as this sample did not contain any template DNA. Because both sets of negative controls experienced this in the midi gel run on day 6 and day 7, the primers were likely contaminated with template DNA either before we started or right at the start of the experiment.

*(KF) CaMV35S/EPSPS Junction Simplex PCR Results*

In Figure 7.3, there is evidence of the CaMV35S/EPSPS junction simplex PCR in the organic tofu, Silk milk, and positive control samples. The expected band size for this primer pair is 447 bp.

In Table 7.1, both the Silk milk and organic tofu have calculated sizes of 459 bp, which is fairly close to the expected size of 447 bp. As mentioned above, the small ladder measurements made very small measurement discrepancies to greatly affect the calculated size of the band. Because of this, 459 bp is not a significant enough deviation to be concerned or determine that it does not agree with the expected. The Silk milk is not at ideal concentration for this primer and it seems like there is a fair amount of extraneous bands present in Figure 7.3. This could be due to the

template having multiple recognition sites for the primer. The organic tofu shows the same pattern as the Silk milk corroborating the fact that there are multiple recognition sites. It is at an even lower concentration than the Silk milk sample.

In Table 7.1, the positive control has a much lower calculated size at 386 bp than the expected at 447 bp. Because the positive control was so highly contaminated with proteins, this could cause unpredictable movement through the gel. While this is nowhere close to ideal and does not particularly agree with the expected result, there is nothing else that could cause a band at this location. So, we can still conclude that this was a positive result. The sample was also at a very high concentration. In Figure 7.3, there is a significant amount of non-specific binding associated with the sample.

The negative control was successful with no band present.

*(KF) CaMV35S Simplex PCR Results*

In Figure 7.3, three  samples showed the presence of a PCR product for the CaMV35S promoter. The positive control, the organic tofu, and the original Silk milk. The expected band size for the promoter primer pair is 158 bp.

In Table 7.1, both the positive control and Silk milk sample have a calculated size of 162 bp, which is very close to the actual size of 158 bp. This is not a big enough difference to cause concern with the integrity of the PCR reaction. These calculated sizes definitely agree with the expected size. The concentration of the positive control, once again, is too high and has caused non-specific binding to occur as seen in Figure 7.3. The Silk milk sample is at a lower concentration than ideal. Both samples experienced unexplained bands that are likely due to multiple recognition sites on the template.

The organic tofu sample has a calculated band size of 159 bp which is almost identical to the expected size of 158 bp. Because the sizes agree, this is a positive result. The organic tofu is at a low concentration for this sample and should be increased in subsequent PCR reactions to yield a clearer product.

*(KF) Final Conclusions*

The simplex PCR results from the two GM genes, the CaMV35S promoter and the CaMV35S/EPSPS junction, seem to agree on which food samples have been genetically modified. The positive control was to be expected as it was a certified genetically modified soy powder, however the organic tofu and the Silk milk samples were a surprise.

The organic tofu was a USDA certified organic product, which should guarantee that there are no genetically modified soybeans used to formulate the tofu. Because this sample consistently tested positive across all simplex PCR reactions, it is fairly certain that this sample does contain GM soy.

The original Silk milk is a GMO Project certified product, which is a third party not associated with the government. In this final simplex PCR, this sample tested positive for both of the GM genes. However, there was no evidence of this in previous simplex experiments. This could have two explanations. One would be that as the conditions were adjusted, the product was able to be seen more clearly and was not as obscured with primer dimers and nonspecific binding. An alternate explanation is that contamination of this sample occurred with either the positive control or the tofu sample between the primary and final simplex PCR reactions.

The stroopwafel never tested positive across any of the simplex PCR reactions. Because this sample started out with the lowest concentration, that could explain why there was not a distinguishable product produced. However, the sample was a product of Denmark, which generally does not use as many GM products as the United States, although it is not outlawed. So, this sample would have to be tested again at a higher concentration to obtain a clearer result.

*(BL) Soy Lectin Simplex PCR Results*

Soy lectin is an internal positive control and should be contained in all soy products. From Figure 7.5, it can be seen that all four samples with gDNA shows band for soy lectin PCR product, which is consistent with the expectation.

Regression lines were determined for each of the ladder on lane 20 and 24 as shown in Figure 7.6. The $R^2$ values are above or very close to 0.99, so the regression lines fit the data well and can relatively estimate the band size accurately.

The expected band size is 210 kb. Positive control has an estimated band size of 218.3 bp based on ladder 20; organic soy flour has an estimated band size of 209.1 bp based on ladder 20; Silk Next Milk has an estimated band size of 209.1 bp based on ladder 20; Clif Bar has an estimated band size of 209.1 bp based on ladder 20. The sizes are very close to the expected size. All lanes show some amount of smearing around the band, which is a sign of non-specific binding. The negative control with no gDNA also shows a faint band, which is inconsistent with expectation. This indicates that the primers for soy lectin were probably contaminated with gDNA from the beginning.

*(BL) CaMV35S/EPSPS Junction Simplex PCR Results*

CaMV35S/EPSPS junction should appear in GM soy products. From Figure 7.5, it can be seen that only positive control shows a band for CaMV35S/EPSPS junction PCR product. This is consistent with the expectation since positive control is guaranteed to be genetically modified and should have the CaMV35S/EPSPS junction gene.

Regression lines were determined for each of the ladders on lane 12 and 16 as shown in Figure 7.6. The $R^2$ values are above or very close to 0.99, so the regression lines fit the data well and can relatively estimate the band size accurately.

The expected band size is 447 kb. Positive control has a calculated band size of 377.7 bp based on ladder 12, which is lower than the expected size. All lanes show some amount of smearing around the band, which is a sign of non-specific binding. The negative control with no gDNA does not have any product, which is as expected and indicates that the primers are not contaminated.

*(BL) CaMV35S Simplex PCR Results*

CaMV35S promoter should appear in GM soy products. From Figure 7.5, it can be seen that only the positive control showed a band for the CaMV35S promoter PCR product. This is consistent with the expectation since positive control is guaranteed genetically modified and should have the CaMV35S gene.

Regression lines were determined for each of the ladders on lane 4 and 8 as shown in Figure 7.6. The $R^2$ values are above or very close to 0.99, so the regression lines fit the data well and can relatively estimate the band size accurately.

The expected band size is 158 kb. Positive control has a calculated band size of 157 bp based on ladder 4, which is very close to the expected size. All lanes show some amount of smearing around the band, which is a sign of non-specific binding. The negative control with no gDNA does not have any product, which is as expected and indicates that the primers are not contaminated. The negative control lane does show some primer dimer binding, which is normal for CaMV35S primers.

*(BL) Final Conclusions*

The final round of simplex PCR indicates that soy lectin gene shows up in all four products, which confirms that all products contain soy.

CaMV35S and CaMV35S/EPSPS junction gene only appear in positive control sample, which is as expected since the positive control is guaranteed to have genetically modified soy.

The fact that CaMV35S and CaMV35S/EPSPS junction gene do not show up in organic soy flour is as expected, since the product is USDA certified organic and should not contain any genetically modified soy.

CaMV35S and CaMV35S/EPSPS junction gene also do not show up in Silk Next Milk and Clif Bar, which is an indication that both products do not contain GM soy. This is consistent with their branding, since Silk Next Milk has Non-GMO Project Verified label on the package and Clif Bar has a Non GMO sign on their package. The final round of simplex PCR support their claim of lack of GM soy.

**Table 7.3** | Multiplex PCR conditions for the amplification of the CaMV35S, CaMV35S/EPSPS junction, and soy lectin genes

| **PCR Step** | | **Temperature (℃)** | **Time** |
|---|---|---|---|
| Initial Denaturation | | 95 | 5 min |
| 35x Cycles | Denaturation | 95 | 20 sec |
| | Annealing | 59 | 40 sec |
| | Extension | 72 | 1 min |
| Final Extension | | 72 | 10 min |
| Holding | | 4 | Overnight |

**Table 7.3** | Multiplex PCR design for the amplification of the CaMV35S, CaMV35S/EPSPS junction, and soy lectin genes using the *Taq* PCR beads

| **Sample (Initials)** | **gDNA** | **Forward Primer**[a] | **Reverse Primer**[a] | **dd$H_2O$** | **Total Volume** |
|---|---|---|---|---|---|
| Positive Control (KF) | 2 µL | 7.5 µL | 7.5 µL | 8 µL | 25 µL |
| Stroopwafel (KF) | 5 µL | 7.5 µL | 7.5 µL | 5 µL | 25 µL |
| Silk Milk (KF) | 4 µL | 7.5 µL | 7.5 µL | 6 µL | 25 µL |
| Organic Tofu (KF) | 5 µL | 7.5 µL | 7.5 µL | 5 µL | 25 µL |
| Negative Control (KF) | 0 µL | 7.5 µL | 7.5 µL | 10 µL | 25 µL |
| Positive Control (BL) | 2 µL | 7.5 µL | 7.5 µL | 8 µL | 25 µL |
| Clif Bar (BL) | 4 µL | 7.5 µL | 7.5 µL | 4 µL | 25 µL |
| Silk Next Milk (BL) | 4 µL | 7.5 µL | 7.5 µL | 4 µL | 25 µL |
| Organic Soy Flour (BL) | 3 µL | 7.5 µL | 7.5 µL | 7 µL | 25 µL |
| N/A Ctrl (BL) | 0 µL | 7.5 µL | 7.5 µL | 10 µL | 25 µL |

[a] 2.5 µL of each primer added

*PCR Conditions for Multiplex*

The only conditions altered for multiplex were that the volume of template DNA was changed to improve the quality of the products. None of the PCR cycler conditions were altered.

In Table 7.3, it can be seen that the volume of gDNA added for some sample is further reduced or increased from Table 6.6 accordingly to reduce the amount of non-specific binding smear or to increase the brightness of the band.

**Day 8: 12/9/22**

**Goal**

Analyze multiplex PCR results for the CaMV35S, soy lectin, and CaMV35S/EPSPS junction genes.

**Data**

**Figure 8.1** | Multiplex PCR (59 °C annealing temp, 35 cycles) for CaMV35S, soy lectin, and CaMV35S/EPSPS junction products on 1.5% agarose mini gel (KF)

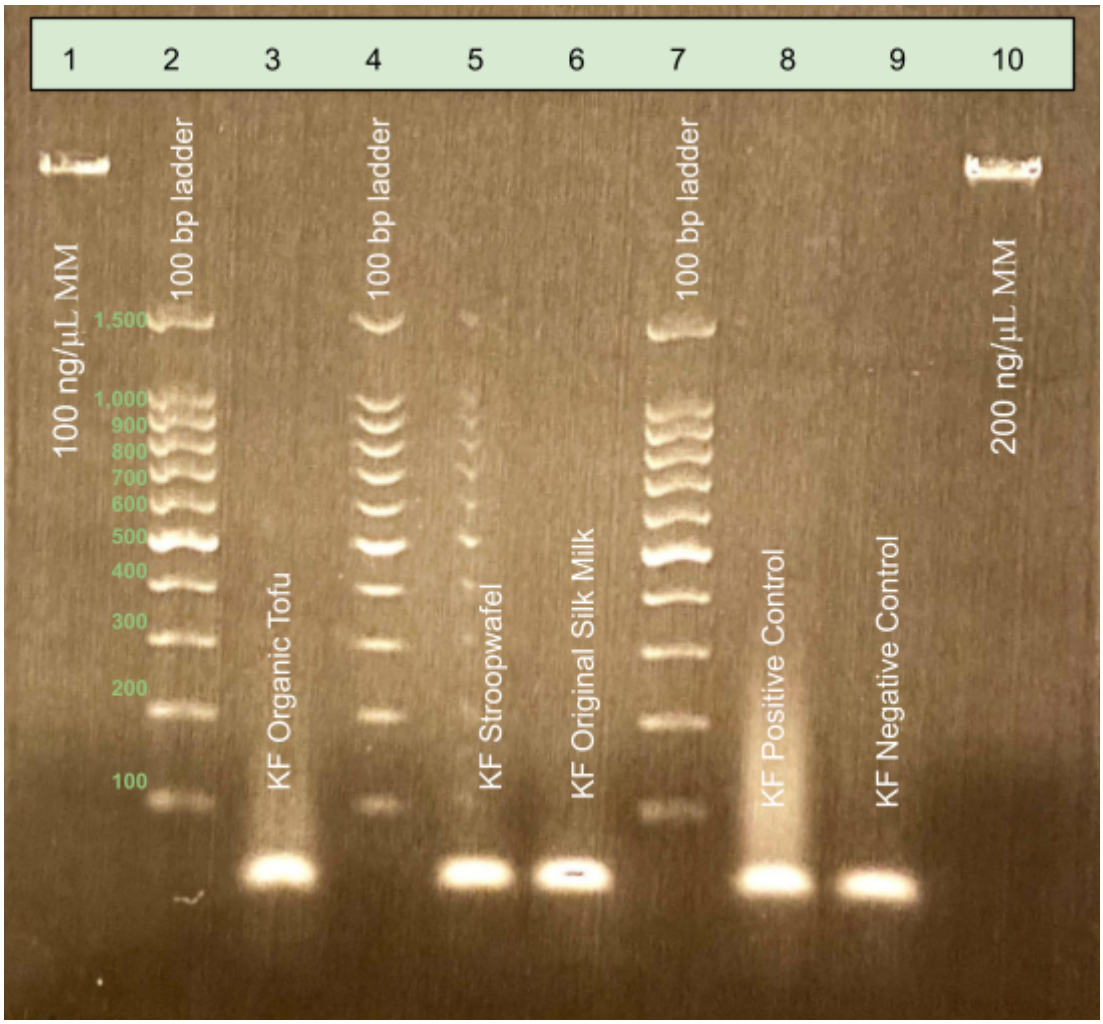
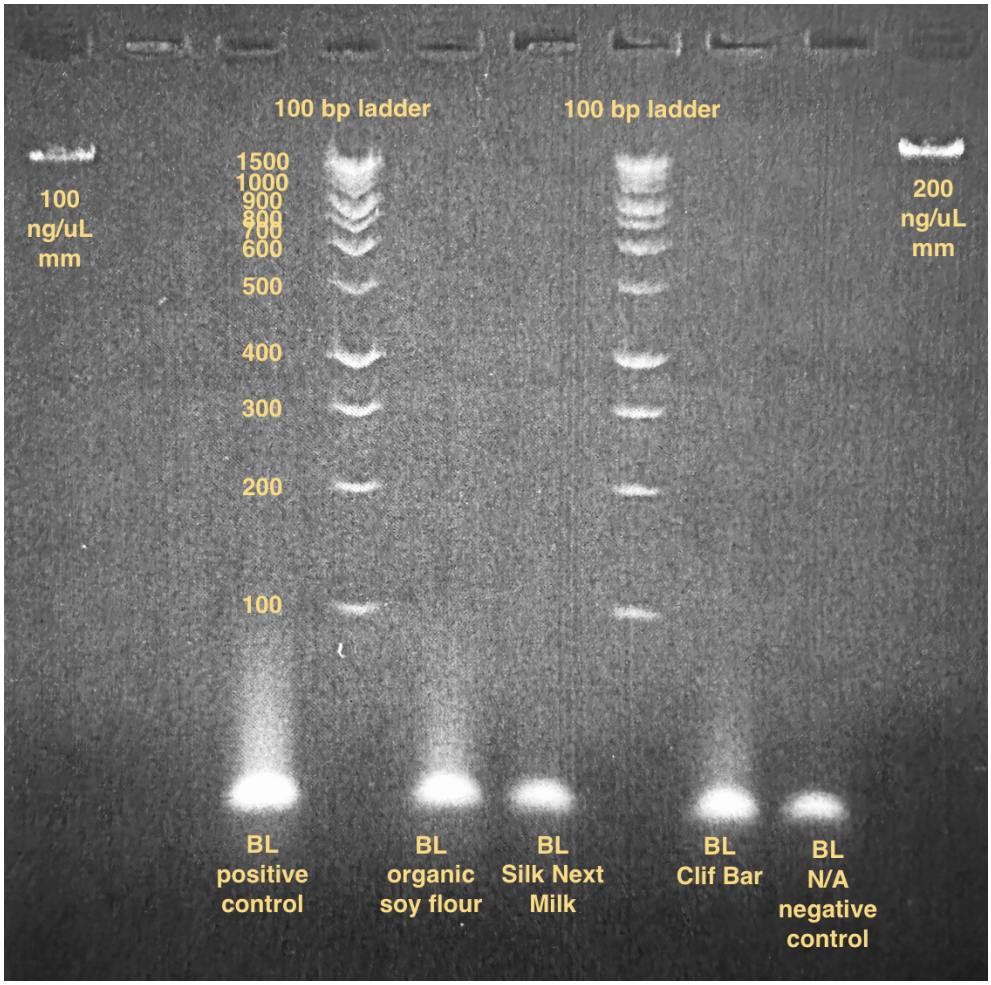


**Figure 8.2** | Multiplex PCR (59 °C annealing temp, 35 cycles) for CaMV35S, soy lectin, and CaMV35S/EPSPS junction products on 1.5% agarose mini gel (BL)




**Review**

*(KF & BL) Multiplex PCR*

In Figure 8.1 and Figure 8.2, there was no evidence of PCR products. The DNA present on the gel is likely just template and primer DNA that ran below the 100 bp band of the ladder. We were later informed that one of the PCR cyclers had not properly wired the night our samples were supposed to run. All conclusions about our food products were made from the simplex PCR results.

**Extension Questions**

1. *Was the goal accomplished?*

   The goal was not accomplished. While all conditions for the final multiplex PCR were tested and determined, the PCR failed to function properly with the final sets of multiplex PCR samples.

2. *Simply list which techniques worked, and those that did not. For those that did not work, explain why----note: if you explained this in the discussion, tell the grader where this is discussed, and then simply list the techniques that worked and those that did not. Provide possible explanations for your success/ or less than perfect result. NOTE: this question is simply a reminder to you—did you discuss all work? Procedures that did not work? If you didn't, discuss them here. If you did already in the report, then simply list the techniques that worked, and ones that didn't.*

   The techniques that worked including retried gDNA extraction using NucleoSpin prep kit, multiple rounds of simplex PCR using different sets of primers and different conditions, and multiple rounds of mini/midi agarose gel electrophoresis. The techniques that did not work include the first round of gDNA extraction, first agarose gel electrophoresis on PCR products, and multiplex PCR.

   The first round of gDNA extraction using NucleoSpin prep kit was unsuccessful. Seven out of the eight samples had very low concentration and intense smearing. It was possibly caused by a long time of vortexing (30s as instructed by the NucleoSpin prep manual) and forceful pipetting. The second round of gDNA extraction with NucleoSpin prep was more successful with shortened vortexing (10s) and slower pipetting. Detailed analysis can be found in Day 2 discussion.

   The first time testing PCR products for promoters was unsuccessful. The products were run on a double lane midi gel, and was not given enough time or space for the ladders to separate or the products band to separate. It was rerun on single lane midi gel and was given more time. Detailed analysis can be found in Day 4 discussion.

   The final multiplex PCR did not work because of technical difficulties. The cycler did not function properly and PCR was not performed on any of the samples. Detailed analysis can be found in Day 8 discussion.

3. *Critique the quality of your work. Did your lab work affect the results of any of your procedures? (yes, it happens sometimes). Did you have to repeat any protocols? Discuss specific examples. Can you make conclusions based on the validity of your data? Did your data allow you to make conclusions and proceed to the next step of the project?*

   The quality of our work improved gradually throughout the project. At first, we failed to perform gDNA extraction with NucleoSpin prep kit with high concentration and good quality, and the protocol had to be repeated. We then failed to perform the first round of agarose gel electrophoresis for promoter PCR products, and the products were rerun a second time. Repeating these protocols with improved techniques guaranteed better and more reliable result, which allowed us to make analysis and conclusions based on the data and proceed to the next step.

4. *What did you learn from the project? (this can be technical AND non-technical).*

   From a technical standpoint, we gained deeper understanding of PCR procedure. From performing and analyzing multiple simplex PCRs as well as designing the final multiplex PCR, we were able to deeply understand entire process of PCR procedure, including the PCR conditions, primer selection, PCR product preparation, etc. We were also able to become quite familiar with running agarose gel electrophoresis and using NucleoSpin prep kit to extract genomic DNA.

   From a non-technical standpoint, we learned the importance of patience and the value of repetition. At the beginning of our project, we had to redo protocols like NucleoSpin gDNA extraction and agarose gel electrophoresis. Such setbacks caused us to lose entire lab sessions' worth of work, and the frustration was overwhelming. We learned that it was important to remain calm and patient, and repeat protocols when necessary. Failures and setbacks are part of sciece. We also realized that "practice makes perfect" applies to lab work as well, and repetition of procedures results in improved outcomes.

5. Consider the goal of your project. What is its broader scientific impact? Describe an application for your project beyond the scope of what you planned to complete in class. Provide at least one Journal reference.

   This project could be repeated with any food samples and any genes of interest. One real-world application for the detection of genes in food products using PCR is the detection of gluten contamination in certified gluten free foods. Gluten free food samples can be extracted and multiple gluten gene primers can be used to amplify sequences within the food product. This would allow for the identification of contaminated samples that could potentially harm people with severe celiac disease. This paper uses simplex

PCR, but this could easily be modified for a multiplex PCR protocol. It also serves as a proof of concept for gluten detection with PCR.

Sandberg, M., Lundberg, L., Ferm, M. et al. Real Time PCR for the detection and discrimination of cereal contamination in gluten free foods. Eur Food Res Technol 217, 344–349 (2003). https://doi.org/10.1007/s00217-003-0758-4

6. Now that the project is done, address the design (outline) of your project. Would you approach it differently if you had to go back into the lab and do it all over again? What steps or methods would you change?

   We would not approach this project differently if we had to start it over again. While we did not always have the best results following certain procedures, they were necessary to the conclusion of our project. If we did it again, we would know which procedures to be more careful with, such as the DNA extraction, and know the optimal conditions for PCR. In that way we would reduce the number of days that we would spend on the project. If we were able to decrease the number of days this project takes, we would ensure that multiplex runs successfully and have a day to repeat if necessary.

7. *If you had one more month to work on the project, how would you continue/improve it? Be sure to state the next logical experiment.*

   First of all, we would rerun the multiplex PCR and obtain correct gel photo from the multiplex products. Then based on the result from the gel, we can further adjust the amount of gDNA added for each PCR sample, and PCR conditions like temperature and cycle numbers. Adjust the conditions until non-specific binding smear is minimized, and PCR products bands are bright and condensed. Then if time permitting, more sets of primers can be added into the multiplex PCR to detect more gene segments, such as EPSPS gene and NPT II gene. The new optimal conditions can be obtained with the same process of repeating simplex PCRs for each set of primers, and the conditions can be integrated into the multiplex final conditions.