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

Detection of the Cry1Ab/Cry1Ac protein using enzyme linked immunosorbent assay (ELISA)

Laboratory Manual (2)

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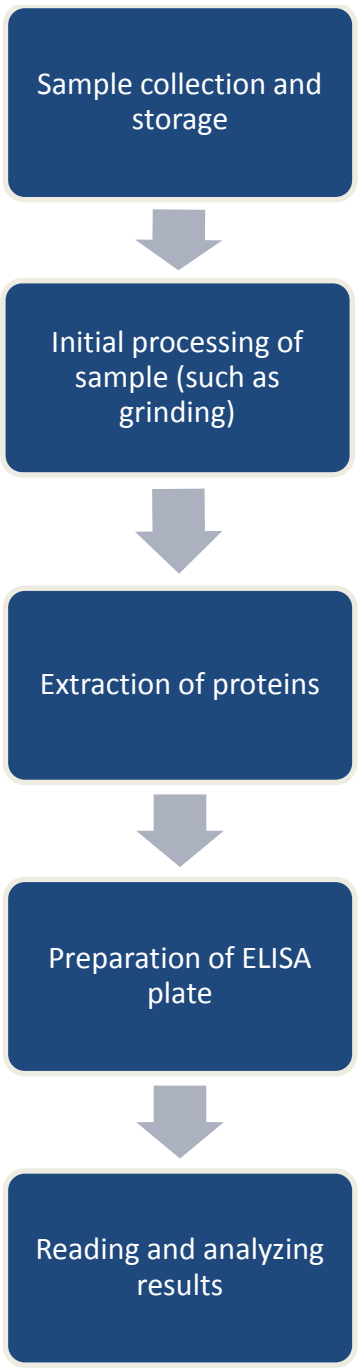
Glossary of terms

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

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

Manual 2:

Detection of the Cry1Ab/Cry1Ac protein using enzyme linked immunosorbent assay (ELISA)



Workflow of ELISA analysis

2.1 Introduction

Enzyme-linked immunosorbent assay (ELISA) also known as enzyme immunoassay, is a widely used tool for the detection and quantification of specific antigens such as transgenic proteins present in a sample. The technique utilizes antibodies conjugated to easily-assayed enzymes and thereby exploits the specificity of antibodies and the sensitivity of simple enzyme assays [3]. The assay is typically performed in a 96-well polystyrene plate, which passively bind antibodies and proteins. There can be several variations to the basic procedure of the assay such as direct, indirect, competition and sandwich detection, nevertheless, sandwich assays are the most sensitive and robust format [3].

To perform the sandwich ELISA, antigen-containing sample is applied to a plate containing immobilized antibodies (capture antibodies) that capture the antigen (Fig. 2.1). The plate is washed to remove unbound antigen and another antibody (detection antibody) specific to the antigen is added. This antibody may be directly conjugated to an enzyme that allows detection, or an enzyme-linked secondary antibody that specifically binds to the detection antibody may be applied. The plate is washed to remove the unbound antibody-enzyme conjugates. A chemical is added to be converted by the enzyme into a colored, fluorescent or electrochemical signal which is measured to determine the presence and quantity of antigen. The sandwich ELISA system quantifies antigens between the capture and detection antibodies and therefore the antigen to be measured must contain at least two antigenic epitopes capable of binding to both antibodies. The sandwich ELISA system can be specific and therefore there is no need to purify the samples before analysis; also the system is very sensitive [3, 4].

In this chapter, EnviroLogix QualiPlate Kit ELISA assay (catalog number AP 003 CRBS) would be used to detect and quantify the Cry1Ab protein in corn samples [5]. The ELISA test is of a sandwich design and can detect both the Cry1Ab and Cry1Ac proteins. In the test, proteins would be extracted from ground corn samples and added to an ELISA plate coated with antibodies raised against Cry1Ab/ Cry1Ac toxins. If present, the Cry1Ab/ Cry1Ac proteins would bind to the antibodies which would then be detected by addition of horseradish peroxidase labeled Cry1Ab/ Cry1Ac antibody. Horseradish peroxidase is a commonly used enzyme that converts particular substrates into colored products which can be measured to determine the

presence and quantity of the transgenic protein where color formation is proportional to the Cry1Ab/ Cry1Ac concentration in the sample.

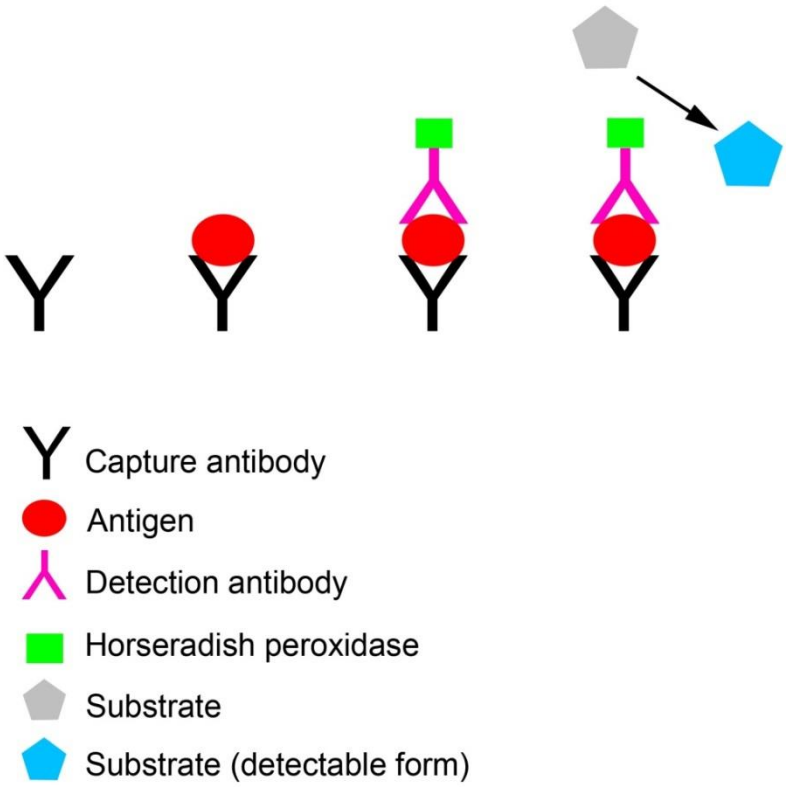


Fig. 2.1 Sandwich ELISA assay

2.2 Precautions and notes

1. Allow all reagents to reach room temperature before use (at least 30 min). If fewer than all twelve strips are used, reseal the unneeded strips and the desiccant in the foil pouch provided and refrigerate.

2. Use caution to prevent sample-to-sample cross-contamination with grain, fluids, or disposables. Thoroughly clean blender parts and containers to remove dust and residue prior to preparation of a second sample.
3. Organize all reagents, sample extracts, and pipettes so that the steps can be performed quickly. The use of a multichannel pipette is strongly recommended.
4. Store all kit components at 4 °C to 8 °C. Do not expose components to temperatures greater than 37 °C or less than 2 °C; do not expose to sunlight.
5. Safety goggles, lab coat, closed toe shoes and gloves must be worn.
6. The stop solution is 1.0 N hydrochloric acid; handle carefully
7. Protein standards must be supplied by the user. Standards are available from the European Commission Joint Research Centre, Institute for Reference Materials and Measurements. It is imperative that the samples be ground to the same consistency as the standards, and that both are extracted with the same extraction buffer, buffer-to-sample ratio, and extraction time. Alternatively, pure Cry1Ab or Cry1Ac protein can be used as the standard.

2.3 Initial processing of samples, extraction of proteins and preparation of ELISA plate

The components present in the EnviroLogix QualiPlate Kit for Cry1Ab/Cry1Ac (catalog number AP 003 CRBS) are stated below. To prepare the wash buffer, add the contents of the wash buffer salt packet to 1 L of distilled or deionized water and stir to dissolve. Store at 4 °C when not in use and warm to room temperature prior to assay.

12 strips of 8 antibody-coated wells and plate frame
Cry1Ab/ Cry1Ac positive control
Cry1Ab/ Cry1Ac enzyme conjugate
Substrate
Stop solution
Wash buffer salt packet

Procedure [5]

1. Determine the number and size of sub-samples (see EnviroLogix's instructions and Appendix 1 for additional guidelines).
2. Weigh out corn sample and grind with a Waring blender (or equivalent) on high speed for about 30 s or until all the grains are ground.
3. Add 10 mL of distilled or deionized water to 0.2 g of ground sample and vortex for 30 s.
4. Incubate the suspension for 1 h at room temperature, vortex for 10 s and allow solids to settle.
5. Transfer the extract to a Falcon tube and centrifuge for 5 min at 5000 x g.
6. Transfer the supernatant to a new tube and dilute to 1 in 5 with wash buffer (mix 100 µL extract with 400 µL wash buffer) and vortex.
7. Add 50 µL of Cry1Ab/ Cry1Ac enzyme conjugate to the wells of the plate immediately follow by 50 µL of standards, diluted sample, negative control sample and extraction buffer blank as shown in Fig 2.2 (perform each reaction in duplicate). NOTE: a multi-channel pipette should be used and the order in adding reagents should be maintained for the remaining steps.
8. Thoroughly mix the contents of the wells by moving the plate in a rapid circular motion on the benchtop for 30 s, cover the wells with tape or Parafilm and incubate on an orbital plate shaker for 45 min at 200 rpm.
9. Carefully remove the cover and vigorously shake the contents of the wells into a sink. Slap the plate on a stack of paper towels to remove residual sample.
10. Flood the wells with wash buffer and discard the contents as before. Repeat this step three times.
11. Add 100 µL of substrate to the wells, thoroughly mix the contents, cover and incubate on an orbital plate shaker for 25 min at 200 rpm.
12. Add 100 µL of stop solution to the wells and mix thoroughly.
13. Read absorbences at 450 nm using a plate reader within 30 min of the addition of stop solution. If the plate reader has dual wavelength capability, use 600, 630 or 650 nm as the reference wavelength. Set the plate reader to blank on the extraction buffer blank wells. If this cannot be done, record the optical density (OD) of each well's contents and subtract the average OD of the blank wells from each of the readings.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1										
B	S2	S2										
C	S3	S3										
D	S4	S4										
E	U	U										
F	N	N										
G	EBB	EBB										
H												

Fig. 2.2 ELISA plate setup

S1 – 0.0% standard

S2 – 0.1% standard

S3 – 1% standard

S4 – 2% standard

U – Unknown corn sample

N – Cry1Ab absent in corn sample (negative control)

EBB – Extraction buffer blank

2.4 Interpretation of results

Qualitative analysis

Compare the OD of sample extracts to those of the positive control to determine presence or absence of Cry1Ab/ Cry1Ac endotoxin. Samples with absorbences close to that of the blank wells and less than positive control are presumed to be free of the endotoxin. Samples with absorbences significantly higher than blank wells are positive for endotoxin content.

Quantitative analysis [6]

1. Calculate the average OD of each set of standards and samples and subtract the average OD of the buffer blank wells from all (if the plate reader has not automatically done so).
2. Plot the mean OD of each standard against its % Cry1Ab content with a quadratic curve fit. The linearity of the standard curve (R2) should be greater than 0.990 for an acceptable assay.
3. Extrapolate the % Cry1Ab content of each sample from the standard curve. Note this is only possible if the OD of the sample falls within the range of OD's of the standards. If the OD of a sample is lower than that of the lowest standard (0.1% Cry1Ab), the sample must be reported as less than 0.1% GM corn. If the OD of a sample is higher than that of the highest standard (2% Cry1Ab), the sample must be reported as greater than 2% GM corn.

Note:

The mean OD of the negative Cry1Ab corn should not exceed 0.2. The coefficient of variance (% CV) of the duplicate wells should not exceed 15% where % CV = (Standard deviation of ODs x 100)/ mean OD.

Reference

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Appendices

Appendix I Determination of number and size of sub-samples

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

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

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

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

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

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

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

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

Appendix II Safety in molecular biology laboratories

General laboratory safety

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

Safety in an end-point PCR laboratory

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

Appendix III Preparation of solutions

100 mL 0.5 M EDTA (pH 8.0)

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

1 L 10X TAE

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

1 L 5X TBE

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

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

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

1 mL ethidium bromide (10 mg/ mL)

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

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

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

Note:

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

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

$C_1V_1 = C_2V_2$

Where:

C_1 = initial concentration of solution

V_1 = initial volume

C_2 = final concentration of solution

V_2 = final volume

Appendix IV DNA quantification

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

Procedure [16]

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

<u>Example of calculation</u>

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

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

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

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

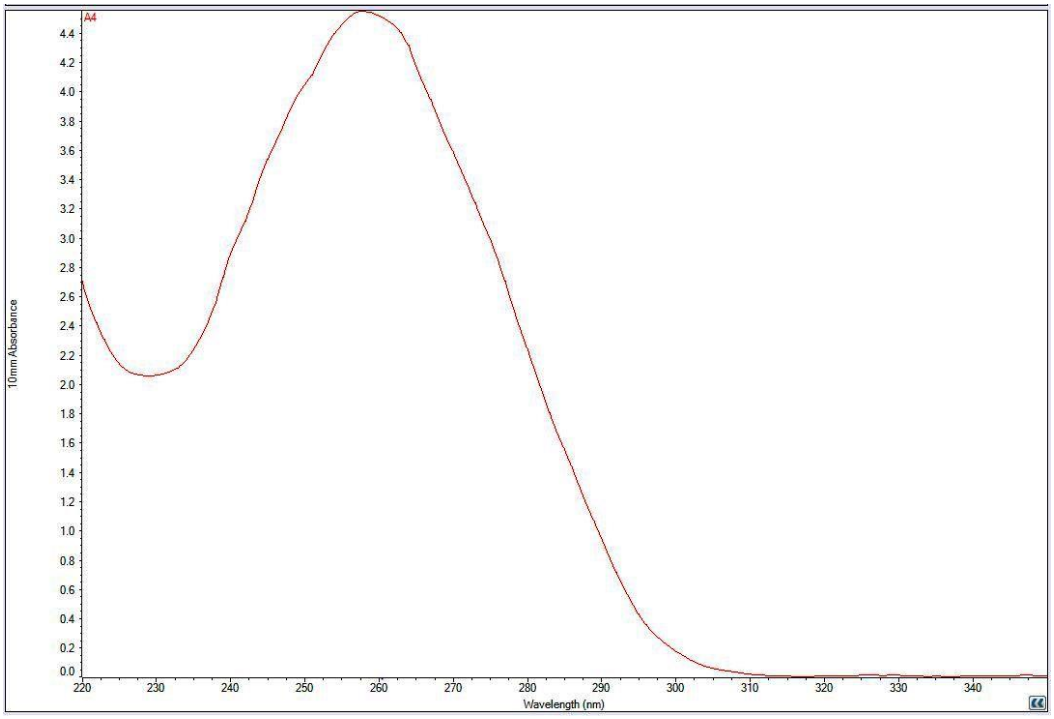

Fig. A4 The wavelength of maximum absorption for DNA is 260 nm. Graph obtained using a NanoDrop 2000 UV-Vis spectrophotometer

Appendix V Agarose gel electrophoresis

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

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

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

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

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

Appendix VI How to use a micropipette

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

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

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

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

Withdraw sample from reagent tube:

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

Expel sample into reaction tube:

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

Preventing cross-contamination of reagents:

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