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

Quantitative measurement of genetically modified corn DNA using real-time quantitative polymerase chain reaction (qPCR)

Laboratory Manual (4)

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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 4:

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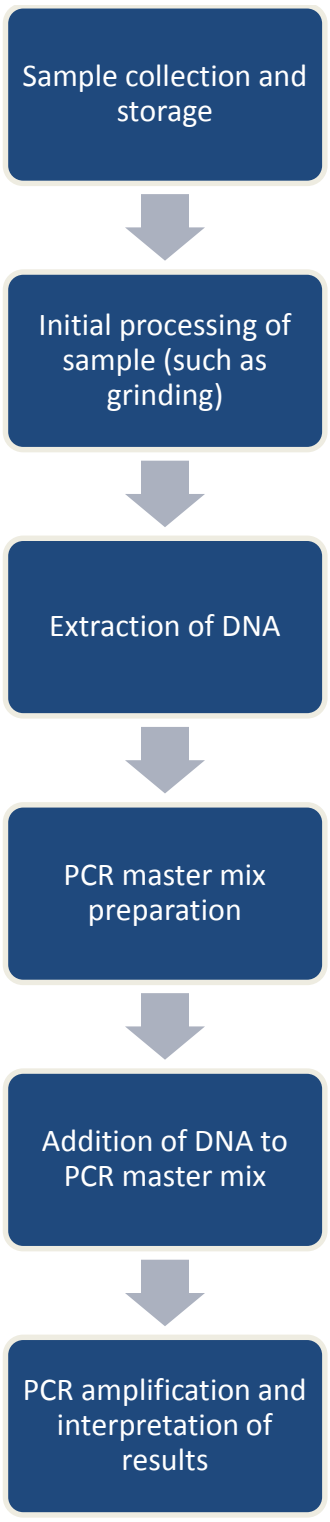

Real-time PCR analysis workflow

4.1 Introduction

Real-time PCR is a type of PCR assay, where the progress of the PCR is monitored as the reaction occurs and data is collected throughout the process rather than at the end. The amount of DNA is measured after each cycle via fluorescent dyes that yield increasing fluorescent signal that is directly proportional to the number of DNA molecules generated [11, 12]. Hence, as the number of copies of the gene increases during the reaction, the fluorescence signal also increases. The change in fluorescence over the course of the reaction is measured by an instrument that combines thermal cycling with fluorescence detection. By plotting fluorescence against the cycle number, the real-time PCR instrument can generate an amplification plot that represents the accumulation of product over the duration of the entire PCR reaction, and data collected in the exponential phase, can yield highly accurate quantitative information on the starting amount of the target DNA. Additionally, real-time PCR has an increased dynamic range of detection and both amplification and detection occurs in a single tube eliminating the need for post-PCR manipulations [11].

There are several types of fluorescent reporter systems available that can be used to monitor the progress of the PCR. These systems can be characterized as being either double-stranded DNA binding dyes such as SYBR green or fluorescence probe based assays such as TaqMan. In this chapter, the GMO*Quant* (LR) 35S Screen Corn kit, which utilizes probe technology, would be employed to detect and quantify the 35S promoter DNA sequence in corn samples [13]. Essentially, a probe is a short segment of DNA that anneals to the template DNA, between the forward and reverse primers, during PCR amplification [11]. The probe has a high-energy dye at the 5' end called a reporter and a low-energy molecule at the 3' end called a quencher. When the probe is intact and is excited by a light source, the reporter molecule fluoresces, however, the emission is suppressed by the quencher molecule when it is in close proximity. During PCR amplification, when DNA polymerase encounters the annealed probe, it degrades it causing the reporter to become separated from the quencher and the suppression of energy to stop. This results in the fluorescent emission in the PCR tube to increase and is captured by the real-time PCR instrument [11]. The amount of reporter signal increase is proportional to the amount of product being produced for a given sample. When the fluorescent signal increases to a detectable level it can be captured and displayed as an amplification plot

which contains valuable information that can be used to determine the starting quantity of the amplified DNA.

4.2 Precautions and notes

1. Use caution to prevent sample-to-sample cross-contamination with grain, fluids, or disposables. Thoroughly clean blender parts and containers to remove dust and residue prior to preparation of a second sample.
2. Safety goggles, lab coat, closed toe shoes and gloves must be worn.
3. Use optical PCR plates and caps or seals as described by the manufacture.

4.3 Initial processing, DNA extraction, master mix preparation and addition of DNA

The following reagents are present in the GMO*Quant* (LR) 35S Screen Corn kit (catalog number 5121203510) [13]:

MasterMix (LR) GSE-P-07.24·p35S (2 tubes)
MasterMix (LR) Corn HMGa (2 tubes)
Corn Standards 1-4 (4 tubes)
35S Standards 1-4 (4 tubes)
1.0% NK603 Corn DNA (1 tube)

MasterMix (LR) GSE-P-07.24·p35S and MasterMix (LR) Corn HMGa are master mix solutions. Each master mix contains primers, probes, nucleotides (dNTPs), hot-start DNA polymerase and reaction buffer. The MasterMix (LR) GSE-P-07.24·p35S contains primers and probes that would specifically amplify the CaMV 35S-Promoter sequence, while MasterMix (LR) Corn HMGa contains primers and probes that would specifically target corn DNA. The detection of the 35S-promoter sequence in a sample strongly indicates the presence of a genetic modification and

therefore the percentage of genetically modified DNA present can be determined by quantifying the percentage of the 35S-promoter present.

Example: Quantitative measurement of genetically modified corn DNA in unknown corn sample

Procedure [13]

1. Process the sample, extract and quantify the DNA as described in chapter 3.3 and adjust the concentration to 40 ng/ µL using AE buffer. For the unknown sample, perform two independent DNA extractions ("A" and "B").
2. Aliquot the MasterMixes into the wells of the optical PCR plate (20 µL each) as shown in Fig. 4.1.
3. Add 5 µL of DNA solution (standards, controls or unknowns). Analyze each DNA extraction at two different dilutions ("dilution 1" and "dilution 2") to assess for inhibitory effects. Also, all reactions should be performed in at least duplicate. Additionally include:
    a. An extraction control to determine if contamination or impurities from the samples or foreign DNA was introduced during DNA extraction and further handling.
    b. A quantification control with 1.0% GMO corn (NK603) DNA
    c. A no template control to determine if the reagents or PCR reactions were contaminated with DNA during pipetting.
4. Carefully close the plate with an optical seal. Mix the contents of the plate and centrifuge at a low speed. Insert the plate into the real-time PCR machine (Fig 4.2A).
5. Select FAM as the detector and "None" as the quencher and choose ROX as the passive reference. Perform the PCR with the temperature profile shown in Table 4.1. At the end of the run automatically determined the baseline and quantitative cycle (Cq or Ct) values using the system detection software (Fig 4.2B). Note: the threshold line is the point at which a reaction reaches a fluorescent intensity above the background and is set in the exponential phase of the amplification. The cycle at which the sample reaches this level is called the quantitative cycle.

	1	2	3	4	5	6	7	8
A	S1-35S	S1-35S	EC-D1	EC-D1	S1- corn	S1- corn	EC-D1	EC-D1
B	S2-35S	S2-35S	EC-D2	EC-D2	S2- corn	S2- corn	EC-D2	EC-D2
C	S3-35S	S3-35S	QC	QC	S3- corn	S3- corn	QC	QC
D	S4-35S	S4-35S	NTC	NTC	S4- corn	S4- corn	NTC	NTC
E	UA-D1	UA-D1			UA-D1	UA-D1		
F	UA-D2	UA-D2			UA-D2	UA-D2		
G	UB-D1	UB-D1			UB-D1	UB-D1		
H	UB-D2	UB-D2			UB-D2	UB-D2		

Fig. 4.1 Real-time PCR plate setup

Master mix layout

- MasterMix (LR) GSE-P-07.24·p35S

- MasterMix (LR) Corn HMGa

DNA layout

S1-35S - 35S standard 1

S2-35S - 35S standard 2

S3-35S - 35S standard 3

S4-35S - 35S standard 4

UA-D1 – Unknown, A extraction, dilution 1

UA-D2 – Unknown, A extraction, dilution 2

UB-D1 – Unknown, B extraction, dilution 1

UB-D2 – Unknown, B extraction, dilution 2

EC-D1 – Extraction control, dilution 1

EC-D2 – Extraction control, dilution 2

QC – Quantification control (1.0% NK603 Corn DNA)

NTC- no template control

S1-corn - corn standard 1

S2- corn - corn standard 2

S3- corn - corn standard 3

S4- corn - corn standard 4

Table 4.1 PCR temperature profile

Temperature	**Time**
95 °C	10 min
95 °C	15 s
60 °C	90 s

45 cycles

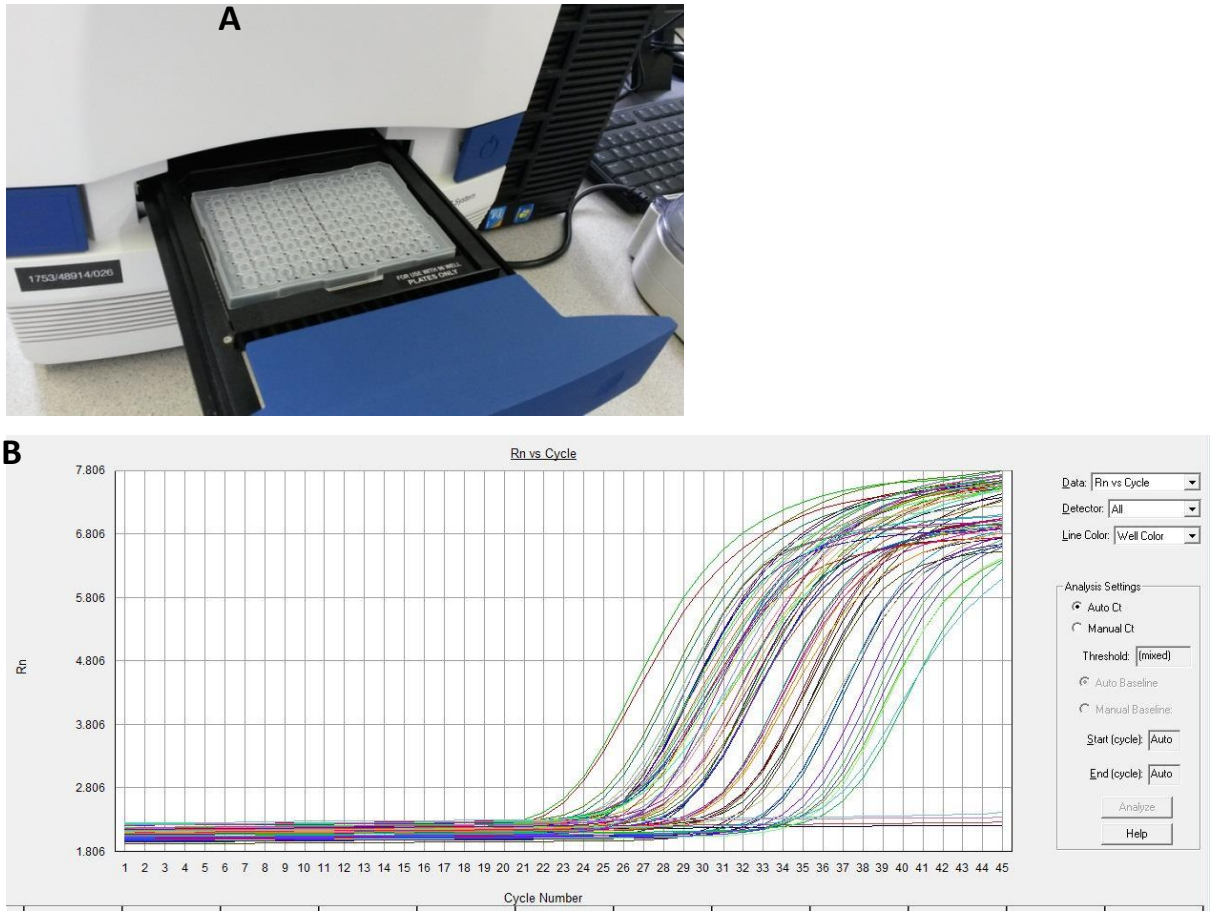

Fig. 4.2 Real-time PCR. A, optical plate being inserted into Applied Biosystems 7500 real-time PCR machine; B, baseline and quantitative cycle values automatically determined using the system detection software

4.4 Interpretation of results

1. Prepare the 35S and corn DNA standard curves by plotting Cq values against log copy number (Table 4.2).
2. Extrapolate the number of copies of 35S and corn DNA from unknown sample.
3. Determine the relative GM ratio (%) using the following:
   GM ratio (%) = number of copies of 35S/ number of copies of corn DNA
4. Compare the GM ratio (%) among all extraction, dilution and technical replicates.

Note

1. The unit of measurement of GM ratio (%) is "% GM DNA".
2. DNA from 1.0% NK603 serves as a control for the calibration and the quantification process and should not show a deviation > 30% from the expected result.
3. If the extraction controls show significant deviations between "A" and "B", this may be due to sample material non-homogeneity or to non-uniformities in the DNA-extraction efficiency. Therefore repeat DNA extraction and homogenize sample more thoroughly.
4. At least two dilutions of the DNA test sample should be used to detect inhibitory effects. If GMO quantities significantly differ between the two dilutions (> 30%), inhibitory effects may be responsible and the analysis should be repeated with either more purified DNA or more diluted DNA.

Table 4.2 Copy number and Cq values

Reaction	**Copy number of 35S**	**Log (copy number)**	**Cq**
S1-35S	10240	4.0103	
S1-35S	10240	4.0103	
S2-35S	1280	3.1072	
S2-35S	1280	3.1072	
S3-35S	160	2.2041	
S3-35S	160	2.2041	
S4-35S	40	1.6021	
S4-35S	40	1.6021	
Reaction	**Copy number of corn DNA**	**Log (copy number)**	**Cq**
S1- corn	81920	4.9134	
S1- corn	81920	4.9134	
S2- corn	10240	4.0103	
S2- corn	10240	4.0103	
S3- corn	1280	3.1072	
S3- corn	1280	3.1072	
S4- corn	160	2.2041	
S4- corn	160	2.2041	

Reference

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3. Gan, S.D. and K.R. Patel, *Enzyme immunoassay and enzyme-linked immunosorbent assay.* Journal of Investigative Dermatology, 2013. **133**(9): p. e12.

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11. Kubista, M., et al., *The real-time polymerase chain reaction.* Molecular Aspects of Medicine, 2006. **27**(2): p. 95-125.

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13. Eurofins GeneScan GmbH, *GMOQuant (LR) 35S screen corn test kit for quantification of the CaMV 35S promoter in corn DNA, V26 (6.11.2014)*, 2014.

14. Freese, L., J. Chen, and R.D. Shillito, *Sampling of grain and seed to estimate the adventitious presence of biotechnology-derived seeds in a lot.* Cereal Foods World, 2015. **60**(1): p. 9-15.

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

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

Appendices

Appendix I Determination of number and size of sub-samples

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

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

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

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

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

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

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

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

Appendix II Safety in molecular biology laboratories

General laboratory safety

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

Safety in an end-point PCR laboratory

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

Appendix III Preparation of solutions

100 mL 0.5 M EDTA (pH 8.0)

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

1 L 10X TAE

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

1 L 5X TBE

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

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

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

1 mL ethidium bromide (10 mg/ mL)

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

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

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

Note:

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

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

$C_1V_1 = C_2V_2$

Where:

C_1 = initial concentration of solution

V_1 = initial volume

C_2 = final concentration of solution

V_2 = final volume

Appendix IV DNA quantification

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

Procedure [16]

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

<u>Example of calculation</u>

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

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

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

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

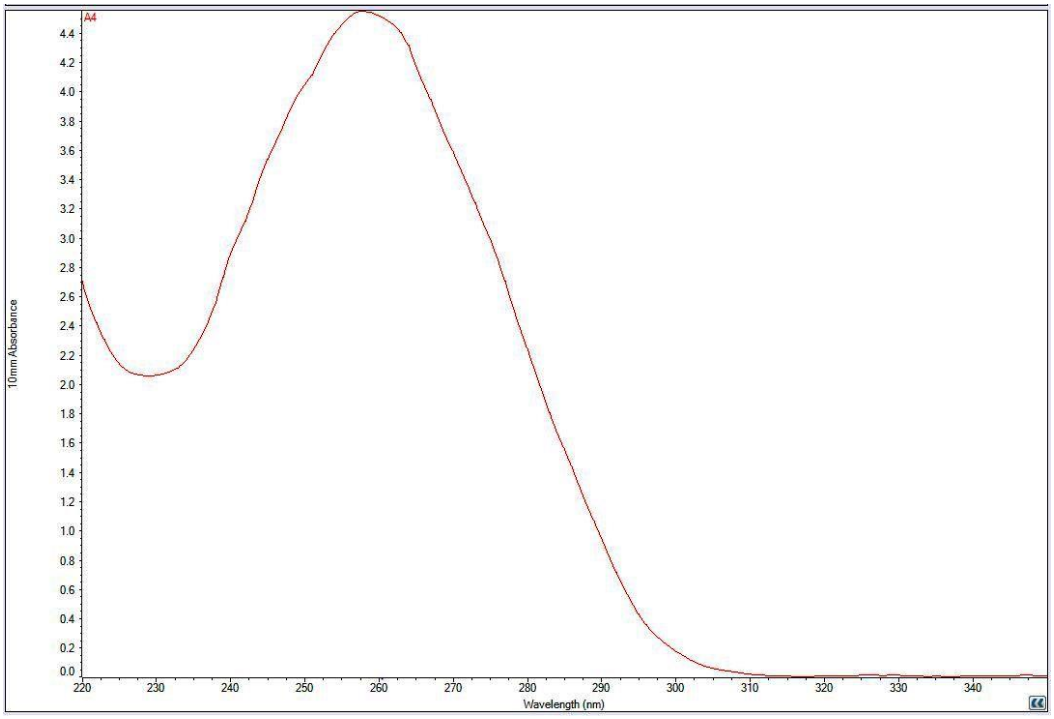

Fig. A4 The wavelength of maximum absorption for DNA is 260 nm. Graph obtained using a NanoDrop 2000 UV-Vis spectrophotometer

Appendix V Agarose gel electrophoresis

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

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

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

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

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

Appendix VI How to use a micropipette

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

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

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

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

Withdraw sample from reagent tube:

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

Expel sample into reaction tube:

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

Preventing cross-contamination of reagents:

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