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

Detection of Cry1A protein using lateral flow strips

Laboratory Manual (1)

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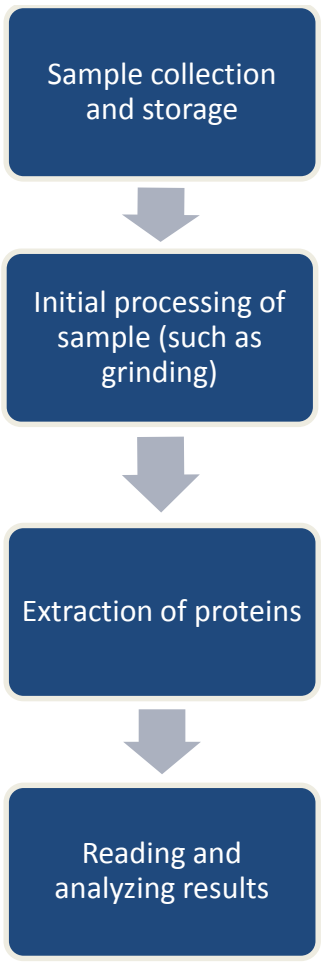
Glossary of terms

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 1:

Detection of Cry1A protein using lateral flow strips



Lateral flow strip analysis workflow

1.1 Introduction

Lateral flow strips, also known as immunochromatographic lateral flow strips, are popular devices utilized in the detection of analytes. They are inexpensive, simple to use and do not require highly trained personnel and expensive equipment, can rapidly detect analytes of interest and are stable over a wide range of climates. A test strip typically consists of porous materials in four zones: a sample application pad, a conjugate pad, a detection membrane and an absorbent pad [1] (Fig. 1.1).

To perform the test, a sample is placed on the sample pad which acts like a sponge and absorbs an excess of sample fluid. Once soaked, the fluid migrates via capillary action to the conjugate pad which contains antibodies that are specific to the transgenic protein and have been conjugated to colored particles. The sample fluid would dissolve the conjugate and if the transgenic protein is present in the sample, it would bind to the antibodies. The fluid mixture would then continue further up the strip into the detection membrane. This material typically has two lines of antibodies immobilized on it, the test line and the control line. The test line contains antibodies that specifically capture the transgenic protein. If the transgenic protein/ conjugated antibody complex is present, the antibodies would capture the complex and as more complex particles accumulate the test line would change color. On the other hand, the control line captures excess conjugate and produces a second visible line on the membrane indicating that the reaction worked. Excess buffer along with any reagents not captured at the test or control lines would move into the absorbent pad.

In this chapter, lateral flow strips from EnviroLogix (QuickStix kit for Cry1Ab bulk grain; catalog number AS 003 BG) would be used to screen corn samples for the presence of the Cry1A protein [2]. The samples would initially be ground and the proteins would be dissolved in water. Lateral flow strips would subsequently be added to the extracted proteins which would migrate to the detection membrane. Two clear lines on the membrane is a positive result for Cry1Ab, while a single line in the control zone is a negative result.

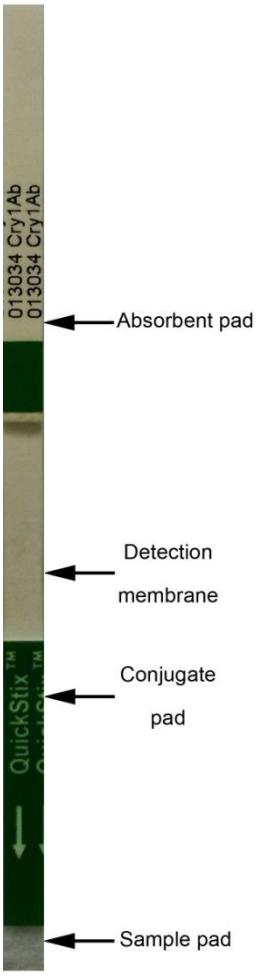


Fig. 1.1 Lateral flow strip

1.2 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. Use a new pipette tip and reaction vial for each sample.
2. This kit is designed to screen for presence or absence only, and is not meant to be quantitative.

3. QuickStix can be stored at room temperature, or refrigerated for a longer shelf life. The kit may be used in field applications; however, prolonged exposure to high temperatures may adversely affect the test results.
4. Allow the refrigerated canisters to come to room temperature before opening and do not open until needed. Reseal the canister immediately and avoid bending the test strips.
5. Safety goggles, lab coat, closed toe shoes and gloves must be worn.

1.3 Initial processing of sample, extraction of proteins and reading results

Procedure [2]

1. Determine the number and size of sub-samples (see EnviroLogix's instructions and Appendix 1 for additional guidelines).
2. Determine the average weight of an individual corn grain to be tested by weighing 100 seeds and dividing by 100.
3. Calculate the weight of the number of grains to be tested using the following:
   Weight of grains to be tested (g) = number of grains x average weight/grain.
4. Weigh out sample and grind with a Waring blender (or equivalent) on high speed for about 30 s or until all the grains are ground.
5. Add an appropriate amount of tap water [volume of water (mL) = weight of sample (g) x 1.5].
6. Mix the ground sample with the water with vigorous shaking for at least 30 s, or longer if needed, until the entire sample is thoroughly wet.
7. When the sample settles, transfer liquid from the top to a reaction vial until it is filled; avoid suspended particles (Fig. 1.2A).
8. Allow the extract to settle in the reaction vial for 30 s then add a test strip. The protective tape with the arrow indicates the end of the strip to insert into the reaction vial (Fig 1.2B). After 5 min, analyze the result.
9. To retain the strip, cut off and discard the bottom section covered by the arrow tape.

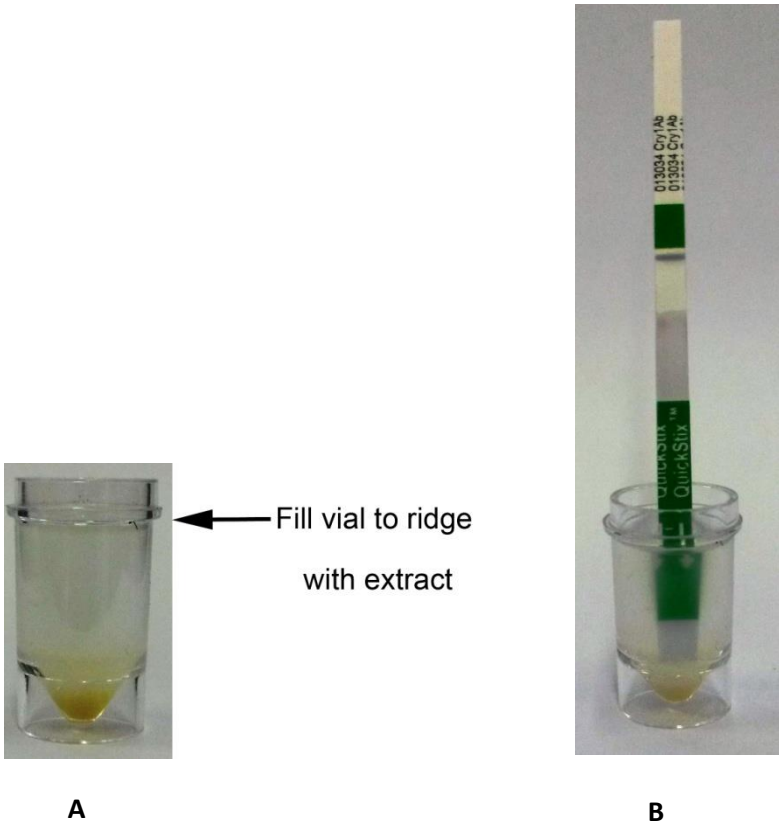


Fig. 1.2 Reaction vial filled to the ridge with protein extract

1.4 Interpretation of results

Development of the control line within 5 min indicates that the strip has functioned properly (Fig. 1.3). Strips that do not develop a control line should be discarded, and the sample re-tested using a new strip. If the extract is from a sample containing 0.8% Cry1Ab modified corn, a second line (test line) would develop on the membrane strip between the control line and the protective tape. The results should be interpreted as positive for Cry1Ab protein expression. If the extract is from a negative sample, the strip will only show the control line. If only the test line appears, or if no lines appear, it is invalid and must be repeated.

Fig. 1.3 Lateral flow strip results

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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.

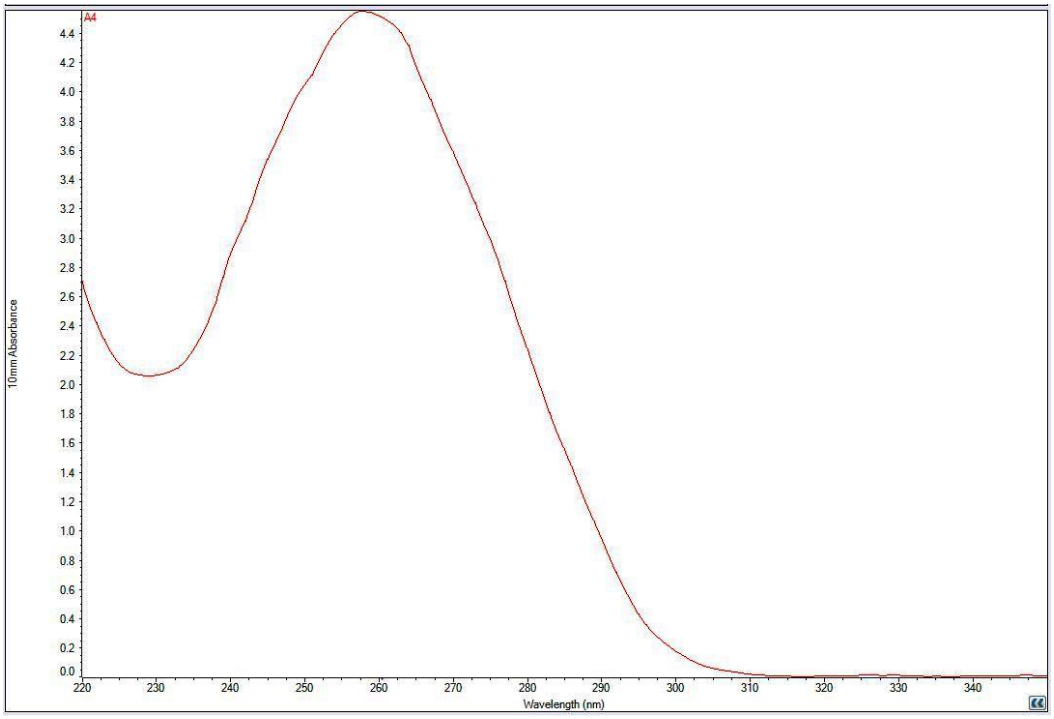

Fig. A4 The wavelength of maximum absorption for DNA is 260 nm. Graph obtained using a NanoDrop 2000 UV-Vis spectrophotometer

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.