Lesson 26

Title of the Experiment: Detection of plant viruses
(Specially designed to train teachers on plant virus detection techniques in the unit 14 of the syllabus, as a part of the activity number of the GCE Advanced Level Practical Guide 38)

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Introduction

In the previous experiments, we discussed the methodologies for identification of plant pathogenic fungi and bacteria by culturing them on synthetic growth media. Isolation into pure culture and identification is required for efficient disease management and it is widely applied for most of the fungal and bacterial pathogens. Since most of the plant diseases – around 85 %– are caused by fungal or fungal-like organisms, isolation has traditionally been useful in identification and implementing control strategies. However, not every pathogen can be isolated and cultured on synthetic media. Plant pathogenic organisms including viruses, phytoplasmas, biotrophic fungi (rusts, powdery mildews) and fastidious prokaryotic plant pathogens, which can cause severe economic damage to important crops cannot be cultured on artificial media and therefore, identification and applying control strategies is challenging. On the other hand no effective economical control measures are available for such organisms especially for the virus diseases. Therefore, early detection and prevention is the key in managing these diseases. For virus detection, Enzyme-linked immunosorbent assay (ELISA), an immunological method has been a very popular technique. With the development of gene technology over the past decade, nucleic acid based pathogen detection methods have also become popular and enabled accurate identification and early detection of both culturable and unculturable plant pathogens. This procedure involves total nucleic acid extraction from the infected plant material or from a pure culture of the pathogen (if pure cultures are available in the case of bacteria and fungi), amplify a specific region of the nucleic acid (DNA/RNA) using specific oligonucleotides, separate the amplified fragments using gel electrophoresis and observe under UV light to detect the presence of the desired product. In nucleic acid based identification, 16S ribosomal RNA (rRNA) gene and internal transcribed spacer region (ITS) has been widely used for bacterial and fungal species identification respectively. In the case of viruses, viral coat protein sequence has been preferred. For an accurate species and strain identification, one can go beyond this point by sequencing the DNA fragment (DNA barcoding) and comparing it to a sequence data base or using strain specific oligonucleotides (primers).

This experiment was specially designed to demonstrate a nucleic acid (DNA/RNA) based virus detection method in the laboratory. The overall aim of the experiment is to give teachers a hands-on experience on modern laboratory techniques; nucleic acid extraction, polymerase chain reaction (PCR) to amplify the coat protein region of a selected plant virus using specific primers and confirm the presence of the pathogen by visualizing DNA fragments on agarose gels under UV light.

Theory

Enzyme-Linked Immunosorbent Assay (ELISA) for virus detection:

Enzyme-linked immunosorbent assay (ELISA) and PCR have been widely applied techniques in plant virus diagnosis since classical light microscopy, isolation and culturing is not feasible or time consuming for viruses. ELISA has been very popular among disease diagnosticians since it was first introduced to plant virology by Clark and Adams in 1977, due to its adaptability, sensitivity and the

ability to test a large number of samples in relatively short period of time. The technique utilizes the ability of antibodies (Ab) raised in animals to recognize antigens (Ag)/proteins, usually the coat protein of the virus of interest. The Antigen (in this case it is virus coat protein present in the infected plant extract) will elicit a specific immune response when injected in an appropriate manner into a warm-blooded animal (rabbits are usually used for this purpose, although mice, chicken, sheep, and goats can be used) resulting in the production of virus-specific antibodies in the animal's blood. Then collected blood is allowed to clot and the cells are centrifuged out to obtain the clear serum with antibodies (called primary antibodies). These antibodies can be used in ELISA tests. ELISAs are typically performed in 96-well (or 384-well) polystyrene plates, which can passively bind antibodies and proteins.

Many formats of ELISA have been developed and fall into two broad categories: "direct" and "indirect" ELISA procedures. They differ in the way the antigen–antibody complex is detected, but the underlying theory and the final results are the same. In the **direct ELISA** test, which is considered to be the simplest form of ELISA, the antigen is adsorbed to a plastic plate. Then an excess of another protein (normally bovine serum albumin) is added to block all the other binding sites of the plastic plate. The next step is the addition of an antibody that binds only to the targeted antigen. An enzyme is linked to this antibody in a separate reaction and this joining of the enzyme to the antibody is often called conjugation and the enzyme-antibody complex is called an "enzyme conjugate". Then this enzyme-antibody complex is adsorbed to the antigen and it is detected by using a color development scheme so that wells with virus samples will display a specific color, depending on the substrate used. One advantage of direct ELISA is that it needs minimum procedure. However, direct ELISA requires labeling of all primary antibodies, which is not economical and not every antibody is suitable for labeling.

The second type of ELISA is **indirect ELISA**, which is a variant of the direct ELISA. In indirect ELISA, the primary antibody used to detect the antigen is not conjugated to an enzyme (primary antibodies are not labeled). Instead, an enzyme-conjugated secondary antibody, which is reactive against the primary antibody is added to the mixture**.** That is why the method named "indirect ELISA"**.** Use of a secondary antibody has many advantages. A secondary antibody amplifies a weak signal, allowing a previously undetectable signal to be visualized and lowering the detection threshold of the assay in question and also the labeled secondary antibodies are commercially available.

In addition to the above described basic ELISA formats, different variants of ELISAs are available and can buy as commercial "kits". Most commercially available ELISAs are sandwich format assays. One such format is called double antibody sandwich ELISA (DAS-ELISA) and it is so named because the antigen is "sandwiched" two different antibodies. In DAS-ELISA the antigen is **NOT** directly immobilized on the plate. Instead a **"capture"** antibody is immobilized on the surface of the wells of the plate. The "capture" antibody binds and retains antigen. An enzyme conjugated **"detector" antibody**, raised against a different epitope on the antigen is added to the plate. The standard colorimetric detection method (described above) is used to detect and quantify the antigen in the sample. Sandwich ELISA and Competitive ELISA are some other variants of ELISA, which we will not discuss much into details in this manual.

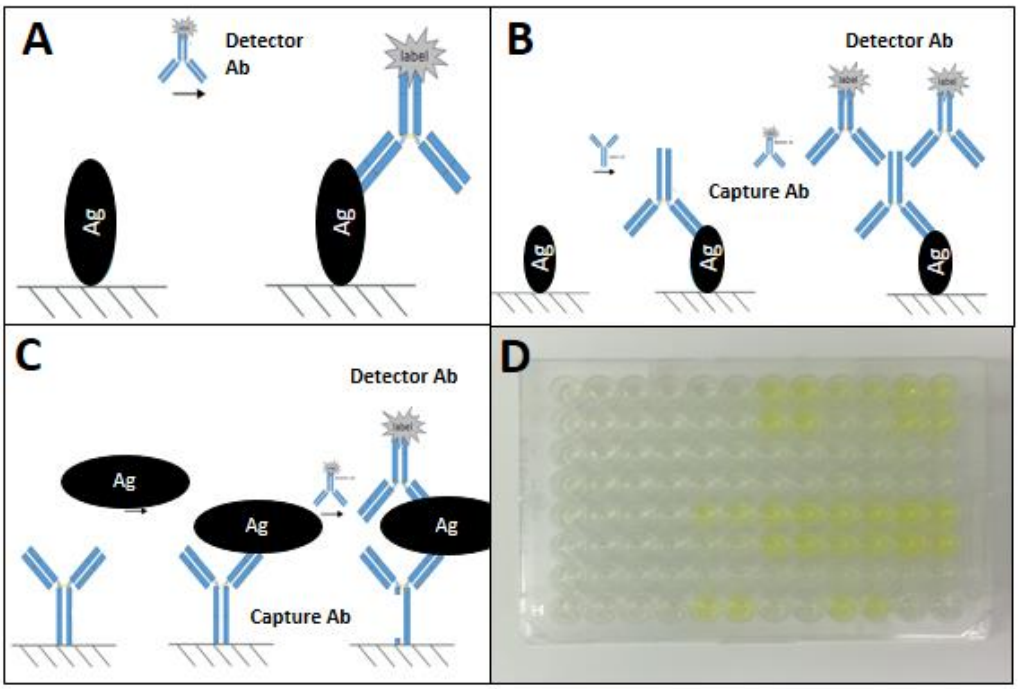

Figure 1: Schematic diagram of ELISA. A: Direct ELISA, B: Indirect ELISA, C: DAS-ELISA (Figure modified from, http://www.antibodiesonline.com). D: An ELISA plate showing the color development on virus infected samples (Pic. by Dr S. Bag, OSU, OR, USA).

Polymerase Chain Reaction (PCR) for virus detection:

In this experiment we will focus mainly on nucleic acid based virus disease diagnosis. However, one has to remember that direct PCR can only be used with DNA viruses and many plant viruses have RNA as their genetic material. In such cases, converting RNA into cDNA using reverse transcriptase enzyme prior to PCR is the common practice. Nucleic acid based disease diagnosis is not limited to viruses, it can be applied to any pathogen. In any case first step is the extraction of nucleic acids.

Nucleic acid extraction:

For virus detection, total nucleic acids can be extracted from various types of infected tissues such as seedlings, leaves, cotyledons, seeds, endosperm, tissue culture callus or roots. The most commonly used basic plant nucleic acid extraction protocols are those described by Dellaporta et al., 1983 and Saghai Maroof et al., 1984, along with many others with modifications of the components of these protocols to suit a particular tissue type or plant species. In addition to these basic protocols, wide range of DNA/RNA isolation kits based on either anion exchange chromatography or silica gel membranes are available commercially. In general, any DNA extraction protocol comprises of the basic steps of disruption of the cell wall, and membranes to release the DNA into solution and precipitation of DNA while ensuring removal of the contaminating biomolecules such as the proteins, polysaccharides, lipids, phenols and other secondary metabolites.

a) The extraction buffer:

The first step is grinding the selected tissue sample in the presence of extraction buffer. The buffer is consisted with a detergent such as cetyl trimethyl ammonium bromide (CTAB) or sodium dodecyl sulfate (SDS) to help disrupt the cell membranes by removing the lipids of the cell membranes.

Ethylene diamine tetra acetate (EDTA) in the buffer helps removing Mg^{2+} ions that are essential for preserving the overall structure of the cell membrane and it also helps chealating Mg2+ ions required for DNase activity. The buffer usually contains a reducing agent such as β-mercaptoethanol, which helps in denaturing proteins by breaking the disulfide bonds and helps in removing the tannins and polyphenols present in the crude extract. When handling β-mercaptoethanol one has to take extra precautions due to its extreme toxicity and grinding step should be carried out in a fume hood. Next step is the removal of insoluble cell debris and partially digested organelles by centrifugation, leaving the cell extract as a reasonably clear supernatant.

b) Precipitation of proteins:

DNA extracts commonly contain undesirable contaminants that are chiefly made of proteins and RNAs. A variety of procedures can be used to remove these contaminants, leaving the DNA in a pure form. The standard way to deproteinize a cell extract is to add phenol or a mixture of phenol:chloroform. These organic solvents precipitate proteins leaving the nucleic acids in aqueous solutions. Centrifugation is performed intermittently and the upper aqueous phase is transferred to a new tube while avoiding the interphase. The contaminants are denatured and accumulate in the organic phase or in the marginal layer between the two phases and the nucleic acids are preserved in the aqueous phase. Another way of removing proteins is by using the enzyme proteinase K, which however again is denatured by phenol via phenol chloroform extraction.

c) Precipitation of DNA:

The most frequently used method of DNA concentration is ethanol precipitation. This requires diluting the nucleic acid with a monovalent salt, adding alcohol to it and mixing gently. In the presence of salt and at a temperature of -20 °C or less, absolute ethanol will efficiently precipitate nucleic acids. With a concentrated solution of DNA one can use a glass rod to spool the adhering DNA strands. For dilution purposes the precipitated DNA can be collected by centrifugation and re-dissolving in an appropriate volume of water. The salts and alcohol remnants are removed by washing with 70% alcohol. Ethanol or isopropanol are the standard alcohols used for nucleic acid precipitation. The nucleic acid pellet can be re-suspended in either sterile distilled water or TE buffer (10 mM Tris: 1mM EDTA). The DNA is further purified by incubating the nucleic acid solution with RNaseA (10mg/ml) at 37° C, which will rapidly degrade RNA molecules into ribonucleotide subunits.

Polymerase Chain Reaction (PCR):

Polymerase Chain Reaction (PCR) is a revolutionary technique developed by Dr Kary Mullis in the 1980s. It is based on the ability of DNA polymerase to synthesize a new strand of DNA *in-vitro* when dNTPs (mixture of all four nucleotides), buffer with $MgCl_2$, forward and reverse primers, Taq polymerase and template DNA are present under appropriate conditions.

The role of each component is given below.

DNA template - The sample DNA that contains the targeted sequence to amplify.

DNA polymerase - A thermo-stable enzyme that synthesizes new strands of DNA complementary to the template sequence. The most commonly used enzyme is Taq polymerase isolated from a bacterium, *Thermus aquaticus,* found in the hot springs of the US Yellowstone National Park.

Primers – Generally 18-22 long oligonucleotide sequences, which serve as a starting point for DNA synthesis. However, the length of a primer can vary depending on the type of reaction carrying out

under specific circumstances. When the proper annealing temperature is given, primer binds to its complementary strand of the template DNA initiating the synthesis of new DNA strands.

Nucleotides (dNTPs or deoxynucleotide triphosphates) – these are single units of the bases A, T, G, and C, which are essentially the "building blocks" for newly synthesizing DNA strands.

When all these components are present under optimum conditions, new DNA strands can be synthesized *in-vitro*. In general, PCR is carried out in three steps, denaturation, annealing and extension, using a thermal cycler, which can heat and cool the tubes with the reaction mixture in a very short time. A short description of each step is given below.

a) **Denaturation at 94° C**: At this step, double stranded DNA melts and becomes single stranded DNA.

b) **Annealing**: Selection of the annealing temperature depends on the nucleotide composition and length of primers. When the temperature lowers to annealing temperature, primers bind with its complementary site of the template strand initiating the synthesis of a new DNA strand.

c) **Extension at 72° C:** At this temperature, synthesis of a new DNA strand by the enzyme Taq polymerase takes place because this is the ideal working temperature for the polymerase.

These steps are repeated for ~35cycles. DNA strand made in one cycle will serve as a template for synthesis of a new DNA strand in the next cycle. This results in an exponential increase in PCR product as a function of cycle number (Refer Figure 2 for a schematic diagram of PCR).

Detection of PCR products using gel electrophoresis:

The PCR product can be detected using gel electrophoresis, a process in which an electrical current is used to separate molecules in a gel, based on their size and electric charge. Most commonly used gel type is agarose, a purified form of agar isolated from seaweeds. At the boiling temperature agarose melts and solidifies at the room temperature. The gel is made by dissolving agarose powder in TAE/TBE buffer and bring it to the boiling point. The solution is then cooled to approximately 55°C and poured into a casting tray which serves as a mold. A comb is placed across the end of the casting tray to form wells when the gel solution solidifies. The gel is then submerged in “running buffer” and the PCR products are loaded into each well. A loading dye is mixed with the PCR product/DNA before loading. Loading dye serves two purposes. First, it gives a color to the DNA sample facilitating the loading process and helps visual tracking of the sample migration. Second, it gives a density to the DNA sample so that DNA will not diffuse out of the wells. A Direct Current (D.C.) power source is connected to the electrophoresis apparatus and electric current is applied. Charged molecules in the sample enter the gel through the walls of the wells. Since DNA molecules have a net negative charge, those molecules migrate towards the positive electrode (anode). The buffer serves as a conductor of electricity and to control the pH. The rate at which linear DNA molecules migrate within the gel depends on the fragment size; larger the molecules, slower the rate of migration and smaller the molecules, faster the migration rate. Gels separating DNA require staining in order to be visualized.

The most commonly used stain for visualizing DNA is ethidium bromide (EtBr). Ethidium bromide is a mutagen and must be handled and disposed carefully. Visualization also requires a short wave ultraviolet light source (trans-illuminator). Gel can be stained with EtBr after running or

alternatively you may add EtBr into the gel before casting. This fluorescent dye intercalates between the nucleic acids and makes them visible under ultraviolet light and the occurrence of a band of the appropriate size indicates the presence of the virus for which the primers were specially designed for.

Learning outcomes:

At the end of this laboratory, you should be able to,

- Explain the principles involve in DNA extraction, polymerase chain reaction and agarose gel electrophoresis,
- Extract total nucleic acid, setup a PCR reaction and detect virus infection of a plant specimen.

Nucleic acid extraction

Materials:

Virus infected plant materials
Permanent marker
Micro Pipettes & micro pipette tips
1.5 ml microfuge tubes – 3 per sample
Mortar & Pestle
Container with ice
Dellaporta extraction Buffer w/ 0.1% β-mercaptoethanol
Extraction buffer composition
100 mM Tris (pH 8.0)
8.5mM EDTA
0.5mM NaCl
10mM β-mercaptoethanol (add **just before** use)
20% SDS
5M Potassium Acetate (KOAc)
100% Ethanol or Isopropanol – ice cold
Water bath @ + 65 ℃
Gloves
Centrifuge
Vortex

Procedure:

Variety of protocols are available and here we describe a very simple and fast protocol. However, one should remember that depending on the type of plant material being used, extraction method should be modified.

- Familiarize yourself on how to handle micropipette correctly following the **Figure 3.**
- Carefully observe infected tissue and compare it with healthy plant tissues. Make necessary notes on the symptoms. Grind about 50 mg of plant material in 500 µl of extraction buffer (add 0.1% βME just prior to use).
    - Ex: For 10 ml ext. buffer – remove 10 µl, then add 10 µl β-mercaptoethanol

- Note: You can increase amounts proportionally (i.e. 0.1 g plant material in 1 ml extraction buffer), yet after grinding, ONLY remove 500 µl of extract into 1.5 ml conical tube

- Pour extract (500 µl) into 1.5 ml microfuge tube (labeled)
- Add 40 µl of 20% SDS (sodium dodecyl sulfate)
- Vortex briefly then incubate in water bath at 65**°**C for 10 min.
- Bring to room temp., add 160 µl of 5 M Potassium Acetate
- Vortex, then centrifuge at 13,000 rpm for 10 min.
- Remove (pour) supernatant into another labeled tube. When removing supernatant, measure the amount being put into labeled (for -20**°**C storage) 1.5 ml microfuge tube
- Add Ice-cold Isopropanol or Ethanol (100% stock) in correct proportion
  *Proportions are critical. If isopropanol is used, use 1 : 2 ratio of isopropanol : supernatant. If ethanol is used, use 1:1 ratio of ethanol: supernatant
- Mix gently and **keep on ice** at 4**°**C for 15 to 20 minutes
  - Note: Longer incubation time at this step is better, > 30 minutes.
- Centrifuge at 13,000 rpm for 10 min. to precipitate the DNA
- Decant the supernatant gently (can pour off) and ensure the pellet is not disturbed
- Add 500 µl of 70% ethanol (ensure pellet is not disturbed), then centrifuge at 13,000 rpm for **5** min
- Carefully decant (can pour off) the supernatant and ensure the pellet is not disturbed.
- Repeat this step if necessary
- Air dry DNA at room temperature until no trace of alcohol can be seen in tube.
- Re-suspend DNA in 50 µl TE buffer and store at -20 **°**C
  **DNA can be re-suspended in Nuclease-free, sterile H_20 from a non-contaminated source

Polymerase Chain Reaction (PCR)

Materials:

PCR tubes
Micropipettes and tips
Microfuge tubes
70% Ethanol
Template DNA
PCR master mix

Master mix is consisted with the following components (per reaction)

1x PCR buffer + Mg^{+2}
0.2 mM dNTPs
10 µM each primer
0.2 U/µl Taq polymerase
Nuclease-Free H_2O to make 25µl
Template DNA 2µl

Procedure:

- You are provided with pre made master mix
- Add 2 µl of the extracted template DNA into each PCR tube
- Dispense 23 µl of master mix into each PCR tube and mix well
- Give a quick spin to bring total volume dawn to the tube

- Place the tubes in a thermal cycler and program the machine to complete PCR reaction as described below. This will take approximately 2-3 hours to complete the reaction

PCR program:

1	95 ºC : 3 min	Initial denaturation
2	95 ºC : 30 sec	Denaturation
3	52 ºC : 45 sec	Annealing temp. depends on the primers used
4	72 ºC : 30 sec	Extension – Time depends on length of product
5	72 ºC : 7	min: final extension
6	4ºC : ∞	

Repeat the steps 2-4 for 35 times

Gel electrophoresis

Materials:

Micropipettes and tips
Loading buffer
Parafilm
0.8% Agarose
TAE buffer
Gel apparatus

Procedure

- Refer Figures 3 and 4.
- Prepare a 0.8% agarose by measuring 0.8 g of agarose and mix with 100 ml of 1x TAE buffer in a 250-ml flask
- Melt agarose
- Pour molten Agarose (cooled to 55-60**°**C) into the sealed gel tray with comb
- Let the gel cool for 20 minutes and solidifies
- Once the gel is solidified, put 1X TAE buffer into the gel apparatus till the top of the gel is covered
- Carefully load 5 µl of PCR product with 5 µl of 1X loading buffer into each well.
- Load the molecular weight standard
- Run the gel at a constant voltage (~90 v) until the dye front reaches anode
- After electrophoresis is completed, stain the gel for 10 minutes in an ethidium bromide staining solution (0.25 µg/ml) if the ethidium bromide was not added to the gel prior to the casting
- Put the gel in UV trans illuminator and observed the presence and absence of bands in diseased and control healthy plants respectively
- Make necessary notes and draw a schematic diagram of your gel figure.

What can you conclude from the experiment?

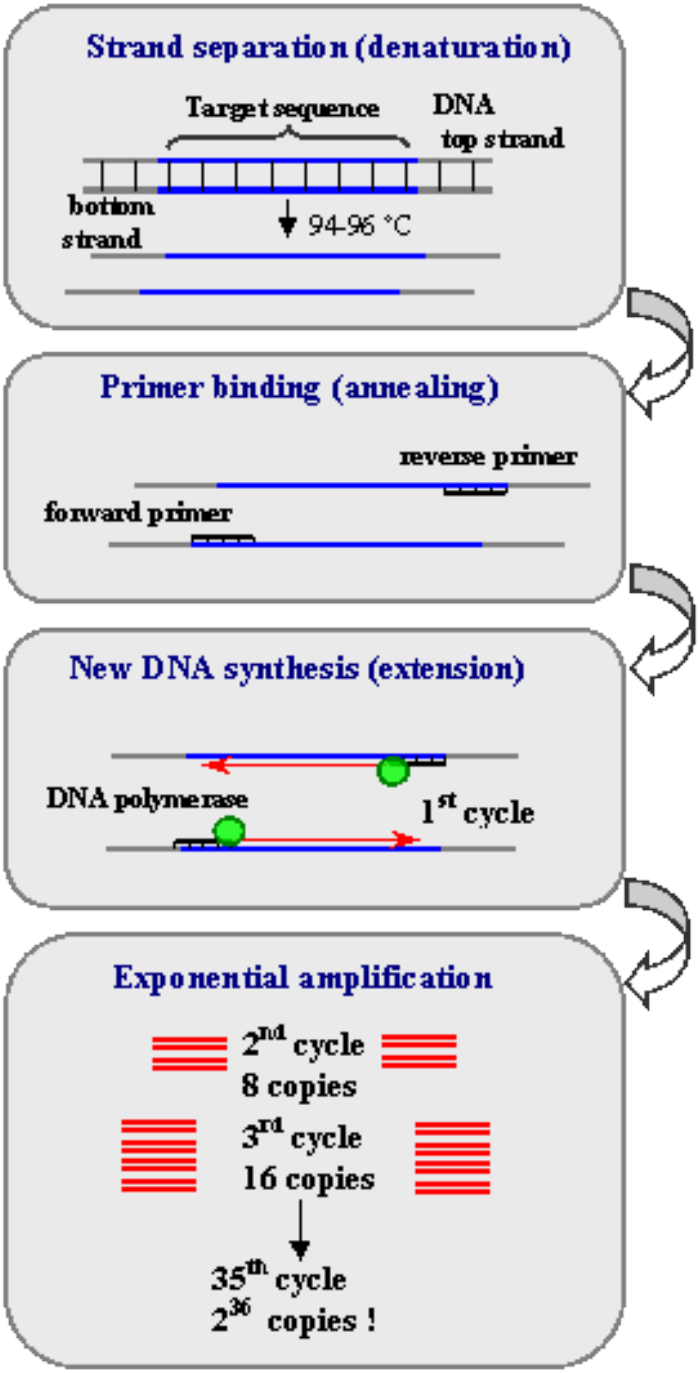

Figure 2: Schematic diagram of PCR reaction. Denaturation annealing and extension steps are shown in each box. Figure source: http://www.ncbi.nlm.nih.gov/genome/probe/doc/TechPCR.shtml

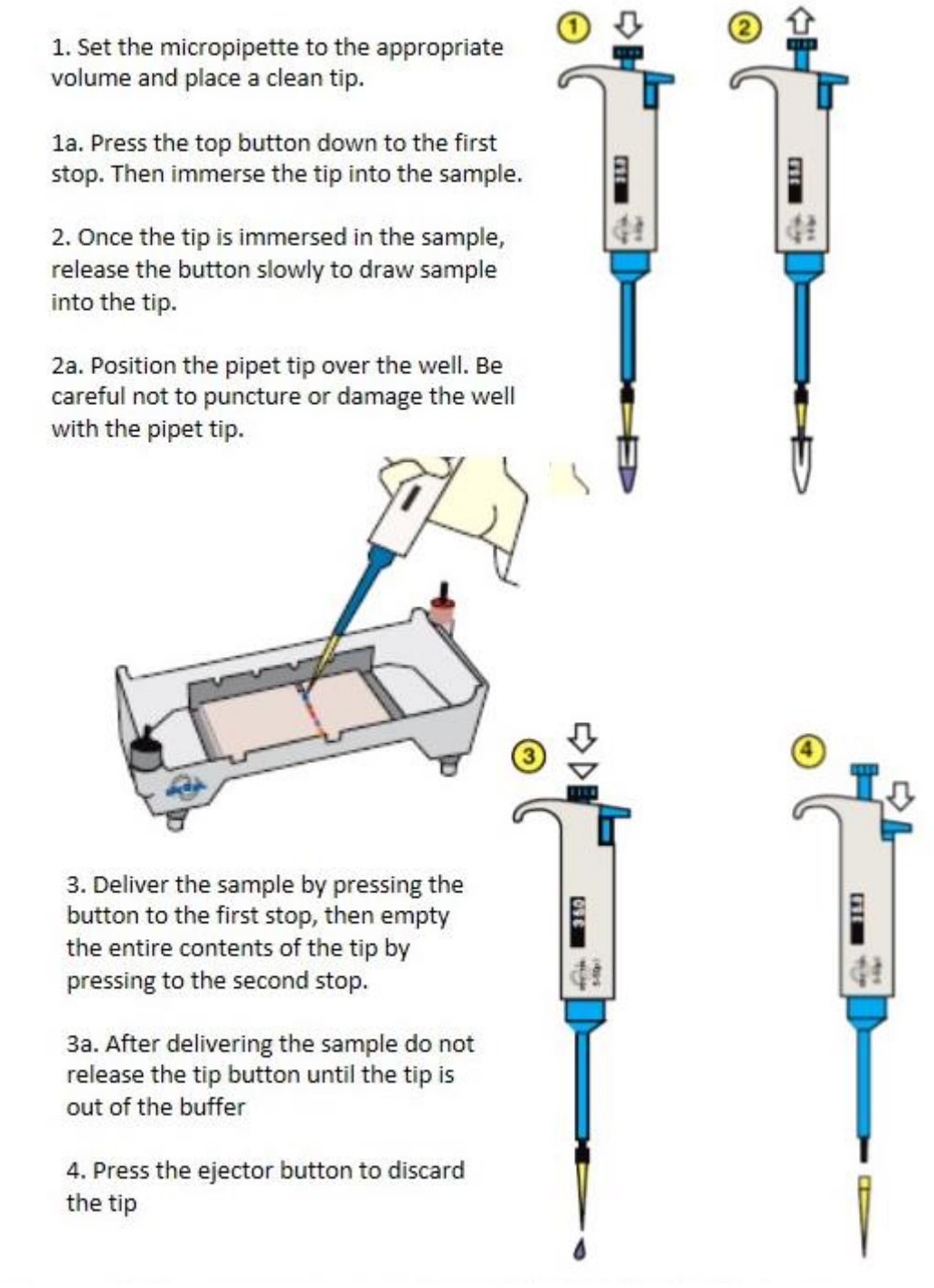

Figure 3: Schematic diagram of proper handling of micro pipette and loading the gel
Figure modified from: https://www.wou.edu/las/physci/ch462/Gel%20Electrophoresis.pdf

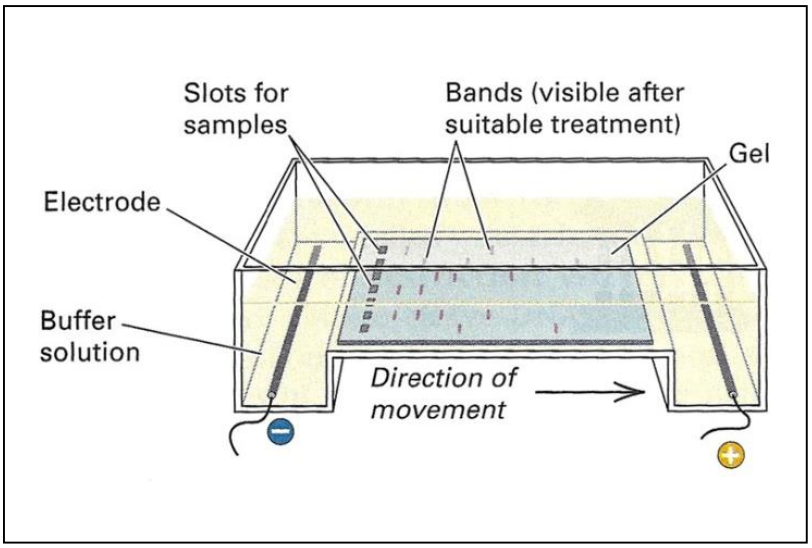

Figure 4: Agarose gel electrophoresis of PCR products. A schematic diagram of a gel tray.
Figure source: https://www.wou.edu/las/physci/ch462/Gel%20Electrophoresis.pdf

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