LABORATORY MANUAL ON DETECTION OF GENETICALLY MODIFIED ORGANISMS

SAFETY ASPECTS, PRINCIPLES AND PROCEDURES

Smegnew Melese
Belete Getnet
Alemayehu Esayas
Amare Genetu
Adugnaw Admas

April 2022
Addis Ababa, Ethiopia

Ethiopian Environment & Forest Research Institute

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I Preface

The Biosafety Laboratory of the Ethiopian Environment and Forest Research Institute (EEFRI) was established mainly to serve as detection laboratory for Genetically Modified Organisms (GMO) as well as to conduct molecular biotechnology research. Well documented and suitable methods are necessary to carry out a reliable GMO detection in a variety of samples including important export food items. In this regard, the Environmental Laboratory Directorate has a goal to get ISO certification for analysis of GMOs in different samples. One of the requirements for this certification is to have a clear manual on GMO detection. Therefore, this manual is meant to serve as a standard manual for the laboratory personnel and also to other interested professionals from other institutions. The clear procedures given in the manual will be serving as a training material and so as to obtain reliable analytical results by following the established procedures stated in the material,

Basically the manual consists of a variety of techniques for GMO detection, identification, characterization, and quantification. The main parts of the manual It has nine main parts: introduction, general instructions, laboratory safety procedures, rules and general guidelines, preparation of solutions and their uses, equipment description, detection of GMO in food samples, agarose gel electrophoresis, polymerase chain reaction (PCR), and laboratory service, and standard operational procedures (SOP). Wherever possible, attempt has been made to incorporate principles, procedures and protocols that are currently in use in modern GMO detection laboratories.

Finally, it is important to note here that the manual will provide theoretical knowledge and practical skills needed by researchers, lab technicians, and others who are interested to use the manual. The authors welcome constructive comments from readers and users of the manual to come up with a better version in the future.

Authors

I. Foreword

As Deputy Director General of the Ethiopian Environment and Forest Research Institute (EEFRI) for the past six years or so, I have been playing a pivotal role in leading research and capacity building. In this regard, we have initially set a goal to be among the best five similar research institutes in Africa in 2025. Since its establishment in December 2014, EEFRI has strategically been working towards achieving this goal by crafting problem solving mega projects, carrying out a rigorous research review process, conducting quality research, delivering information and technologies in the fields of environment, forest and climate change which are published in local and international journals, annual proceedings and package of practices. Moreover, the institute has secured government budget and started the construction of two laboratory complexes which, when completed, are expected to significantly improve the work environment at EEFRI. I believe our researchers shall enjoy spacious offices as well as equipped laboratories enabling them to conduct more advanced scientific research and produce accurate and reliable laboratory data.

In line with the human and material resources development plan, it is essential that documents compiling standard methodologies are available. Such documents are a must to have to carry out standard laboratory analysis and request ISO certification for the analysis of different parameters. Recognizing this demand, different manuals have already been prepared by different scholars in our institute. This manual, prepared by a team of dedicated researchers with complementary knowledge and skills, is an attempt to have a standard manual on GMO detection in suspected samples such as crops planned for export. In this respect, our institute has already agreed with the Commission for Environment, Forest and Climate Change to carry out the detection of GMOs in agricultural export products. Thus it is clear that the manual shall have a significant contribution in generating accurate and reliable analytical data and develop trust from the customers.

I believe the contents, order of presentation and style of writing this manual is up to the standard. I would like to take this opportunity to thank and appreciate the authors of this manual for their significant inputs and contribution. Users of the manual are highly encouraged to provide critical feedback so that the authors will have the opportunity to produce a revised version. It is hoped that the manual will serve not only EEFRI researchers but also other higher learning and research institutions with interest on GMO detection. Enjoy reading the manual and perform a reliable GMO detection!

Agena Anjulo (Ph.D.)
Deputy Director General
October 2022

II. Acknowledgements

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The information used in this manual has been adapted from different published and unpublished (web based information) sources. Thus all the sources used for preparing the manual are also acknowledged. Finally, the authors would like to acknowledge the contribution of manual publication committee members (Dr Yigardu Mulatu, Dr Alemayehu Negasa and Dr Abayneh Derero) for providing critical comments and suggestions for the improvement of the manual.

III. Acronyms

CTAB	Cetyl trimethyl ammonium bromide
cDNA	Complementary DNA
ddH_2O	Double distilled water
dH_2O	Distilled water
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked ImmunoSorbent assay
EtBr	Ethidium bromide
GMOs	Genetically modified organisms
LMOs	Living modified organisms
M	Molarity
MSDS	Material safety data sheet
PCR	Polymerase chain reaction
qPCR	Qualitative PCR
RT-PCR	Real Time PCR
SDS	Sodium dodecyl sulfate
SOP	Standard operational procedures
TAE	Trise Acetate EDTA
Taq	*Thermus Aquaticus*
TBE	Trise Borate EDTA
TE	Tris EDTA
UV	Ultraviolet

Table of Contents

1 Introduction

Agricultural products have been improved through saving and selecting crop seeds with specific traits for the past 10,000 years. During the past 150 years, farmers and scientists have used crossbreeding through selection, hybridization, and mutation technology to shorten the duration of plant reproductive cycles. However, transferring and recombining of entire genomes instead of single gene of interest, and the subsequent development of high quality and genetically stable variety is a slow process. To alleviate this drawback, novel techniques such as genetic transformation, genomic selection, high throughput phenotyping, and marker assisted selection breeding have been developed in biotechnology. These novel techniques have allowed scientists to introduce very specific traits via gene insertion that comes from the same or different species of plants, or even from animals or bacteria. An organism whose genetic material (DNA) has been modified using genetic engineering techniques is called transgenic organism or genetically modified organism (GMOs). Products such as preserved foods, food additives, vaccines, drugs, enzymes, and others produced by genetic engineering process from any organism are known as GM products.

The use of modern biotechnology for producing GMO or sometimes called living modified organism (LMO) can serve four general purposes:

(1) Agricultural purpose: to produce novel and new ornamental plants, disease resistant plants or animals, insect and herbicide resistant plants, to increase yield, nutrition and quality of plants or animals
(2) Environmental purpose: for bioremediation, biodegradation, biotransformation of wastes, to reduce use of pesticides, herbicides, fertilizers and fossil fuels
(3) Industrial purpose: to produce enzymes, amino acids, antibiotics, and metabolites; and
(4) Medical purpose :for prevention of diseases by producing edible vaccines, for production of biopharmaceuticals, therapeutic proteins and monoclonal antibodies, and for diagnostic purposes

Despite the claimed benefits of GMOs, societal concerns on the possible potential risks of using GMO products have increased internationally. To address the societal concern, GMO detection mechanisms have been developed, improved and adopted in many countries. Transgenic products are required to undergo safety assessments to fulfill the regulatory requirements prior to commercialization. The aim of the assessment is to evaluate the impact of transgenic products on humans, animals, and the environment.

In Ethiopia, the Commission for Environment, Forest and Climate Change is mandated with providing a GMO free certificate to exporters of agricultural products. In accordance with this, the Ethiopian Environment and Forest Research Institute (EEFRI) agreed to provide analytical service for checking absence or presence of GMOs in samples that are delivered to the Biosafety Laboratory. Therefore, this manual is written to demonstrate that EEFRI has made the necessary

preparation to discharge its responsibility. The manual assures our customers that standard methodologies and procedures are implemented in the GMO detection process.

The manual consists of nine main parts: introduction, general instructions, laboratory safety procedures, rules and general guidelines, preparation of solutions and their uses, equipment description, detection of GMO in food samples, agarose gel electrophoresis, polymerase chain reaction (PCR), and laboratory service, and standard operational procedures (SOP).Wherever possible, attempt has been made to incorporate principles, procedures and protocols that are currently in use in modern GMO detection laboratories. The technical manual also contains format of a laboratory notebook that includes sample type, date and page number on the top of the page for all GMO detection analysis samples.

2 Laboratory Safety Rules and Safety Equipment

Before starting to work in the Biosafety Laboratory, all personnel involved should be familiar with laboratory safety, principles and rules that are essential to avoid hazards before an accident occurs.

2.1 Safety Rules

2.1.1 General Laboratory Safety Rules

Laboratory safety rules cover the following safety aspects:

- What to do in the event of an emergency
- Where proper signage and safety equipment are located
- How to use laboratory equipment
- Apply basic common sense rules

The following general safety rules should also apply whenever you work in the Biosafety Laboratory.

- If you are going to work in the lab for the first time, you must get orientation before commencing any activity in the lab.
- Be aware of where the laboratory's safety equipment such as first aid kit(s), fire extinguishers, eye wash stations, and safety showers are located, and know how to properly use them. See details on safety equipment in section **2.2** below.
- Be sure to observe where laboratory exits, fire alarms and safety signs are located and follow the instructions in the event of an accident or emergency.
- Use the list of important emergency phone numbers posted on the laboratory wall and contact first the laboratory supervisor or higher officials in case of emergency.
- Note that laboratory areas containing different chemicals are properly marked with the appropriate warning sign.
- Never use open flames in the laboratory unless and otherwise you have got permission from the laboratory supervisor.
- Always work in properly-ventilated areas.
- Do not chew gum, drink, or eat while working in the lab.
- Do not use laboratory glassware as food or beverage containers.
- Do not store food items in laboratory refrigerators or freezers.
- Be sure to check for chips and cracks whenever you use laboratory glassware and notify the laboratory supervisor about any damaged glassware so that it can be properly disposed of.
- Never use laboratory equipment that have not been approved for use and before you are trained to operate the equipment.

- If an instrument or equipment fails during use, or isn't operating properly, report the issue to the laboratory supervisor right away. Never try to repair equipment on your own.
- If you are the last person to leave the lab, remember to lock all the doors and turn off all ignition sources.
- Do not work alone in the laboratory unless you get a special permission from the laboratory supervisor.
- Never leave an ongoing experiment unattended.
- Never lift any glassware, solutions, or other types of apparatus above eye level.
- Never smell or taste chemicals.
- Do not pipette by mouth.
- Make sure that you always follow the proper procedures for disposing laboratory waste.
- Report all injuries, accidents, and broken equipment or glass right away, even if the incident seems small or unimportant.
- If you have been injured, please call for help immediately and as loud as you can to ensure you get help.
- In the event of a chemical splashing into your eye(s) or on your skin, immediately flush the affected area(s) with running water for at least 20 minutes (min). Seek medical attention afterwards if the case is serious.
- If you notice any unsafe conditions in the laboratory, let the laboratory supervisor be informed about it as soon as possible.

2.1.2 Dress Code Safety Rules

As you would expect, laboratory dress codes set a clear policy for the clothing that laboratory users should avoid wearing to prevent accidents or injuries.

- Always tie back hair that is chin-length or longer.
- Make sure that loose clothing or dangling jewelry is secured, or avoid wearing it.
- Never wear sandals or other open-toed shoes in the laboratory. Your feet should be covered completely.
- Never wear shorts or skirts in the laboratory.
- When working with Bunsen burners, lighted splints, matches, etc., artificial (acrylic) nails are not allowed.

2.1.3 Personal Protection Safety Rules

Personal protection safety rules refer to what researchers, technicians and laboratory assistants should be wearing in the laboratory to protect themselves from various potential hazards and follow basic hygiene rules to avoid any sort of contamination.

- Always wear face shields or safety glasses when working with laboratory equipment, hazardous materials, glassware, heat, and/or chemicals.
- Always wear appropriate gloves when handling any toxic or hazardous agents.
- Always wear a smock or laboratory coatwhen performing laboratory experiments.
- Be sure to keep hands away from the body, mouth, eyes, and face when using laboratory equipment and chemicals.
- Always wash your handsbefore leaving the laboratory or eating.

2.1.4 Electrical Safety Rules

The electrical safety rules thathelpus prevent the misuse of electronic instruments, electric shocks and other injuries are the following:

- Make sure that any damaged equipment, cables, or plugs are reported to the appropriate authority so that they can be repaired or replaced.
- Make sure that permission is obtained from the laboratory head before using any high voltage equipment (voltages above 50Vrms ac and 50V dc).
- Make sure all electrical panels are unobstructed and easily accessible.
- Be aware that high voltage equipment should never be changed or modified in any way.
- Use only one hand if you need to adjust any high voltage equipment. It's the safest to place the other hand either behind back or in a pocket.
- Avoid using extension cords whenever you can.

2.1.5 Housekeeping Safety Rules

- The following important housekeeping safety rules should exercise:
- Keep all common areas tidy and clean, andfree of dirty dishes and disordered equipment.
- Label all solutions and other containers to be kept ina refrigerator and incubator.
- Make sure that all eye wash stations, emergency showers, fire extinguishers, and exits are always free and accessible.
- Do not keep materialsother than thatrequired for the present work in the working area. .
- Keep lightweight items on top of cabinets, and heavier items at the bottom.
- Keep solids out of the laboratory sink.
- Keep any equipment that requires air flow or ventilation in a clear area to prevent overheating.

2.1.6 Chemical Safety Rules

Safe handling and storage of chemicals has a vital role in a laboratory setting for new and senior users. Therefore, you should apply the following chemical safety rules:

- Read the material safety data sheet (MSDS) before you start working with chemicals.
    - MSDS is a technical bulletin that describes detailed potential hazard chemicals, reactivity, physical data and properties, safe handling, chemical name, chemical abstract service (CAS#), spill or leak and precautionary information procedures for commercially available chemical products.
    - MSDS information can be accessed on the web of the Biological Sciences Home Page (BSHP).
- Treatevery chemical as though it was dangerous.
- Do not allow any chemical to come into contact with the skin.
- Labelall chemicals clearly. The label should contain:
    - Name of the chemical
    - Concentration
    - Molecular weight
    - Grade
    - Storage temperature
    - Date it was received.
- Read the label on the chemical container before taking any of the contents.
- Never take more chemicals from a bottle than needed for the required work.
- Do not put unused chemicals back into the original container.
- Never takechemicals or other materials out of the laboratory.
- Nevermix chemicals in sink drains.
- Ensure that all chemical waste is disposed of properly.
- Do not put chemicals near the sink.
- Inform janitors not to clean empty chemical containers wherever they find them.

2.1.7 Other Safety rules

Special attention must be given to safety rules related to electricity, ultraviolet light, glassware, plastics, tubes, micropipette, microcentrifuge tube and other chemicals or media. These include the following:

- Cover the buffer tanks during electrophoresis.
- Always turn off the power supply and unplug the leads before removing a gel.
- Always wear appropriate eye protection when using UV lamps.
- Cleanand rinse glass and plastic wares with distilled water carefully and autoclave or bake at 150°C for 1 hour.
- Autoclave micropipette tips and micro-centrifuge tubes before use.
- Treat glassware and solutions with diethylpyrocarbonate for RNA analysis to inhibit RNases, which can be resistant for autoclaving. Discardany uncontaminated, solidified agar or agarose in the trash, not in the sink, and rinse the bottles well.

- Autoclaveany contaminated medium promptly before it is discarded.
- Use flammable and volatile chemicals such as phenol and chloroform only in a fume hood.
- Handle the chemical EtBr as follows:
    - Storeitin a brown or foil-wrapped bottlebecause it is light sensitive.
    - Always wear gloves, goggles, and a laboratory coat during preparation involving EtBr since it is highly toxic. .
- Do not forget to treat it before disposal in a labeled container and wear gloves during this process.

Safety measures to be taken while running horizontal gel electrophoresis:

- Read the instructions before using the apparatus.
- Always remove electrophoresis units from the power supply before removing the safety cover.
- Remove the power supply from the mains first, then disconnect the leads.
- Do not exceed the maximum operating voltage or current.
- Do not operate the electrophoresis units in metal trays.
- Following the replacement of a platinum electrode have the unit inspected and approved by your safety officer prior to use.
- Do not fill the unit with running buffer above the maximum fill lines and
- Do not move the unit when it is running.
- Be cautious about the following:
    - *During electrophoresis very low quantities of various gases are produced at the electrodes. The type of gas produced depends on the composition of the buffer employed. To disperse these gases make sure that the apparatus is run in a well-ventilated area*.

Safety conventions used in Applied Biosystems 7500/7500 Fast Real-Time PCR System:

In this instrument there are safety alert words: Four safety alert words appear in ABIs operation manual at points in the document where you need to be aware of relevant hazards. Each alert word implies a particular level of observation or action as shown below:

Important! - Indicates information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

Caution!- Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

Warning!-Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

Danger! -Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

Except for IMPORTANTs, each safety alert word in ABIs document appears with an open triangle figure that contains a hazard symbol. These hazard symbols are identical to the hazard icons that are affixed to ABIs (for further references about "Safety Symbols" see the link: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/4387783c.pdf).

General safety labels on instrument:

In ABIs, users must consider safety, and read the MSDSs about the disposal of hazardous wastes, chemicals, handling of the instrument, hot surface, replacing of lamp, high voltage etc. Therefore, when working with ABIs users must be careful for the following hazard types:

- **Physical injury hazards**: It occurs on work such as moving and lifting the instrument, moving and lifting standalone computers and monitors, operating and cleaning or decontaminating the instrument.
- **Chemical hazards and safety**:Users must consider chemical hazards warning, storage, safety guidelines, waste storage and disposal.
- **Electrical safety and hazard**:Users should be careful from exposure to electrical shock hazards, fuse fire, fire, power, overvoltage and rating.
- **Biological hazards (Biohazards) safety**:biological samples such as tissues, body fluids, infectious agents, and blood fromhumans and animals have the potential to transmit infectious diseases. Hence users must follow and exercise all applicable local, state/provincial, and/or national regulations, and wear appropriate protective equipment (protective eyewear, face shield, clothing/lab coat, and gloves).
- **Workstation safety**:Correct ergonomic configuration of the workstation can reduce or prevent effects such as fatigue, pain, and strain. Minimize or eliminate these effects by configuring workstation to promote neutral or relaxed working positions.
- **Protocol Sheet**:Read installation handbook, usage and maintenances manual, and paying careful attention to the "safety information" and "important notes" supplied by the manufacturer's instrument procedures before doing anything.

Safety guidelines when using thermal cycler:

Read the following guidelines before using the instrument and be sure to conform to the following basic security measures:

- Do not use the instrument in humid, dusty, high temperature and magnetic environment.
- Do not touch the inner device of the instrument when opening the cover.
- Do not block the air flow vents.
- Keep the instrument clean and maintain it regularly.

2.2 Safety equipmenttypes, uses and their operations

The availability of different types of safety equipment in a laboratory is essential to the practice of safe science. Safety equipment should be present in well-marked, highly visible, and easily accessible locations in or near all laboratories that use hazardous chemicals. Essential emergency laboratory safety equipment that should be present in a Biosafety Laboratory is described in the following sections.

2.2.1 Fire Extinguishers

Laboratory personnel working with flammable liquids must receive training on the types and proper use of fire extinguishing equipment. Laboratories have different classes of fire extinguishers among which liquid CO_2 and H_2O extinguishers are the common ones.

To use a fire extinguisher, follow the steps below:

- **P**: Pull and twist the locking pin to break the seal.
- **A**: Aim low, and point the nozzle at the base of the fire.
- **S**: Squeeze the handle to release the extinguishing agent.
- **S**: Sweep from side to side until the fire is out.
- Be prepared to repeat the process if the fire breaks out again.

2.2.2 Eyewashes and Safety Showers

Emergency eyewash and shower equipment should be provided wherever there is a risk of accidental splashes of chemicals onto the skin or eyes.

Eyewash and safety showers include the following:

- Know the locations of safety showers and eyewashes, and how to use them; keep routes free and areas under showers clear.
- Only potable water should be supplied to eyewashes and safety showers.
- Delivered hot water should not be at the extremes(comfortable range is 15-35^{o}C).
- It should be easy to locate and identify with a clearly visible sign.
- Small eyewash units attached to the laboratory sink are not as good as the special-use units.
- Eyewash bottles should be to the acceptable standard to deliver sufficient amounts of water and avoid contamination with microorganisms.
- All users who might be exposed to chemical splashes and dust particles or objects entering the eye must be trained in the proper use of emergency eyewashes and showers.
- In case of emergency such as chemical spill on face and eyes, flush your eyes for at least 15 min, holding the eyelids open; rinse the eyes completely under and behind the eyelid.

- If only one eye is splashed, take care to avoid contamination of the other eye.
- For splashes to the body, take a shower for not less than 15 min, and soak completely the removed clothes during showering process. A helper may be required to ensure complete cleansing.
- Always seek medical attention as soon as possible after the 15 min wash period.
- Be aware that, in some situations, you may need to dispose of the shower water and clothing as hazardous waste.
- Activate weekly the showers, and eye/face washes to flush the line and verify proper operation; let the water flow for at least 3 minutes.

2.2.3 First Aid

First-aid services must be provided to laboratory users in accordance with factories and other places of work. Requirements of these directives include reporting procedures, training, and the provision of first-aid attendants, kit, and rooms.

Be aware and practice the following when the need arises:

- Users sustaining injuries or illness shall report to a first-aid attendant as required.
- Up-to-date first-aid attendants' name and location schedule must be posted.
- Arrangement for first-aid training is the responsibility of the employer.
- Place first-aid kits in the laboratories and at designated first-aid stations.

2.2.4 Fire blankets

Fire blankets are recommended in all laboratories that use flammable liquids. It should be easily accessible and unobstructed. It can also be used to keep shock victims warm.

The following STOP-DROP-and-ROLL method should apply in case of fire accident:

- In the event that a person's body or clothing catches fire, the person should immediately drop to the floor and roll to help extinguish the fire.
- It should also be used only as a last resort to help smother a body or clothing fire.

2.2.5 Safety Cans

A safety can is a container of not more than five-gallon (ca.20 L) capacity, having a spring closed lid, spout cover, and flame arrestor and so designed that it safely relieves internal pressure. Itmust be compatible with the chemical that they are to contain.Safety cans with damaged parts such as corroded spark arrestor screens or insufficient springs must be taken out of service and replaced immediately.

2.2.6 Chemical Spill Kit

Every laboratory that uses hazardous chemicals should have access to a spill control kit.The keys to an effective spill kit are location and contents.

- Locate spill kits around work areas in fixed spots so that it will be easily accessible.
- Label the drawer or cabinet as “Spill Kit”if ithas been placed in a drawer or cabinet.
- Aspill kit should contain absorbent material, appropriate personal protective equipment, a container for spill residue, and a plastic dust pan and scoop.
    - Absorbents: Universal spill absorbent material- 1:1 mixture of sodium bicarbonate: Flor-Dri (unscented kitty litter) or a 1:1:1 mixture of Flor-Dri: Sodium Bicarbonate: Sand. This all-purpose absorbent material is good for most chemical spills including solvents, acids, and bases.
    - Use the following as remedy for different types of chemical spills:
        - For Acid spill, use sodium bicarbonate, sodium carbonate, or calcium carbonate.
        - For Alkali (base) spill, use sodium bisulfate.
        - For solvents/other organic liquids, use inert absorbents such as vermiculite, clay, sand, and chemicals supplied by Flor-Dri, and Oil-Dri companies.

2.2.7 Personal Protective Equipment

Goggles and face shield, plastic vinyl booties, disposable coveralls and apron, disposable vinyl gloves and heavy neoprene glovesshould wear as required.

2.2.8 Clean-Up Material

The laboratory should provide available plastic dust pan and scoop, plastic bags (30 gallon, 3 millimeter thick), one empty 5 gallon, plastic bucket with lid for spill and absorbent residues.

3 Chemicals Used in the GMO Detection and Preparation of their Solutions

3.1 Common chemicals needed in the GMO Detection

The following chemicals are commonly used for GMO detection in the Biosafetylaboratory. These are:

- **Phenol**: It is used to denature the proteins.It helps to remove non-polar proteins and lipids from the solution.
- **Chloroform**:It increases the efficiency of phenol for denaturation of the protein. It also allows the proper separation of the organic and aqueous phases,and solubilizes lipids and a lot of proteins to remove them from the DNA.
- **Iso-amyl alcohol**: It is used to prevent from phosgene and reaction of chloroform with air.
- **Tris-HCl**: It is used to remove unwanted cellular components and precipitation, and used to maintain a stable pH in DNA extraction. It controls the pH under buffering systems for electrophoresis applications.
- **Ammonium acetate**: It is used for DNA & RNA precipitation from very dilute solutions after centrifugation.
- **Glycogen**: In the presence of salts, it forms a precipitate that traps the target nucleic acids and quantitatively precipitates nucleic acids from diluted solutions with a higher efficiency of DNA/RNA.
- **Ethanol**: It is a dehydrating agent and used for the precipitation of DNA molecule in DNA isolation. It removes salts and other water soluble impurities but does not re-suspend the DNA. It also traps water and facilitates Na^{+} to interact with PO_4^{3-} and then precipitate DNA.
- **CTAB (Cetyltrimethyl ammonium bromide)**:It is a cationic detergent that facilitates the separation of polysaccharides during purification.
- Sodium chloride: Phosphates of DNA molecule repel one another. Na^{+} ions form an ionic bond with phosphates and neutralize the negative charge allowing the DNA molecules to group in.
- **EDTA (Ethylene diamine tetraacetic acid)**: It works as ligand and chelating agent in the DNA extraction. By chelating the metal ions, it deactivates the enzyme, and therefore, reduces the activity of DNase and RNase, i.e., especially useful for sequestering calcium (Ca^{2+}), magnesium (Mg^{2+}) and iron (Fe^{3+}) ions.
- **RNaseA**: An enzyme that breaks down RNA and shall be added in the final solution to ensure that no RNA is in the solution that contaminates during extraction of pure DNA.
- **Proteinase K**: It is used to digest contaminating proteins or nucleases that might interfere or inhibit downstream application of DNA. Addition of it rapidly inactivates nucleases that might otherwise degrade the DNA or RNA during purification.
    - **TE (Tris EDTA)**: It is used to re-suspend the extracted DNA in a buffer to ensure stability and long term storage. It is used to solubilize DNA or RNA, while protecting it from degradation.

- **TAE (Trise Acetate EDTA)**: It is a very common buffer used for Agarose gel electrophoresis of DNA. It provides faster electrophoretic migration of linear DNA and better resolution of supercoiled DNA. It contains Tris-acetic acid and EDTA, tris-acetate provides electrical conductivity and maintains pH.
- **TBE (Tris Borate EDTA)**:TBE is one of the other very common buffers, used for Agarose gel electrophoresis of DNA. It contains Tris-Boric acid and EDTA; Tris-borate provides electrical conductivity as well as maintains the pH.
- **DNA loading dye**: It is used to help to track how far the DNA sample has traveled. It allows the sample to sink into the well of the gel that is created by gel comb. It is also used to estimate the migration rate of molecules (nucleic acid and protein).
- **EtBr (Gel Red)**: It is a molecule used to visualize DNA in Agarose gel electrophoresis.

3.1.1 Common Concentration Units

Solution is a homogeneous mixture created by dissolving one or more solutes in a solvent to obtain the desired final concentration. Concentration is a general measurement unit stating the amount of solute present in a known amount of solution:

$$\text{Concentration} = \frac{\text{Amount of Solute}}{\text{Amount of Solution}}$$

Molarity (M), percent (%) and mole fraction (X) are the most common units used in the GMO detection or molecular biology laboratory.

- **Calculation of molar concentrations**

Molarity is the concentration of a particular chemical species in solution expressedin moles of solute per liter of solution.

$$\text{Molarity(M)} = \frac{\text{moles of solute(n)}}{\text{volumes of solution(V)}} \text{ or } \frac{\text{mass of solute(g)}}{\text{molecular weight}\left(\frac{\text{g}}{\text{mol}}\right) * \text{molecular weight)on(V)}}$$

  - **Example1**: If you are required to prepare a stock of 5M NaCl(formula mass = 58.456) solution in 1L needed for DNA extraction, how would you do that?

Answer: 5 M NaClmeans 5molof NaCl dissolved in 1 L of solution. Since 1 mol of NaCl is equivalent to its formula mass expressed in grams, 5 mol of NaCl/L = 5 x 58.456 g NaCl/L = 292.28 g NaCl/L Thus, to prepare 5 M NaCl solution, first, dissolve 292.28 g NaClin 700mL H_2O; here don't add NaCl all at once, it will never go into solution.And finally, add the remaining 300 mL of distilled water to bring to 1L final volume.

  - **Example2**: What is the molarity of a solution made by dissolving 2.5g of ammonium acetate in water and diluting to a final volume of 50.0 mL?

Molar mass of $NH_4CH_3CO_2$ = 77.083 g/mol.

Answer: $\text{Molarity} = \frac{\text{Given mass (g)}}{\text{Molecular mass } \left(\frac{\text{g}}{\text{mol}}\right) \text{xV(L)}} = \frac{2.5\ \text{g}}{77.083\ \text{g/mol} * 0.05\text{L}} = 0.65 \frac{\text{mol}}{\text{L}} = 0.65\ \text{M}$

Calculation of percent (%) concentrations

Weight by weight percent (% w/w), volume by volume percent (% v/v) and weight to volume percent (% w/v) expressconcentration as units of solute per 100 units of solution. A solution in which a solute has aconcentration of 23% w/v contains 23 g of solute per 100 mL of solution.

- **Example1**: In a GMO detection laboratory,agarose gel electrophoresis is a common technique. If you are required to prepare a 0.7% solution of agarose in TAE buffer, how would you do it?

Answer: The answer is you weigh 0.7g of agarose and transfer this to a 100 mL flask, and then bring up the final volume to 100ml mark with the TAE buffer.

- **Example2**: During DNA extraction steps of GMO detection, how can you prepare a 70% ethanol solution?

Answer: The answer is you transfer 70 mL of absolute ethanol from its container to a 100 mL flask and then add 30 mL of distilled water. Then shake the mixture to homogenize the solution.

- **Example3**: During DNA extraction, how do you prepare a 20% (w/w)NaClsolution in 250g solution?

Answer:20% NaCl of 250g solution is equal to 50 gNaCl.Thus you weigh exactly 50 g ofNaCl, transfer it to an appropriate container, and add distilled water until the total mass of the solution becomes 250 grams.

Calculation of mole fraction (X) concentrations

Many buffers are prepared as concentrated solutions, e.g., 5X or 10X which means 5 or 10 times the concentration of working solutions. These solutions are diluted 5 or 10 times so that the final concentration of the buffer in the reaction is 1X.

- **Example1**: In a typical GMO laboratory,how do you prepare 1X TE buffer of 1L from 10X TE buffer stock available in the laboratory.

Answer: The answer is you add900 mL of distilled water to 100 mL of 10X TE and label it as "1X" TE buffer.

3.1.2 General Steps in Solution Preparation

- Weigh out the desired amount of chemical(s). Use an analytical balance if the amount is less than 0.1 g.
- Pour the chemical(s) into an appropriate size beaker.
- Add less than the required amount of water. Prepare all solutions using double-distilled water.

- Dissolve the chemicals using magnetic stirrerbar on a stirrer and when the chemical is dissolved, transfer to a graduated cylinder and add the required amount of distilled water to achieve the final volume. An exception is when preparing solutions containing agar or agarose; weigh the agar or agarose directly in the final vessel, because the volume is already known.
- If the solution needs to be at a specific pH, check the pH meter with fresh buffer solutions and follow the instructions for using a pH meter.
- Autoclave, if possible, at 120°C for 20 min (in the case of automated and semi-automated autoclave) and at 121°C for 15 min (in the case of manual autoclaves). Some solutions cannot be autoclaved; for example, SDS. These should be filter-sterilized through a 0.22 mm filter.
- When needed, the agar can be dissolved in a microwave.
- Concentrated solutions, e.g., 1 M Tris-HCl, pH = 8.0, 5 M NaCl, can be used toprepare working stocks by adding autoclaved double-distilled water in a sterile vessel to the appropriate amount of the concentrated solution.

3.1.3 Preparation ofstock and working solutions

Stock solutions are solutions with accurately known higher concentrations of stable analyte(s) that can be stored under specific conditions for a long time. These stock solutions are used to prepare working solutions of lower concentrations, for instance to prepare standard solutionsneeded for analysis of the target analyte(s) on daily basis. Stock solutions are used to reduce error, save time and minimize risk in any laboratory.

Dilution is one of the main preparation processes that follow preparation of stock solutions. The solutions that are obtained after dilution are known as working solutions because they are the ones that are used directly for the analysis required. The formula used to calculate unknown variable (volume or molar concentration)for dilution of stock solutions are:

$$C1 \times V1 = C2 \times V2,$$

Where: C1= initial concentration, or concentration of stock solution;
V_1= initial volume, or amount of stock solution needed
C_2 = final concentration, or concentration of desired solution;
V_2= final volume, or volume of desired solution

Calculations of the stock solutions

- **Example1**: For pH adjustment of 0.5MEDTA solution, howdo you prepare 200mL solution with concentration of 1% (w/v) NaOH from 20% NaOH?

Answer:Use the dilution formula: $C_1 \times V_1 = C_2 \times V_2$, and substitute the given values in the equation. To find the unknown, rearrange the equation;accordingly, $V1 = C2\ x\ V2/C1$ 1% x 200 ml/20 % = 10 mL. Therefore, 10 mL of 20 % (w/v) NaOH solution is taken and diluted to 200 mL final volume for preparing 200 mL 1 % (w/v) NaOH solution.

- **Example2**:TE buffer is used for preserving extracted DNA. How do you prepare 100mL TE buffer from 1M Tris and 0.5M EDTA?

The answer is just to combine 1mL of a 1MTris solution and 0.2mL of 0.5M EDTA and 98.8mL distilled sterile water.

3.2 Materials and Proceduresfor Preparation of Solutions

Ina GMO detection analysis, different chemical solutions of different concentrations have different roles and preparation procedures.The role of each chemicalused in a GMO laboratory has already been describedabove **section3.1**.

3.2.1 Chemicals used in phenol-chloroform DNA extraction methods

3.2.1.1 Preparation of 10mMTris-HCl solution from 1MTris-HCl

- **Required items**:

Beaker	Balance	0.22µm sterile filter
Graduated cylinder	Magnetic stirrer pH meter	

Composition of 1MTris-HCl:

Tris-(hydroxymethyl) aminomethane and Concentrated HCl and Nuclease-free water.

Preparation procedure:

1. For a 1M solution, dissolve 12.1g of Tris base in 80mL of nuclease-free water.
2. Adjust the pH to 7.4 by slowly adding 6-7 mL concentrated HCland allow the solution to cool to room temperature before making final adjustments to the pH. *Remember always to add an acid to an aqueous solution; never add an aqueous solution to an acid.*
3. Adjust the volume of the solution to 100 mL with distilled sterile water.
4. Filter Tris-HCl with 0.22µm sterile filter (Nylon membrane filters).
5. To obtain a 10mMTris-HCl pH 7.4 solution, dilute 1M Tris-HCl pH 7.4 1:100 with nuclease-free water. For example, add 1mL of 1M Tris-HCl pH 7.4 to 99mL of nuclease-free water.

Note that Tris Base or Tris Hydrochloride by itself does not provide adequate buffering capacity when the pH is between 7 and 9 without adequate mixing of the buffer.

3.2.1.2 Preparation of 7.5 M ammonium acetate

- **Required items**:

Beaker	Filter	Magnetic stirrer
Flask	pH meter	

- **Composition of 7.5 M ammonium acetate**:Ammonium acetateand Nuclease free water.

Preparation procedure:

1. Dissolve 57.81g ammonium acetate in water to final volume of 100 mL.
2. Sterilize by filtration by using a0.2µm filter.
3. Adjust the final pH to 5.5.

3.2.1.3 Preparation of 20 mg/mL Glycogen

- **Required items**:

Microcentrifuge (Temperature adjustable)	Flask	Microcentrifuge tubes
Beaker	Filter	Speed vacuum concentrator
	pH meter	
	Magnetic stirrer	

Composition of 20 mg/mLglycogen:

Chloroform	100% D-Glycogen
Ethanol	Iso-amyl alcohol

Preparation procedure:

1. Add 5g of glycogen to 30mL of ddH_2O and stir for up to 1-2 huntil the glycogen is fully dissolved.
2. Aliquot the glycogen solution into 2 mL microcentrifugetube, approximately 1mL per tube. Add an equal volume of phenol: chloroform (25:24 vol:vol) to the glycogen solution and vortex thoroughly. After vortexing, the phenol phase should be in the lower phase.
3. Centrifuge at 14,000rpm for 10min at 4^{o}C. Transfer the upper aqueous phase containing the glycogen into new microcentrifuge tube. Discard the lower phenol phase.

4. Add an equal volume of cold (4^{o}C) of chloroform: iso-amyl alcohol (50:1 vol:vol) into the glycogen phase and vortex thoroughly. After vortexing, the chloroform phase should be the lower phase, and the glycogen should be in the upper aqueous phase.
5. Centrifuge at 14,000rpm for 10min at 4^{o}C. Transfer the upper glycogen phase into new microcentrifuge tube. Discard the lower chloroform phase.
6. Add an equal volume of room temperature absolute (100%) ethanol into the glycogen phase, and mix well by inverting 4-5 times.
7. Centrifuge at 14,000rpm for 10min at 4^{o}C. Discard the supernatant.
8. Dry the precipitate in the SpeedVac®for 8 h or overnight at 30-40^{o}C with the vacuum on until the glycogen is completely dried.
9. Pool the pellets and weigh the pooled pellet.
10. Dissolve the pellet inddH$_2$Oat concentration of 20mg/mL. Getting the glycogen into solution may require frequent shaking at room temperature for about 1-2 h.
11. Store at –20^{o}C.

3.2.1.4 Preparation of 80% of 500 mL Ethanol

- **Required items**:

  Measuring cylinder
  Funnel Bottle
- **Reagents**: 100% ethanol and ddH$_2$O

Preparation procedure:

1. Obtain a graduated cylinder with 100mL of ddH$_2$O.
2. Obtain a graduated cylinder with 400mLethanol.
3. Transfer 100mLof ddH$_2$O into a wash bottle.
4. Transfer 400mL of ethanol into the wash bottle, minimizing any splashing of the solution.
5. Close the wash bottle and swirl it in circular motion to mix.

3.2.2 Chemicals used in CTAB DNA extraction methods

3.2.2.1 CTAB extraction buffer

- **Required items**:

  Beaker Measuring cylinder pH meter
  Flask Magnetic stirrer
- **CTAB extraction buffer composition in 1L**:

  2% w/v (20g) CTAB Tris-HCl pH 8.0
  1.4M (81.8g)NaCl 20mM (7.44g)EDTA
  100mM (12.1g)

Preparation procedure:

1. Add12.1gTris HCl, 81.8g NaCl, 7.44g EDTA and 20g of CTAB respectively to a2L flaskand add around 800mL ddH_2O to this flask.
2. Precaution(s): Do not dissolve in 1L ofdH_2O. In most cases, the volume of solution increases when the large amount of solute dissolves in a solvent.
3. Mix well by magnetic stirrer and adjust pH to 8.0 by HCl.
4. Then fill the bottle up to a total volume to 1L with ddH_2O.
5. Finally store buffer at 4°C for maximum of 6 months.

3.2.2.2 CTAB precipitation buffer

- **Required items**:

  Beaker Measuring cylinder pH meter
  Flask Magnetic stirrer

- **Composition of CTAB precipitation buffer in 1L solution**:

  0.5% w/v (5g) CTAB,0.04M (2.3g) NaCl

Preparation procedure:

1. Pour 800ml ddH_2O into 2 L flask.
2. Add 5g CTAB and 2.3gNaCl to the flask and mix well.
3. Adjust the pH to 8.0 with 1MNaOH, fill to 1000mL and autoclave.
4. Finally store the solution at 4°C for a maximum of 6 months.

3.2.3 Other chemicals

3.2.3.1 Preparation of 20mg/mL Proteinase K

- **Required items**:

  Measuring cylinder Beaker pH meter
  Flask Bottle0.22 µm filter

- **Reagents**:

  Tris Buffer Calcium chloride ($CaCl_2$)
  100% glycerol
  Tris-$CaCl_2$ Buffer Proteinase K

Preparation procedure:

1. Measure 5mL of 200mMRNase free* Tris Buffer (pH 8.0).
2. Add 1.66mg (3mM) calcium chloride ($CaCl_2$) to the Tris Buffer and dissolve.
3. Measure 500µL of the Tris-$CaCl_2$ Buffer (pH 8.0) into a centrifuge tube.

4. Add 20mg Proteinase K and mix gently to dissolve it.
5. Sterilize the solution by filtering with a 0.22µm filter.
6. Fill to a final volume of 1mL with 100% glycerol.
7. Mix until completely dissolved.
8. Store in aliquots at -20°C for a maximum of one year.

3.2.3.2 Preparation of 1xTE (Tris EDTA)

- **Required items**:

Measuring cylinder	Flask	pH meter
Beaker	Magnetic stirrer	

- **Composition of 1X TE buffer in 500mL**:
  10mMTris-HCl: pH 8.31mM EDTA; pH 8.0

Preparation procedure:

1. Pour 300mL dH_2O into 1000 mL beaker.
2. Add 5 mL of 1M Tris-HCl and 1ml of 1mM EDTA to the beaker.
3. Mix well by magnetic stirrer.
4. Finally addddH_2O to 500mL.

3.2.4 Chemicals used in Gel electrophoresis

3.2.4.1 Preparation of 0.5M EDTA

- **Required items**:

Measuring cylinder	Magnetic stirrer	Volumetric flask
Balance	Funnel	

- **Composition of 0.5M EDTA in 1L**:
  186.1gEDTA and 16-18g NaOHpellets

Preparation procedure:

1. Add 186.1g EDTA into about 750 mL ddH_2O of 2L volumetric flask.
2. Mix and stir the solution vigorously using a magnetic stirrer until the EDTA is completely dissolved.
3. Adjust the pH to 8.0 by using 10MNaOH (40g NaOH in 1L).
4. Fill to a final volume of 1Lwith ddH_2O.
5. Filter the solution through a 0.5-micron filter.
6. Dispense into containers as needed and sterilize in an autoclave.
7. Store at room temperature.Note that the EDTA will not dissolve until the solution is sufficiently alkaline.

3.2.4.2 Preparation of 50X TAE Buffer

- **Required items**:

  Measuring cylinder conical flask / Beaker Magnetic stirrer

- **Composition of 50X TAE buffer in 1L**:
  40mM (242g)Tris acetate
  20mM (57.1 mL) glacial acetic acid
  1mM (100 mL) EDTA pH 8.2

Preparation procedure:

1. To prepare 1Lof 50X TAE buffer, weigh 242gofTris base and transfer it to 2Lbeaker.
2. Add 750 mL ddH_2O and mix by magnetic stirrer until all Tris base dissolves completely.
3. Add 100mL of 1mMEDTA and 57.1mL of 20mM glacial acetic acid solution.
4. Mix the solution well again.
5. Adjust the pH to 8.3 at room temperature (20°C).
6. Adjust the solution volume to 1L with ddH_2O and mixthe solution well again.
7. Filter the solution with 0.22µm that removes any undissolved materials (Optional).
8. Sterilize the solution by autoclaving (20min at 15lb/sq.in. (psi) from 121-124°C on liquid cycle) to inactivate most enzymes except some (e.g., RNases).
9. Store at 15-25°C (room temperature) for three weeks.

To prepare 50X TAE electrophoresis buffer of various volume (100, 250, 500 and 1000 mL) follow **Table1** below.

Table 1: Preparation of 50X TAE electrophoresis buffer of various volumes (100, 250, 500 and 1000 ml).

Volume / Reagents	100 mL	250 mL	500 mL	1000 mL
Tris base	24.2g	60.5g	121g	242g
Glacial acetic Acid	5.71 mL	14.27 mL	28.55 mL	57.1 mL
0.5 M EDTA (pH 8.0)	10 mL	25 mL	50 mL	100 mL
Adjust volume using Water	100mL	250mL	500 mL	1000 mL

Note: *To prepare a 1XTAE working solution from 50X TAE stock buffers, mix 50X stock buffer with DNase free* ddH_2O*at 1:4 ratios or use 40mMTris (pH 7.6); 20mM acetic acid and 1mMEDTA. Then dissolve in 600mL*ddH_2O*and fill to 1L.*

3.2.4.3 Preparation of 10X TBE buffer

- **Required items**:

  Measuring cylinder conical flask / Beaker Magnetic stirrer

- **Composition of 10XTBE electrophoresis buffer**:

  0.89 M Tris borate 0.02 M EDTA pH 8.2 - 8.4 (at 25°C)

Remark: *The preparation procedure and storage of TBE is the same as that of TAE buffer.*

To prepare 10X TBE electrophoresis buffer of various volumes (100, 250, 500 and 1000ml), follow **Table2** below.

Table 2: Preparation of 10X TBE electrophoresis buffer of various volumes (100, 250, 500 and 1000 ml).

Volume / Reagents	100 mL	250 mL	500 mL	1000 mL
Tris base	10.8g	27g	54g	108g
Boric acid	5.5g	13.8g	27.5g	55g
0.5 M EDTA (pH 8.0)	4 mL	10 mL	20 mL	40mL
Adjust the final volume using water	100 mL	250 mL	500 mL	1000 mL

Note: *To prepare a 1XTBE working solution from 10XTBE stock buffer, mix 10X stock buffer with DNase free* ddH_2O*at 1:9 ratio or use 89mMTris (pH 7.6); 89mM boric acid and 2mMEDTA. Then dissolve in 600mL*ddH_2O*and fill to 1L.*

3.2.4.4 Preparation of 6X DNA loading dye

A DNA loading dye must contain at least one tracking dye (orange G, Bromophenol blue, Xylene cyanolFF or bromocresol green) and a high density reagent (glycerol, sucrose or Ficoll 400).

- **Required items**:

  Measuring cylinder Magnetic stirrer Conical flask or Beaker

- **Composition of 6X DNA loading dye**:

  0.042% (w/v) Bromophenol blue 0.042% (w/v) Xylene cyanolFF 6.67% (w/v) sucrose and Deionized/Nuclease free water

To prepare 6X loading dye of various volumes (5, 10, 25 and 50 mL); follow**Table3**below.

Table 3: Preparation of 6X loading dye of various volumes (5, 10, 25 and 50 mL).

Volume / Reagents	5 ml	10 mL	25 mL	50 mL
Bromophenol blue	12.5 mg	25 mg	62.5 mg	125 mg
Xylene cyanolFF	12.5 mg	25 mg	62.5 mg	125 mg
Sucrose	2 g	4 g	10 g	20 g
Adjust the final volume byNuclease free Water	5 mL	10 mL	25 mL	50 mL

Preparation of 10-mg/ml stock solution of Ethidium Bromide

- **Required items**:

  Graduated cylinder Balance Beaker
  Magnetic stirrer Volumetric flask

Preparation procedure:

1. Add 1g EtBr to a 100ml beaker.
2. Add 1mL of 95% ethanol and use a magnetic stirrer to mix and dissolve it for about 5minutes.
3. Bring the volume to 100mL withdH_2O.
4. Store at 4°C in dark.

51 Types, Uses and Operations of Facilities in a GMO Detection Laboratory

51.1 Spatial Layout of GMO Detection Laboratory

Routine testing for the same DNA fragments often leads to cross contamination. It is caused by dust accumulating from the milling of samples, dispersion of DNA molecules during extraction and sample handling, and aerosols of DNA amplicons (if reaction tubes are opened post PCR). Good layouts of the laboratory, equipment arrangement, and safe use of equipment/operation procedures are the key things to reduce the chances of contamination and prevent hazard.

To reduce the above constraints and DNA contamination, at least five rooms are required. These are:

1. Sample storage and preparation areas
2. Chemical preparation and PCR-setup (reagent only) areas
3. DNA extraction areas
4. Gel electrophoresis and PCR amplification areas and
5. Chemical storage and general cleanup area.

51.2 Equipment in Sample Storage and Preparation Area

In a sample storage room, the following equipment/items are available:

Shelf-Box:Is used for storage of the received samples.

Fridge/Freezer: Depending on the day time room temperatures in the sample storage area,a fridge may be necessary for some sample types.

Agenda: It is used to record the received samples.

In this part, the equipment used in a sample storage room along with their uses and operation procedures are described.

High speed multi-functional crusher/ blender:

- It is used to crush received food sample.
- Is operated as follows:
    - Plug in the crusher to the power supply.
    - Then press the “ON”button.
    - Put your sample on top of crusher.
    - Then adjust the“frequency” and time. Then press the “start” button.

Weight Balance:

- It is used for weighing samples.
- Is operated as follows:
    - Plug in the weighing balance to the power supply.
    - Then press the “ON”button.
    - Change the unit.
    - Put your weighing boat on top of your balance.

- Then press “ZERO” or TARE; until displays 0.0.
- Put your sample on the top of your balance.

Fume Hoods (Dust Cabinet):

- It is used to remove dust, gases, vapors, mists and dirt during the operation of chemical substances and sample powders.
- The following recommendations should be observed when operating chemical fume hoods.
- Each hood must be checked for adequate face velocity upon installation at least once annually in accordance with applicable testing standards. The correct face velocity should be prominently displayed on the fume hood (for conventional hoods this is normally 0.40 and 0.50 m/s with a sash opening of 30cm).
- A maintenance program must be developed and include the following: every 6 months the baffles, sash and interior surfaces should be cleaned; every 12 months additional maintenance including inspection for corrosion and correct operation, testing of all systems and alarms.
- The face velocity should be verified before commencing work; the indicator should be checked, if present. Attaching a strip of paper to the opening only tells you if there is inward (in face) airflow.
- An alarm warning both visual and audible installed in the fume hoodwould also be beneficial to alert users to know incorrect face velocities or abnormal airflow conditions. The warning system must also be maintained.
- Stop working in the fume hood if the ventilation system fails and immediately report the problem to the laboratory supervisor.
- Have an emergency plan in case of spills or power failure.
- When working in the fume hood or performing maintenance, personal protective equipment is required (gloves, eye/face protection).
- Materials should be placed at least 15cm from the edge of the fume hood; large apparatus can obstruct airflow.
- Keep all objects away from the air foil and baffle openings.
- Adjust the tie to the smallest opening that can able to work easily to maximize face velocity, minimize exposure and provide additional physical protection from splattering or explosion.
- Always open the sash slowly and use slow arm movements while working in the hood.
- Only equipment and chemicals involved in the experiment should be present in the fume hood.
- Avoid cross drafts in front of the hood (i.e. open windows, opening and closing doors etc.)
- Never remove the air foil or modify a chemical fume hood in any way.

- Don’t lean your head into the hood. It will disturb air flow and risk of chemical exposure, and when the hood is not in use the sash should be closed.*For further reference about fume hood refer the link:* https://protect.iu.edu/doc/environmental-health/lab-chp/lab_chp_sop_3-2.pdf.

51.3 Equipment in Reagent Preparation and PCR-Setup Area

Thereagent preparation area should consist of only specific chemicals that are used for reagent preparation, not anything else. The prepared reagents can then be taken to the specific laboratory areas for use. Reagents used in the reagent preparation should not be taken back into where they were originally kept in the laboratory. That is to say no reverse flow of items allowed.

51.3.1 Equipment in Reagent preparation room

In this section, the equipment used in a reagent preparation room along with their uses and operation procedures are described below.

Weight Balanceand **Fume Hood**have already been described in **section 4.1** above.

Bench top and portable pH meter:

- Is an instrument that is used to measure how acidic or basic a sample solution is. A pH scale ranges from 0 to 14. Biological functions are very sensitive to changes in pH, and hence buffers are used to stabilize the pH.
- Is operated as follows:
  - Expose the hole on the side of electrode by sliding the neck down. Make sure there is sufficient electrode filling solution in the electrode. If not, fill with ROSS filling solution only.
  - Ensure that sample to be at room temperature and is stirring gently on the stir plate.
  - Calibrate the pH meter with the two solutions oftarget pH 4 and 7 or 7 and 10.
  - Press the “CAL” key to initialize the calibration sequence and calibration range will be displayed, e.g.7-4. Press “YES” to accept by using the scroll keys to select a different range.
  - The number 7 will light up on the left hand side of the screen indicating that the meter is ready to accept the pH 7 standard buffer. Rinse off the electrode and place in fresh pH 7 standard buffer solution. The “READY” light will come on when the value has stabilized. Press YES to accept the value. Use number 4 or10 like number 7 above.
  - SLP will be displayed. The meter will then go “MEASURE mode”.
  - Rinse electrode and place into the sample. The “READY” light is displayed when signal is stable.

Hot plate magnetic stirrer-

- It is used to mix or keep liquids circulating as they are heated for a faster, more even reaction.
- It's operatedas follows*:*
    - Choose a beaker that is 2-4 times the volume of the solution.
    - Weigh the beaker and solution before heating.
    - Add room temperature or chilled buffer and a stir bar to the beaker.
    - Sprinkle any premeasured powder while the solution is rapidly stirred to prevent the formation of clumps.
    - Cover the beaker with plastic wrap or aluminum foil.
    - Pierce a small hole in the plastic wrap for ventilation.
    - Bring the solution to a boil while stirring.
    - Maintain gentle boiling until the powder is dissolved (~5 -10 min).
    - Add sufficient hot distilled water to obtain the initial weight.
    - Mix thoroughly; and cool the solution to 60°C prior to use.

Pipettes and or micropipettes:

- Used to measure and deliver accurate volumes of liquid in the laboratories.
- The difference is that micropipettes measure a much smaller volume, starting at 1µL, while pipettes generally start at 1mL.
- Execute the following general safety guidelines:
    - Choose the appropriate pipettes, pipette, or pipette aid for the required volume.
    - Choose the appropriate tips, or pipettes for the type of pipette aid to be used.
    - Always work in the appropriate area, biosafety cabinet, fume hood, etc.
    - Never use your mouth to operate a pipette.
    - Always use caution when attaching a tip or pipette (especially glass) to a pipette aid.
    - Never hold thepipette aid or pipettor upside down when used pipette or tip is in place.
    - Wear the appropriate personal protective equipment for the handling materials (lab coat, gloves, eye protection etc.)
    - Dispose the waste materials according to the biosafety hazardous waste standard.
    - Ensure having an appropriate disinfecting agent for the biological material being used.
- Operational procedures for pipettes or micropipettes are as follows:
    - Determine the appropriate type of pipettor, or pipette aid for protocol.
    - If working with Risk Group2 material, it is recommended to conduct work inside biological safety cabinet (BSC),and follow guidelines for working inside BSC.
    - Pipettor racks will avoid placing the pipettor on the BSC work surface.
    - Do the pipetting procedure as follows:

- Always depress the plunger to the 1st stop prior to immersing the tip into liquid sample, and then aspirate sample in the vertical position. After thatdispense the sample while holding the tube on a considerable angle to avoid splashing of sample in to the tube. The tip should touch the side of the vessel while dispensing, butdon't immerse tip into liquid while dispensing (can create aerosols).
- When dispensing, carefully depress the plunger to the 1st stop; pause, and then depress plunger to the 2nd stop to eject (blow-out) remaining liquid. There afterejectthe pipette tip carefully into the proper waste container.
- When removing any device from the BSC, spray the device with disinfectant first.
- Monitor the pipette aid/pipettor accuracy monthly - calibration can be completed either in the lab or sent away to calibration company.

51.3.2 Equipment in PCR-setup (reagent only) room

This area includes all PCR reagents that are used for PCR amplification, with the exception of the DNA template. No DNA is allowed to be broughtinto this area from other rooms such as the gel electrophoresis and PCR amplification areas either forhandling or storage purposes.Equipment that should be available in PCR-setup (reagent only) room is described below along with their respective uses and operational procedures.

Pipette/micropipette:It is already been described in **section 4.3.1** above.

Centrifuge (Fixed speed, 0.2-2.0 mL):

- It is used for the separation of fluids, gases or liquids, based on density by spinning a vessel containing material at high speed using centrifugal force pushes.
- It maypose hazard because of the creation of infectious aerosols and hazardous chemical leaks. Therefore,carefully read the safety guidelines and attentions for installation and maintenance, electric system, fireproofing, safety in mechanical parts, safety in chemistry and biology, the use of suitable rotor and adaptor.
- Consider the following important points on centrifuge operation:
  - Use Stoppard tubes that have been inspected to be free of cracks or chips; stress lines can develop at the junction of the sides and bottom of tubes.
  - Ensure that the cups are properly balanced and attach the rotor and lid.
  - Use sealed safety cups or rotors that are loaded and unloaded in fume hood. The outside of the cups should be disinfected when removed from the cabinet.
  - Floor model centrifuges used for biologics should ideally be located in a separate room with a high efficiency particulate air(HEPA)filter. However,BSC will only provide containment for some small, low speed centrifuges.

- For centrifuging solvents, a mobile exhaust hood should be positioned over the centrifuge to capture vapors when a sample tube breaks.
- The use of free spinning centrifuges is prohibited.
- All centrifuges must be equipped with a locking mechanism so that the lid is not opened while the rotor is turning on. Older models, which don't have a lockout mechanism, should have a warning sign and this warning shallbe part of the operating instructions.
- Power should be switched off and machine left unopened for at least 30 min to allow aerosols or vapors to settle.
- Wearing suitable protective cloth (strong gloves, gasmask in some cases), and by using long forceps, remove carriers and any broken glass.
- Swab all contaminated surfaces, including cups or buckets, turn-on and rotor with a non-corrosive disinfectant; autoclave as many parts as possible.
- If breakage involves a biologic, disinfect bowl of centrifuge by allowing sufficient contact time with disinfectant prior to clean up.

- Is operated as follows:
  - Plug in the centrifuge to the power supply and then press the"ON"button.
  - Put your sample on the top of your centrifuge in balanced way and closed the lid.
  - Then adjust your rpm/rcf and time.
  - Press the start button

Vortex:

- It is used to mix or blend liquid samples gently.
- It is operated as follows:
  - Plug inthe vortex to the power supply and then press the "ON"button.
  - Put the tube containing your sample on the top of your vortex.
  - Scroll the start button.

51.4 Equipment in DNA Extraction Area

The uses of centrifuge (16,000rpm, 12 x 1.5mL tubes);vortex and pipette/micropipetteand their operational procedures are described in **section(4.3.1** and **4.3.2)** above.

Water Baths:

- It is used to improve solubility of the sample solution in DNA extraction.
- It should be cleaned regularly and a disinfectant (quaternary ammonium compounds or phenolics) should be added to avoid contamination with infectious agents.
    - Is operated as follows: Fill with the optimum volume of water.
    - Plug in the water bath to the power supply and then press the ‘‘ON’’ button.
    - Setyour temperature and time, start it.
    - Put your sample in the appropriate rack and put in thebath.

Incubator:

- Is an insulated and enclosed device that has the following advantages in DNA extraction:
    - Incubation at 65^{o}Cin CTAB ensures complete lysis of cells in the suspension. These digest proteins, suspend lipids, and also digest/breakdown celluloid material and purify the DNA.
    - DNA pellet will be kept at 37oC heat block before and after suspension, i.e.,
        - Before suspension - to evaporate the remaining alcohol (ethanol, propanol whichever is used), and
        - After suspension - to ensure all the DNA is dissolved. This depends on the appearance of the DNA pellet if it is translucent it should be ok.
- Is operatedas follows:Before using the incubator, make sure that no remaining items are present in the incubator from the previous cycles.
    1. Open the door and check the incubator free of any items.
    2. Keep the door of the incubator closed.
    3. Connect and switch on the power supply.
    4. Turn on the red color power knob.
    5. Use the arrow on the incubator up or down to set the required temperature and time.
    6. Touch the start button after setting it.
    7. Heat up the incubator to the desired temperature.
    8. Put the sample in the incubator and keep it closed.

Refrigerator (+4 to -20^{o}C):

- Is an instrument used to storeany reagent or solution for laboratory purpose, and to maintain temperatures a few degrees above the freezing point of waterto transfer heat from its inside to its external environment i.e. its inside is cooled to a temperature below the room temperature.
- Is operated as follows:
    1. Connect the power plug to the main power and switch on the power supply.

2. Put a thermometer in the refrigerator compartment and freezer.
3. Set the thermostat control knob to the normal position.
4. Load the refrigerator with the items to be stored between 2-6^{o}C.
5. Open the door of refrigerator to take out the items and close the door;
6. Monitor the temperature once a day.

Horizontal deep freezer (-40 to -80^{o}C):

- Is similar to a refrigerator, the difference is deep freezer maintains a temperature below the freezing point of water; it is used for long time storage of any items. The operational procedures are the same asrefrigerators.

51.5 Equipment in Gel Electrophoresis and PCR Amplification Area

51.5.1 Gel Electrophoresis room

Equipment used in the gel electrophoresis areas are:

Water bath- has already been described in **section 4.4** above.

Microwave oven:

- It is used to dissolve agarose, agar, etc. into solutions.
- It is operated as follows:
  - Weigh and measure the agarosee and the chilled buffer followed by ddH_2O by balance and measuring cylinder respectively.
  - Pour the agarose into Erlenmeyer flask and add chilled buffer and water.
  - Cover the Erlenmeyer flask with parafilmand pierce a small hole in the parafilmfor ventilation.
  - Sprinkle while the solution is rapidly stirred to prevent the formation of clumps.
  - Power "ON" the microwave oven.
  - Heat the Erlenmeyer flask in the microwave oven on medium power for 1 minute.
  - Remove the solution from the microwave then swirl.
  - Reheat the Erlenmeyer flask on high power for 1-2min until the solution becomes clear.
  - Remove the Erlenmeyer flask from the microwave oven.
  - Cool the solution to 60°C prior to casting on gel tray.

Horizontal agarose gel electrophoresis apparatus

- A device used to run electrophoresis using agarose gels in running buffer. A gel is casted in a horizontal orientation and submerged in running buffer within the gel box.
- It is operated as follows:
  - ***While Gel Pouring*:**
    - Slot gel casting tray.
    - Place the gel casting gates in the slots provided in the ends of the casting tray.
    - Place the gel unit on a leveled surface.

- Position the required comb(s) into the slot(s) in the gel casting tray.
- Prepare the desired percent of agarose based on the required depth of the gel.
  ***Note**: The type of running buffer used should always be the same as that used for preparation of the gel.*
- Pour in the agarose smoothly so as not to generate bubbles. If bubbles do occur, these can be smoothed to the side of the gel and dispersed using a clean gloved hand.
  ***Important**: ensure that the agarose has cooled to between 50 and 60°C to prevent apparatus distortion before pouring to the tray.*
- Allow the agarose to set, ensuring that the gel remains undisturbed.
- Carefully remove the gel casting gates and comb(s).
- Place the gel casting tray in the running position such that the wells are nearest to the black electrode (cathode).
- Fill the tank with appropriate volume of running buffer until the gel is completely covered by the buffer.

- ***While Runningthe Gel***:
  - MixDNA or RNA samples with a loading dye prior to loading. This aids sinking of the sample into the well, and visualization of how far the samples have traveled. *Note: Consult your laboratory manual for details on loading buffers*.
  - Load the samples into the wells taking care not to damage the sides or bottoms of the wells.
  - Replace the lid correctly beforeconnecting the leads to the power supply.
  - Set the voltage and current to suit the electrophoretic application. As a guide, to obtain the optimum resolution of DNA fragments, agarose gels should not be run greater than 5V/cm.
    ***Note**: Refer to the equipment's instruction to see recommended voltage and current settings. Important: Do not exceed the recommended voltage or current as this may result in poor band resolution and may result in damage to the unit.Long runs may require buffer re-circulation, to prevent overheating and or buffer depletion. Important: When re-circulating buffer, remember that the buffer flowing through the tubing is live. Take all necessary precautions.*
  - The cooled gel units have a built-in cooling block at the base. The side connectors can be attached, via tubing, to the mains water supply or to a circulating water bath.
- ***At the End of the Run***:
  - Turn the power supply settings to zero, turn off mains supply and disconnect the power leads.
  - Visualize the run progression or final separation on a UV transilluminator.
  - At the end of the run, rinse the apparatus with distilled water only.

- Ensure that the connectors are clean and dry before usage or storage.

Gel documentation system:

- It is laboratory equipment used to visualize/ capture DNA image after completion of electrophoresis.
- It is operated as follows:
    1. Plug in the gel doc to the power supply and then pressthe “ON”button.
    2. Put your gel- inside of the gel doc and then turnon the white light.
    3. Open the software on the monitor and click on “LIVE” to see your gel position.
    4. White light should be switch OFF and UV-light should be “ON”.
    5. Click on “LIVE”; click on “SNAP” hassle; and click on “SAVE”.
    6. Finally, dispose the gel following disposal procedures.

51.5.2 PCR Amplification room

Pipette/ micropipette: Please refer to **section 4.3.1** above.

Laminar Air Flow Hood or Cabinet (LAF):

- It is a device used to protect sample, but not the users from any contamination. It pulls air from the environment; the air goes through a filter and then blows smoothly out the front of the hood back into the room.
- Start-up proceduresin LAF are as follows:
    1. Get training on the correct use and maintenance of laminar air flow hood.
    2. Turn on the fluorescent light and cabinet blower if off.
    3. Check the air intake and exhaust grilles for obstructions.
    4. Confirm inward air flow by holding a tissue paper at the middle of panel edge and ensuring that it is drawn in at an angle of 45^{o}C.Allow the cabinet to operate unobstructed for 10 minutes.
    5. Disinfect the interior surfaces with 70% ethanol, or a suitableandnon-corrosive disinfectant.
    6. Assemble all required materials and load into the cabinet, and do not block the air grilles.
    7. Depending on the work performed it may be necessary to line the working surface with absorbent paper orplastic backing and also segregate "clean" items from “contaminated” items; and then wait five minutes to purge airborne contaminants from work area.
- Follow these procedures for working in the cabinet:
    1. Wear protective clothing and gloves as appropriate.
    2. Perform operations as far to the endof the work area as possible.
    3. Avoid moving materials or excessive movement of hands and arms through the front access opening because itdisrupts the laminar air flow at the opening of the cabinet which can allow contaminants to enter or escape from the cabinet; and also when enter and exit the cabinet, allow the cabinet to stabilize before

resuming work.*Keep discarded contaminated material to the rear of the cabinet; do not discard materials in containers outside of the cabinet.*

4. If there is a spill during use, all objects in the cabinet should be surface decontaminated and disinfect the working area of the cabinet while it is still in operation (do not turn the cabinet off).

- Follow the following procedures upon completion of the work:
    1. Allow the cabinet to run for five minutes with no activity.
    2. Objects in contact with contaminated material should be surface disinfected before removal from the cabinet.
    3. Open containers should be closed or covered before being removed from the cabinet.
    4. Remove contaminated gloves and dispose as appropriatethenwash your hands.
    5. Do on clean gloves and remove materials from cabinet to biohazard bag for autoclaving,or incubator, etc.
    6. Using 70% ethanol, or a suitable non-corrosive disinfectant, disinfect interior surfaces of the cabinet, andperiodically remove the work surface then disinfect the area beneath it.
    7. Turn off the fluorescent light and the power when appropriate (some cabinets must be left on at all times; if you are unsure, check with your cabinet certifier, safety officer, or building maintenance staff).

Ultraviolet (UV) Radiation in LAF:

- The use of UV Radiation germicidal lamps in LAF is strongly discouraged. Any userswishing to use the UV in the LAFmustreceive training on the safe work practices,and hazards of UV radiation before using UV implanted LAF.Therefore, before using theUV radiation in LAF, beaware of the hazards, cause of UV light exposure and the limitations is important.

Applied Biosystems 7500/7500 Fast Real-Time PCR System (ABI FRT-PCR):

- The ABI FRT-PCR System is a 96-well, five-color platform that uses fluorescence-based polymerase chain reaction reagents to provide:
    - Quantitative detection of target nucleic acid sequences using real-time analysis.
    - Qualitative detection of targets using post-PCR (endpoint) analysis.
    - Qualitative analysis of the PCR product (achieved by melt curve analysis that occurs in post-PCR) etc.
- It is operated following the general work flow indicated in **Figure1** below. Moreover, details of the procedure can be referred to in the instrument manual which you can download from:

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/4387783c.pdf.

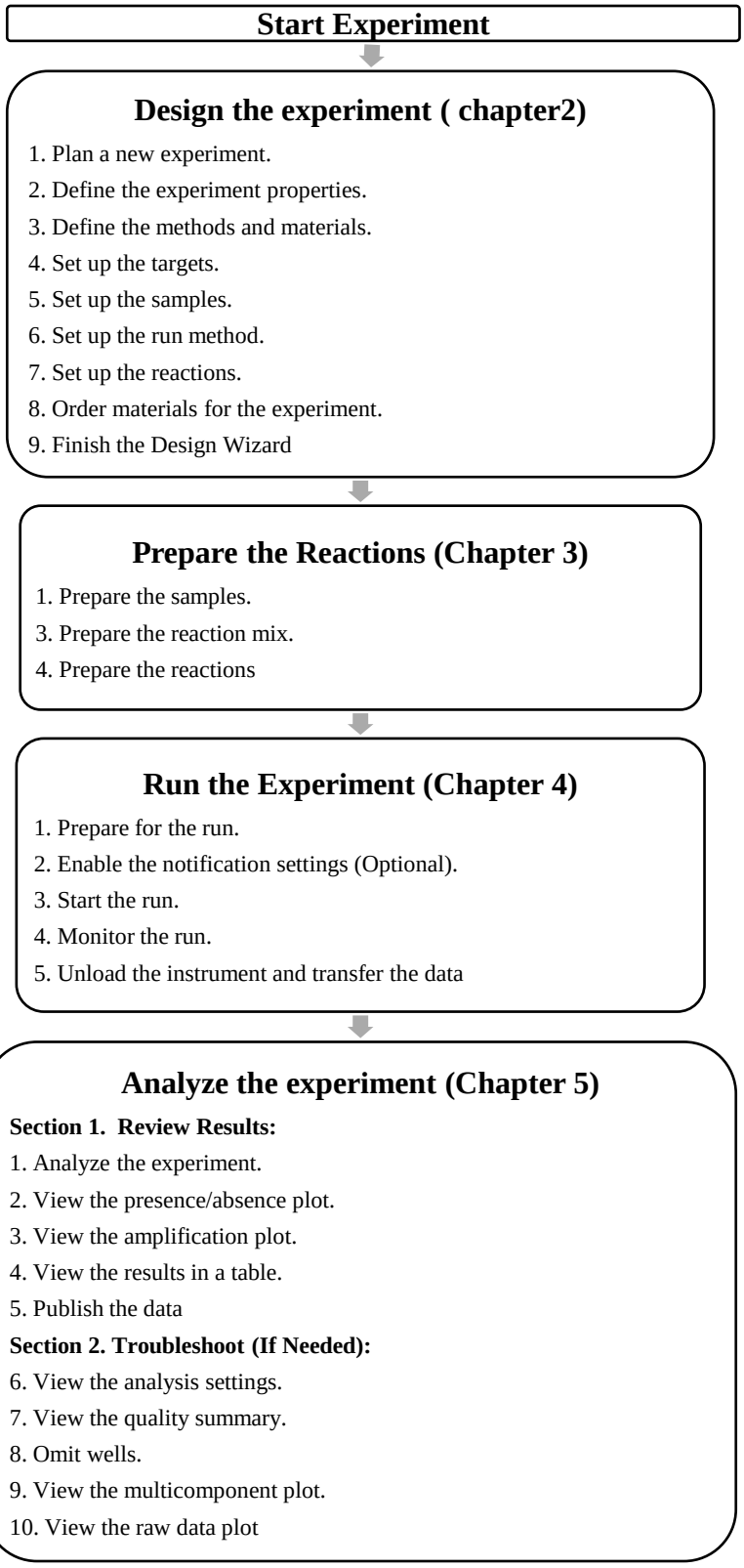

Figure1.Demonstrative experimentalworkflow for the analysis of PCR products using ABIs fast real time PCR

PCR thermal cycler:

- Also known as gene amplification or PCR amplification instrumentthat is old conventional and versatile laboratory equipment used to carry out amplification of selected segment or region of DNA molecule.
- It is operated as follows:
    1. Plug in the thermo cycler to the power supply and then click on the “ON”button.
    2. Adjust the PCR program depending on the required protocol.
    3. Put theprepared sample in the machine.
    4. Close the lid.
    5. Click onthe“START” button.
    6. Wait until all the cycles are completed.
    7. Finally, open and take out the sample for the next step.

51.6 Chemical Storage and General Cleanup Area

51.6.1 Equipment in Chemical storage room

Shelving or storage cabinets:

- It is used for storage of the chemical reagents. There are legal requirements if thestore chemicals in chemical cabinets, cupboards or on shelves within laboratory.
- Store chemicals safely: any hazardous chemical must be stored in a suitable container that is:
    - Sealed securely to prevent spills
    - Resistant to the effects of the substance and
    - Strong enough to cope with handling
    - Store flammable chemicals (cleaning fluids, adhesives, thinners, aerosols and paints) in a fire-proof steel cabinet or chemical storage cupboard.
- Userscan always check the chemical's safety data sheet (SDS) to see whetherit is hazardous or not. The SDS contains information about the chemical, including details of how to store, use and dispose of it safely. Separate all incompatible chemicals (e.g. hydrocyanic acid with nitric acid, hydrofluoric acid with ammonia, sodium nitrite withammonium nitrate, nitrates with acids,acetic acid with chromic acid etc.). If incompatible chemicals arestoredtogether and there is a leak or spill, it could cause a violent reaction. A chemical's SDS will tell which chemicals shouldn't be mixed with each other.

51.6.2 Equipment in general cleanup room

Cleaning and sterilization products and instruments such as autoclaves, industrial cleaners, mini cleaners, lab ware washers, ultrasonic bath cleaners, biological and temperature indicators, steam cleaners, high frequency cleaners, irradiation products, and stand-alone water treatment systems are used in any laboratories. This usesphysical methods such as flaming, red heat, incineration,

hot air oven, and chemical methods using liquids such as H_2O_2 and per-acetic acid or gases such as ethylene oxide are applied. Equipment used in general cleanup room include:

Autoclave:

- An autoclave is a device used to eradicate microorganisms using steam sterilization under specific pressure and temperature for a specific period of time. The autoclave is structured by container, lid, pipe, control system, heating system and security system, etc.
- Is operated as follows:
    - Wear personal protective equipment including heat and cut resistant gloves, a rubberapron, rubber sleeve protectors. Before beginning to use the autoclave, check it if there is any item left from the previous cycle.
    - Put a sufficient amount of water inside the chamber.Now, the materials to be sterilized are placed inside the chamber.
    - The lid is then closed, and the screws are tightened to ensure an airtight condition, and the electric heater is switched on.
    - The safety valves are adjusted to maintain the required pressure in the chamber.
    - Once the chamber water boils, the vapour(mixof air and water) is allowed to escape through the discharge tube, and then all the air inside to be removedwhich can be ensured once the water bubbles cease to come out from the pipe.
    - The drainage pipe is then closed, and the steam inside is allowed to reach the desired levels (15 Psiin most cases).
    - Once the pressure is reached, the whistle blows to remove excess pressure from the chamber.
    - After the whistle, the autoclave is run for a holding period, which is 15 minutes.
    - Now, the electric heater is switched off, and the autoclave is allowed to cool until the temperature reaches up to 60-70 ^{o}C.
    - The discharge pipe is then opened to allow the entry of air from the outside into the autoclave.
    - Finally, the lid is opened, and the sterilized materials are taken out of the chamber.

Deionizer:

- Used to purify water throughevaporation and condensation of the steam. It removes organic as well as inorganic material.
- It is operated as follows:
    1. Plug in the power.
    2. Switch on the water, and always check that there is no water leakage from the hose connection; the drain water flows freely; the flow rate of the water

supply is sufficient; the reservoir is empty. Thereafter,a safety cut-out should switch off both the electricity and the water supply when the reservoir is full.

3. Ensure water spigot is fully open.
4. Check water quality and verify that the water quality light indicates acceptable.
5. Press and hold button for 2 s. Green color Light should be observed.***Note****: If light indicates unacceptable water quality (red light), cartridge must be replaced.*

Large plastic bins for transporting dirty lab-ware:

- Plastic bins are any storage boxes made of plastic and designed for long term storage or distribution of large objects. It can also protect the contents and resist damage caused by contact with extreme temperatures or with other objects while in transit or storage.
- Polypropylene and sometimes polycarbonate and polyvinyl chloride (PVC) are used for heavy-duty plastic storage materials because of their strength and durability.
- The average shelf life of a medium to large-sized plastic storage container is intended to last as long as possible, andare not generally designed or marketed as disposable products.

52 Detection of GMOs in Food Samples

52.1 Principle

Genetic manipulation in crops for food means the direct manipulation ofthe genetic makeup of the plant genes to produce improved or novel plants or crops. It is done by transferring of genes within and across species by either isolating or copying the genetic material of interest using recombinant DNA technology or by artificially synthesizing the DNA.

There are two methods totransfer the gene of interest into host organisms:

- The biological indirect method (e.g. *Agrobacteriumtumefacians)*
- The direct methodincluding chemical(polyethyleneglycol-mediated transfer, calcium precipitation method, etc.) and physical(gene gun or biolistic, electroporation, microinjection etc.).

The biological vector system is the most commonly used method and has various advantages over the physical and chemical transformation methods because it reduces the copy number of the transgene, potentially leading to fewer problems with transgene co-suppression and instability.*Agrobacterium*mediated gene transfer technology– uses *Agrobacteriumtumefacians*as biological vector to transfer the gene of interest into the host plant. In order for the transgene to work effectively in its new host, it needs to be controlled by a promoterand terminator sequence along with the selectable marker gene and transgene.A **promoter sequence**must be added for the

gene to be correctly expressed (i.e., translated into a protein product). It is the on/off switch that controls when and where in the plant the gene will be expressed. While a**terminator sequence** initiatessignals to the cellular machinery that the end of the gene sequence has been reached.A **selectable marker gene** is added to the gene "construct" in order to identify plant cells or tissues into which the transgene has successfully integrated. This is necessary because achieving incorporation and expression of transgene in cells is a rare event, occurring in just a small portion of the targeted tissues or cells. **Transgene** is the gene that has been transferred or introduced naturally or by any of genetic engineering techniques from one organism to another. This grouping is called a gene cassette as shown in **Fig.2**, where the promoter and terminator regulatory regions influence when a gene will be expressed. These regulatory regions enable strong and generalized transcription of the transgene across all tissues of the host plant.

The most commonly used promoter and terminator in engineered plants,selected because of their ability to be recognized in most plant species,are:

- CaMV35S promoter which isderived from the cauliflower mosaic virus that infects cauliflower.
- NOS terminator obtainedfrom the Ti plasmid in *A. tumefactions*. Itis the most commonly used terminator.

Hence, most improved GM food crops have been developed through the insertion of 5-enol pyruvylshikimate-3-phosphate synth*ase (EPSPS).* In the process of transformation,*Cauliflower Mosaic Virus promoter (P-CaMVE35S)* and *A. tumefactions*nopaline synthase terminator (T-NOS) have been used invariably in most GM crops.

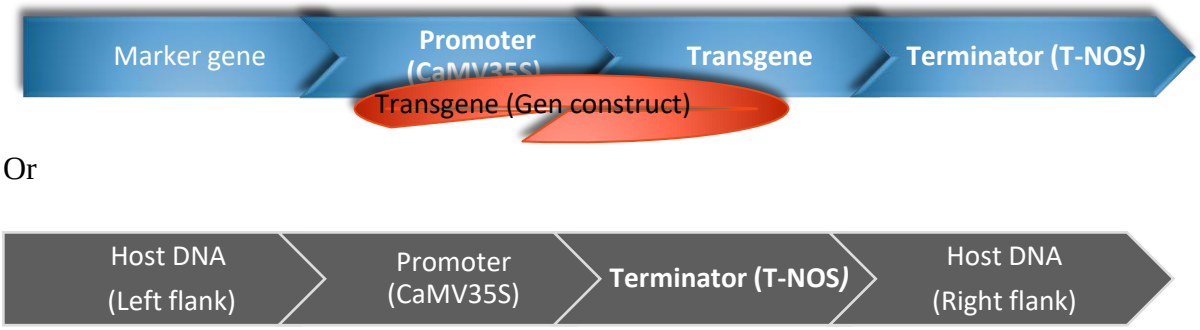

Figure 2. Schematic representation of a transgenic cassette used to generate GMOs

52.2 Why and how to detect GMOs in Food Samples

As types of genetically modified crops andtheir demand is increasing, detection of GMOs in foodsamples is an important issue for all the subjects involved in food control and customer's right. Detection of food samples isrequested by traders and their customers because it has a particular significance for implementation of labeling policy which is important for monitoring export and import of unapproved GM products.

In the context of Ethiopia, any cereal crops that are exported to other countries must be free from GMOs, and thepresenceof GMOsin cereal crops is not acceptable. Therefore, it is necessary to use appropriate qualitative methods for GMO screening in different cereal based food products. This can be done by detecting the new transgenic DNA that has been inserted, or the new protein expressed, or (if the protein acts as an enzyme), by using chemical analysis to detect the product of the enzymatic reaction.

More than 95% of currently available GMO crops are positive for either the 35S promoter or the NOS terminator, or both. As a reference and control for DNA extraction efficiency, a plant-specific gene is used because the plant-specific primers will be used to confirm the presence and quality (amplifiability) of the DNA extracted from the samples.

The two most common scientific approaches used at present for detecting genetic modification in products such as soybeans, corn, cotton and others areELISA (Enzyme-Linked ImmunoSorbent Assay) and PCR (Polymerase Chain Reaction):

- **ELISA**involves testing for the presence of specific proteins by exploiting the specificity of binding between expressed antigen and target antibody.
- **PCR**is based on the detection of novel DNA sequences inserted into the crops genome.

Both methods show the absence or presence of GMOs in the sample but PCR methods can also give indication of quantity (percentage) and quality in a tested sample. Therefore, the PCR methods of detection are the most commonly used methods in the world.
The general DNA-based screening, detection and quantification of GMOs in cropsfollows sampling, crushing, nucleic acid extraction, amplification, and interpretationof the data obtained. For DNA extraction, the crop samples should be collected in the normal and optimum harvesting seasonto achievegood quality and quantity of DNA.

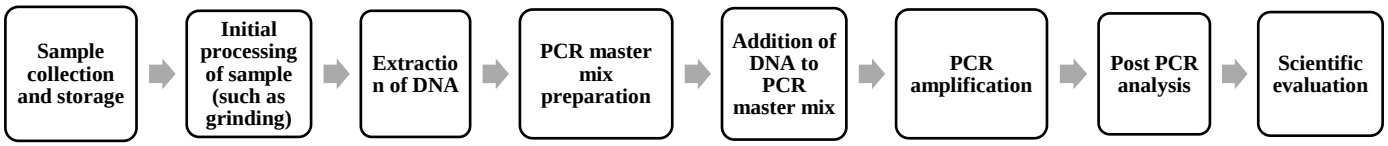

Figure 3. Diagram of DNA extraction for GMO detection and PCR analysis workflow

The objective of DNA-extraction prior to PCR analysis is to isolate, purify and detect genomic DNA from the given crops whether the crops are genetically altered or not.
The five essential steps of DNA extraction for the achievement of good quality of DNA are described below:

1. **Cell breakage for cell lysate creation**

The first step in any nucleic acid purification reaction is to rapidly and completely disrupt cells in a sample to release the DNA or RNA into solution by using different techniques. The four general techniques used for lysing cells are:

- **Physical methods**typically involve some type of sample grinding or crushing to disrupt the cell walls or tough tissue by using grinder machine.
- **Chemical methods**canbe used alone with easy-to-lyse materials. Cellular disruption is accomplished with a variety of agents that disrupt cell membranes and denature proteins by using commonly used chemicals like CTAB buffer, phenol, SDS etc.
- **Enzymatic methods:** The enzymes utilized help to disrupt tissues and tough cell walls. Depending on the starting material, typical enzymatic treatments can include: lysozyme, zymolase and liticase, proteinase K, collagenase and lipase, among others.
- **Combination method**:In many protocols, a combination of chemical disruption and another is often used since chemical disruption of cells rapidly inactivates proteins, including nucleases.

2. Clearing of lysate to remove proteins and other debris

In this step, unwanted materials (proteins, lipids and saccharides) couldbe removed prior to nucleic acid purification. Usually clearing is accomplished by centrifugation and filtration. Centrifugation can require more hands-on time, but it is able to address large amounts of debris. Filtering can be a rapid method, but samples with a large amount of debris can clog the filter. This procedure is also called deproteinization that is removal of proteins fromthe DNA solution which depends on differences in the physical properties (solubility, partial specificvolume,sensitivity to digestive enzymes)between nucleic acidsand proteins.

- **Deproteinization using organic solvents**

The most frequently used methods for removing proteins explore the solubility differences between proteins and nucleic acids in organic solvents. Nucleic acids are predominantly hydrophilic molecules and are easily soluble in water. Proteins, on the other hand, contain many hydrophobic residues making them partially soluble in organic solvents.Based on this difference,there are several methods of deproteinization that vary by the choice of organic solvents. The commonly used organic solvents are phenol and chloroform containing 1%iso-amyl alcohol. The use of phenol as the deproteinization is based on the following principle. Phenol molecules are extremely hydrophobic, and more soluble in the hydrophobic cores of the protein than in water.Hence, phenol molecules diffuse into the core of the protein causing the protein to swell and eventually to unfold or denature. The denatured protein is far more soluble in the phenol phase than in the aqueous phase. As a result proteins are separated into the phenol phase leaving the nucleic acids in aqueous phase.

Nucleic acids do not have hydrophobic groups at all and are insoluble in the phenol phase. Application of the phenol method does require mixing the phenol phase with the water phase. This introduces some shearing of DNA molecules. The application of chloroform: iso-amyl alcohol (CIA) mixture deproteinization method is based on a characteristic of this organic solvent that differs from phenol. The chloroform is not miscible with water and, therefore, even numerous extractions do not result in DNA loss into the organic phase. The deproteinization

action of chloroform is based on the ability of denatured polypeptide chains to partially enter or be immobilized at the water chloroform interphase.

A substantial improvement in the method can be accomplished by limiting the number of extractions. This saves time and limits DNA shearing. This can be done by enzymatically removing most of the protein before extraction. Another modification frequently used is combining phenol and chloroform extraction into one-step. The efficient use of the phenol and chloroform methods of deproteinization of DNA requires prior enzymatic digestion of protein.

- **Deproteinizationusing enzymes**

Proteins can be removed from DNA preparation step using a protease that can digest all proteins, i.e. a general-purpose protease. Two of such enzymes are in use, proteinase K and pronase which are very stable and secreted by fungi commercially.They are free of DNase contamination which makessafe to use in the purification of nucleic acids. Proteases are active in the presence of low concentrations of anionic detergent, high concentrations of salts, and EDTA and exhibit broad pH (6.0-10.0) and temperature (50-67°C) optima. They can digest intact (globular) and denatured (polypeptide chain) proteins, and do not require any co-factors for their activities. Pronase is a self-digesting enzyme, whereas proteinase K is not. Therefore, proteinase K is a more convenient enzyme than pronase, because there is no need of continuous addition like pronase.

The major drawback in using these enzymes is that enzymatic treatment can only remove 80-90% of the proteins present because protein digestion is an enzymatic reaction that is dependent on substrate and enzyme concentrations. The characteristics of enzymatic removal of proteins make the enzymatic deproteinization an ideal and indispensable or critical first step in nucleic acid purification. This treatment is used when a large amount of protein is present, i.e. right after cell lysis. The remaining proteins can be removed with a single extraction using organic solvent.

3. Removal of RNA

The removal of RNA from DNA preparations is usually carried out using an enzymatic procedure. Consequently, this procedure does not remove all RNA and, therefore, yields DNA preparations with a very small amount of RNA contamination. Ribonuclease A (RNase A) and ribonucleaseT1 that can be easily and cheaply prepared free of DNase contamination are used for removal of RNA. RNase A is an endoribonuclease that cleaves RNA after C and U residues. RibonucleaseT1 (RNaseT1) is an endoribonuclease that is very similar to RNase A in its reaction conditions and stability, but it cleaves both double and singlestranded RNA after G residues. Because of the RNA cleaving specificity, it is recommended to use both enzymes together for complete RNA removal from DNA samples.

4. Washing for concentrating the DNA

Precipitating with alcohol (ethanol and isopropanol) is usually performed for concentration of DNA from the aqueous phase of the deproteinization step. Alcohol precipitation is based on the phenomenon of decreasing the solubility of nucleic acids in water. Polar water molecules

surround the DNA molecules in aqueous solutions. The positively charged dipoles of water interact strongly with the negative charges on the phosphodiester groups of DNA. This interaction promotes the solubility of DNA in water. Ethanol is completely miscible with water, yet it is far less polar than water. Ethanol molecules cannot interact with the polar groups of nucleic acids as strongly as water, making ethanol a very poor solvent for nucleic acids. Replacement of 95%of the water molecules in a DNA solution will cause the DNA to precipitate. Making a DNA solution of 95%volume ethanol is not practical because it requires the addition of a large volume of 100%ethanol.

To precipitate DNA at a lower ethanol concentration, the activity of water molecules must be decreased. This can be accomplished by the addition of salts to DNA solutions. Moreover, the presence of salts will change the degree of charge neutralization of the DNA. These changes, simultaneous with water elimination, will cause the separation of the DNA phase, i.e. precipitation, of complete neutralization of DNA molecules. DNA precipitation is customarily carried out with 70%ethanol (final concentration) in the presence of the appropriate concentration of sodium or ammonium salts. Usually ethanol precipitation is carried out at temperatures of -20°C or lower. It is the reason that low temperature and the presence of salts further lower the activity of water molecules, thereby facilitating more efficient DNA precipitation.

5. Determination of the purity and quantity of DNA

This is the last step of DNA isolation procedure, and used to evaluate the final result that means determination of DNA concentration and evaluation of the purity of the DNA. UV spectrophotometer and or Nano Drops are used for the determination of DNA concentration. InEEFRI's biosafety laboratory, the isolated DNA is checked by agarose gel electrophoresis.

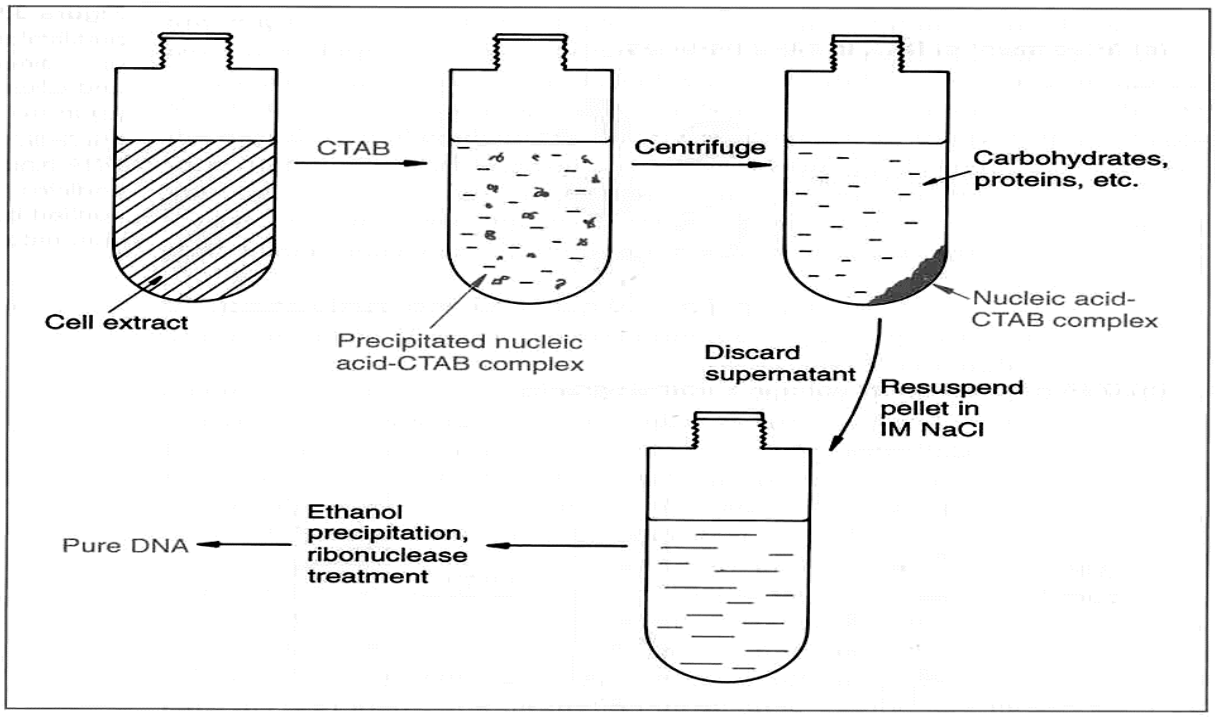

Figure 4. General DNA isolation procedure from any sources

52.2.1 Extracting DNA by Using Phenol-Chloroform Methods

Required Reagents:

- Phenol/chloroform/iso-amyl alcohol (PCI) solution (25:24:1),DNase (RNase and Protease free), pH 7.8-8.2. A basic pH is chosen becauseacidic pH makes the DNA go into the phenolic layer while RNA goes into the aqueous layer.
- Chloroform/iso-amyl alcohol, 24:1
- Elution Buffer (EB) (10mMTris-HCl, pH 8.5)
- NH4OAc, concentrated solution (5M – 7.5M) or powder
- Glycogen, 20mg/mL, available from most biological reagent
- 100% Ethanol
- 80% Ethanol

***Remark**: All chemicals should be of molecular biology grade; ddH_2O and buffers should be autoclaved prior to use. In addition, all chemicals should be DNA and DNase free.*

Procedure:

1. Mix 1.2mg of sample powder is with EB to the volume of 200µL tube (labeled No.1).
2. Add an equal volume of the PCI solution (25:24:1) to tube (No.1).
3. Vortex the tube (No.1)vigorously for one minute.

4. Swirl tube-1 solution at high speed (13, 000rpm) for 5minutes.
5. Remove ~180µLof the top aqueous solution and place into a new tube, tube (labeled No.2). Avoid picking up any of thePCI phase.
6. Add 200µLof EB to tube (No. 1).
7. Vortex tube (No. 1)vigorously for one minute.
8. Swirl tube (No.1) solution at high speed (13000rpm) for 5 minutes.
9. Remove as much of the top aqueous solution as possible from tube No-1 without picking up any of the PCI phase. Add the solution to tube (No. 2).
10. Add equal volumes of the chloroform/isoamyl alcohol solution (24:1) to, tube (No. 2).
11. Vortex, tube (No 2) vigorously for 1minute.
12. Spin, tube (No. 2)solution at high speed (13,000rpm) for 5minutes.
13. Remove as much of the top aqueous solution as possible and place into a new tube, tube (labeled No. 3). Avoid picking up any of the PCI phase.
14. Add 10µl NH4OAc to a final concentration of 0.75 M.
15. Add 1µLof glycogen (20µg).
16. Mix the solution well.
17. Add 2.5X (1.2mL) volume of 100% ethanol and mix well.
18. Keep at 20°C (optional).
19. Spin for 20 min in a 4°C centrifuge at 13,000rpm.
20. Decant the supernatant carefully without disturbing the pellet.
21. Wash by adding 300µLof 80% ethanol and vortex 3 times.
22. Spin for 15min in a 4°C centrifuge at 13,000rpm.
23. Decant the supernatant carefully without disturbing the pellet.
24. Repeat steps 21-23 for a second 80% ethanol wash.
25. Quick spin on table top centrifuge to draw residual ethanol to the bottom.
26. Remove residual ethanol with a P20 pipette. Be careful not to disturb the pellet.
27. Air dry for 1-2min by usingWhatman® paper or clear cotton.
28. Re-suspend in 100µLvolume EB (based on input amount).

52.2.2 CTAB extraction and purification method

The CTABprotocol is an appropriate method for elimination of polysaccharides and polyphenolic compounds, and purification of DNA from plants and plant derived foodstuff. This procedure has been widely applied in molecular genetics of plants and already been tested in validation trials in order to detect GMOs.

Reagents:

- *CTAB extraction buffer*(for 200mL): add the following components

- 20g/L CTAB, 4g
- 1.4mol/LNaCl, 16.4g
- 0.1mol/l Tris-HCl, 3.15g
- 20mmol/LNa2EDTA), 1.5g.

- Then, add 100mLof ddH_2O adjust pH to 8.0 with 1MNaOH, fill up to 200mL and autoclave. Finally, store the buffer at 4°C for max. of 6 months.
- *CTAB precipitation buffer*(for 200mL): add the following components:
  - 5g/LCTAB, 1g
  - 0.04mol/LNaCl, 0.5g.
- Then, add 100mLof ddH_2O adjust pH to 8.0 with 1MNaOH, fill up to 200mLand autoclave.Finally, store this solution at 4°C for maximumof 6 months.

Other chemicals:

- 70% ethanol (v/v): 70mLof pure ethanol is mixed with 30mLof sterile ddH_2O.
- 20µLof RNase A (10mg/mLstored at –20°C)
- 20µLof Proteinase K (20mg/mL) and stored at –20°C

Remark:*All chemicals should be of molecular biology grade. Deionised water and buffers should be autoclaved prior to use. In addition, all chemicals should be DNA and DNase free.*

Procedure:

1. Transfer 100mg of a homogeneous sample into a sterile 1.5mLmicrocentrifuge tube.
2. Add 300µLof sterile deionized water, mix with a loop.
3. Add 500µLof CTAB-buffer, mix with a loop.
4. Add 20µLProteinase K (20mg/mL), shake and incubate at 65°C for 30-90 minutes.
5. Add 20µLRNaseA (10mg/mL), shake and incubate at 65°C for 5-10 minutes.
6. Centrifuge for 10 min at about 16,000rpm.
7. Transfer supernatant to a microcentrifuge tube containing 500µLchloroform, shake for 30 s.
8. Centrifuge for 10 min at 16,000rpmuntil phase separation occurs.
9. Transfer 500µLof upper layer into new tube containing 500µLchloroform, shake.
10. Centrifuge for 5 min at 16,000rpm.
11. Transfer upper layer to a new micro-centrifuge tube.
12. Add 2 volumes of CTAB precipitation solution, mix by pipetting.
13. Incubate for 60 min at room temperature.
14. Centrifuge for 5 minutes at 16,000rpm.
15. Discard supernatant.
16. Dissolve precipitate in 350µLNaCl (1.2 M).
17. Add 350µL chloroform and shake for 30 s.
18. Centrifuge for 10 min at 16,000rpmuntil phase separation occurs.
19. Transfer upper layer to a new micro-centrifuge tube.

20. Add 0.6 volumes of isopropanol, and then shake it.
21. Centrifuge for 10 min at 16,000rpm.
22. Discard the supernatant.
23. Add 500µLof 70% ethanol solution and shake carefully.
24. Centrifuge for 10 min at 16,000rpm.
25. Discard supernatant.
26. Dry pellets and re-dissolve DNA in 100µLof TE buffer.

Note*:Any contamination should be avoided during sample preparation by using single-use equipment, decontamination solutions, and by avoiding the formation of dust. For both protocols, the DNA solution may be stored in a refrigerator for a maximum of two weeks, or in the freezer at – 20°C for longer periods.*

52.3 Agarose gel electrophoresis

Electrophoresis is defined as the migration of charged particles or molecules through a solution under the influence of an electric field. During electrophoresis, water is electrolyzed, which generates protons at the anode (+), and hydroxyl ions at the cathode (-). The use of a buffering system (TAE or TBE) is therefore required when charged molecules are electrophoresed through a separation medium. Because the pH of these buffers is neutral, the phosphate backbone of DNA has a net negative charge and migrates towards the anode.

In molecular biology and biochemistry,agarose gel electrophoresis is the most effective standard method which is used to separate, identify, and purify DNA fragments through agarose gels. The technique is simple, rapid to perform, and capable of resolving mixtures of DNA fragment that cannot be separated adequately by other procedures. It is used to separate and analyze fragments of DNA based on their size and charge under the thin gel supported medium made up of agarose, which is widely used for larger molecules like DNA, RNA, and proteins. Furthermore, the location of DNA within the gel can be detected by direct examination of the gel in ultraviolet light.

Samples (DNA solutions) are pipetted into the sample wells, followed by the application of an electric current at the cathode (negative, black, acidic) end, which causes charged DNA to migrate towards the bottom anodes (positive, red, basic) end. The rate of migration is proportional to the size of the macromolecule: smaller fragments move faster, the amount of voltage plug in, and wind up at the bottom of the gel. Agarose is commercially available but not completely pure; it is contaminated with other polysaccharides, salts and proteins. These differences can affect both, the migration of the DNA and the ability of the DNA recovered from the gel.Agarose gels are cast by melting the agarose in the presence of the desired buffer until a clear, transparent solution is achieved. The melted solution is then poured into a mold and allowed to harden. Upon hardening, the agarose forms a matrix, the density of which is determined by the concentration of the agarose. When an electric field is applied across the gel,

DNA, which is negatively charged at neutral pH, migrates toward the anode.The objective of doing gel electrophoresis is to separate DNA fragments or molecules in a solid support medium such as an agarose gel.

52.3.1 Separation of DNA fragments

The migration (separation performance) of DNA through agarose gels depends upon or affected by the following factors:

The molecular size of the DNA:The rate of migration of DNA molecule decreases for larger molecules and the reverse is true, that is inversely proportional to the logarithm of the number of base pairs (bp). It is due to increase frictional and electrostatics forces.

The agarose concentration: Agarose gel electrophoresis can be used for the separation of DNA fragments ranging from 50bp to several millions of bases using specialized apparatus. Increasing the agarose concentration of a gel reduces the migration speed and enables separation of smaller DNA molecules and the reverse is true. In general lower concentrations of agarose are better for larger molecules because they result in greater separation between bands that are close in size. The disadvantage of higher concentrations is the long run times (sometimes days) requirement. Instead high percentage agarose gels should be run with a pulsed field electrophoresis (PFE), or field inversion electrophoresis. The following table offers suggested agarose concentration for separating various fragment sizes.

Table 4. Gel concentration required for DNA separation

Agarose gel concentration (%)	Effective resolution of linear DNA fragments (Kb)
0.50	1-30
0.75	0.8-12
1.00	0.5-10
1.25	0.4-7
1.50	0.2-3
2-5*	0.01-0.5

* Sieving Agarose such as AmpliSize® Agarose

The concentration of intercalating dye: The concentration of intercalating dye such as Ethidium bromide which intercalates into circular DNA can change the charge, length, as well as the superhelicity of the DNA molecules; therefore, its presence in gel during electrophoresis can affect its movement.

The applied voltage: At low voltages, the rate of migration of linear DNA fragments is proportional to the voltage applied. At high voltage, the faster the DNA moves and produces heat, this can generate gel artifacts such as S-shaped migration fronts, and can also even melt the agarose gel. However, as the electric field strength is raised, the mobility of high-molecular-weight fragments of DNA is increased differentially. Thus, the effective range of separation of agarose gels decreases as the voltage is increased. Gels should be run at no more than 5 V/cm.

Buffer composition: The most common buffers for agarose gel are: TAE, TBE and sodium borate (SB). TAE has the lowest buffering capacity but provides the best resolution for larger DNA >4 kb in length; this meansa lower voltage and more time, but a better product. While for 0.1-3kb fragments, TBE is selective and it has both a higher buffering capacity and lower conductivity than TAE; and therefore it should be used for high-voltage electrophoresis. Additionally, TBE buffer generates less heat than TAE at an equivalent voltage and does not allow a significant pHdrift.

Note*: Because of its lower buffering capacity, TAE should be circulated or mixed from time to time for full-length electrophoresis, especially at higher voltages.*

Materials Required:

- Gelcasting tray, gel platform, comb, power packs, gel tank, sterilemicro tips and UV transilluminator or gel documentation system

Reagents Required:

- DNA sample, agarose special, 1X TAE or TBE, EtBr(50 mg/ml stock), 6X loading dye, sterile water

Protocol:

- The general steps and diagrammatic illustration for AGEis shown below (**Fig 5**).

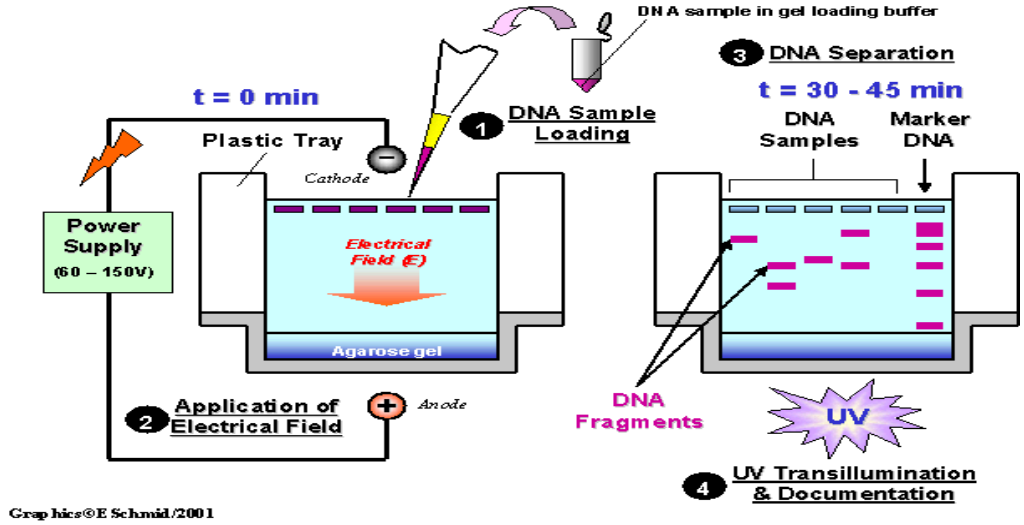

Figure 5. Steps in Agarose gel electrophoresis.

Based on a report by Lee(2012), the protocol for gel electrophoresis follows the following 3 steps:

a) Preparation of the gel

1. Weigh out the appropriate mass of agarose into an Erlenmeyerflask. Example: for a 1% agarose gel, add 1g of agarose to 100mLof 1Xelectrophoresis buffer.

Table 5. Protocol for making an agarose gel

Amount of 0.5%TBE (mL)	gel red (µL)	Preparation of agarose gel concentration in grams (g)		
		0.8 %	1.0 %	2.0 %
50	1.5	0.4	0.5	1.0
75	2.25	0.6	0.75	1.5
100	3.0	0.8	1.0	2.0

2. Add running buffer to the agarose-containing flask. Swirl to mix.
3. Melt (dissolve) the agarose/buffer mixture. This is most commonly done by heating in a microwave, but can also be done over a Bunsen flame. At 30seconds intervals, remove the flask and swirl the contents to mix well. Repeat until the agarose has completely dissolved.
4. Add EtBr to a concentration of 0.5µg/mL. Alternatively, the gel may also be stained after electrophoresis in running buffer containing 0.5µg/mLEtBr for 15-30minutes, followed by destaining in running buffer for an equal length of time.

***Note**:EtBr is a suspected carcinogen and must be properly disposed of as per the institution's regulations. Gloves should always be worn when handling gels containing EtBr. Alternative dyes for the staining of DNA are available; however EtBr remains the most popular one due to its sensitivity and cost.*

5. Allow the agarose to cool either on the bench top or by incubation in a 65°C water bath. Failure to do so will warp the gel tray.
6. Place the gel tray into the casting apparatus. Alternatively, one may also tape the open edges of a gel tray to create a mold. Place an appropriate comb into the gel mold to create the wells.
7. Pour the molten agarose into the gel mold. Allow the agarose to set at room temperature. Remove the comb and place the gel in the gel box. Alternatively, the gel can also be wrapped in plastic wrap and stored at 4°C until use.

b) Setting up of gel apparatus and separation of DNA fragments

1. Add loading dye (3-5µL) to the DNA samples to be separated.
2. Program the power supply to desired voltage (1-5V/cm between electrodes).
3. Add enough running buffer to cover the surface of the gel. It is important to use the same running buffer as the one used to prepare the gel.
4. Attach the leads of the gel box to the power supply, thenturn on, and verify thatboth gel box and power supply are working.
5. Remove the lid. Slowly and carefully load the DNA sample (5-10µL) into the gel. An appropriate DNA size marker (3µL) should always be loaded in one lane preferably in the first lane.
6. Replace the lid to the gel box. The cathode (black lead) should be closer to wells than the anode (red lead). Double check that the electrodes are plugged into the correct slots in the power supply.
7. Turn on the power, andrun the gel until the dye has migrated to an appropriate distance.

c) Observing separated DNA fragments

1. When electrophoresis has completed, turn off the power supply and remove the lid of the gel box.
2. Remove gel from the gel box. Drain off excess buffer from the surface of the gel. Place the gel tray on paper towels to absorb any extra running buffer.
3. Remove the gel from the gel tray and expose the gel to UV light. This is most commonly done using a gel documentation system. DNA bands should show up as orange fluorescent bands. Take a picture of the gel.
4. Properly dispose of the gel and running buffer as per the safetyregulations.

The following figure shows different DNA fragment band lengths separated on the gel during electrophoresis.

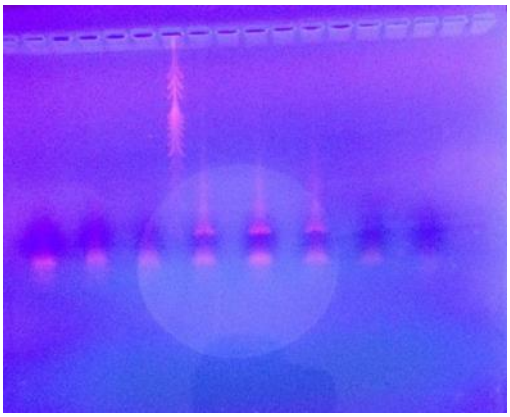

Figure 6. Diagrammatic illustration showing DNA bands of the PCR product.

52.3.2 Troubleshooting Guide for DNA Electrophoresis

When preparing agarose for electrophoresis, it is best to sprinkle the agarose into room-temperature buffer, swirl and let it sit at least for one minutebefore microwaving. This allows the agarose to hydrate first, which minimizes foaming during heating. Electrophoresis buffer can affect the resolution of DNA.

Migration of DNA is retarded and band distortion can occur when too much buffer covers the gel. The slower migration results from a reduced voltage gradient across the gel; loading DNA in the smallest volume possible will result in sharper bands; you can preserve DNAfromagarose gels for long-term storage using 70% ethanol. Electrophoresis at high temperature can cause the DNA to denature and deform agarose gel. Cool the gel with a small fan during the electrophoresis; for the supercoiled DNA ladder electrophoresed on <1% agarose gels, add 3µLof 20g/mL ethidium bromide to the gel. Otherwise, smeared bands and extra bands will be seen because of different degrees of supercoiling.

Most problems can be avoided by reading and following the instructions given in **Table 6** that contains good information along with suggestions.

Table 6.Suggested guides for gel electrophoresis

Problems	solutions
Agarose leaks into chamber when pouring gel	Check if the gasket is firmly seated in the grooves on the ends of the UV transmittance (UVT) gel tray. Reseat gasket if necessary by removing and rinsing under warm running water, then reseat evenly in the tray groove.
Bands seem to be running at an angle	Check andmake sure the casting is being done on a level surface. A flattening platform may be required. Make sure the gel tray is pressed all the way down and rests level on the casting chamber platform (the bubble in the bubble level should rest in the center circle). Adjust the leveling screws to make the casting chamber (D4- CST) level.
Samples seem to be running unevenly in certain areas.	Check to be sure the platinum electrode wire is intact and running evenly across the base of the chamber and upside to the junction of the banana plug. If there is appearanceto be a break in the electrode connection, contact Technical Services immediately. This problem may also be caused by regular casting with very hot agarose gel (>60°F) which may damage the gel tray over time. Always cool the melted agarose to below 60°F before casting to avoid warping the UVT gel tray. Warping the gel tray will cause all subsequent gels to be cast unevenly.
Samples do not band sharply and appear diffuse in the gel.	Gels should be no more than 5mm thick, and allowed to solidify completely before running. For standard agarose, this would be about 30 minutes, but if low melting point agarose is used, it may be necessary to completely solidify gels at a cooler temperature in the refrigerator or cold room. Gels should be submerged in 3-5mm of buffer to avoid gel dry out, but excess buffer >5mm can cause decreased DNA mobility, and band distortion.
Samples are not moving as expected through the gel, remaining in the wells, running “backwards” or diffusing into the gel.	Check and make sure that a complete power circuit is achieved between the unit and the power supply. Platinum wire and banana plugs should be intact. To test, simply fill the unit with running buffer, and attach to the power supply without a gel or gel tray in the unit then if the current passes through it, small bubbles are produced on both sides of the platinum wires unit. If a complete circuit does not exist, there will be little to no bubbles. Contact technical services to schedule a repair.Samples that appear to run backwards through the gel are caused by the tray being placed in the chamber in the reverse direction. Hence, the tray should be replaced correctly in the chamber with the comb at the edge of the tray closest to the cathode.
When the comb is removed from the gel, the sample well is	Always make sure to allow enough time to solidify the gel completely before moving the tray, unit, or removing the comb.To avoid damage to the sample wells, gently rock the comb back and forth lightly to loosen,

ripped and damaged.	and then slowly pull the comb straight up out of the gel tray. This rocking helps to avoid suction as the comb is removed.
The gel seems to run slower under usual running conditions.	The volume of running buffer used to submerge the gel should only be between 3-5mm over the gel surface. The gel should be completely submerged to avoid the gel from drying out, which can smear the bands and possibly melt the gel due to overheating. If excessive running buffer is added, the mobility of the DNA decreases and band distortion may result. Excess buffer causes heat to build up and buffer condensation inside the unit may result.
If abnormal DNA band migrations appear	Improper electrophoresis conditions might beused. Hence, check and make sure the voltage doesnot exceed~20 V/cm, thetemperature maintained at<30°C andthe electrophoresis buffer used had sufficientbuffer capacity.
If faint or no bands appear on the gel	There might havebeen insufficient quantity or concentration of DNA loaded on the gel or the degraded DNA or electrophoresed offDNA from the gel orused improper W light source for visualization of EtBrstained DNA, hence increase the amount of DNA,but do not exceed 50ng/band; avoid nuclease contamination; electrophorese the gel for less time and use a lower voltage, oruse a higher percent gel; and use a short wavelength (254 nm) W light for greater sensitivity.Note: For preparative gels, using a longer wavelength (312 nm) W light will minimize DNA degradation.
If smeared DNA bands appear	There might have either degraded DNA, or too much DNA loaded on the gel, orimproper electrophoresis conditions, or excess temperature. To solve either of these problems, avoid nuclease contamination,decrease the loaded amount of DNA, do not allow voltage to exceed ~20V/cm, and maintain the temperature<30°C during electrophoresis.In addition, check that the electrophoresis buffer capacity is sufficient which is done by checking the pH at the anode and cathode of the chamber. If there is toomuch salt in the DNA, use ethanol precipitation to remove excess salts or the DNA contaminated with protein, use phenol extractions to remove protein prior to electrophoresis orand small DNA bands diffused during staining then add the EtBr during electrophoresis.

52.4 Polymerase Chain Reaction (PCR)

PCR is a molecular biology technique that was developed in 1987 by Kary Mullis. It is an *invitro* technique used to amplify a single or a few copies of a piece of DNA up to several orders of a particular DNA sequence to obtain multiple copies of target DNA fragments using *Taq*DNA polymerase in a temperature dependent reaction. Its result is a selective amplification of a target region of a DNA or RNA molecule. PCR technique has several applications such as

cloning specific DNA fragments, genetic matching, genetic engineering, detecting and identifying genes in diagnostics and forensics, and in the investigation of gene expression patterns, classification of organisms, genotyping, molecular archaeology, mutagenesis, mutation detection and sequencing.

Today, PCR has acceptable mechanism for examination and detection of the presence or absence of genetically modified DNA for the validity of foodstuffs, because PCR process identifies sequences of DNA that have been inserted into the genetically modified food. In PCR techniques, detecting genetically modified DNA from food samples, how to extract DNA and amplify markers used in the genetic modification process, and how the presence or absence of GMOs in food samples can be established. DNA is a relatively stable molecule, so viable DNA fragments suitable for amplification can be isolated even from highly processed goods, like corn chips or vegetable burgers.

Basic components of PCR:

Six core ingredients and nuclease free water are required to set up a PCR reaction. These are:

1. **DNA template**: the sample DNA that contains target DNA to be amplified.A template for replication can be genomic DNA (gDNA), complementary DNA (cDNA), and plasmid DNA sources. E.g. 0.1–1ng of plasmid DNA is sufficient, while 5–50ng of gDNA may be required as a starting amount in a 50µL PCR.
2. **Oligonucleotide primers**: short stretches of DNA that initiate the PCR reaction, designed to bind to either side of the section of DNA thatneedsto be amplified. E.g. primers are added to the reaction in the range of 0.1–1µM. For primers with degenerate bases or those used in long PCR, primer concentrations of 0.3–1µM are often favorable.
3. **DNA nucleotide bases (dNTPs)**: DNA bases (A, C, G and T) which are the building blocks of newly synthesized DNA strand.E.g. in common PCR applications, the recommended final concentration of each dNTP are generally 0.2mM.
4. **Thermo stable Taq DNA polymerase enzyme (Taq DNA)**: a type of enzyme that synthesizes new strands of DNA complementary to the target sequence.E.g.in a typical 50µL reaction, 1–2 units of DNA polymerase are sufficient for amplification of target DNA.
5. **PCR buffer solution**: to provide a suitable chemical condition for optimum activity and stability of the DNA polymerases.The buffer pH is usually between 8.0 and 9.5 and is often stabilized by Tris-HCl. For Taq DNA polymerase, a common component in the buffer is potassium ion (K^+) from KCl or may replace KCl by ammonium ion (NH_4^+) from $(NH_4)_2SO_4$. Since Mg^{2+} has a stabilizing effect similar to K^+, the recommended $MgCl_2$ concentrations are generally lower when using a KCl buffer (1.5 ± 0.25mM) but higher with an $(NH_4)_2SO_4$ buffer (2.0 ± 0.5mM). Due to antagonistic effects of NH_4^+ and Mg^{2+}, buffers with $(NH_4)_2SO_4$ offer higher primer specificity over a broad range of Mg^{2+}.

6. **Divalent cations (e.g. Mg^{2+})**: act as a co-factor for increasing Taq DNA polymerase activity by enabling incorporation of dNTPs during polymerization.The Mg^{2+}at the enzyme's active site catalyzesphosphodiester bond formation between the 3′-OH of a primer and the phosphate group of a dNTP. In addition, Mg^{2+} facilitates formation of the complex between the primers and DNA templates by stabilizing negative charges on their phosphate backbones. E.g.a typical final concentration for Mg^{2+} in PCR is in the range of 1-4mM, with recommended 0.5mM titration increments for optimization.
7. **Nuclease free water**:for dissolving the above six components or ingredients.

The objective of using polymerase chain reaction is to amplify the target DNA molecules or a segment of DNA of interest exponentially by making huge number of copies.PCR uses DNA polymerase enzyme that directs the synthesis of DNA from deoxynucleotide substrates on a single-stranded DNA template. DNA polymerase adds nucleotides to the 3` end of a custom-designed oligonucleotide when it is annealed to a longer template DNA. Thus, if a synthetic oligonucleotide is annealed to a single-stranded template that contains a region complementary to the oligonucleotide, DNA polymerase can use the oligonucleotide as a primer and elongate its 3` end to generate an extended region of double stranded DNA. PCR thermal cycler is a machine that rapidly heat and cool the reaction mixture, allowing for heat-induced denaturation of duplex DNA (strand separation), annealing of primers to the plus and minus strands of the segregated DNA template, and elongation of the PCR product.

PCR involves a process of heating and cooling called thermal cycling which is carried out by machine. It is based on the mechanism of DNA replication *in vivo*: dsDNA is unwound to ssDNA, duplicated, and rewound. This technique consists of repetitive cycles of three main stages:

1. ***Denaturing (94-95^oC)***: melting of DNA through at elevated temperature to convert double-stranded DNA to single-stranded DNA.
2. ***Annealing or hybridization (cool to 50-65^oC)*:**the temperature is lowered to enable the DNA primers to anneal to the complementary sequences of the template DNA.
3. ***Extending (heat at 72^oC)*:** extension of the DNA chain by nucleotide addition from the primers using DNA polymerase as catalyst in the presence of Mg^{2+} ions.

The three major steps in a PCR amplification process is illustrated below (**Fig. 7**) which comprises a single "cycle" in the PCR amplification methodology.After each cycle, the newly synthesized DNA strands can serve as templates in the next cycle(**Fig.8**). The major product of this exponential reaction is a segment of dsDNA whose termini are defined by the 5' termini of the oligonucleotide primers and whose length is defined by the distance between the primers. The products of a successful first round of amplification are heterogeneously sized DNA molecules, whose lengths may exceed the distance between the binding sites of the two primers. In the second round, these molecules generate DNA strands of defined length that will

accumulate in an exponential fashion in later rounds of amplification and will form the dominant products of the reaction. Thus, amplification, as a final number of copies of the target sequence, is expressed by the following equation:

$$(\mathbf{2^n - 2n})\mathbf{X} \qquad \text{Equation (1)}$$

Where:

- $2^n$is the first product obtained after the first cycle and second products obtained after the second cycle with undefined length.
- Xis the number of copies of the original template.

Full information about PCR amplification is given in the following paragraphs (http://mbg.jrc.ec.europa.eu/capacitybuilding/documentation.htm).

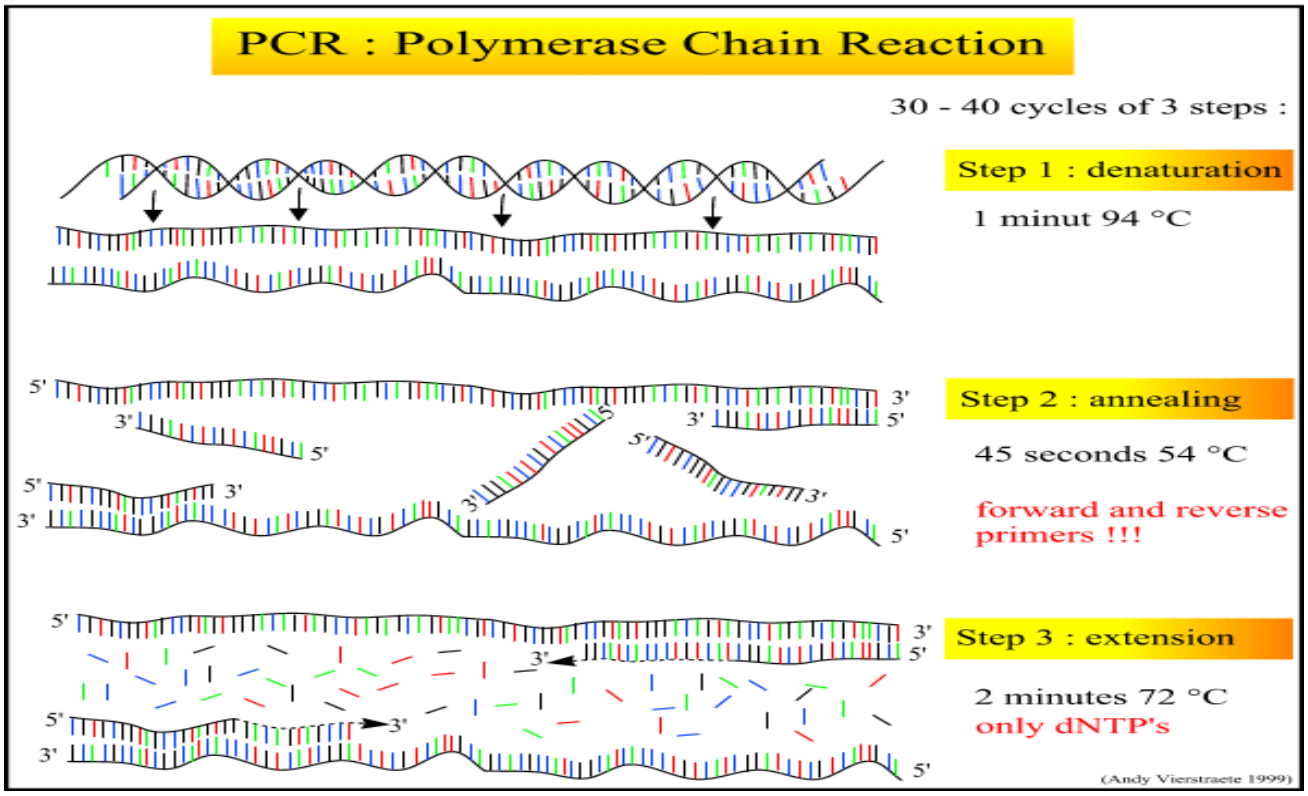

Figure 7. Basic steps of PCR amplification (picture taken from: Andy Vierstraete 1999)

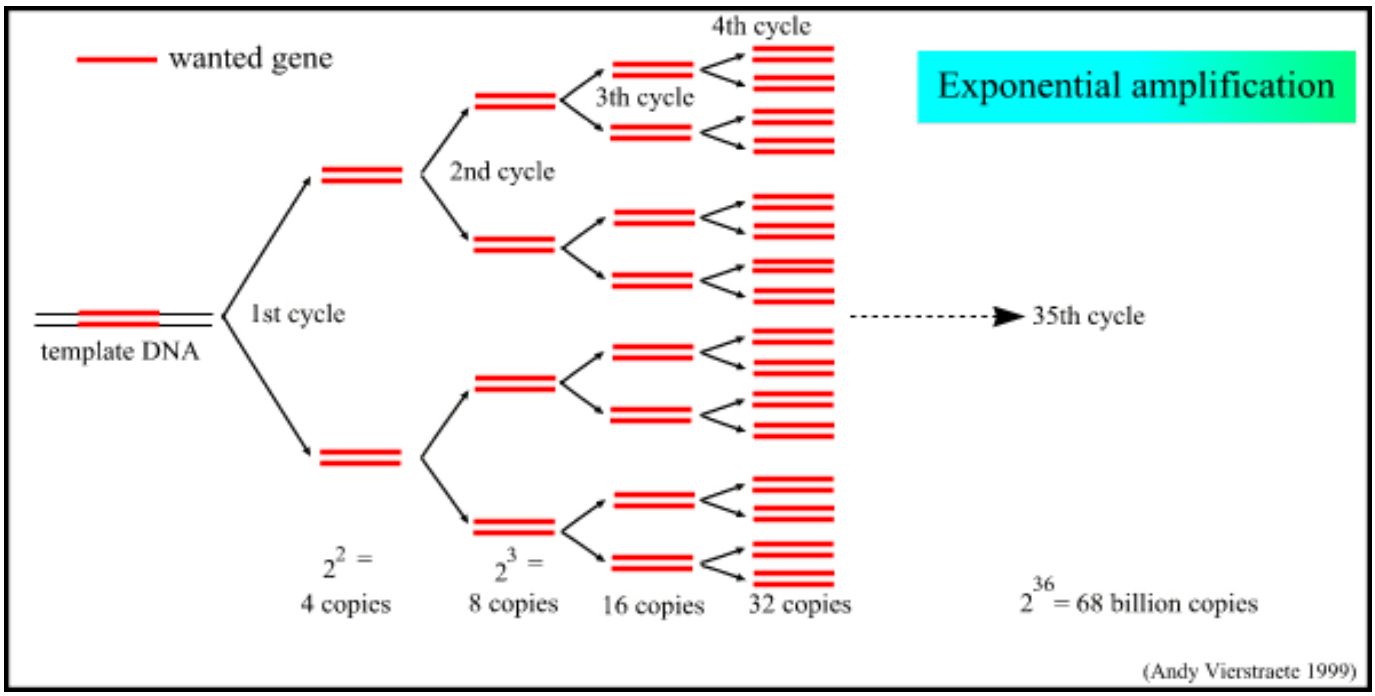

Figure 8. The exponential cycle of DNA (picture taken from: Andy Vierstracete 1999).

Table7: Oligonucleotide primer sequences that are used for PCR amplification at EEFRI's GMO detection laboratories

Order number	Name	Sequence(5'-3')	Length	MW	Tm	GC%	OD	Tube	nmol	Water /Tube	Purification
Primers common for most GMO crops											
0827-017	35S-1/2_F	GCTCCTACAAATGCCATCA	19	5716.78	55.41	47.37%	2	5	11.54	115.45	QPC
0827-018	35S-1/2_R	GATAGTGGGATTGTGCGTCA	20	6228.12	57.80	50.00%	2	5	10.60	105.97	QPC
Primers for soybean crops											
0827-021	LE103/104_F	GCCCTCTACTCCACCCCCATCC	22	6472.20	67.53	68.18%	2	5	10.20	101.97	QPC
0827-022	LE103/104_R	GCCCATCTGCAAGCCTTTTTGTG	23	6981.58	61.95	52.17%	2	5	9.45	94.53	QPC
Primers for maze crops											
0827-023	IVR1/2_F	CCGCTGTATCACAAGGGCTGGTACC	25	7643.00	66.90	60.00%	2	5	8.64	86.35	QPC
0827-024	IVR1/2_R	GGAGCCCGTGTAGAGCATGACGATC	25	7732.07	66.90	60.00%	2	5	8.54	85.36	QPC

Materials required:

- Thermocycler machine, micropipettes, micropipette tips, microcentrifuge, vortex mixer, PCR tubes, rack for PCR tubes, laminar air flow hood.

Reagents required:

- PCR buffer, dNTPs, $MgCl_2$, Taq DNA polymerase enzyme, forward and reverse oligonucleotide primers, and Nuclease free water.

PCR Protocol:

Master mix preparation is the first work to be executed before anything in PCR protocols.Because master mix preparation significantly reduces time, labor, and errors while also increasing reproducibility.

Table 8. PCR master mix preparation

No.	Components	For 1 tubes 20µl	For 4 tubes 60 µl
1	ddH_2O	6.6 µl	26.4 µl
2	10X PCRBuffer	2µl	8µl
3	25mM $MgCl_2$	1.6µl	6.4 µl
4	2.5mMdNTPs	1.6µl	6.4 µl
5	Taq DNA polymerase	0.2µl	0.8 µl
Post master mix preparation			
6	10 µM F Primer	2µL	8 µL
7	10 µM R Primer	2µL	8 µL
8	Template DNA	4 µL	16 µL
	Total volume	20 µL	80 µL

1. Prepare 2mLPCR tubes that are free of any contaminants to prevent nuclease activity and nonspecific priming;
2. Pipette and add the PCR reagents in order according to **Table 8** into a 2mLPCR tubes.

Note1*: Since experiments should contain a control, in this case the PCR negative control (no DNA), GMO negative control (certified or Non-GMO sample DNA), Positive control (certified GMO sample DNA) and suspected sample DNA (unknown sample DNA) are required for detection.*

Note2:*TaqDNA polymerase is typically stored at -20°C refrigerator and for complete dispersal in the reaction; the mix requires gentle mixing of the PCR reagents by pipetting up and down at least 20 times. The micropipette should be set to about half the reaction volume of the master mix when mixing, and care should be taken to avoid introducing bubbles.*

3. In a separate 2mLPCR tubes, add all the reagents with the exception of template DNA for a negative control (*Note:* increase the water to compensate for the missing volume). In another

reaction add all the reagents with or without the template DNA that should contain a positive control (*Note*: increase the water to compensate for the missing volume).

4. Place each of the labeled PCR tubes on 96 well plate microtubeholders with caps open and or place them into the thermal cycler. Start the program after once the lid to the thermal cycler is firmly closed.
5. After completion of the reaction, the 2mLPCR tubes may be removed and stored at 4°C. PCR products can be detected by loading aliquots of each reaction into wells of an agarose gel, and then visualized with a UV transilluminator.

52.4.1 PCR Troubleshooting

52.4.1.1 "No Bands" and "Nonspecific Bands''

In PCR processes, many failures will happen which include getting the no bands, incorrect size of product, extraneous bands, inconsistent results, multiple products and no specific bands. For this failure, there are many causes such as master mix preparation, primer design, thermo cycler parameters, or nonspecific binding to other template sequences, etc. Therefore, this section presents some possible solutions for these problems.

The first one is to make a checklist similar to prepare the master mix in their order as shown above (**Table 8**).

***Note**:If these reagents are not added in their order, it is no coincidence that the template is listed at the end. Adding the template last helps prevent DNA contamination of your stocks during the preparation. The template (in the case of master mix) and the enzyme (in the case of single reaction) are added at the last to prevent unspecific reaction.*

The second and most reliable (but depends onthe manufacturer) way to prevent errors is to use a PCR pre-mix such as BioMix™. BioMix™ is a complete ready-to-use2Xreaction mix containing an ultra-stable DNA polymerase. It is developed to perform PCR assays of many common genomic and cDNA templates, and the user has simply to add water, template and primers. BioMix dramatically reduces the time required to set up reactions, thereby minimizing the risk of contamination. Greater reproducibility is ensured by reducing the number of pipetting steps that can lead to errors. This is also thebest approach to relative quantitative PCR applications.

Concentrations of each reagent such as dNTPs, $MgCl_2$, primers, template DNA and enzyme should be on the range of recommended working concentrations. Similarly, be aware that many chemicals used in every preparation of DNA (phenol, chloroform, alcohol, detergents) can strongly inhibit PCR process and therefore if they are carried over into the final product it may cause to fail or slow the PCR process considerably. If it could be verified that thetemplate is of sufficient purity, different reagents (phenol, alcohol, EDTA, detergents, and other)that were carried out through DNA extraction process added through the process will strongly inhibit PCR, then hence it is the time to lookthe available and alternative strategies under the microscope.

Design of primer is the other critical parameters for successful PCR products. A common error when doing PCR off genomic DNA is that the introns might be present between the primer sites, which, if long enough, will result in incomplete product, and thus no amplification. It is also possible one or both primers are matching homologous sequences far away from the target sequence; it might be even on completely different chromosomes. Checking the designedprimers in the National Center for Biotechnology Information Basic Local Alignment Search Tool(NCBI BLAST) database to make sure there are no other homologous sequences in your species is a good idea. Generally, a poorly designed primer can result in little or no product due to nonspecific amplification and/or primer-dimer formation, which can become competitive enough to suppress product formation. Therefore, numerous variables such as:

- **Primer length:** Since specificity, temperature and time of annealing partly depend on primer length, this parameter is critical for successful PCR. In general, oligonucleotides between 18 and 24 bases are extremely sequence-specific, provided that the annealing temperature is optimal. Primer length is also proportional to annealing efficiency; the longer the primer, the more inefficient the annealing. With fewer templates primed at each step, this can result in a significant decrease in amplified product. The primers should, however, not be too short unless the application specifically requires it.
- **Melting temperature (T_m):** there are two primers added to a target directed PCR reaction, both of these primers should have similar Tm. If primers are mismatched in terms of Tm, amplification will be less efficient or may not work at all. Since the primer with the higher Tm will misprime at lower temperatures, the primer with the lower Tm may not work at higher temperatures. Hence, Tm calculations are required to get a good product in PCR amplifications. There are different Tm calculation methods which differ based on thermodynamic relationships, standard approximations, salt concentration adjustments, etc.

Thermodynamic Calculations: The Tm calculations based on the thermodynamic relationship are between entropy, enthalpy, free energy and temperature. The Tm of oligosare most accurately calculated using nearest neighbor thermodynamic calculations with the formula:

$$\mathbf{Tm} = \Delta\mathbf{H}\left[\Delta\mathbf{S} + \mathbf{Rln}\left(\frac{\mathbf{c}}{\mathbf{4}}\right)\right] - \mathbf{273.15°C} + \mathbf{16.6log10}[\mathbf{K}+]\boldsymbol{equation}\ (\mathbf{2})$$

Where H is the enthalpy and S is the entropy for helix formation, R is the molar gas constant and c is the concentration of primers. This is most easily accomplished by using primer design software packages already available on the market.

- **Standard approximation calculation methods:** there are two basic formulas
    1. ***For sequences less than 14 nucleotides the Marmur formula:***

$$\mathbf{Tm} = (\mathbf{wA} + \mathbf{xT}) * \mathbf{2} + (\mathbf{yG} + \mathbf{zC}) * \mathbf{4equation}\ (\mathbf{3})$$

Where w, x,y,z are the number of bases A,T,G,C in the sequence, respectively.

2. ***For sequences longer than 14 nucleotides, the Wallace equation***:

$$\mathbf{Tm} = \mathbf{64.9} + \mathbf{41} * \frac{\mathbf{yG} + \mathbf{zC} - \mathbf{16.4}}{(\mathbf{wA} + \mathbf{xT} + \mathbf{yG} + \mathbf{zC})} \mathbf{equation}\ (\mathbf{4})$$

- ***Salt adjusted T_m calculations*:** These equations assume the annealing occurs under the standard conditions of 50nM primer and pH 7.0.

1. ***For sequences less than 14 nucleotides the Nakano formula:***

$$\mathbf{Tm} = (\mathbf{wA} + \mathbf{xT}) * \mathbf{2} + (\mathbf{yG} + \mathbf{zC}) * \mathbf{4} - \mathbf{16.6} * \mathbf{log10}(\mathbf{0.050}) + \mathbf{16.6} * \mathbf{log10}([\mathbf{Na}+])\boldsymbol{eq}\ (\mathbf{5})$$

Where w,x,y,z are the number of the bases A, T, G, C in the sequence, respectively. The term 16.6*$\log_{10}$ ([Na^+]) adjusts the Tm for changes in the salt concentration, and the term $\log_{10}$ (0.050) adjusts for the salt adjustment at 50mMNa^+.

2. ***For sequences longer than 13 nucleotides, the Howley equation:***

$$\mathbf{Tm} = \mathbf{100.5} + \left(\mathbf{41} * \frac{\mathbf{yG} + \mathbf{zC}}{(\mathbf{wA} + \mathbf{xT} + \mathbf{yG} + \mathbf{zC})}\right) - \left(\frac{\mathbf{820}}{(\mathbf{wA} + \mathbf{xT} + \mathbf{yG} + \mathbf{zC})}\right) + \mathbf{16.6} * \mathbf{log10}([\mathbf{Na}+])\mathbf{eq}(\mathbf{6})$$

When calculating the melting temperatures of the primers, care must be taken to ensure that the melting temperature of the product is low enough to obtain 100% melting at 92°C.

- **Specificity:** As mentioned above, primer specificity is at least partly dependent on primer length. Primers must be chosen and had a unique sequence within the template DNA that is to be amplified. A primer designed with a highly repetitive sequence will result in a smear when amplifying genomic DNA. However, the same primer may give a single band, if a single clone from a genomic library is amplified. Primer extension will occur at the lower temperatures of annealing; because Taq DNA polymerase is active over a broad range of temperatures. If the temperature is too low, non-specific priming may occur, which can be extended by the polymerase if there is a short homology at the 3' end. In general, a melting temperature of 55-72°C gives the best results (note that this corresponds to a primer length of 18-24 bases using Wallace's rule.
- **Complementary primer sequences:** Primers need to be designed with absolutely no intra-primer homology beyond 3 base pairs. If a primer has such a region of self-homology, “snap back”, or “hair-pin”, partially double-stranded structures can occur, which will interfere with annealing to the template. Another related danger is inter-primer homology. Partial homology in the middle regions of two primers can interfere with hybridization. If the homology occurs at the 3' end of either primer, primerdimer

formation will occur, which, more often than not, will prevent the formation of the desired product via competition.

- **G/C content and polypyrimidine (T, C) or polypurine (A, G) stretches**: The base composition of primers should be between 45% and 55% GC. The chosen primersequences have no poly-G or poly-C stretches that can promote non-specific annealing.The primer sequence must be chosen so that there is Poly-A and poly-T stretches are also to be avoided, as these will "breathe" and open up stretches of the primer-template complex; thiscan lower the efficiency of amplification. Polypyrimidine (T, C) and polypurine (A, G) stretches should also be avoided. Ideally, the primer will have a near random mix of nucleotides, a 50% GC content, and be ~20 bases long;this will put the Tm in the range of 56-62°C.
- **3'-end sequence**: the 3' terminal position in PCR primers is essential for the control of mispriming. The problem of primer homologies occurring in these regions has already been explored. Another variable is the inclusion of a G or C residue at the 3' end of primers. This "GC Clamp" helps to ensure correct binding at the 3' end, due to the stronger hydrogen bonding of G/C residues. This also helps to improve the efficiency of the reaction by minimizing any "breathing" that might occur.

A final strategy would be to include a "Hot Start" in the givenprogram. Usually a hot start is used to prevent extraneous bands in thePCR products but it definitely can be a help in some circumstances that may prevent bands from forming altogether. To do a hot start, there are two methods:

- The "old school" way is to add everything to each PCR tube (or well) except the DNA polymerase and then bring the reactions to 95°C. Then the polymerase is added to each sample at 95°C. The idea is to prevent unwanted polymerase activity when the samples are heating up from room temperature to 95°C. The problem with this method is, it is very laborious for many samples, it's inconsistent (not possible to add polymerase for all samples at a time), and it allows serious pipetting errors which would make procedures such as qPCR completely unreliable (adding only 0.5 or 1µl to each sample will cause great variability in the actual amount).
- However, using a "Hot Start" polymerase solves these issues easily because polymerase activity is suppressed until the denaturing temperature is reached.

Hence, examining the primer selection/strategy is good because most primer design programs generally take care of things such as primer hair pinning and dimerization.

52.4.1.2 Troubleshooting guide for PCR

Table 9.Checklist guides that can be used to troubleshoot PCR reactions

Observation	Possible causes	Solutions
Sequence errors	Low fidelity polymerase	Choose a higher fidelity polymerase such as Taq DNA Polymerases.
	Suboptimal reaction conditions	Reduce number of cycles. Decrease extension time. Decrease Mg^{2+} concentration in the reaction.
	Unbalanced nucleotide concentrations	Prepare fresh deoxynucleotide mixes.
	Template DNA has been damaged	Start with a fresh template. Limit UV exposure time when analyzing or excising PCR product from the gel.
Incorrect product size	Incorrect annealing temperature	Recalculate primer T_m values using the NEB Tm calculator.
	Mispriming	Verify that the primers have no additional complementary regions within the template DNA.
	Improper Mg^{2+}concentration	Adjust Mg^{2+} concentration in 0.2–1mM increments.
	Nuclease contamination	Repeat reactions using fresh solutions.
No product	Incorrect annealing temperature	Recalculate primer T_m values using the NEB T_m calculator. Test an annealing temperature gradient; starting at 5°C below the lower T_m of the primer pair.
	Poor primer design	Check specific product literature for recommended primer design. Verify that primers are non-complementary, both internally and to each other. Increase length of primer.
	Poor primer specificity	Verify that oligos are complementary to proper target sequence.
	Insufficient primer concentration	Primer concentration can range from 0.05–1µM in the reaction. Please see specific product literature for ideal conditions.
	Missing reaction component	Repeat reaction setup.
	Suboptimal reaction conditions	Optimize Mg^{2+} concentration by testing 0.2–1mM increments. Thoroughly mix Mg^{2+} and buffer solution prior adding to the reaction. Optimize annealing temperature by testing an annealing temperature gradient, starting at 5°C below the lower T_m of the primer pair.
	Poor template quality	Analyze DNA via gel electrophoresis before and after

		incubation with Mg^{2+}.
	Presence of inhibitor in reaction	Further purify starting template by alcohol precipitation, drop dialysis or commercial clean up kit. Decrease sample volume.
	Insufficient number of cycles	Rerun the reaction with more cycles.
	Incorrect thermocycle reprogramming	Check program, verify times and temperatures.
	Inconsistent block temperature	Test calibration of heating block.
	Contamination of reactiontubesor solutions	Autoclave empty reaction tubes prior to use to eliminatebiological inhibitors.Prepare fresh solutions or use new reagents and new tubes.
	Complex template	Use high fidelityTaqDNA Polymerases. For GC-rich templates, use high fidelityTaqDNA polymerases. Include the appropriate GC enhancer.For longer templates, we recommend Hot-Start high fidelity DNA Polymerase.
Multiple ornon-specificproducts	Premature replication	Use a hot start polymerase, such as one TaqHot Start DNAPolymerase. Set up reactions on ice using chilled components and addsamples to thermo cycler preheated to the denaturationtemperature.
	Too low Primer annealing temperature	Increase annealing temperature.
	Incorrect Mg^{2+}concentration	Adjust Mg^{2+}in 0.2–1mM increments.
	Poor primer design	Check specific product literature for recommended primer design. Verify that primers are non-complementary, both internallyand to each other. Increase length of primer. Avoid GC-rich 3´ ends.
	Excess primer	Primer concentration can range from 0.05–1µM in thereaction. Please see specific product literature for idealconditions.
	Contamination withexogenous DNA	Use positive displacement pipettes or non-aerosol tips. Set-up dedicated work area and pipettes for reactionsetup. Wear gloves during reaction setup.
	Incorrect template concentration	For higher complexity templates (i.e. genomic DNA), use 1ng–1µg of DNA per 50µLreaction.

53 GMO Laboratory Service and Standard Operational Procedures

This section contains SOP to be followed at Central Ethiopia Environment and Forest Research Center (CEE-FRC) of Ethiopian Environment and Forest Research Institute (EEFRI) GMO laboratory to provide service for customers, and agreement format that has to be filled by the customer and service provider.

53.1 SOP for GMO Laboratory Service

	Ethiopian Environment and Forest Research Institute **Central Ethiopia Environment and Forest Research Center**	
	Genetically Modified Organism (GMO) Detection Laboratory	
Standard Operating Procedures for GMO Laboratory Service		
Lab SOP No.: EEFRI- 1.0		Version No.:1.0
Date prepared:		Date adopted:

I. PURPOSE

This SOP guideline describes about the basic consideration of standard operating procedures for GMO laboratory service to accept or reject samples to be done in the laboratory that delivered from public and private crop export or import customers, and other developmental agents with full biological description of samples and legal supportive letters from home institute to take legal agreement with our laboratories.

II. POLICY

It is the policy of Biological Division of GMO Laboratory at CEE-FRC to check the acceptability of sample of food crops (soybean, maize, apple juice, chocolate, and other) prior to investigation to avoid confusions on the identity of laboratory samples and to manage the integrity test samples during laboratory custody.

III. RESPONSIBILITY

Custodian, lab managers, lab technicians and workers, and store man are responsible for samples received in Biological Division of GMO Laboratory at CEE-FRC.

IV. TECHNIQUE

To evaluate whether to accept the sample materials or not, Memorandum of understanding (MoU) or other agreements and background interview information are considered.

V. SAMPLE SIZE

In harmony with the work load of the laboratory and the analytical requirements, the size of the sample must be 500g to 1000g for each cereal crops and 300mLto 500mL for each liquid samples such as, apple juices.

VI. APPARATUS

Each individual numbers and amounts are counted manually and weighed using analytical balance for each commodity, respectively. And also liquid matters are measured using micropipette or measuring cylinder.

VII. PROCEDURE

During sample acceptance the following parameters will be checked and considered.

- Corresponding labels and age of the cereal crops.
- Proper packing, labels (production and expiry date) and batch number of the liquid samples.
- The physical integrity and packing materials used.
- Storage condition.
- Make sure the sample is free from pests such as; weevil and pests.
- Sample must be clean; and
- Make sure the samples are not cross contaminated with each other during sampling.

VIII. ATTENTION PLEASE

During sampling:

- Carefully read the sample preparation procedure before sampling.
- Use sampling hook for cereal crop samples.
- Glove must be worn during sampling.
- Use blade/scissors during random sampling to till sack.
- Mask must be worn during sampling to protect the entry of bad matters into the intestinal and respiratory pathway.
- Make sure the samples have free from right contact with other cereal crops for any bias.
- Read more about sample management.

IX. RECORDS

- Sample and Biohazard Disposal EEFRI /CEEFRC-2.0
- Follow up EEFRI / CEEFRC-3.0
- Service Request EEFRI/CEEFRC-4.0
- Contract review EEFRI / CEEFRC-5.0

X. REFERENCE

- Clean harbors los angles, LLC laboratory manual

53.2 SOP for Sample and Biohazard Disposal

	Ethiopian Environment and Forest Research Institute **Central Ethiopia Environment and Forest Research Center**
	Genetically Modified Organism (GMO) Detection Laboratory
Standard Operating Procedures for GMO Laboratory Service	
Lab SOP No.: EEFRI- 2 .0	Version No.:1.0
Date prepared:	Date adopted:

I. PURPOSE

This SOP should be applied to all activities that could be done in the CEE-FRC laboratory which is subjected to the Biological Division Laboratory biosafety guidelines. The principal purpose of this SOP guideline is used to clarify the basic requirement for the right disposal of samples/biohazards after the completion of analysis. In here, the term "biohazard" applies to any waste material (chemical waste, gel waste, EtBr, chloroform, phenol and others) that is potentially hazardous to humans, plants, animals and the environment.

II. POLICY

It is the policy of CEE-FRC to decide on the means of how to dispose the sample material of cereal crops (soybean, maize, corn etc.) and juices (apple juice, chocolate etc.) and/or waste after analysis.

III. RESPONSIBILITY

Custodian, Laboratory manager, Laboratory technician are responsible for samples/waste disposals received at CEE-FRC.

IV. General Guidance for Biohazardous Materials

General guidelines for biohazardous materials and their associated appropriate methods of disposal are described below:

- Biohazardous waste for off-site treatment must be collected in specific containers;

- Biohazardous waste that is autoclaved on-site to render it non-infectious or non- viable must have autoclave tape affixed to the container prior to treatment (select a lead-free autoclave tape);
- Biohazard labels and markings must be avoided following treatment and prior to disposal;
- Red-bags must be placed in unmarked outer bags that are not translucent following autoclave, and; all liquids must be absorbed onto a solid media prior to placement in refuse containers or may be disposed via the sanitary septic tank.
- Biohazardous waste containing live organisms must be decontaminated in autoclaves that have been validated;
- Do not use red biohazard bags for any collected wastes other than biohazardous waste(i.e., regular trash, radioactive materials not contaminated with biohazards); and
- Laboratory waste from infectious agents (i.e., culture plate and broths) must be autoclaved prior to disposal. Chemical disinfection is generally suitable for contaminated disposable and non-disposable laboratory equipment and consumables (i.e., pipette tips, glass, cuvettes, etc.). This material must not be incinerated on-site.

V. Procedures for Sample Disposal after Analysis

- Be sure that whether the samples are to be disposed or retained;

- Samples with proper labeling will only be subjected for retainingin the laboratory;
- Samples having holding time in the sample storage SOP should be disposed appropriately after its expiry time.
- Even if it's holding time is over in the sample storage SOP, samples can be kept and available for analysis if required.
- After a three-month holding period, DNA, RNA, Protein, and other unwanted materials can be disposed according to the laboratory procedure.
- Samples will be retained until threemonth review unless visual inspection revealed changes on the samples such as bad color; bad smell, but if it is changed, the sample will be disposed
- If decisions are made to retain some of the samples, they will be retained at room temperature and
- Generally, keep the rule of the laboratory whether to get or not to get the sample; if the availability and getting of the sample is rare, it will be stored beyond the holding time.

VI. RECORDS

It should have forms for properly documenting the disposal of the sample. Laborers who treat and dispose wastes onsite in accordance with the guidelines described in this SOP must keep the following records: • date of treatment (also time for incineration) • amount of waste treated •

method/conditions of treatment • name (printed) and initials of person(s) performing treatment etc.

VII. REFERENCE

- University of Nebraska Lincoln, 2011/2012.
- Ethiopian Institute of Agricultural Research (EIAR) and Restoration service, Inc. Water sample neutralization and disposal work plan (WP-11141), 2011.

VIII. APPROVAL

The signature below constitutes the approval of all those SOP for use in the laboratory.

Prepared By:

Name: _____
Designation:
E-mail:
Signed: ----------Date: ----------

Approved By:

Name: _____
Designation:
E-mail: _____
Signed: ----------Date: ----------

53.3 Laboratory Administrative Format

53.3.1 Guidelines for Service Request

	Ethiopian Environment and Forest Research Institute **Central Ethiopia Environment and Forest Research Center**
	Genetically Modified Organism (GMO) Detection Laboratory
Standard Operating Procedures for GMO Laboratory Service	
Lab SOP No.: EEFRI- 3.0	Version No.:1.0
Date prepared:	Date adopted:

የጥያቄው ቁጥር

Request No.፡_____

በአገልግሎትጠያቂውየሚሞላ **TO BE FILLED BY REQUESTING BODY**

ለ

1. TO_____

አገልግሎትተጠያቂው

2. Requested by_____

የሚፈለገውአገልግሎት

3. Service requested_____

Sample Type:_____	Sample No._____

4.አባሪየሆኑመረጃቸውዓይነትእናየገጽብዛት

Attached document (Title and No. of pages)

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_____
_____
_____
_____
_____
_____
_____

5.ተጨማሪመግለጫ
Additional information:

_____
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ጥያቄውንያቀረበውጥያቄውንየተረከበው

Requested by

Name:_____
Sign:_____
Date:_____

Request received by

Name:_____
Sign:_____
Date:_____

53.3.2 Guidelines for Contract Review

	Ethiopian Environment and Forest Research Institute **Central Ethiopia Environment and Forest Research Center**
	Genetically Modified Organism (GMO) Detection Laboratory
Standard Operating Procedures for GMO Laboratory Service	
Lab SOP No.: EEFRI- 4.0	Version No.:1.0
Date prepared:	Date adopted:

1. Customer name_____
2. Customer request No._____
3. Sample Code_____
4. Purpose of the Request_____
5. Sample description_____
6. Date of Sampling_____
7. Address: Tel No._____Fax.No._____email_____
8. Activity to be done

Activity to be done	**Method to be followed**
Cereal crop grinding	
DNA extraction	
Centrifugation	
Incubation	
Agarose Gel electrophoresis	
PCR	
RT-PCR	
Gel Documentation	

9. Other activities/Methods/Requirements/Discussions/Request/comments:
_____
_____
_____
_____
_____
_____
10. Date of Appointment _____
11. Service Fee _____
12. Transmissions of Result
_____
_____
13. Record of acceptance by the customer
Customer Name_____ Sign._____
Date_____
Custodian Name_____ Sign._____
Date_____

53.3.3 Guidelines for Follow Up

	Ethiopian Environment and Forest Research Institute **Central Ethiopia Environment and Forest Research Center**
	Genetically Modified Organism (GMO) Detection Laboratory
Standard Operating Procedures for GMO Laboratory Service	
Lab SOP No.: EEFRI- 5.0	Version No.:1.0
Date prepared:	Date adopted:

TO BE FILLED BY THE CUSTODIAN

Sample Type _____	Sample No.:_____
Sample Code:_____	

No.	Requested Activity	Method to be followed

Submitted by: _____ Sign_____ Date: _____

FOR LABORATORY USE

S.N ተ/ቁ	Received by/ Submitted to የተላለፈለት/ የተመራለት	To be delivered/ service delivered የሚከናወን/ የተከናወነ	Date/Time ቀን/ ሰዓት	ID/Sign. መ/ቁ/ ፊርማ

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About the authors

Smegnew Melese obtained his B.Sc. degree in Biotechnology from the University of Gondar. He has been working on GMO detection and analysis at the Biosafety Laboratory of EEFRI for the past two years. He has the experience of working in molecular biology, vaccine production technology, and food quality analysis. Before he joined EEFRI, he worked at the National Veterinary Institute (NVI), Kality Share Company and Universal Food Complex Plc. 21melese@gmail.com

Belete Getnet obtained his M.Sc. degree in Plant Biotechnology from Jimma University. He has been a research staff at the Tree Seed Technology Coordination Unit of EEFRI since December, 2019. He has an experience in the area of plant tissue culture, molecular biology, molecular breeding, and plant breeding. Before he joined EEFRI, he worked in the Ethiopian Sugar Corporation, Research and Development Center at Sugarcane Biotech and Breeding Directorate. beletebiot@gmail.com

Alemayehu Esayas (Ph.D.) is currently a researcher and director of Environmental Laboratory at EEFRI. He has expertise in the area of analytical chemistry, environmental chemistry and ecotoxicology. Before he joined EEFRI, he served as a lecturer of chemistry and analytical chemistry at Hawassa University. alemayehu.esayas@gmail.com

Amare Genetu obtained his B.Sc. degree in Biotechnology from the University of Gondar. He has been a research staff at EEFRI working on GMO detection at the Biosafety Laboratory of EEFRI for the past two years. amarebiotech2008@gmail.com

Adugnaw Admas obtained his M.Sc. degree in Medical Biotechnology from the University of Gondar. He has been a research staff at EEFRI working on GMO detection at the Biosafety Laboratory for the past five years. He has experience in molecular biology and microbiology techniques. Before he joined EEFRI, he has worked at the Ethiopian Environmental Protection Authority. adu.biot@gmail.com

EEFRI