Manual on GMO Detection and Good Laboratory Practices

Implementation of the National Biosafety Framework of Bangladesh Project

Department of Environment

Ministry of Environment, Forest and Climate Change

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CONTENTS

Abbreviations

LMOs	: Living Modified Organisms
DNA	: Deoxyribonucleic acid
GMOs	: Genetically Modified Organisms
GM	: Genetically Modified
PEG	: Polyethylene Glycol
T-DNA	: Transfer Deoxyribonucleic acid
mRNA	: Messenger Ribonucleic acid
ELISA	: Enzyme-linked Immunosorbent Assay
PCR	: Polymerase Chain Reaction
Taq	: *Thermus aquaticus*
qRT-PCR	: quantitative Reverse Transcriptase Polymerase Chain Reaction
RT-PCR	: Real Time Polymerase Chain Reaction
Ct	: **Cycle threshold**
FRET	: Forster Resonance Energy Transfer
TMB	: 3,3',5,5'- Tetramethylbenzidine
PBS	: Phosphate-Buffered Saline
CTAB	: Cetrimonium bromide
dsDNA	: double stranded DNA
TRIzol	: Total RNA Isolation reagent
M-MuLV RT (page 29)	: Murine Leukemia Virus Reverse Transcriptase
AMVRT(page 29)	: Avian Myeloblastosis virus Reverse Transcriptase
cDNA	: Complementary DNA
dNTP	: deoxyribonucleotide triphosphate
EDTA	: Ethylenediamine Tetra Aacetic acid
SDS	: Sodium Dodecyl Sulfate
BSA	: Bovine Serum Albumin
PBST	: Phosphate Buffered Saline with Tween 20

BCIP	: 5-Bromo-4-Chloro-3-Indolyl Phosphate
SDS-PAGE	: Sodium Dodecyl Sulfate- Polyacrylamide Gel electrophoresis
HRP	: Horseradish Peroxide
Ab	: Antibodies
Ag-Ab	: Antigen-Antibodies
DEAE (page 40)	: Diethylamino ethanol
PVP	: Polyvenyl pyreledon
CaMV35S	: Cauliflower Mosaic Virus 35S

INTRODUCTION

The United Nations Conference on Environment and Development, also known as the "Earth Summit", held in Rio de Janeiro in 1992 recognized the potential of biotechnology in contributing enhancement of food security, health and environment. To achieve environmentally sound application of biotechnology several documents have been made following that summit which shaped the basis of international law on biosafety. The documents include Agenda 21, the Rio Declaration on Environment and Development and the United Nations Convention on Biological Diversity, etc.

The term biosafety means protection of the environment and human health from potential harm due to biological agents. Under the Convention on Biological Diversity and more specifically under the Cartagena Protocol on Biosafety this biological agent is LMOs.

According to the Cartagena Protocol on Biosafety Article 3, paragraphs (g) and (i):

(a) "Living modified organism" means any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology;

(b) "Modern biotechnology" means the application of:

(i) *In vitro* nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles; or

(ii) Fusion of cells beyond the taxonomic family; that overcome natural physiological reproductive or recombination barriers and that are not techniques used in traditional breeding and selection.

Modern biotechnology techniques include, but are not limited to, *in vitro* DNA and RNA techniques for the modification of genetic material (eg. by insertion, modification or deletion of genes or other nucleic acid sequences) in all types of organisms, such as plants, animals, microbes and viruses.

Detection and identification of GMOs/LMOs becomes a vital element in contributing to the implementations of the objectives of the Cartagena Protocol, with respect to the safe transfer, handling and use of living modified organisms resulting from modern biotechnology. Detection and identification of LMOs would hold in implementation of several Articles of the Cartagena Protocol on Biosafety. For example, risk assessment

and risk management; unintentional transboundary movements and finally emergency measures, etc. This manual will be an important document for the researchers and biosafety regulators which will help significantly to detect GMOs/LMOs.

It may be noteworthy to mention, keeping up with the growing complexity of the transgenic crops, GMO detection is a long-term future challenge. Majority of the approved GM crop varieties can be detected using a common 35S/NOS PCR-based detection method, but new transgenic crops are likely to have different promoters and terminators, even with native promoter and terminator, no selectable marker or reporter gene, or the selectable markers would be eliminated by recombination. Moreover, transgene homologous to the native gene using antisense/sense suppression technology which will complicate GMO detection approaches. As a result, new technologies need to be developed to cope with the progressive complexity, providing accurate GMO testing results at a reasonable cost and in a reasonable amount of time.

METHODS OF GENETIC MODIFICATION OF PLANTS

In the agricultural science, the term GMOs/LMOs refers to the plants where foreign genetic material (from plant or non-plant sources) has been introduced through modern biotechnology techniques. This genetically modified plant is then called "transgenic" plant.

Genetically modified (GM) or transgenic plants are playing an important role in crop germplasm improvement. Transgenic technologies started in mid 1980s. Genetic engineering of crop plants though apparently simple in principle but is a complex, sophisticated and costly technology, which requires a certain degree of expertise. Genetic engineering of crops plants comprises of following steps:

1. Isolation of gene
2. Vector construction
3. Transformation
4. Efficient regeneration system
5. Gene integration and expression analysis
6. Inheritance
7. Field evaluation

There are several methods for introducing foreign DNA into plants. Following methods are commonly being used to improve plants through biotechnology:

A. Indirect DNA Delivery System (Vector mediated): *Agrobacterium* mediated transformation.

B. Direct Delivery of DNA

B.1. Protoplast based transformation

- PEG-mediated
- Electroporation
- Microinjection
- Liposome mediated

B.2. Non-protoplast based transformation

Gene gun/ Biolistic **Method**

However, among all the methods *Agrobacterium*-mediated genetic transformation and gene gun method are most commonly used in case of plant genetic transformation.

Agrobacterium-Mediated Transformation:

Engineering crops with desired traits depends upon the reliable and efficient means of transferring Gene of Interest into plants. The gram negative soil bacteria, *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* are natural genetic engineers capable of transforming a range of dicotyledonous plants by transferring plasmid encoded genes into recipient plants genomes. Both bacteria contain a large plasmid (more than 200Kb), which is required for initiation of the neoplasia (crown gall and hairy root). In the case of *A. tumefaciens*, the plasmid is called the tumor inducing Ti plasmid whereas, it is called the root inducing or Ri plasmid in the case of *A. rhizogenes*. During the establishment of infection, a region of either the Ti or Ri plasmids termed the T-DNA is transferred into the plant cell and becomes stably integrated into one of the chromosomes in the nucleus (Figure 1).

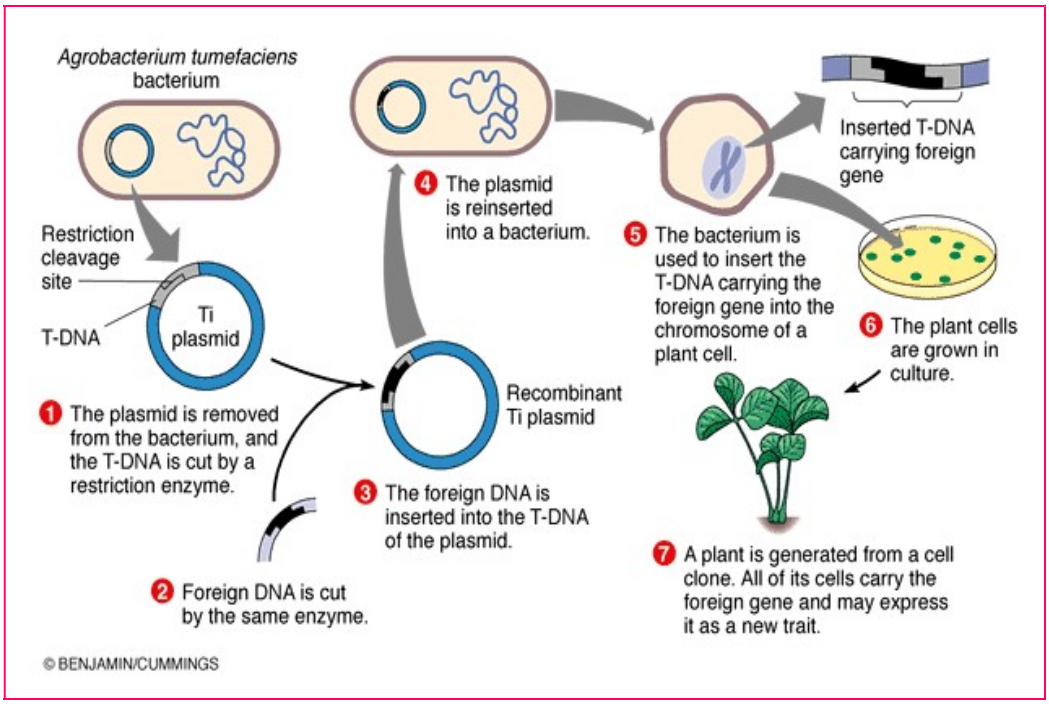

Figure 1: Agrobacterium-mediated genetic modification in plants (Source: https://lh3.googleusercontent.com; Date visited: 16.02.2020)

Gene gun or Biolistic Transformation:

One way to circumvent the limitations of regeneration of *Agrobacterium* mediated transformation method is to use micro-projectile bombardment, also called particle gun or biolistic method of transformation. In this method, tissues or cells are bombarded with DNA typically bound to gold or tungsten particles and the plants are then regenerated. The merit of this procedure is that almost any kind of tissue having the potential to regenerate plants can be used as the target/recipient for foreign DNA. Thus particle bombardment provides best method for achieving genotype independent transformation in plants. After the development of first apparatus based on gun-powder charge, different types of guns have been developed/used by varying the mode of the acceleration of particles like compressed air, nitrogen gas, helium gas and electric discharge (Figure 2). Recently, a hand held particle gun 'Heliose' has been introduced in the market.

The first report of transgenic plant production through particle bombardment appeared in 1988 when transgenic soybean plants were produced. By 1990, transgenic cotton, maize and tobacco were reported. In the following years, almost all major crops like rice, wheat, and sugarcane were transformed using particle bombardment.

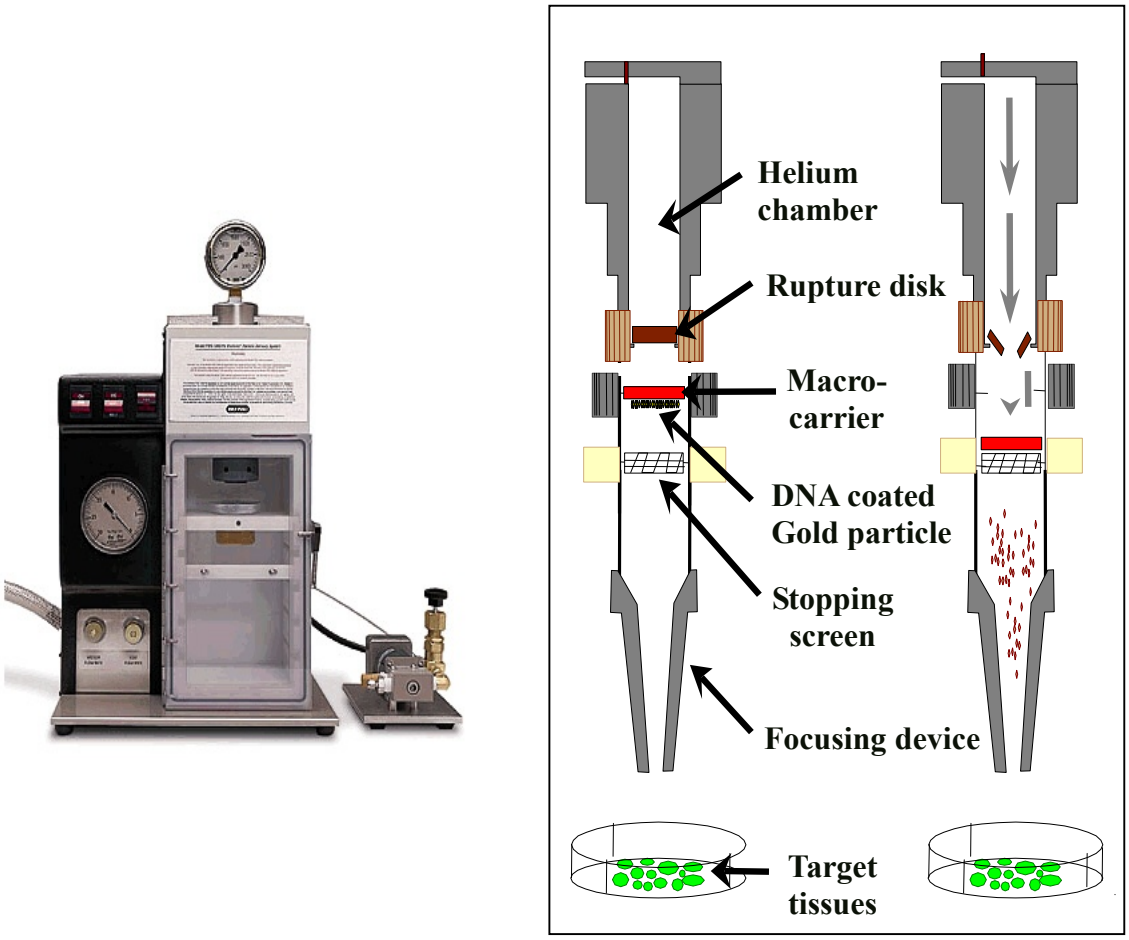

Figure 2: Genetic modification of plants through particle Gene gun delivery system (Source: https://geneticliteracyproject.org/wp-content/; Date visited: 16.02.2020)

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The ideal transformation system should be based on a DNA delivery technology that is simple, efficient and inexpensive. This process should enable efficient, high fidelity integration of transgene sequence(s) (nuclear, plastid or mitochondrial). Target cells for DNA delivery should be easy to isolate and exhibit totipotency from a wide array of genotypes within a species. The tissue culture process required for selection of transgenic cultures and regeneration of plants from there should be minimized to reduce the frequency of deleterious mutations. These guidelines will differ from species to species. Improvements to these DNA delivery systems will assist in increasing the frequency of stable transformation and promote utility of such systems in industry.

METHODS OF GMO DETECTION

Background Information

The detection of GMO/LMOs, more importantly the genetically modified (GM) crops of Bangladesh has become necessary for risk assessment, risk management, unintentional transboundary movements and finally to address emergency measures. Identification of these allows consumers to make an informed choice. Although legislation governs the release of new foods, and a strict approval process includes the safety assessment of every new GM crop, there is still reluctance on the part of consumers to knowingly buy plant-derived GM products. Public concern and awareness of the issue have been significantly increased. Food safety is concerned with consumers' right to know what is in the products they buy. Without transparent information flow of GM products, informed choice cannot be sustained. Lack of information also negatively impacts the development of biotechnology. Although biotechnology presents considerable potential for food production and crop improvement efficiency, but food derived from biotechnology will face constant challenges from consumers around the world until information and evidence of the issues raised can be resolved. Some countries have introduced mandatory-labeling legislation of GM foods to give their consumers a choice in selecting the foods they feel comfortable with. An agreement, the 'Cartagena Protocol on Biosafety', governs the trade and transfers of GMOs across international borders and allows governments to prohibit importation of GM food when there is concern over its safety. Universal legislation makes it imperative for governments, the food industry, crop producers and the testing laboratories to develop ways to accurately quantitate GMOs in crops, foods and food ingredients to assure compliance with threshold levels of GM products required for labeling.

Basics of GMO Detection

A genetically modified organism (GMO) is a living organism, e.g. a plant, whose genetic composition has been altered through recombinant DNA technology. The

genetic modification usually involves insertion of a foreign DNA into the genome of the organism. The most common genetic modifications in crops confer herbicide or insect resistance to the plant. This resistance is achieved through production of a novel protein encoded by the inserted DNA sequence. In plants that are genetically modified for commercial agricultural purposes, the recombinant DNAs that are artificially inserted into the natural plant genome have some common genetic elements. Each inserted DNA sequence consists of at least a promoter, a protein-coding site (the structural gene) and a terminator. The promoter is a sequence of DNA that acts like an "on switch" for the transcription of DNA into mRNA. The terminator marks the end point for this transcription procedure. The structural gene determines the particular protein that is to be made.

Since, GM products contain an additional trait encoded by introduced gene(s), which generally produce additional protein(s) that confers the trait of interest. Therefore, raw materials (e.g. grains) and processed products (e.g. foods) derived from GM crops might thus be identified by testing for the presence of introduced DNA, or by detecting expressed novel proteins encoded by the genetic material.

Methods of GMO Detection

There are several commonly used GMO testing protocols, which include protein and DNA based detection. Of these methods, DNA-based polymerase chain reaction (PCR) with different formats achieves the greatest sensitivity, selectivity, and ability to screen a wide range of GM products. While among protein-based, Enzyme-linked immunosorbent assay (ELISA) is a quicker, less expensive and simpler to perform. Also the range of tests vary from fast and cost-effective, such as the lateral flow assay and endpoint polymerase chain reaction (PCR), to more complex experiments, such as, RT PCR and genome sequencing. But the suitability of these test or selection of test depends on the testing sample. The immunological assay is based on the specific binding between a protein and an antibody and therefore, any conformational changes in the tertiary structure of the protein render the test ineffective. Such conformational changes are induced frequently during food processing and hence, processed foods are generally analyzed with PCR method. Although much progress has been achieved in the development of genetic analysis methods, several other analytical technologies are

emerging that can provide solutions to current technical issues in GMO analysis. Those include, mass spectrometry, chromatography, near infrared spectroscopy, micro fabricated devices, DNA chip technology and nanoscale GMO analysis.

A) Protein-Based GMO Detection

The specific detection of a novel protein synthesized by a gene introduced during transformation constitutes an alternative approach for the identification of genetically modified plants. Protein detection methods are based mainly on immunoassays, which can be used for the detection of many types of proteins in different matrices when the target analyte is known. Both monoclonal (highly specific) and polyclonal (often more sensitive) antibodies can be used. On the basis of typical concentrations of transgenic material in plant tissue (more than 10 µg per tissue), the detection limits of protein immunoassays can predict the presence of modified proteins in the range of 1% GMOs. There are also some limitations of protein based GMO testing because genetic modification is not always specifically directed at the production of a new protein and does not always result in protein expression levels sufficient for detection purposes. In addition, certain proteins may be expressed only in specific parts of the plant or expressed at different levels in distinct parts or during different phases of the physiological development. Since immunoassays require proteins with an intact tertiary or quaternary structure, so these methods are limited to fresh and unprocessed foods.

I) Immuno-assays

Immuno-assays are analytical measurement systems that use antibodies as test reagents. Antibodies are proteins produced in the serum of animals in response to foreign substances (antigens) and specifically bind the substance that elicited their production. In the case of detection of GMOs, the antigen can be the newly synthesized protein. One of the major drawbacks of immunochemical assays is that their accuracy and precision can be adversely affected in complex matrices, such as, processed vegetables and food products. Indeed, many substances present in food matrixes, such as, surfactants (saponins), phenolic compounds, fatty acids, endogenous phosphatases, or enzymes may inhibit the specific antigen-antibody interaction. Moreover, detection capability may be hampered when the transgenic protein is expressed at a very low level, or degraded and denatured by thermal treatment. The newly expressed protein may not be evenly present in all tissue of the plant.

a) ELISA

The Enzyme-Linked Immunosorbent Assay, ELISA, is a type of immunological testing procedure that is based on the use of the specific interaction that takes place between antibodies and their antigens as shown in Figure 3. This technique is used to identify the protein that is synthesized by the newly introduced genes within LMOs or GM plants. The ELISA technique has been widely applied for evaluating the expression level of the protein(s) synthesized by the newly introduced gene. With ELISA we can detect the presence of GMOs in raw material at concentrations ranging between 0.3% and 5%. However, differences may be observed in the expression level of the protein between crop varieties. There are two formats in ELISA: the microwell plate (or strip) format and coated tube format. In an ELISA test for genetically modified agricultural product, the novel protein that is made by the bioengineered gene, is isolated and antibodies are raised against specific surface structures (epitopes) of this protein. If proteins are present, they are bound to the walls of the test kit and react with tagged antibodies resulting in a change of color. In some commercial kits it is possible to get a quantified reading of the amount of targeted protein by preparing a standard curve and using a photometric reader to compare the degree of colour change of the sample to the standard curve.

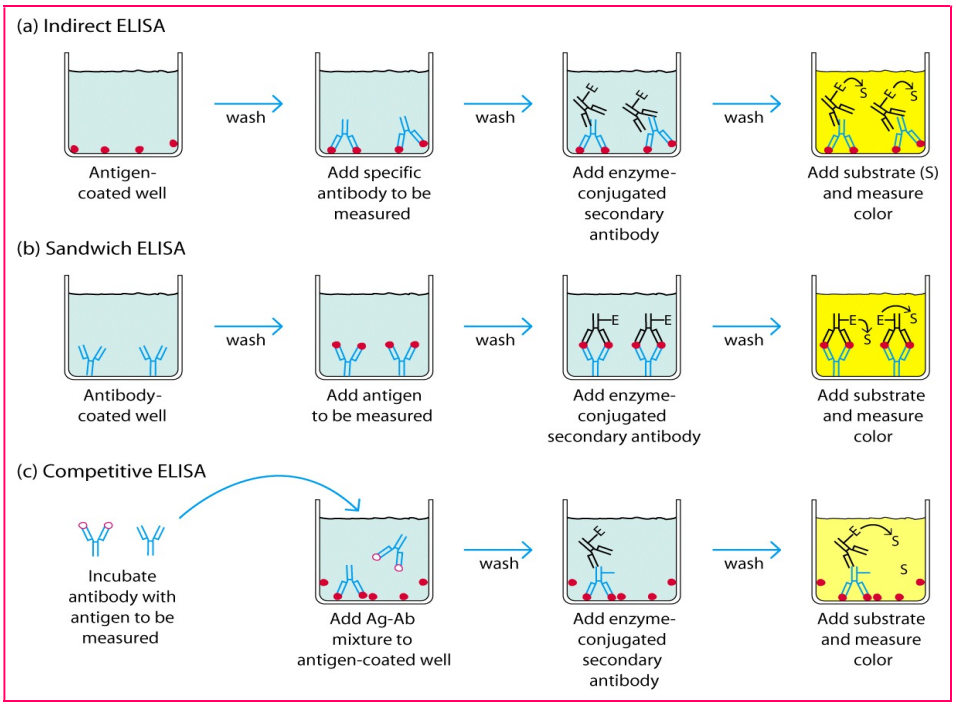


Figure 3. Immunology-based technique using indirect ELISA, sandwich ELISA, and competitive ELISA through schematic diagrams (Source:

https://www.researchgate.net/publication/319354384; Date visited: 16.02.2020).

b) Lateral Flow Strip

A lateral flow assay is a simple and easy-to-use detection method that is based on the use of immuno-chromatography. This assay very rapidly confirms the presence or absence of a specific analyte (new gene expressed protein). The assay is available in the form of a test strip impregnated with antibodies. Here immobilized two antibodies are coupled to a color reactant and incorporated into a nitrocellulose strip. When the strip is placed in a plastic Eppendorf vial containing an extract from plant tissue or seed harboring a transgenic protein, it leads to an antibody sandwich with some of the antibody that is coupled to the color reagent. This colored sandwich flows to the other end of the strip through a porous membrane that contains two captured zones, one specific for the transgenic protein sandwich and another specific for host natural antibodies (control) coupled to the color reagent. Presence of only one (control) line on the membrane indicates a negative sample, and presence of two lines indicates a positive result (Figure 4).

The lateral flow format gives results within a few minutes, but these results are qualitative because there is no quantitative internal standard within the assay and no extra information can be obtained concerning the presence of GMOs at the ingredient level in food. It is economical, more amenable to point-of-sale application, and suitable as an initial screening method early in the food chain. These test strips are fast, cheap and require minimal training and equipment.

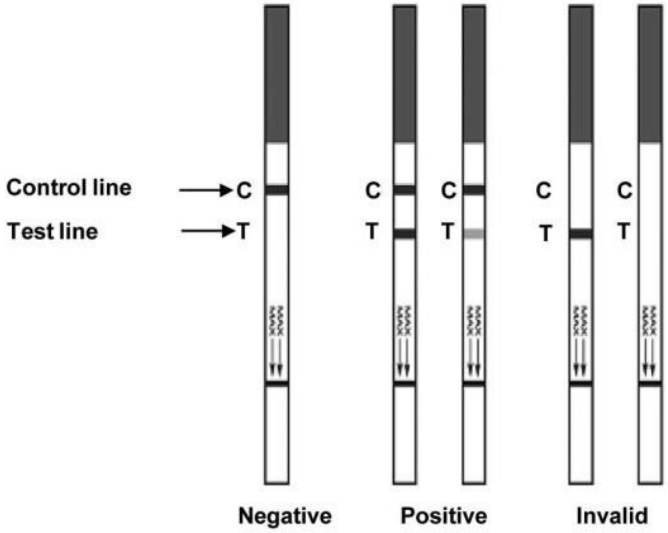
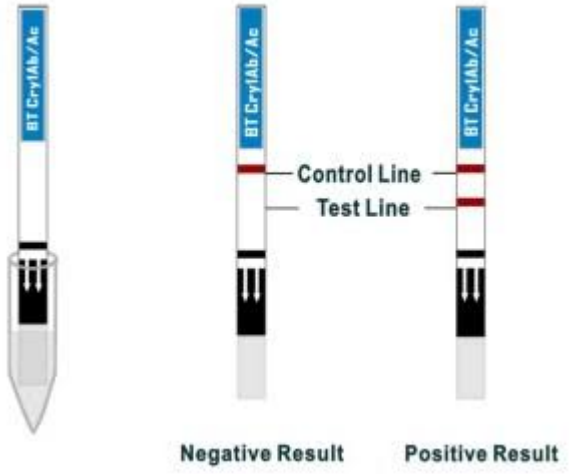


Figure 4: Schematic diagram of the structure and components of a lateral flow assay test strip. Source: Adapted from https://www.spandidos-publications.com/ijmm/30/5/1041 (Date visited: 16.02.2020).



B) DNA-Based GMO Detection

As DNA is a rather stable molecule it is the preferred analyte for almost any kind of sample (raw materials, ingredients, processed foods). Irrespective of the variety of methods used for DNA analysis, only PCR in its different formats has been widely applied in GMO detection/analysis and generally accepted for regulatory compliance purposes. PCR is very sensitive and therefore, very small amount of material is required for the analysis.

PCR-Based Detection

PCR allows the million-fold amplification of a specific target DNA fragment framed by two primers. In principle, the PCR is a multiple-process with consecutive cycles of three different temperatures, where the number of target sequences grows exponentially according to the number of cycles. In the first step, the template, i.e. the DNA serving as master-copy for the DNA polymerase is separated into single strands by heat denaturation at ~94°C. In the second step, the reaction mix is cooled down to a temperature of 50- 65°C (depending on the GC-content) to allow the annealing of the primers to the target sequence. In the third step, the annealed primers are extended usually by a *Thermus aquaticus* (*Taq*) polymerase at the optimum temperature of 72°C. With the elongation of the primers, a copy of the target sequence is generated. The cycle is then repeated about 40 times (Figure 5).

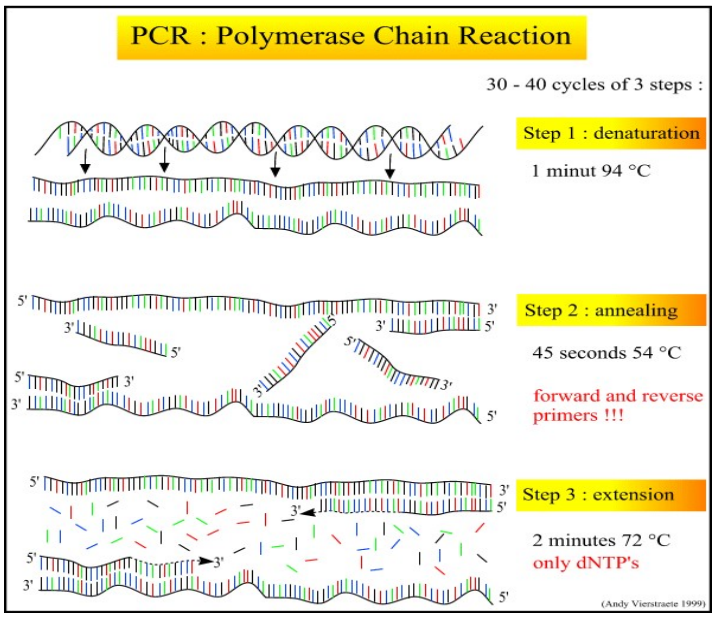


Figure 5: Steps of Polymerase Chain Reaction (Source: http://users.ugent.be/~avierstr/principles/pcr.html; Date visited: 16.02.2020)

Any PCR-based GMO detection strategy will thus depend on the selection of the oligonucleotide primers and the detailed knowledge of the molecular structure and transgenic DNA sequences used in the development of all GMOs.

Real-time PCR

qRT-PCR (quantitative RT-PCR) is the most sensitive technique for mRNA detection and quantification from much smaller samples. The development of novel chemistries

and instrumentation platforms enabling detection of PCR products on a real-time basis has led to widespread adoption of real-time RT-PCR as the method of choice for quantitating changes in gene expression.
Any PCR-based GMO detection strategy will thus depend on the selection of the oligonucleotide primers and the detailed knowledge of the molecular structure and transgenic DNA sequences used in the development of all GMOs.

In theory, production of PCR products should proceed exponentially. However, in practice it reaches a plateau between 30 and 40 cycles because certain reaction components become limited. In conventional PCR, products of the reaction are measured at a single point in the reaction profile. Plotting the concentration of products present at this point as a function of the initial amount of DNA present in each of those reactions shows that proportionality between DNA concentration (dynamic range) and PCR products occurs over a limited range of DNA concentrations, leading to loss of precision in quantification. However, it has been shown empirically that the concentration of DNA in real-time PCR reaction is proportional to PCR cycle number during the exponential phase of PCR. Therefore, if the number of cycles it takes for a sample to reach the same point in its exponential growth curve is known, its precise initial DNA content can be determined. Real-time PCR also allows for detection of low copy DNA number. A unique feature of this PCR technique is that the amplification of the target DNA sequence can be followed during the whole reaction by indirect monitoring of the product formation. Therefore, the conventional PCR reaction has to be adapted in order to generate a constant measurable signal, whose intensity is directly related to the amount of amplified product. Real time detection strategies rely on continuous measurements of the increments in fluorescence generated during the PCR reaction. The number of PCR cycles necessary to generate a signal that is significantly and statistically above noise level is taken as a quantitative measure and is called cycle threshold (Ct). As long as the Ct value is measured at the stage of the PCR where the efficiency is still constant, the Ct value is inversely proportional to the log of the initial amount of target molecules. Currently four different chemistries, TaqMan® (Applied Biosystems, Foster City, CA, USA), Molecular Beacons, Scorpions® and SYBR® Green (Molecular Probes), are available for real-time PCR. All of these chemistries allow detection of PCR products via the generation of a fluorescent signal. TaqMan

probes, Molecular Beacons and Scorpions depend on Förster Resonance Energy Transfer (FRET) to generate the fluorescence signal via the coupling of a fluorogenic dye molecule and a quencher moiety to the same or different oligonucleotide substrates. SYBR Green is a fluorogenic dye that exhibits little fluorescence when in solution, but emits a strong fluorescent signal upon binding to double-stranded DNA.

SYBR Green provides the simplest and most economical format for detecting and quantitating PCR products in real-time reactions. SYBR Green binds double-stranded DNA, and upon excitation emits light. Thus, as a PCR product accumulates, fluorescence increases proportionately. The advantages of SYBR Green are that it is inexpensive, easy to use, and sensitive. The disadvantage is that SYBR Green will bind to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products, which results in an overestimation of the target concentration. For single PCR product reactions with well-designed primers, SYBR Green can work extremely well, with spurious non-specific background only showing up in very late cycles. However, detection by SYBR Green requires extensive optimization. Since the dye cannot distinguish between specific and non-specific product accumulated during PCR, follow up assays such as dissociation-curve or gel analysis are needed to validate results. A dissociation curve (also known as melt curve) is a graph that displays dissociation data from the amplicons of quantitative PCR runs. To generate this curve change in fluorescence, due to a dye or probe interacting with double-stranded DNA is plotted against temperature.

Blotting Techniques:

The blot analysis technique is a powerful method to detect specific biomolecules in samples of complex composition. It can be applied to biomolecules that will adhere stably to a support material such as a nitrocellulose, nylon or paper membrane and are still able to bind their cognate ligand. Usually, the biomolecules of interest are first separated according to size and/or charge before transfer to the membrane. The method was originally developed for DNA by Edwin M. Southern, who demonstrated that DNA restriction fragments that had been electrophoretically fractionated in agarose gels could be transferred to a solid support (nitrocellulose) and detected as discrete

bands following hybridization to a complementary DNA probe. When the Southern blotting method was applied to RNA it was termed Northern blotting, whereas Western blot analysis refers to the transfer of proteins to membranes and their detection with antibody probes.

A) Southern Blotting

Southern blotting is a hybridization technique for identification of particular size of **DNA** from the mixture of other similar molecules**.** This technique is based on the principle of separation of DNA fragments by gel electrophoresis and identified by labelled probe hybridization. Basically, the DNA fragments are separated on the basis of size and charge during electrophoresis. Separated DNA fragments after transferring on nylon membrane, the desired DNA is detected using specific DNA probe that is complementary to the desired DNA. A hybridization probe is a short (100-500bp), single stranded DNA. The probes are labeled with a marker so that they can be detected after hybridization.

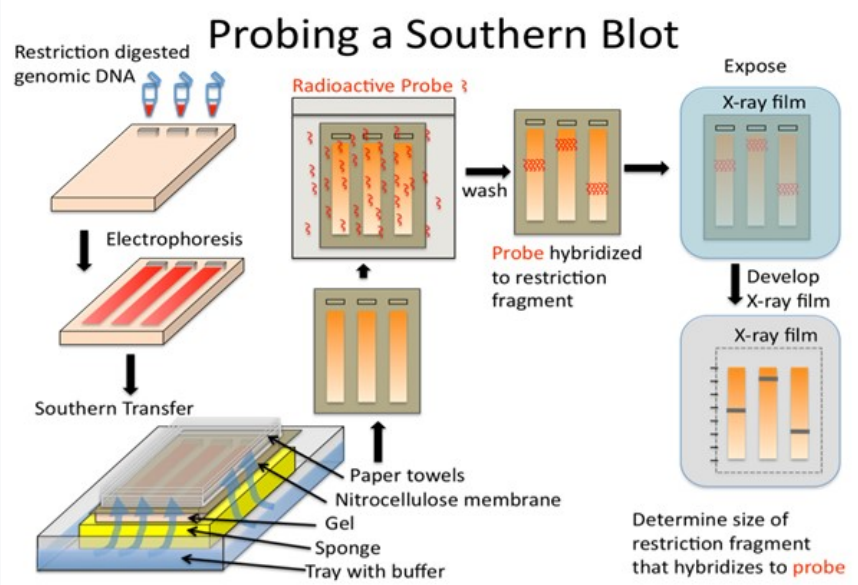


Figure 6. Flow diagram outlining the general procedure for DNA detection by Southern blotting (Source: Todd Nickle and Isabelle Barrette-Ng; *Original-J. Locke-CC:AN;* Date visited: 16.02.2020*)*

B) Northern Blotting

Northern blotting is a technique for detection of specific RNA sequences. Northern blotting was developed in 1977 by James Alwine, David Kemp and George Stark at Stanford University and was named such by analogy to Southern blotting.

The northern blot is a technique used to study gene expression via mRNA transcripts. The northern blot was named after the southern blot, which was developed to study DNA. The two techniques are the same except that the northern blot is used to detect RNA while the southern blot is used to detect DNA. The northern blot protocol, in brief, involves gel electrophoresis to separate mRNA by size, a blotting step to transfer the separated mRNA to a membrane, and a probe hybridization step to identify the mRNA sequence of interest. Even with the advent of powerful RNA analysis techniques such as RT-qPCR and sequencing, the northern blot is still useful for comparing gene expression between samples. The northern blot protocol is relatively inexpensive and makes it easy to visualize the results on a single membrane.

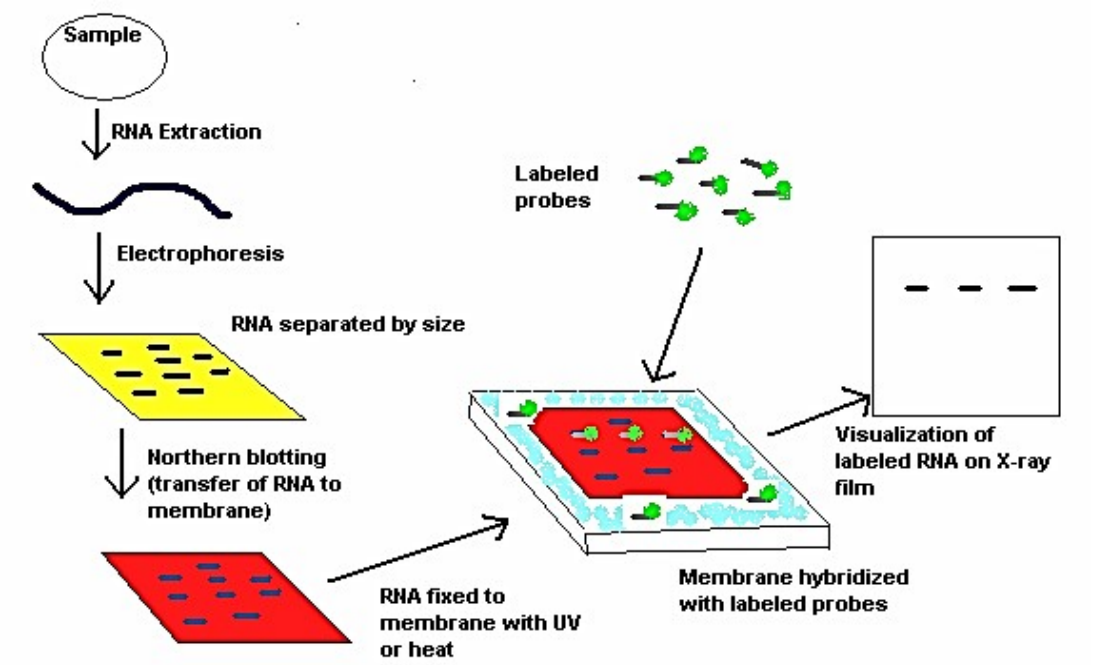


Figure 7. Flow diagram outlining the general procedure for RNA detection by northern blotting (Source: https://en.wikipedia.org/wiki/Northern_blot; Date visited: 16.02.2020)

C) Western blotting technique

Western blotting technique is used for identification of particular protein from the mixture of protein. In this method labelled antibody against particular protein is used identify the desired protein, so it is a specific test. Western blotting is also known as immunoblotting because it uses antibodies to detect the protein.

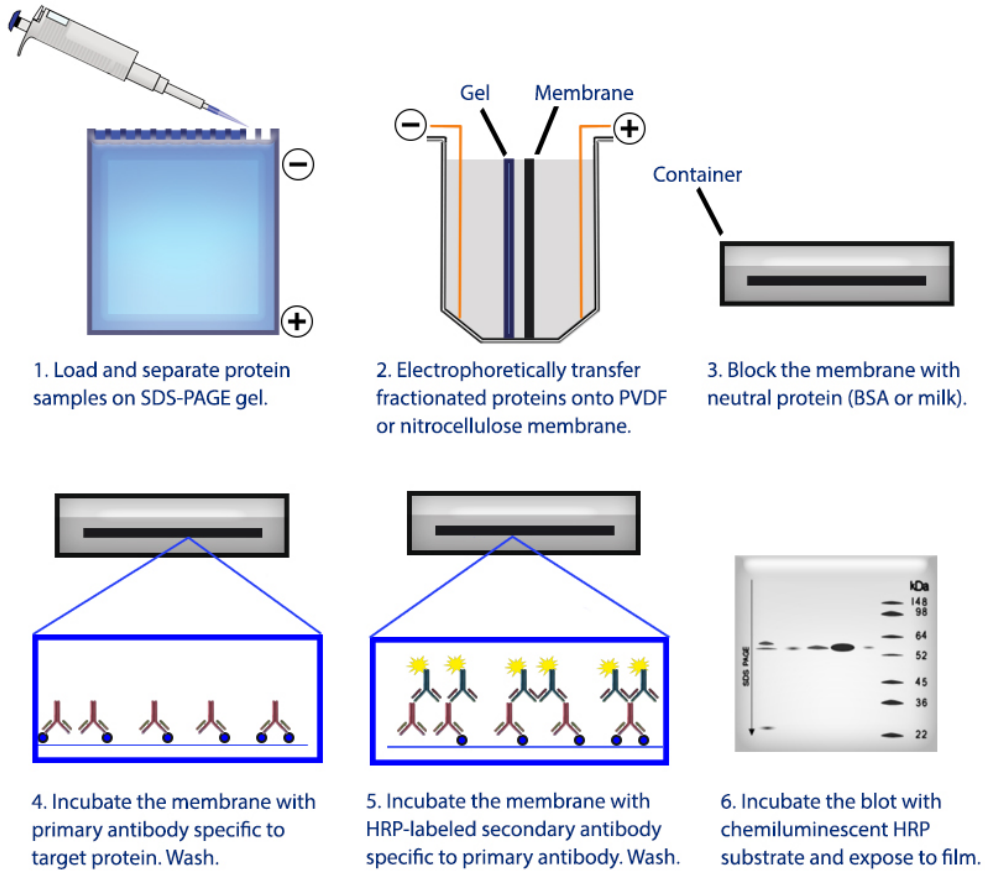


Figure 8. Flow diagram outlining the general procedure for protein detection by Western blotting (Source: https://www.biolegend.com/en-us/western-blot; Date visited: 16.02.2020).

Annexure-I

Experimental Protocols

Protocols

A. Protein based GMO Detection Method:

Protocol 1: ELISA Method

Protocol 2: Lateral flow strip method

B. DNA/RNA based GMO/LMO Detection

B.1: PCR based Method:

Protocol 3: Isolation of Genomic DNA Using CTAB Method

Protocol 4: DNA quantification and quality check: Spectrophotometry

Protocol 5: Polymerase Chain Reaction (PCR)

Protocol 6: Gel electrophoresis and DNA visualization

B.2: **Gene Expression Analysis by Quantitative Real-Time PCR** :

Protocol 7: RNA isolation from plant tissue

Protocol 8: RNA quantification and quality check by Spectrophotometry

Protocol 9: Reverse Transcription: first strand cDNA synthesis

Protocol 10: Real-Time PCR

C. GMO/LMO Detection using Blotting Techniques:

Protocol 11: Southern Blotting Technique

Protocol 12: Northern Blotting Technique

Protocol 13: Western Blotting Technique

A. Protein based GMO Detection Method:

Protocol 1: Enzyme-Linked ImmunoSorbent Assay (ELISA) method

Requirements: (Manufacturers protocol should be followed)

1. Microtiter plate
2. Buffers (Coating buffer, wash buffers, blocking buffers etc.)
3. Secondary antibody
4. Chromogenic substrate (usually TMB)
5. Absorbance-based microplate reader

Procedure :

1. 100µl peptide (@4µg/ml) in coating buffer is added to individual wells of a microtiter plate. Incubate the plate for 2 hours at 37°C or overnight at 4°C.
2. Remove the coating solution and wash the plate three times by filling the wells with 100 µl PBS-0.05%Tween20. The solutions or washes are removed by flicking the plate over a sink. The remaining drops are removed by patting the plate on a paper towel.
3. Block the remaining protein-binding sites in the coated wells by adding 100µl blocking buffer, 3% skim milk in PBS per well. Incubate for 1 hour at RT with gentle shaking.
4. Wash the plate three times with 100ul PBS-0.05% Tween 20.
5. Add 50µl of diluted antibody to each well. Incubate the plate at 37°C for an hour with gentle shaking.
6. Wash the plate six times with 100ul PBS-0.05% Tween 20.
7. Add 50µl of conjugated secondary antibody, diluted at the optimal concentration (according to the manufacturer) in blocking buffer immediately before use. Incubate at 37°C for an hour.
8. Wash the plate six times with 100µl PBS-0.05% Tween20.

9. Prepare the substrate solution by mixing acetic acid, TMB and 0.03% H_2O_2 with the volume ratio of 4:1:5.
10. Dispense 50µl of the substrate solution per well with a multichannel pipe. Incubate the plate at 37°C in dark for 15-30mins.
11. After sufficient color development, add 100µl of stop solution to the wells (if necessary).
12. Read the absorbance (optical density at 450nm) of each well with a plate reader.

Protocol 2: Lateral flow strip method

Requirements:

1. Samples (plant materials)
2. Clean tubes, mortar pestle, tips etc.
3. Test strips and extraction buffer.

Procedure

1. Take the sample into a clean, dry grinding jar and grind with a blender on high speed for about 30 second (seed samples) or placed in mortar pestle and crushed (other plant parts) until all the samples are grinded.
2. Place 5 g of each sample into a clean tube.
3. Add an appropriate amount of extraction buffer [volume buffer (mL) = weight of sample (g) x 3].
4. Mix the grinded sample with the extraction buffer by vigorous shaking for at least 15 seconds.
5. Allow the sample to settle for at least 1 minute before testing with the strip.
6. When the sample settles, transfer liquid from the top to a reaction vial until it is filled, avoid suspended particles.
7. Add a test strip to the reaction vial, the protective tape with the arrow indicates the end of the strip to insert into the reaction vial. Test strips must not be submerged more than 0.5cm. If too much of the strip is submerged the antibodies will be released into the sample rather than being wicked up into the strip.
8. After 10 minutes, remove the test strip and analyse the result.
9. To retain the strip, cut off and discard the bottom section covered by the arrow tape.

B. DNA/RNA based GMO/LMO Detection

B.1: PCR based Method:
Protocol 3: Isolation of Genomic DNA Using CTAB Method

Basic principle of DNA extraction

The basic principle of DNA extraction consists of releasing the DNA present in the matrix removal of polysaccharides (pectin, cellulose, hemi-cellulose, starch, etc.), RNA and proteins. Further, concurrently or subsequently, purifying the DNA from PCR inhibitions are done. Purification can be achieved, by fractionated precipitation, using solvents, such as, phenol, chloroform, ethanol, isopropanol, and/or by adsorption on solid matrices (anion exchange resin, silica or glass gel, diatomaceous earth, membranes, etc.). In order to obtain a good quality and purified DNA, it is advised, where relevant, to remove: Polysaccharides (pectin, cellulose, hemi-cellulose, starch, etc.) with depolymerases (pectinase, cellulase, hemi-cellulase, a-amylase, etc) before precipitation of the DNA; RNA and/or proteins by an appropriate treatment, such as enzymatic treatment; lipid fractions by initial treatment with enzyme solutions or solvents (e.g. n-hexane) and salts (e.g. from the extraction/lysis buffer, from the precipitation step) otherwise these interfere with the subsequent analysis. DNA is re-suspended in water in a buffer solution, which prevents DNA from degradation. The DNA extracted shall be stored under such conditions that the stability is ensured to perform the subsequent analyses. Repeated freezing and thawing of DNA solutions should be avoided.

Requirements

1. CTAB buffer
2. Micro centrifuge tubes
3. Mortar and Pestle
4. Liquid Nitrogen
5. Absolute Ethanol (ice cold)
6. 70 % Ethanol (ice cold)
10. Agarose
11. 6x Loading Buffer
12. 1x TBE solution
13. Agarose gel electrophoresis system
14. Ethidium Bromide solution
15. 55^{o} C water bath

7. 7.5 M Ammonium Acetate
8. Chloroform : Iso amyl alcohol (24:1)
9. Water (sterile)
16. Centrifuge and rotor capable of reaching up to 12,000 × g

Procedure

1. Grind 200 mg of plant tissue to a fine paste in approximately 1500 µl of CTAB buffer.
2. Transfer CTAB/plant extract mixture to a microcentrifuge tube.
3. Incubate the CTAB/plant extract mixture for about 15 min at 55^{o}C in a recirculating water bath.
4. After incubation, spin the CTAB/plant extract mixture at 12,000 g for 5 min to spin down cell debris. Transfer the supernatant to clean microcentrifuge tubes.
5. To each tube add 250 µl of Chloroform: Isoamyl alcohol (24:1) and mix the solution by inversion. After mixing, spin the tubes at 13,000 rpm for 1 min.
6. Transfer the upper aqueous phase only (contains the DNA) to a clean microcentrifuge tube.
7. To each tube add 50 µl of 7.5 M Ammonium Acetate followed by 500 µl of ice cold absolute ethanol.
8. Invert the tubes slowly several times to precipitate the DNA. Generally, the DNA can be seen to precipitate out of solution. Alternatively, the tubes can be placed for 1 hr at -20 ^{o} C after the addition of ethanol to precipitate the DNA.
9. Following precipitation, the DNA can be pipetted off by slowly rotating/spinning a tip in the cold solution. The precipitated DNA sticks to the pipette and is visible as a clear thick precipitate. To wash the DNA, transfer the precipitate into a microcentrifuge tube containing 500 µl of ice cold 70 % ethanol and slowly invert the tube. Repeat. (Alternatively, the precipitate can be isolated by spinning the tube at 13,000 rpm for a minute to form a pellet. Remove the supernatant and wash the DNA pellet by adding two changes of ice cold 70 % ethanol).
10. After the wash, spin the DNA into a pellet by centrifuging at 13,000 rpm for 1 min. Remove all the supernatant and allow the DNA pellet to dry (approximately 15 min). Do not allow the DNA to over dry or it will be hard to re-dissolve.

11. Resuspend the DNA in sterile DNase free water (approximately 50-400 µl H_2O; the amount of water needed to dissolve the DNA can vary, depending on how much is isolated). RNaseA (10 µg/ml) can be added to the water prior to dissolving the DNA to remove any RNA in the preparation (10 µl RNaseA in 10 ml H_2O).
12. After resuspension, the DNA is incubated at 65^{o}C for 20 min to destroy any DNases that may be present and store at 4^{o} C.
13. Agarose gel electrophoresis of the DNA will show the integrity of the DNA, while spectrophotometry will give an indication of the concentration and cleanliness.

Protocol 4: DNA quantification and quality check: Spectrophotometry

The quantity of DNA was assessed by acquiring spectrophotometric absorbance at the wavelength of 260 nm and 280 nm. Concentration of DNA was further calculated using OD_{260nm} formula (OD_{260} = 1, corresponds to 50 µg/ml of dsDNA). Quality of DNA was considered to be good enough if A_{260}/A_{280} ratio was above 1.8 for DNA.

Protocol 5: Detection of Transgene by Polymerase Chain Reaction (PCR)

a) Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is a technique to copy, sequence or quantify DNA. In short, PCR is a biochemical technique that uses thermocycling and enzymes to quickly and reliably copy of DNA.

PCR components

1.	dH_2O	3.	dNTP	5.	Forward primer
2.	10X Buffer	4.	Taq Polymerase	6.	Reverse primer

Reaction setup

It is recommend assembling all reaction components on ice and quickly transferring the reactions to a thermocycler preheated to the denaturation temperature (95°C).

Component	25 µl reaction	50 µl reaction	Final Concentration
10X Standard *Taq* Reaction Buffer	2.5 µl	5 µl	1X
10 mM dNTPs	0.5 µl	1 µl	200 µM

10 µM Forward Primer	0.5 µl	1 µl	0.2 µM (0.05-1 µM)
10 µM Reverse Primer	0.5 µl	1 µl	0.2 µM (0.05-1 µM)
Template DNA	variable	variable	<1,000 ng
Taq DNA Polymerase	0.125 µl	0.25 µl	1.25 units/50 µl PCR
Nuclease-free water	to 25 µl	to 50 µl	

Note: Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Overlay the sample with mineral oil if using a PCR machine without a heated lid.

Transfer PCR tubes from ice to a PCR machine with the block preheated to 95°C and begin thermocycling.

Protocol 6: Gel electrophoresis and DNA visualization

Requirements:

1. Agarose gel (1xTAE , Agarose powder and ethidium bromide)
2. Loading dye (Bromophenol blue and Xylene cyanole FF).

Buffer (TAE)

Chemicals	Amount
Tris-Acetate	10x buffer/litre
Tris Base	242 g
Glacial acetic acid	57.1ml
0.5M Na_2 EDTA (pH 8.0)	100 ml

B.2: **Gene Expression Analysis by Quantitative Real-Time PCR**

Protocol 7: **RNA isolation from plant tissue**

Isolating high quality, intact RNA is the first and usually the most critical step in performing quantitative PCR and Real-time PCR. Finding the most appropriate method of cell or tissue disruption for the specific starting material is important for maximizing the yield and quality of RNA preparation. In order to prevent RNA degradation, it is crucial that the denaturants must be in contact with the cellular contents at the moment when the cells are disrupted. A common solution to these problems is to grind tissues with a mortar and pestle in the presence of liquid nitrogen into a fine powder, to which

the solution containing the denaturant(s) is added. For many plants and most of the applications, TRIzol reagent, which is a ready-to-use, monophasic solution of phenol and guanidine isothiocyanate, gives optimum results. RNA isolation has four fundamental steps:

A. Homogenization
B. Phase Separation
C. RNA precipitation
D. RNA wash and dissolution

Requirements

1. Diethyl pyrocarbonate (DEPC)
2. TRIzol reagent (Invitrogen)
3. Chloroform, Propan-2-ol (Isopropanol)
4. Ethanol, Nuclease-free water
5. Centrifuge and rotor capable of reaching up to 12,000×g
6. Polypropylene microcentrifuge tubes
7. Water bath or heat block (55–60°C)

Procedure

1. Grind ≤100 mg of plant tissue in liquid nitrogen to a fine powder and add 1ml of TRIzol reagent. Grind quickly and mix the homogenate intermittently.
2. Add 0.2 ml chloroform for each 1 ml of initial Trizol. Shake tubes vigorously by hand for 15 seconds, and incubate for additional 10 min. at room temp.
3. Centrifuge the samples for 15 min. at 12000 X g using a microfuge. The mixture separates into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The upper aqueous phase is usually ~50% of the total volume.
4. Transfer the aqueous phase carefully to a fresh microfuge tube avoiding contamination from the interphase or the organic layer.
5. Add 0.6 volume of propan-2-ol to the aqueous phase. Incubate for 10 min. at room temperature.
6. Centrifuge at 12000 X g for 10 minutes. Discard the supernatant.
7. Wash the RNA pellet with 75% ethanol (prepared in nuclease-free water) by centrifuging at 7500 X g for 10 minutes.
8. Air dry the RNA pellet. Avoid completely drying the pellet as this will decrease the solubility of the RNA.

9. Dissolve the RNA in 30-40 µl of nuclease-free water and keep it in the freezer (–80 ^{o}C) until further use. Dissolution should be facilitated by tapping.
10. For long term storage, precipitate the RNA in 75% Ethanol and store at -80^{o}C.

Protocol 8: RNA quantification and quality check by Spectrophotometry

In order to determine the concentration of RNA sample, its absorbance at 260 nm is measured and an absorbance of 1.0, when the path length is 1.0 cm, is equivalent to about 40 µg/ml of RNA. For pure RNA sample, the A_{260}/A_{280} ratio should be 2.0. A ratio below 1.8 indicates protein contamination. Besides, for pure RNA sample the A_{260}/A_{230} ratio should be ≥2.0. A ratio quite lower than this indicates contamination by phenolate ion, thiocyanates, and other organic compounds mostly used during RNA extraction. The organic contaminants and chaotropes can interfere with subsequent steps such as reverse transcription. Hence, it is often necessary to remove them which can be done by an additional wash with 75% ethanol after re-precipitation using propan-2-ol.

Protocol 9: Reverse transcription: first strand cDNA synthesis (Manufacturers protocol should be followed)

cDNA is a more convenient way to work with the coding sequence than mRNA because RNA is very easily degraded by omnipresent RNases. Reverse transcription is a first step in two step reverse transcriptase PCR (RT-PCR**)**. The RNA templates are reverse transcribed into cDNA which can then be amplified using PCR or used for several applications like real-time PCR and semi-quantitative RT-PCR. Alternatively, radioactively and non-radioactively labelled nucleotides can be incorporated into first strand cDNA for use as a probe in hybridization experiments. The enzymes used are reverse transcriptases (RT) of different origins; most popular types being M-MuLV RT and AMVRT.

Primers

Synthesis of first strand cDNA can be primed with either oligo$(dT)_{18}$ primer, random primers or gene-specific primers. Oligo $(dT)_{18}$ primes cDNA synthesis from the poly(A) tail usually present at the 3'-end of eukaryotic mRNAs. Random primers initiate cDNA synthesis from the total RNA population (rRNA and mRNA). If we want to produce pieces of cDNA that were scattered all over the mRNA, then we can use a random primer cocktail that would produce cDNA from all mRNAs but the cDNAs

would not be full length. The major benefits of random priming are the production of shorter cDNA fragments and increasing the probability that 5' ends of the mRNA would be converted to cDNA. Because reverse transcriptase does not usually reach the 5' end of long mRNAs, random primers can be beneficial.

Procedure

A. Removal of genomic DNA contamination from the RNA sample

For RT-PCR applications, template RNA must be free of DNA contamination. Prior to cDNA synthesis, RNA can be treated with DNaseI (RNase-free) to remove trace amounts of genomic DNA. Volume the reaction with Nuclease free water and incubated at 37 °C for 15 minutes. Add 1 µl of 25 mM EDTA and incubate at 65 °C for 10 minutes to inactivate DNase.

Note: RNA hydrolyzes at this temperature in the absence of a chelating agent such as EDTA.

B. Preparation of 2x RT master mix:

To prepare the cDNA it is recommended to follow the manufacturer instruction.

1. Referring to the table below, calculate the volume of components needed to prepare the required number of reactions.

Components	Volume (µl) per reaction
10X RT Buffer	2.0
25X dNTP Mix (100 mM)	0.8
10X RT Random Primers	2.0
MultiScribe™Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free H_2O	3.2
Total per Reaction	10.0

*Note***:** Prepare the 2x RT master mix on ice.

2. Pipette 10 µL of 2x RT master mix into each well of a 96-well reaction plate or individual tube.
3. Pipette 10 µL of RNA sample (as prepared in step A above) into each well, pipetting up and down two times to mix.

4. Seal the plates or tubes.

5. Briefly centrifuge the plate or tubes to spin down the contents and to eliminate any air bubbles.
6. Place the plate or tubes on ice until you are ready to load the thermal cycler.
7. Program the thermal cycler conditions as follows:

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

*Note***:** The reverse transcription reaction product (cDNAs) can be directly used in PCR applications or stored at -20°C.

Protocol 10: Real-Time PCR

RT-PCR (reverse transcription-polymerase chain reaction) is the most sensitive technique for mRNA detection and quantitation currently available.

Requirements: (Manufacturers protocol should be followed)

1. cDNA samples
2. Primers
3. SYBER Green master mix
4. 96 well plate
5. Sealer
6. Real Time machine

Procedure

1. Add 5 µL of the 10x diluted first strand cDNA sample.
2. Prepare Master mix (for the required no. of wells) as detailed below.

Component	Volume (per well)
Fast SYBR® Green Master Mix (2)	10.0 µL
Forward primer 100 nM (final concentration)	variable
Reverse primer 100 nM (final concentration)	variable
Nuclease free sterile water	variable
Total volume	15 µL

3. Add 15 µl of the mastermix to each well so that the final volume per well is now 20 µl.
4. Seal the plate with an optical adhesive cover, and then centrifuge the plate briefly (3000 X g for 1 minute) to spin down the contents and eliminate any air bubbles.
5. Set the program of the qRT-PCR as follows.

	Hold	PCR (40 cycles)	
		Denaturation	Annealing/Extension
Time	10 min	15s	60s
Temperature (ºC)	95	95	58-60 (depending on the annealing temperature of the primer)

6. Also run a dissociation curve using the default parameters.

D. GMO/LMO Detection using Blotting Techniques:

Protocol 11: Southern Blotting Technique

Requirements:

1. DNA samples
2. Restriction enzymes
3. Chemicals (listed below)

Composition of the buffers and solutions used for Southern Hybridization

1	**10X TBE**		
a.	Tris	107.8 g	Total volume was made up to 1000 ml with distilled water, and pH was adjusted to 8.2.
b.	EDTA	8.41 g	
c.	Boric acid	559 g	
d.	Distilled water	600 ml	
2	**Denaturation Solution**		
a.	NaCl (1 M)	29.22g	Total volume was made to 500 ml with distilled water.
b.	NaOH (0.5 N)	10g	
c.	Distilled water	400 ml	
3	**Neutralisation Solution**		
a.	NaCl	43.83g	The final pH was adjusted to 7.5 with concentrated HCl and the final volume was made up to 500ml.
b.	Tris	30.28g	
c.	Distilled water	350 ml	
4	**20X SSC**		
a.	NaCl	175.3g	The final pH was adjusted to 7 with concentrated HCl and

b.	Na citrate	88.2 g	the final volume was made up to 1000ml.
c.	Distilled water	800 ml	
5	**Maleic acid buffer (10X)**		
a.	1M maleic acid	116g	Final volume was made to 1000 ml, adjust with
b.	1.5 M NaCl	87g	NaOH (solid) to pH 7.5
6.	**Washing Buffer**		1X maleic acid buffer pH 7.5; 0.3% (v/v) Tween 20
7.	**Detection Buffer**		
a.	0.1M Tris Hcl		Final volume was made to 100 ml with pH 9.5
b.	0.1M Nacl		
8.	**Blocking solution (10X)**		Dissolved blocking solution10% (W/V) in maleic acid buffer under constantly stirring on a heating block. The solution remains opaque. Autoclaved. 1X working solution freshly prepared by dilution in Maleic acid buffer.

Procedure

A. Restriction digest:

1. The DNA is fragmentized by using suitable restriction enzyme (RE). RE cuts the DNA at specific site generating fragments
2. The number of fragments of DNA obtained by restriction digest is amplified by PCR

B. Gel electrophoresis:

1. The desired DNA fragments is separated by gel electrophoresis

C. Denaturation:

1. The SDS gel after electrophoresis is then soaked in alkali (NaOH) or acid (HCl) to denature the double stranded DNA fragments.
2. DNA strands get separated

D. Blotting:

1. The separated strands of DNA is then transferred to positively charged nylon membrane (Nitrocellulose paper) by the process of blotting.

E. Baking and blocking:

1. After the DNA of interest bound on the membrane, it is baked on autoclave to fix in the membrane.
2. The membrane is then treated with casein or Bovine serum albumin (BSA) which saturates all the binding site of membrane

F. Hybridization with labelled probes:

1. The DNA bound to membrane is then treated with labelled probe
2. The labelled probe contains the complementary sequences to the gene of interest
3. The probe bind with complementary DNA on the membrane since all other non-specific binding site on the membrane has been blocked by BSA or casein.

G. Visualization by Autoradiogram:

1. The membrane bound DNA labelled with probe can be visualized under autoradiogram which give pattern of bands.

Protocol 12: Northern Blotting Technique

Requirements:

1. RNA samples
2. Restriction enzymes
3. Chemicals (Similar as southern hybridization)

Procedure:

A. Extraction of RNA:

This procedure has been described above (Experiment 7).

B. Gel electrophoresis to separate RNA by size:

Agarose gels containing formaldehyde were traditionally used to denature RNA. The formaldehyde reacts with the imine and amine groups on the nucleic acids, which disrupts the hydrogen bonding between bases and disrupts the secondary structure of the RNA. It is important to disrupt the secondary structure because the RNA must be extended to allow proper binding of probe for identification.

C. Transfer of RNA to blotting membrane:

1. The transfer is necessary because the probes can't enter into the gel matrix. Therefore, the RNA must be transferred to a membrane where they can be accessed by the probes.
2. Transfer is accomplished via a capillary (overnight) or vacuum (15-60 minutes) blotting system.

3. The blotting membrane is positively charged to attract the negatively charged RNA. Nylon is a commonly used membrane.

D. Immobilization of RNA to the blotting membrane:

1. Covalently attached to the membrane by the application of UV light or heat.

E . Application of Probe:

1. Probes have a minimum of 25 bases that are complimentary to the mRNA sequence of interest.
2. Excess probe is washed off

F . Probe visualization:

1. Radioactive isotopes were traditionally used, but have been replaced in favor of safer detection methods. Chemiluminescence is commonly used in the modern northern blot protocol (follow the manufacturer instructions).

Protocol 13: Western blotting Technique

Requirements:

1. Protein samples
2. Nitrocellulose membrane
3. Electroblotting buffer (150 mM glycine, 20 mM Tris and 20% methanol, pH 8.0)
4. PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4, pH 7.3)
5. Blocking solution (5% BSA in PBS)
6. Wash buffer (PBS containing 0.1% tween-20 PBST)
7. Primary antibody
8. BCIP/NBT containing solution (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$ containing 150 µg/ml of NBT and 75 µg/ml of BCIP)

Procedure:

A. Extraction of Protein

1. Cell lysate is most common sample for western blotting.

2. Protein is extracted from cell by mechanical or chemical lysis of cell. This step is also known as tissue preparation.
3. To prevent denaturing of protein protease inhibitor is used.
4. The concentration of protein is determined by spectroscopy.
5. When sufficient amount of protein sample is obtained, it is diluted in loading buffer containing glycerol which helps to sink the sample in well.
6. Tracking dye (bromothymol blue) is also added in sample to monitor the movement of proteins.

B. Gel electrophoresis

1. The sample is loaded in well of Sodium dodecyl sulfate- poly-acrylamide gel electrophoresis (SDS-PAGE).
2. The proteins are separated on the basis of electric charge, isoelectric point, molecular weight, or combination of these all.
3. The small size protein moves faster than large size protein.
4. Protein are negatively charged, so they move toward positive (anode) pole as electric current is applied.

C. Blotting

1. The nitrocellulose membrane is placed on the gel. The separated protein from gel get transferred to nitrocellulose paper by capillary action. This type of blotting is time consuming and may take 1-2 days
2. For fast and more efficient transfer of desired protein from the gel to nitrocellulose paper electro-blotting can be used.
3. In electro-blotting nitrocellulose membrane is sandwich between gel and cassette of filter paper and then electric current is passed through the gel causing transfer of protein to the membrane.

D. Blocking

1. Blocking is very important step in western blotting.

2. Antibodies are also protein so they are likely to bind the nitrocellulose paper. So before adding the primary antibody the membrane is non-specifically saturated or masked by using casein or Bovine serum albumin (BSA).

E. Treatment with Primary Antibody

1. The primary antibody (1° Ab) is specific to desired protein so it form Ag-Ab complex.

F. Treatment with secondary antibody

1. The secondary antibody is enzyme labelled. For eg. alkaline phosphatase or Horseradish peroxidase (HRP) is labelled with secondary antibody.
2. Secondary antibody (2° Ab) is antibody against primary antibody (anti-antibody) so it can bind with Ag-Ab complex.

G. Treatment with suitable substrate

1. To visualize the enzyme action, the reaction mixture is incubated with specific substrate.
2. The enzyme convert the substrate to give visible colored product, so band of color can be visualized in the membrane.
3. Western blotting is also a quantitative test to determine the amount of protein in sample.

DETECTION OF DNA FROM GENETICALLY MODIFIED ORGANISMS (GMOS) DERIVED FOOD MATERIALS

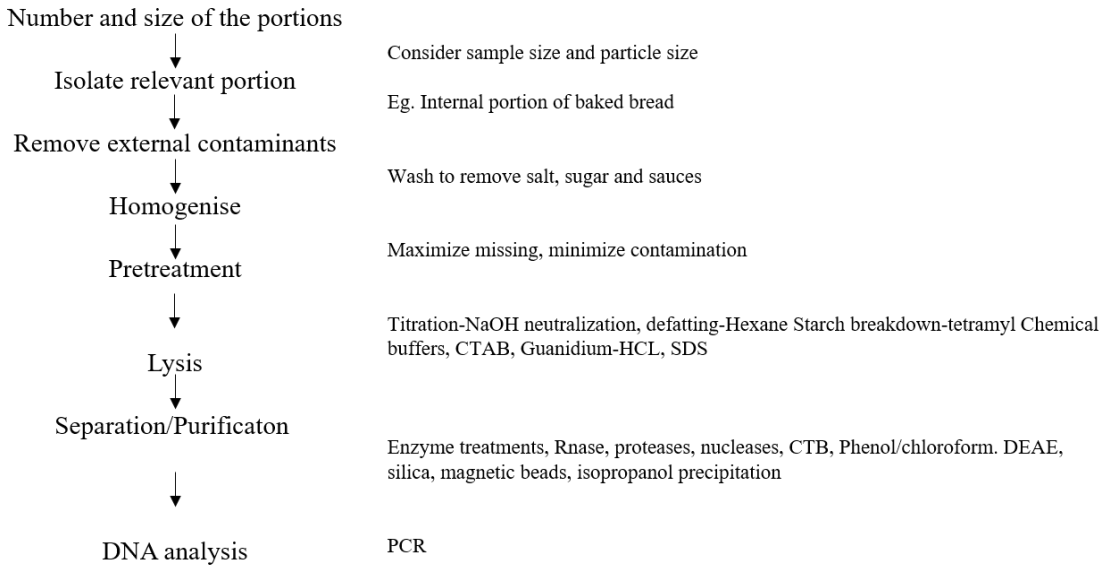
Introduction

There is worldwide debate concerning the safety and desirability of planting and consuming genetically modified foods (GMOs). Methods vary for the different companies and countries, but there are two basic means for detecting genetic modification. One method tests food for the product of the transgene, usually a protein. The other method tests for the presence of DNA from the transgene or another portion of the gene cassette. Proteins are assayed using an ELISA (Enzyme Linked Immuno Sorbent Assay). In the test an enzyme is linked to an antibody bound to the protein, which then reacts with a colored substrate Genetically Modified Foods 75 enabling detection of a specific protein. ELISAs usually cost less than DNA tests, offer quicker results, and can sometimes be done on site. The big drawback is that ELISAs do not work well on processed foods because heat during processing can destroy the protein. In contrast, DNA tests are more expensive, cannot be done on site, and take several hours to complete. Importantly, however, DNA tests are very accurate, work on processed foods, and can be quantified. As a consequence, the number and importance of rapid and cost-effective GMO testing processes have grown significantly. PCR is now the standard process for analyzing food materials for the presence of GMOs. However, a standard method for preparing DNA from a wide variety of foodstuffs is needed to establish a standardized GMO testing program. In the past decade, the rise of molecular diagnostics in research and quality control has greatly increased the need for reliable and reproducible extraction of nucleic acids from food and feed. Food samples are very heterogeneous, and often contain substances which can impair DNA extraction, such as fats, cocoa or polysaccharides. In addition, processed foods often have a very low DNA content, which is of poor quality. It is therefore important that PCR inhibitors are completely removed, to enable even low amounts of partially degraded DNA to be extracted from these complex samples.

DNA extraction and purification methods

In addition to requirements of the food matrix type and target analyte, the numbers of samples analyzed, required speed of throughput, and skills resource availability may also influence decisions on extraction and purification, particularly on the use of commercial kits or more traditional organic reagents. Pretreatment of particularly acidic, fatty, or starchy samples is performed to aid extraction of DNA and improve efficiency of cell lysis (Table 1). Acidic samples are neutralized to pH 7.0 ± 1.00 with 2M sodium hydroxide solution. Fat-containing samples that cannot be dispersed in aqueous lysis buffer are treated with hexane to remove fat before extraction. Samples with a high starch content, which may swell in lysis buffer, are better dispersed by adding termamyl type L and incubating at 65°C for 30 min.

DNA sample preparation steps



Lysis

This step disrupts the cell wall and frees the DNA from cellular and organelle membranes. The initial lysis step for the breakdown of cell walls is performed with a variety of buffers containing sodium dodecyl sulfate (SDS) as detergent and a high ethylenediaminetetracetic acid (EDTA) concentration, and is typically performed at 65°C with incubation of at least 30 min. Proteinase K, α-amylase, and RNase are used in the lysis step to aid breakdown of protein, starch, and RNA, respectively.

Extraction and Purification

After lysis, the aqueous phase is extracted with phenol and/or chloroform to remove any nuclease activity and potential polymerase chain reaction (PCR)-inhibitory compounds, such as lipophylic molecules, polysaccharides, and proteins. In spite of the toxicity of phenol, organic separation is still widely used. However, alternative DNA extraction methods based on co-precipitants such as cetyltrimethylammonium bromide (CTAB), polyvinylpyrolidone (PVP), and binding of DNA to silica matrixes are now used frequently. After the initial lysis step, denatured proteins and polysaccharides complexed with CTAB and/or PVP are removed from the DNA-containing liquid phase. A variety of silica adsorption matrixes are also used to aid DNA recovery and purification; such silica matrixes include commercially available products such as Wizard™ (Promega Corp., Madison, WI) and DNeasy™ (Qiagen, Crawley, UK). DNA binds to these silica resins in the presence of the chaotropic reagent guanidine-hydrochloride, and contaminants are then eluted with isopropanol. Finally, the DNA is eluted from the resin with a low salt buffer. Other methods include adsorption of DNA onto diethylaminoethyl (DEAE) paper. An overall summary of the available methods is shown in Table 2.

Inhibitor Removal

A further consideration in choosing an extraction and purification procedure is its influence on the subsequent analytical quality of the DNA. Co-precipitants, solvents, or adsorption matrixes should not bring nuclease activity or PCR inhibitors and competitors or contain any sequence homology to the PCR target. Potential PCR inhibitors are a particular problem with complex food matrixes. Table 3 lists common sources of potential PCR inhibitors, with respect to both the matrix and the extraction process and presents recommendations for the best extraction and purification steps to eliminate them from the DNA sample. Particular chemical and enzymic treatments steps can be introduced into the extraction process to minimize or remove inhibitors introduced from the sample matrix, such as protein, polysaccharide, fat, phenolics, and salt. Process-introduced PCR inhibitors include CTAB, SDS, and phenol, and removal of these reagents is also required before analysis. The final step in the extraction procedure, therefore, is to precipitate the DNA and wash with ethanol to remove salts and residual chemicals such as chloroform and phenol. However, when degraded DNA

is expected from the extraction process, as is typically the case for processed foods, the inefficiency of ethanol precipitation for short molecules (<200 bp) should also be considered. Precipitation may be improved by adding $MgCl_2$ (to 10mM) or glycogen (to 10 µg/mL).

Table 1. Procedure for homogenization and pretreatment of food samples prior to DNA extraction

Procedure	Method	Usage/results	Advantages	Disadvantages
Homogenize	Stomach blender	Good for flour, protein powder, tofu, or soy sausage	No contamination	Insufficient mixing, with soybean and corn
	Mortar/mill	Good for samples with particles, such soy, corn and grist	Good mixing	Contamination
	Stirring/shaking	Good for liquid samples	No contamination	Liquids only
Pretreatment	NaOH treatment	Neutralizes acidic samples	Improves extraction from soy sauce, ketchup or fruit juice	
	Hexane treatment	Removes fat from a sample allowing dispersion in aqueous buffer	Improves extraction from fats, oils and fat containing samples	Additional step in protocol
	Teramyl treatment	Breakdown of starch allowing dispersion in aqueous buffer	Improves extraction from starch-containing samples	

Table 2. Procedures for DNA extraction and purification from food matrixes

Method	Usages/results	Advantages	Disadvantages
CTAB lysis and purification	Good results with phenol/chloroform or followed by isopropanol precipitation	Effective for a wide range of matrixes. CTAB complexes out polysaccharides and proteins	A 260 nm effect spectrophometric quantitation
SDS	Good except with DEAE	Improves lysis	Inhibitor of PCR
PVP	Good in combination with SDS	Rapid, good quality DNA	
Guanidinium	Good with silica	Wide range of matrixes	
Phenol	Good in combination with CTAB	Nuclease and cell disruption	Hazardous, residual phenol inhibits PCR
Chloroform	Good in combination with CTAB	Removal of starch and fat	Hazardous
Silica			
Qiagen Dneasy	Limited amount of sample material does not yield good results with highly processed samples	Nontoxic	Expense, inhibitor of PCR
Qiagen Qiaex II	Best results with both buffer and enzymes	High throughput/automation	Not good with fat rich matrixes (combine with ligase treatment)

Wizard	Routinely used by a range of laboratories		
Nucleon	Effective for extraction from plant material		
Isopropanol precipitation	Good with chloroform	Concentration of DNA removal of salts and organic compounds	
DEAE	Good with guanidinium buffer and chloroform extraction	Removes EDTA inhibition	Does not work with SDS lysis buffer
Magnetic beads	Not used in studies so far		

Table 3. Sources of potential PCR inhibitors and possible preventive actions

Source of inhibitor	**Inhibitor**	**Action**
Matrix	Protein	SDS, CTAB or guanidium buffers, Proteinase K
	Polysaccharide	Depolymerase (amylase), CTAB buffer and chloroform extraction
	Fat	Lipase or hexane treatment and chloroform extraction
	Phenolics	PVP, PVP/ammonium acetate
	Salt	Wash sample, Silica or DEAE and 70 % ethanol wash
	Acid	Neutralize with NaOH prior to extraction
Extraction procedure	Salt	Silica or DEAE and 70% ethanol wash
	EDTA	DEAE purification
	Organic residue	70 % ethanol wash
	Isopropanol	Dry pellet then resuspend
	SDC/CTAB/guanidium	Silica or DEAE and 70 % ethanol wash

This process of identification of GMOs has three parts: 1) isolation of DNA from food, 2) PCR, and 3) visualization of the results using agarose gel electrophoresis. Polymerase chain reaction (PCR) is used test to detect foreign DNA in genetically modified food, and by using primers that are designed to amplify DNA from the CaMV35S promotor component of the gene cassette. Amplification of this promoter yields DNA fragments 195 base pairs (bp) in length. Since almost all transgenic crops contain the CaMV35S promoter it offers a good target for DNA testing. A positive test for this promoter is not always conclusive, however. Plants in the cabbage family should be treated carefully because these plants may be naturally infected with the Caulimovirus, the source of the CaMV35S promoter. In such cases further PCR tests should be run with primers designed to amplify the specific transgene DNA.

Materials:

Equipment

- Micropipettors (100-1000 µL, 10-100 µL,and 0.5-10 µL)
- Analytical balance
- Autoclave
- pH meter
- Spatulas
- Thermometer
- 55-60º C incubator
- 70ºC waterbath or dri bath
- Timers
- Vortexer
- Microcentrifuge 14,000 x g
- Minifuge
- Microcentrifuge tube racks
- Vacuum line or vacuum pump
- Lab markers
- Thermalcycler
- Horizontal gel electrophoresis boxes
- Gel electrophoresis power supplies
- UV transilluminator
- Staining trays
- Camera
- Assorted beakers, graduated cylinders andstorage bottles
- Stir bars
- Magnetic stirplate
- Hotplate or microwave oven
- Heat-proof gloves
- Refrigerator
- Freezer
- Goggles or safety glasses

Materials and Consumable Lab Supplies

- Microcentrifuge tubes 1.5-mL (sterile)
- Sterile sticks
- Nuclease free water
- Weigh boats
- Aerosol resistant pipet tips (100 - 1000µL, 10- 100 µL, and 0.5 - 10 µL)
- Tris-HCl
- NaCl
- EDTA (disodium salt)
- SDS
- Guanidine-HCl
- Proteinase K
- Labeling tape
- 3-ml syringes (sterile)
- minicolumns
- resin
- Isopropanol
- PCR primers CaMV35S Promoter
- Sterile mineral oil (Sigma M 5904)
- 4% precast agarose gels
- electrophoresis buffer
- PCR Marker, 50-2000 bp
- Blue/Orange Loading Dye, 6X
- Deionized or distilled water
- Film
- Ethidium bromide 10 mg/mL
- Gloves
- Lab coats or aprons

Protocol

DNA Isolation

1. Wearing gloves select your food sample or control sample and weigh out 0.1 g of food and place it into a disposable microcentrifuge tube.
2. Add 200 µL of nuclease free water to the tube.
3. Use a sterile stick to homogenize the food sample to a smooth slurry.
4. Add 860 µL of extraction buffer, 100 µl of 5M Guanidine-HCl and 40 µl of 20 mg/ml Proteinase K to the tube containing the homogenate. Vortex tube.
5. Incubate at 55-60ºC for 3 hours with intermittent mixing.
6. Allow samples to cool at room temperature for 10 minutes.
7. Centrifuge 10 minutes at 14,000 x g in a microcentrifuge.
8. For each sample, attach one labeled 3-mL syringe barrel to the Luer-Lok® extension of a Wizard® minicolumn and attach this minicolumn/syringe barrel assembly to the Vac-Man® Laboratory Vacuum Manifold.
9. Check to ensure all stopcocks are closed before proceeding.
10. Add 1mL of Wizard® resin to each minicolumn/syringe assembly.
11. Carefully remove 300 µl of the cleared supernatant from each sample and transfer it to the barrel of the minicolumn/syringe assembly containing the Wizard® resin.
12. Open the stopcocks and apply a vacuum to pull the resin/supernatant mix into the minicolumn. When the entire sample has passed through the column, close the stopcock and turn off the vacuum. In this step the DNA will stick to the column.
13. Add 2 ml of 80% isopropanol to each minicolumn and reapply the vacuum to draw the solution through the minicolumn. This step washes the column.
14. Remove the syringe barrel and transfer the minicolumn to a 1.5 ml microcentrifuge tube. Centrifuge the minicolumn at 10,000 x g in a microcentrifuge for 2 minutes to remove any residual alcohol.
15. Transfer the minicolumn to a new microcentrifuge tube, add 50 µl of 70ºC nuclease free water to the column and allow it to interact with the resin for 1 minute. This step elutes the DNA from the column.
16. Elute the DNA by centrifugation at 10,000 x g for 1 minute in a microcentrifuge.
17. You may stop here and store the DNA in the refrigerator for about a week. For longer periods, store it in the freezer.

PCR DNA Amplification

Follow the instruction for PCR described before.

Agarose Gel Electrophoresis Analysis

1. Wearing gloves obtain one 4% agarose TAE gel with eight wells, a gel box containing TAE 1X
   buffer, and a power supply.
2. Add 5 µl loading dye to the tubes with your PCR samples, standards and negative control.
3. Load 15 µl of each sample, standard and negative control into separate wells of your gel, avoid
   mixing mineral oil with the sample. Save a lane for the PCR marker on each gel. Record where each sample is located on the gel.
4. Add 1µl loading dye to your PCR marker. Load the marker into one well of the gel.
5. Attach the gel box to the power supply, turn the power on, and set to 100-150 volts. Electrophorese for 40 - 60 minutes or until the bromophenol blue band has travelled one-third the length of the gel. Volt settings and time will vary with different equipment.
6. Wearing gloves, carefully remove the gel and put it into a staining tray. Cover the gel with ethidium bromide stain and stain for 5 - 10 minutes.
7. After staining, decant the ethidium bromide stain from the staining tray back into the storage bottle.
8. Rinse the gel with tap water, in the tray, for several minutes to remove background ethidium bromide stain from the gel.
9. View on ultraviolet transilluminator and photograph. Record your results and share with all other groups.

Data Analysis and Interpretation

- Share a copy of your results with all the other groups.
- Are any bands in the 180 -195 bp range?
- How many lanes show PCR bands?
- Does the 0% control standard show a PCR band? Should it? Why?
- Does your negative control show a PCR band? Should it? Why?

- Does your positive control standard show a PCR band? Should it? Why?
- Can you see the PCR Marker ladder? How many bands should there be? Are all the bands visible?
- Do your organic food samples show a PCR band? If they do what does this mean?

Expected Results

No DNA fragments should appear in the negative control lane except bands at or below 50bp in length are artifacts of the primers. Bands of 195bp indicate presence of the CaMV35S promoter. No DNA fragments of 195bp should appear in the 0% control standard lane. A 195bp fragment will appear in the 2% control standard lane. Student samples may or may not show a band at 195bp. Theoretically, organic food products should be free of genetically modified foods. However, organic definitions vary. If a product indicates that it is 100% organic (Non GMOs), there should be no band. Non-organic food products (GMOs) will probably contain detectable levels of genetically modified corn (Figure 9).

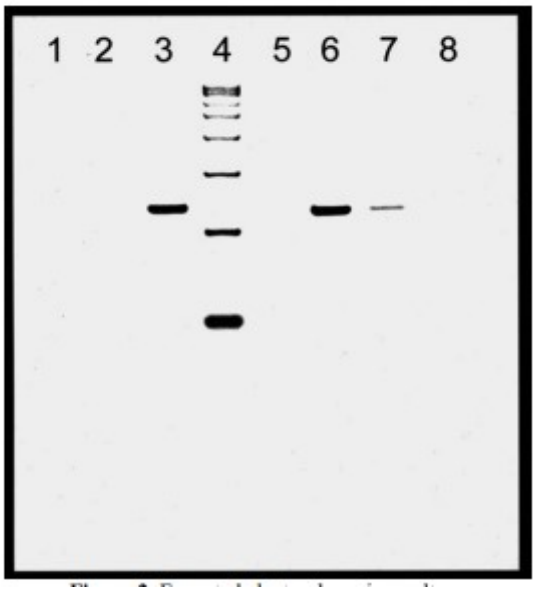


Figure 9: Agarose Gel Electrophoresis

GOOD LABORATORY PRACTICES FOR WORKING IN GMO DETECTION LAB

Introduction

It is very important to learn good laboratory practices before working in any biosafety and GMO detection lab thus biotechnology lab. There are supplies and equipment's that may pose a hazard. Moreover, disciplined laboratory activities also ensure authentic results along with safety. Followings are few measures that such laboratories may opt to achieve those. However, variation in practices depends on the purpose of the laboratory.

General Rules

1. Only authorized personal may enter laboratories
2. Laboratory doors shall be kept closed
3. Keep the laboratory clean and organized
4. Unnecessary materials should not clutter in the laboratory
5. Equipment are well maintained and cleaned
6. Whenever a solution is made the name and date should be labeled properly on the solution container
7. Reagents or solutions needs to be stored at appropriate place. If special storage conditions (for example, some items need to be stored frozen, or wrapped in foil to block light) then special care needs to be done. In case of hazardous substances (such as concentrated acid or flammable solvents) precaution needs to be taken and stored either in the fume hood or in their designated cabinet.
8. Mouth pipetting is prohibited
9. Follow procedures to minimize the formation of aerosols and gases
10. The use of equipment must remain within the framework in which it was designed. Equipment under the ethidium bromide fume hood should remain under it and no other use must be made with it.
11. Do not pour products into the sink if you are not sure whether it is allowed or if another disposal route exists in the laboratory.

12. Store the laboratory notebooks and manuals at the designated area. Keep that area clean and organized.
13. Every day work needs to be documented precisely each day in the notebook. Keep the records factual, concise, clear and complete in all aspects.
14. Disinfect or decontaminate glassware before washing.
15. Decontaminate all regulated waste before disposal by an approved method, usually by autoclaving.
16. Cleaning has to be done at the end of every manipulations
17. Have an insect and rodent control program in effect.
18. Use a laminar flow biological safety cabinet when needed.
19. In case of incident or accident, we must inform the laboratory supervisor, or a competent person.
20. It is also important to record the incident or accident in the registry.

Personal Safety

1. Use personal protective equipment (PPE) at all times and keep all PPE inside the lab.
2. Lab coats have to be worn inside the laboratory. Outside the lab and also during the breaks one should not wear it.
3. The open shoes are prohibited. Always ware closed-toed shoes in lab.
4. Eating, drinking, smoking is prohibited, as well as storing food or drink in the laboratory.
5. To avoid cross contamination the laboratory personal should use gloves. There are two types of gloves in the laboratory
   - Latex gloves for handling but not for ethidium bromide
   - Nitrile gloves for handling ethidium bromide.
6. While working with ethidium bromide, it is compulsory to wear the facial protection to protect from both projections and UV radiation.
7. Always remove gloves before touching phones, doorknobs, light switches, etc.
8. Avoid touching your face with your hands or gloves.
9. Wash hands after handling viable materials and biological subjects, after removing gloves and before leaving the lab.
10. Wear natural fibre clothing (synthetic material melts onto skin in a fire)

LABORATORY WASTE DISPOSAL GUIDANCE

1. Introduction

These laboratory advisory guidelines provide procedural information for laboratory workers to follow when disposing of transgenic materials/waste generated in laboratories. The guidelines should be read properly and materials should be handled properly in order to minimize risks associated with the disposal of laboratory waste.

2. Scope

These guidelines apply to all workers, students and visitors who work within a laboratory environment at the Department of Environment, Ministry of Environment, Forest and Climate Change that generate and/or dispose of any type of waste. Arrangements need to be made with the appropriate disposal authority of the city for their final disposal.

3. General Principles

All potential waste (transgenic materials, chemicals, sharps etc.) streams that arise from laboratory operations needs to be assessed and an appropriate disposal route selected prior to waste being generated. Waste should be collected in a suitable container and labeled.

Main points for consideration are:

1. minimize waste and do not accumulate large amounts in the laboratory. Regular disposal from the laboratories must be part of the regular disposal of the central facility.
2. segregate waste - have a separate residue container if you are generating a large amount of any particular type of waste. Ensure the waste container is compatible with the waste you are collecting.
3. label the waste residue container with the appropriate waste label.

4. store waste in a suitable area prior to collection. For example, chemicals and solvents should be stored in ventilated areas and residue container lids must be secure. Ensure container is not leaking and no spillage on the exterior of the container. Primary container should be placed in a suitable bund.
5. handle waste only if you are aware of the hazards associated with the waste and appropriate risk controls are used.
6. dispose waste as per relevant UOW guidelines.
7. record all disposal on Waste Tracking Log to ensure evidence of correct waste management.

4. Hazardous Waste Streams

4.1 Chemically Contaminated Waste

Bin colour: yellow base with orange lid

Final disposal method: incineration

Figure 10: Bin for disposing chemically contaminated waste

Filter paper, gloves, tissues, paper towel, benchcote, spent silica, and sample vials that have been contaminated with hazardous chemicals should be placed directly into a chemically contaminated waste bin or bag.

4.2 Waste Chemicals

Waste chemicals can take various forms including solvents, aqueous solutions, dry powders, and unwanted old chemicals. The SDS for each chemical must be checked to ensure compatibility of materials for mixed chemical residue containers. Where possible, mixing of chemicals should be avoided to prevent unexpected reactions from occurring. The waste container should be compatible with the residue material

placed within. If the waste is a liquid, residue containers are approved strong, plastic sealable containers. Only containers up to 5 litres can be accepted by the Waste Store. Containers up to 20L can be collected from their location - a Waste Tracking Log is to be taken to the Waste Store in place of the container.

Figure 11: Container for collecting waste chemicals

No hazardous chemical substances should be disposed down drains. Generally chemical waste should be segregated according to its properties, such as:

1. aqueous acidic
2. aqueous alkaline
3. halogenated
4. non-halogenated
5. general hazardous waste – powders etc.
6. toxic
7. cytotoxic

4.3 Cytotoxic Waste

Bin colour: purple base with purple lid

Final disposal method: incineration

Figure 12: Bin for disposing cytotoxic waste

Cytotoxic waste is any substance contaminated with any residue or preparations that contain materials that are toxic to cells principally by their action on cell reproduction. All cytotoxic waste should be placed in an approved purple cytotoxic bag or container. When the residue container is full, place in purple labelled cytotoxic waste wheelie bin kept in secure area. Although the final disposal method for cytotoxic waste is the same as chemically contaminated waste, it must be treated more securely prior to incineration due its mutagenic potential.

4.3.1 Ethidium Bromide

Ethidium bromide can expose users to hazards such as toxic, mutagenic, carcinogenic, and other secondary hazards dependent on its use. The decision to use products such as ethidium bromide should be assessed early as substitutes such as Gel Red may provide a similar outcome with much lower hazards, and final disposal should be considered at this early assessment stage.

Liquid Waste *e.g,* buffer solutions, stock solutions, etc

1. Keep record of amount of EtBr in solution

2. Place in spill proof container with bunding or secondary containment
3. Apply Cytotoxic Waste Disposal Identification Label (and label for any secondary hazards)
4. Dispose via Hazardous Waste Store
5. Solid Waste eg gels, powder, contaminated consumables (eg gloves, paper, used tea bags, etc)
6. Place waste in laboratory bin lined with purple cytotoxic bag
7. If deemed necessary, first put waste in sealed bag/container to avoid unnecessary exposure or contact
8. When bin/bag is full, place in larger secure purple base/purple lid cytotoxic bin which is in a secure location.

4.4 Biological/Clinical, GMO and Biosecurity Waste

Bin colour: yellow base with yellow lid

Final disposal method: autoclave then landfill

Figure 13: **Bin for disposing biological/clinical, GMO and biosecurity waste**

Biological/clinical and GMO waste must be rendered non-viable before disposal. This generally means autoclaving. Where applicable, any biosecurity waste must be effectively contained and disposed in a manner approved by the Department of Agriculture and Water Resources.

Waste that has been chemically treated must NOT be autoclaved.

4.5 Sharps Waste

Bin colour: yellow (general) or purple (cytotoxic) approved sharps container

Final disposal method: dependent upon primary contamination

Sharps are objects or devices that have acute, rigid corners, edges, points or protuberances capable of cutting or penetrating the skin e.g. hypodermic needles, broken glass, scalpel blades, lancets, syringes with needles, razor-blades.

Place any sharps in approved sharps container. Sharps containers should be located adjacent to the work area where sharps are used for easy access.

Sharps may also be contaminated with toxic, infectious or radioactive materials. These sharps should be placed into separate sharps containers which are then labelled appropriately according to the type of primary contamination (chemically contaminated, biohazardous, radioactive, and cytotoxic).

Figure 14: Bins for disposing sharp wastes (yellow for general and purple for cytotoxic)

When the sharps residue container is filled to the "fill line", seal container, affix Hazardous Waste label (if required), and place in appropriate bin.

* Do not overfill container - only fill to "fill line" marked on container.

4.6 Glass Waste

Bin colour:

green base with brown lid (brown glass)

green base with white lid (clear glass)

Final disposal method: recycling

Glass, whether broken or unbroken, should not be placed in general waste bins. The bottle cap can be removed and disposed in the general waste bin. Once clean, place glass in waste bin based on glass colour. When the laboratory glass bin is ¾ full, the lid should be placed on the bin and the contents transferred to the larger solid waste bins.

4.6.1 Broken Glass

Broken glass should be treated as Sharps waste.

If pieces of broken glass are too large for a sharps container, they should be placed into an impervious container with a secure lid, and then placed in appropriate wheelie bin.

Figure 15: Bins for disposing broken glass.

4.6.2 Contaminated Glass

Any glass that has been contaminated, and unable to be safely decontaminated, should be treated as other waste of the same hazard e.g. Chemically Contaminated Waste Biological/Clinical, GMO and Biosecurity Waste, cytotoxic. Contaminated glass containers or laboratory glass such as beakers, volumetric flasks of other Pyrex items cannot be placed in general recycling bins.

4.7 Radioactive Contaminated Waste

Bin colour: red base with red lid

Final disposal method: dependent on primary hazard

Figure 16: Bins for disposing radioactive contaminated waste.

Radioactive waste should be packaged according to its primary hazard eg Chemically Contaminated Waste or Biological/Clinical, GMO and Biosecurity Waste. It will be kept in the Radioactive Waste Store to “delay and decay” prior to final disposal as non-radioactive waste.

Figure 17: Different coloured bins for different types of waste disposal.

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