Manual for Training Course

"DETECTION, IDENTIFICATION & QUANTIFICATION OF BT COTTON"

Funded by PARB through Project # 890

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MESSAGE BY

Director General Agri. (Research), Punjab

It provides me immense pleasure to extend a message on the occasion of Training on detection, identification and quantification of GMOs in agriculture sector, which I am confident, is going to bear its testimony for the research endeavors of the participants and will be an outstanding session. My advice to participants is that the aim behind attending this training should be purely for the sake of growth of the economy of Pakistan through agricultural technology. Ayub Agricultural Research Institute (AARI), Faisalabad, has been demonstrating its excellence in the field of agricultural sciences ever since its inception. Introducing several GM varieties of cotton in market is a clear manifestation of the meticulous planning and incessant efforts by the scientists of AARI.

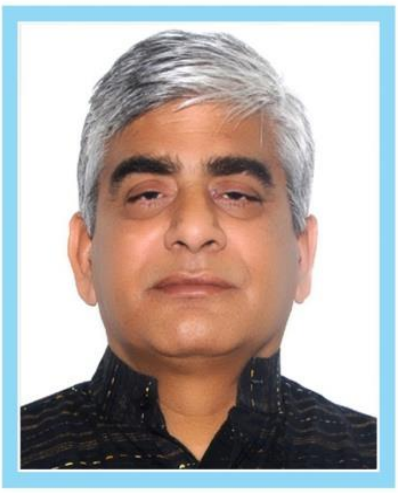
A good institute is thought to be the one that not only nurtures the quality of research but also makes provision for the trainings and exchange of ideas and opinions among the scientific community. Our institution has been fully alive to this responsibility and promises to develop new varieties of crops, technologies for food safety and sustainable generation of exportable surplus for economic safeguard, value addition, and conservation of natural resources and introduction of new plants.

We in AARI believe that the only treasure worth piling up is the true pursuit of excellence, because all other treasures follow automatically. Being in consonance with this belief Agricultural Biotechnology Research Institute (ABRI) has been marching on the road to excellence at a very brisk pace, and the quality work produced by ABRI merits a high measure of appreciation. I have no hesitation in saying that ABRI is one of the prized possessions of AARI.

Dr. Abid Mahmood

MESSAGE BY

Chief Executive PARB



It's a matter of great honor and pleasure for me that PARB is funding a project solely meant for training of extension officers, FSC&RD and private seed sector personnel in detection, identification and quantification of GMOs. Agriculture is the main strength and of course, backbone of the economy of Pakistan.

Pakistan has one of the fastest population growth rates and food security is the major issue for the nation. The total population of the country is around 170 million which is expected to rise to 210 million by 2022. In order to meet the growing demand to produce more food and create crops that are resistant to pests, viruses and climate, Genetically Modified crops are often intentionally utilized for food and feed production. Ayub Agricultural Research Institute was the beacon to the green revolution in the country in late sixties and since then is playing a key role in meeting the post green revolution productivity challenges and ensuring national food security.

I would like to say that higher crop yields are essential, but it is not enough just to grow more, rather, higher quality is also important to maximize the value of crop and to meet rising consumer expectations. ABRI is assisting all breeding institutes of AARI for the development of better quality crop varieties using latest tools of biotechnology including tissue culture, genomics and genetic engineering. GMO testing lab of this institute has been approved for testing of GMOs by Govt. of the Punjab and recently has been recommended for award of ISO-17025 accreditation by a team of PNAC assessors. The training programme will be valuable for all stakeholders in not only creating the awareness but also imparting practical training in testing of GMOs.

Dr. Noor ul Islam Khan

PREFACE

Genetically modified crops are plants whose genetic characteristics have been altered by the insertion of a new gene or sets of genes into their genome using genetic engineering methods. In most cases, the aim is to introduce a new trait to the plant which does not occur naturally in the species. In Pakistan, GM cotton (resistant to some bollworms carrying Cry1AC gene) cultivation was allowed few years back and recently permission for cultivation of GM maize carrying insect and herbicide tolerant genes has been granted by National Biosafety Committee (NBC), Islamabad.

At present more than 95% area of cotton is under Bt but still there is lack of awareness among stakeholders regarding the appropriate testing of Bt cotton to maintain its purity and effectiveness in insect control. Hence, trainings in the field of development, detection and identification of GM crops is the necessity of the day. For this purpose PARB project No.890 entitled "Training of Agriculture Extension, FSC & RD and private seed sector personnel in detection, identification and quantification of Bt cotton" has been approved. Through this project training and capacity building of more than 200-300 persons from Extension wing, FSC & RD and private sector would be carried out.

This training manual has been designed to provide the participants an insight into the methodologies used for detection, identification and quantification of GMOs, which will be followed during three days training organized by the Agricultural Biotechnology Research Institute, AARI, Faisalabad. The primary objective of the training is to provide up-to-date information on testing techniques of GM crops. We hope the course will help to build capacity of technical personnel from extension wing of Agriculture Department, Federal Seed Certification & Registration Department, public/private research institutes and seed companies with skills to test Biotech plants.

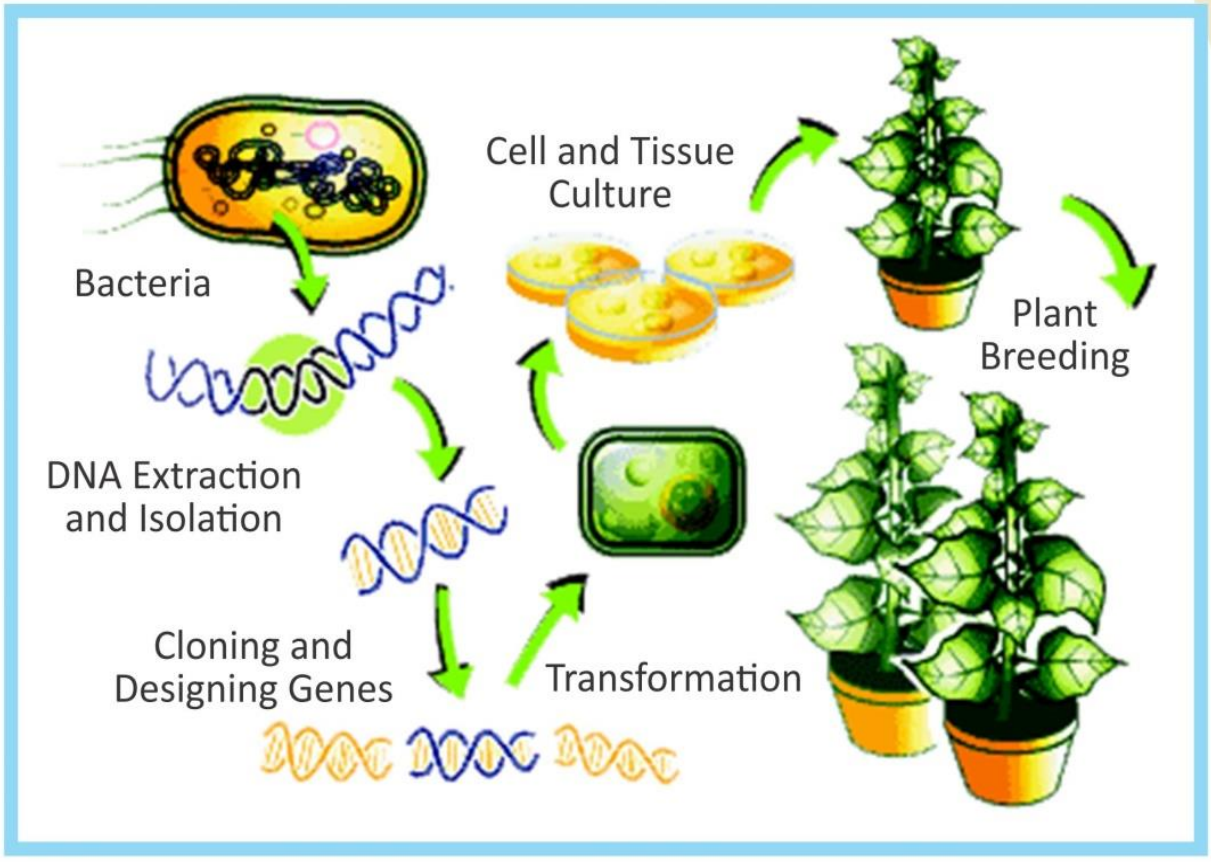
Dr. Muhammad Zaffar
Director, ABRI, Faisalabad.

TABLE OF CONTENTS

BIOTECH (GM) CROPS

A genetically modified organism (GMO) is an organism or microorganism whose genetic material has been altered to a certain segment of DNA from another organism. Modern recombinant DNA technology enables the "stitching together" of pieces of DNA, regardless of the source of the pieces.

Genetically modified crops (GMCs, GM crops, or biotech crops) are plants whose genetic characteristics have been altered by the insertion of a new gene or sets of genes into their genome using genetic engineering methods. In most cases, the aim is to introduce a new trait to the plant which does not occur naturally in the species. Examples in food crops include resistance to certain pests, diseases, or environmental conditions, reduction of spoilage, or resistance to chemical treatments (e.g. resistance to a herbicide), or improving the nutrient profile of the crop. Examples in non-food crops include production of pharmaceutical agents, biofuels, and other industrially useful goods, as well as for bioremediation.



Steps in development of Genetically Modified Plants

TESTING OF BIOTECH CROPS

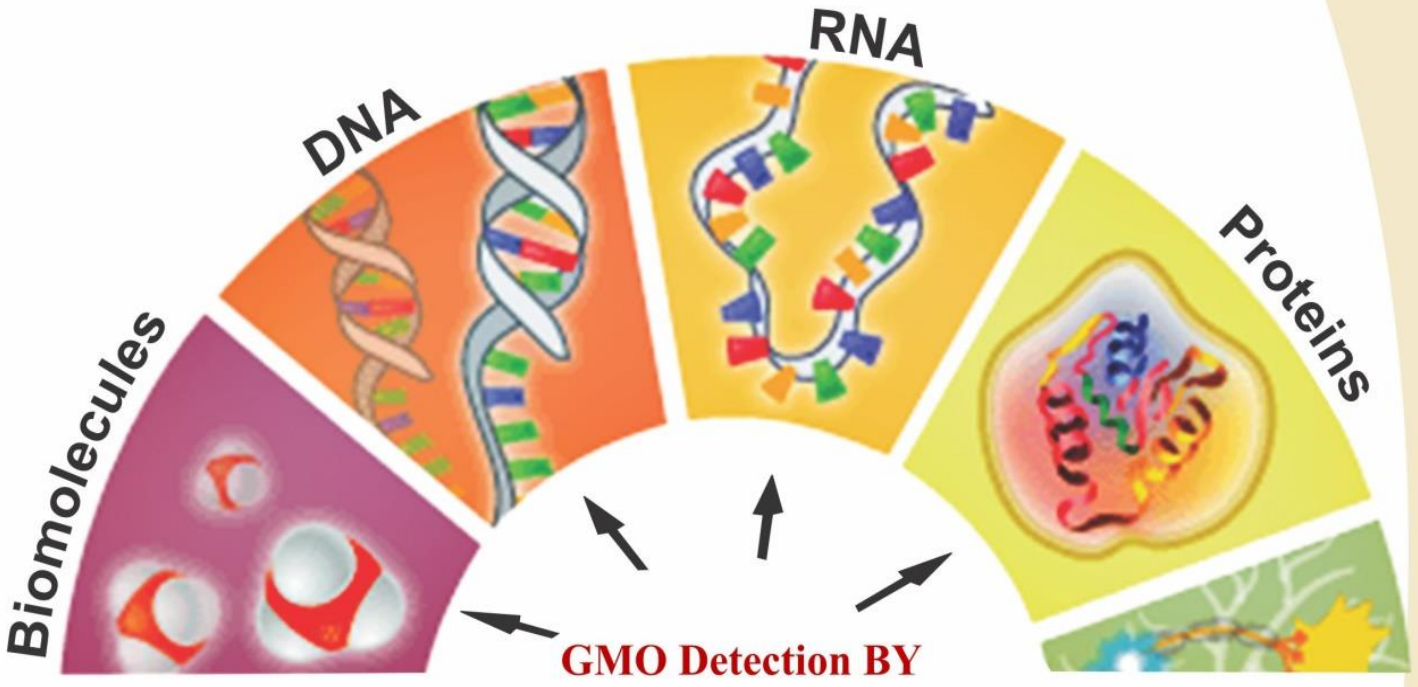
"GM crops are indistinguishable from non- GM crops to the naked eye"

- Basis of detection is to exploit the differences between the unmodified variety and the transgenic plant
- Testing methods need to look
  - for the genes (DNA) introduced into the crop
  - for the proteins produced in the plant by the introduced gene

GM crops detection methods

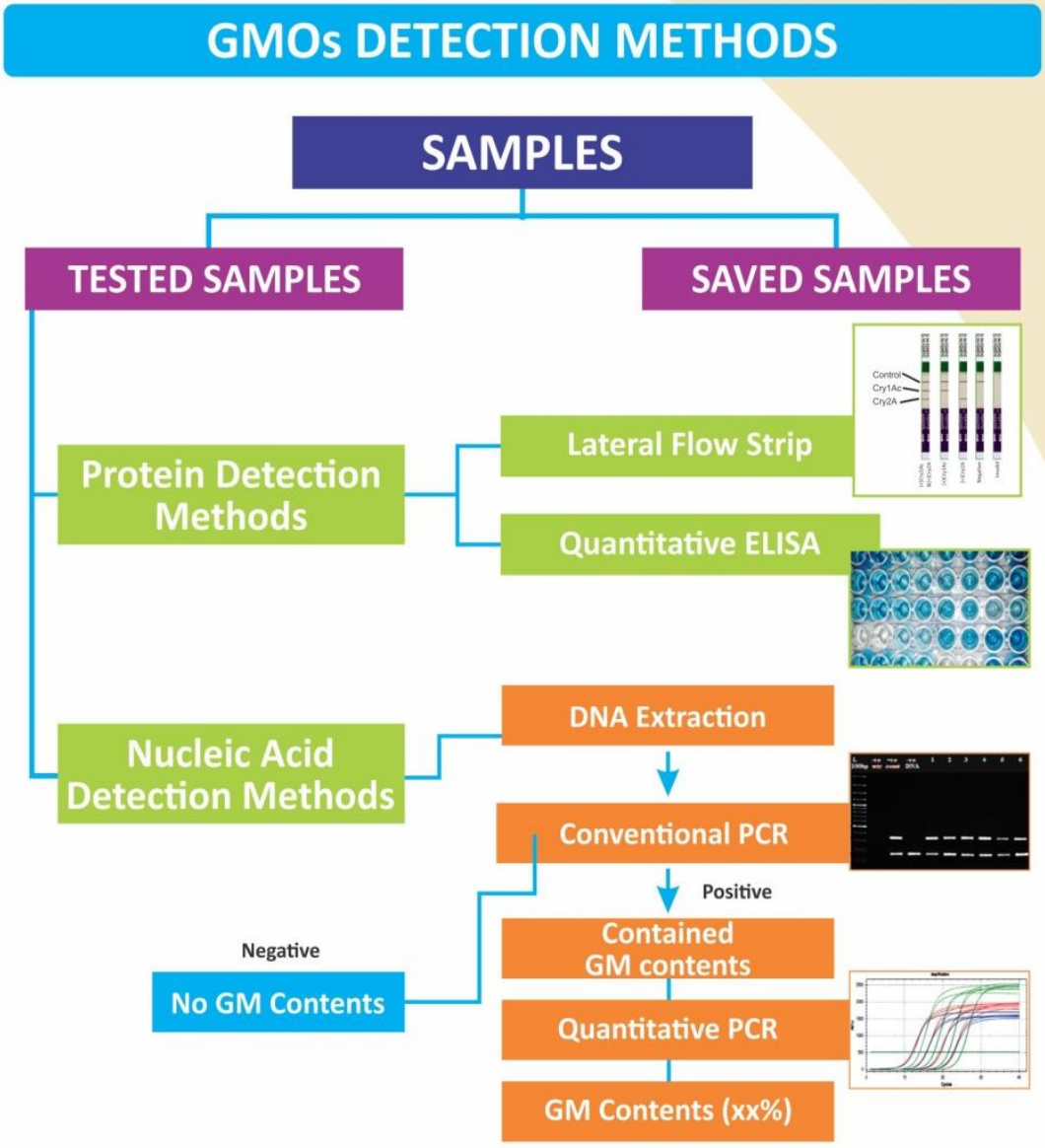
Detecting inserted genetic material at

1. DNA level,
2. Protein level,
3. RNA level,
4. Phenotypic level /Bioassays



But the most common and reliable detection methods are DNA based (PCR analysis) and protein based (strip test and ELISA techniques), so detailed protocols regarding both these detection methods are discussed on following pages.

STANDARD PROCEDURE FOR BIOTECH TESTING



Biotech Testing Assays Needs

- **Presence and integrity of target molecule**
  - DNA template and protein
- **Availability of capture molecules**
  - Primers and probes for nucleic acids testing
  - Antibodies for protein testing
- **Reference materials**
  - Positive and negative controls
  - Calibrants for quantitation

1. DNA BASED TESTING

Qualitative PCR analysis

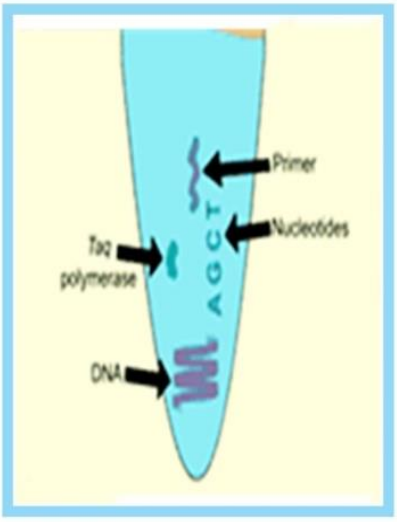
Most of the commercially released Biotech crops contain three genetic elements: the cauliflower mosaic virus (CaMV) 35S promoter, the nopalin synthase (NOS) terminator, and the kanamycin-resistant marker gene (*nptII*). Amplification of amplicons specific for these elements may confirm transgenic status of the sample. These type of assays are referred to as screen specific PCRs. If such PCR assay gives a positive result verification of the identity of the amplicon is also performed using gene specific and event specific PCRs. All these types of PCR reactions follow same principle however; differ in sequence of the primers used and annealing temperatures. Qualitative testing of biotech crop based on conventional PCR technique will involve following steps.

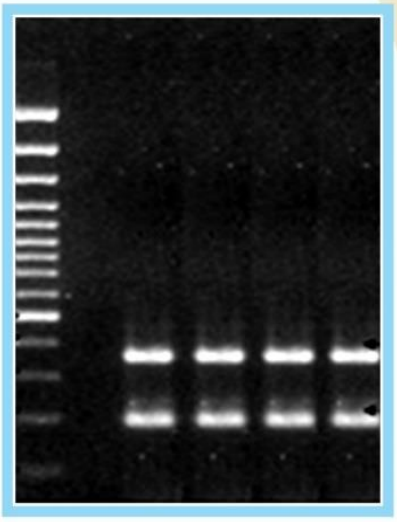
i. Genomic DNA Isolation and quantification

ii. Amplification of the targeted DNA template using PCR

iii. Gel electrophoresis and documentation

Detailed protocols regarding all three steps are mentioned on following pages.





I. GENOMIC DNA ISOLATION FROM PLANT MATERIALS

Isolation of high quality DNA from test sample is starting material for Polymerase Chain Reaction (PCR) based on detection of transgenic elements in biotech crops. An array of methods, using in house recipes as well as commercially available kits, is being utilized to isolate genomic DNA from plant material. However, all the techniques being used follow the same basic principle of DNA extraction which comprised of removal of polysaccharides, proteins, pigments and RNA, and subsequent purification of DNA from PCR inhibitors. Genomic DNA isolation from plant materials is generally achieved through a series of steps including breakage of cell wall by grinding the tissue using beads/mortar and pestle in liquid nitrogen; disruption of cell membranes by a suitable detergents such as CTAB and SDS; degradation of protein, lipids and polyphenols with organic solvents like chloroform and phase separation for their removal through centrifugation; propanol/alcohol/ based DNA precipitation; RNase treatment to remove RNA and finally to dissolve isolated DNA usually in water. Detailed protocol of DNA isolation, using CTAB (Cetyl Trimethyl Ammonium Bromide) method as well as one of the commercially available kits, are detailed below:-

EQUIPMENTS NEEDED

- Water bath
- Centrifuge Machine
- Incubator

A. CTAB (CETYL TRIMETHYLAMMONIUM BROMIDE) METHOD

MATERIALS

- Autoclaved pestle & mortar
- Liquid nitrogen
- 2X CTAB
- Add 0.5-1.0% (v/v) of β- Mercaptoethanol just before use
- Chloroform: Iso-amyl alcohol (24: 1, v: v)
- Chilled 2-propanol
- RNase A
- 3M NaCl
- Absolute ethanol
- 70% ethanol,
- Double distilled de-ionized water (d_3H_2O)

Wear appropriate personal protective equipment; lab coat, safety glasses and gloves where needed.

PROCEDURE

- Turn on the water bath and set at 65°C, preheat 2XCTAB extraction buffer
- Pre-cool the autoclaved mortar and pestel with liquid nitrogen.
- Grind 2-3 leaves in liquid nitrogen into very fine powder and transfer it to 50ml falcon tube.
- Add 15ml of pre-heated (65°C) 2X CTAB extraction buffer and mix contents by gently inverting the tube several times, and then incubate at 65°C for 45 min in water bath.
- After incubation, add 15ml of Chloroform: Iso-amyl alcohol (24: 1, v: v). Centrifuge for 10 min at 4000-5000rpm at room temperature.
- Precipitate DNA by addition of equal volume of chilled 2- propanol.
- Centrifuge for 10 min at 4000-5000rpm at 4°C, decant supernatant and wash the pellet twice with 70% chilled ethanol.
- Dry the pellet thoroughly and dissolve in 500ul of d_3H_2O.
- And add 4ul of RNAase A to digest RNA. After incubation for one hour at 37°C, add one volume of chloroform: iso-amyl alcohol and centrifuge for 10 min at 13000rpm for phase separation.
- Transfer supernatant to a new 1.5ml microcentrifuge tube and mix gently after adding 0.1 volume of 3M NaCl.
- Add two volume of cold ethanol to precipitate DNA. After 10 min centrifugation at 13000 rpm at 4°C, and wash pellet twice with 70% chilled ethanol.
- Air dry the pellet and dissolve in 300ul of d_3H_2O or 0.1X TE buffer.

B. KIT BASED DNA ISOLATION

MATERIALS

- Genomic DNA purification kit
- Autoclaved pestle & mortar
- Liquid nitrogen
- Chloroform
- Absolute ethanol
- 70% ethanol,
- d_3H_2O

PROCEDURE

- Pulverize leaf tissue in liquid nitrogen with mortar and pestle.
- Shift 50-100mg of pulverized material in 1.5 microcentrifuge tube and resuspend in 200µl of d_3H_2O or TE buffer.
- Add 400µl of **lysis solution**, mix and incubate at 65°C for 5 min. Then the sample is incubated at 65°C for 10 min.
- Immediately add 600µl of **chloroform**, gently emulsify by inversion (3-5 times) and centrifuge the sample at 10,000 rpm for 2 min.
- Transfer the upper aqueous phase containing DNA to a new microcentrifuge tube and add 800µl of freshly prepared **precipitation solution**, mix gently by several inversions at room temperature for 1-2 min and centrifuge at 10,000 rpm for 2 min.
- Remove supernatant completely and dissolve DNA pellet in 100µl of **NaCl solution** by gentle vortexing. Make sure that the pellet is completely dissolved.
- Add 300µl of cold ethanol, let the DNA precipitate (10 min at -20°C) and spin down (10,000 rpm, 3-4 min). Remove the ethanol. Wash the pellet once with 70% cold ethanol and dissolve DNA in 100µl of sterile deionized water.

DNA QUANTIFICATION

Good quality DNA of known concentration is needed for subsequent use in PCR. Concentration of DNA is measured at 260nm wavelength on spectrophotometers (1 unit at 260:50 ng dsDNA) whereas A260/A280 reveals quality of DNA.

EQUIPMENTS NEEDED

NanoDrop-2000

MATERIALS

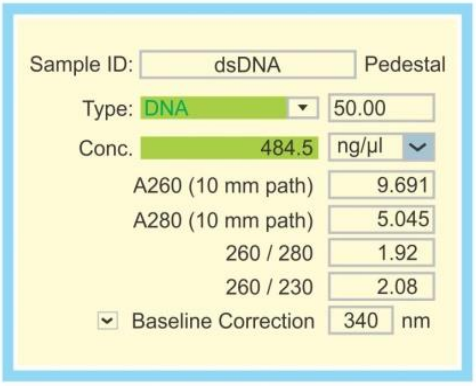
- Double distilled de-ionized water/TE buffer
- Lint less tissue

PROCEDURE

- Double click on icon to open program

- Select DNA in "Type" options
- Raise the sampling arm, pipette 1ul of double distilled de-ionized water onto the lower measurement pedestal, drop the arm and click blank on PC. When the measurement is complete, raise the sampling arm and wipe the sample from both the upper and lower pedestals using a dry, lint-free laboratory wipe.

- After performing blank, place 1µl of DNA sample to be quantified on the lower measurement pedestal and click measure and note concentration of DNA and 260/280 ratio.



ii. PCR (POLYMERASE CHAIN REACTION)

Once high quality DNA of known concentration is in hand, a series of different PCR reactions are performed for testing of transgenic elements in test sample. Firstly, the isolated DNA is subjected to a control reaction using primers specific for house keeping genes or microsatellite for that crop species to test quality of DNA for amplification. Secondly, screen specific PCR is conducted using primer specific for marker/reporter genes, promoters and terminators commonly used in development of transgenic crops. If samples are found positive for any of these elements, the type and source of gene is validated through gene and event specific PCRs, respectively. The PCR technique is very sensitive and may yield false negatives in case of plus minus assays due to reaction failure, hence, a multiplex PCR is assembled for such present absent assays. In multiplex PCR, two different targets (one internal control like housekeeping gene/microsatellite and other desired sequence such as locus specific for NPTII, specific gene or event) are amplified in a single tube.

The following procedure describes the assembling of standard PCR for all these reactions which however will vary only for the primer sequences and annealing temperature used.

MATERIALS

PCR mix per reaction

Reagents	Concentration	Volume
PCR buffer	10 X	2.0 µl
$MgCl_2$	25 mM	1.6 µl
dNTPs	2.5 mM	2.0µl
Primer forward*	30 ng/µl	1.0 µl
Primer reverse*	30 ng/µl	1.0 µl
Taq DNA polymerase	5 units/µl	0.2 µl
Template DNA	15 ng/ul	3.0 µl
d_3H_2O	-	9.2 µl
	Total volume	**20.0 µl**

Basic PCR Profile

Steps	Temperature	Temperature	Number of cycles
Initial denaturation	94 °C	94 °C	1 (first)
Denaturation	94 °C	94 °C	35
Annealing*	variable	variable	
Extension (1 min per 1Kb amplicon)	72 °C	72 °C	
Final extension	72 °C	72 °C	
Hold	4 °C	4 °C	1

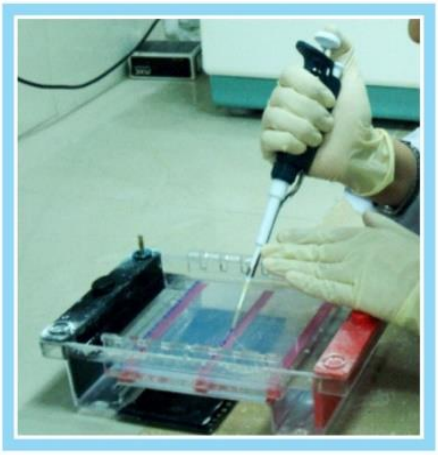
iii. GEL ELECTROPHORESIS AND DOCUMENTATION

a. Agarose Gel Electrophoresis and Ethidium Bromide Staining

Electrophoresis is a technique used to separate and sometimes purify macromolecules especially proteins and nucleic acids that differ in size, charge or confirmation**.** Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through an agarose matrix. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel. This phenomenon is called sieving. Proteins are separated by charge in agarose because the pores of the gel are too large to sieve proteins.This procedure describes the preparation of agarose gels, gel electrophoresis of the amplified products and post-staining with ethidium bromide.

EQUIPMENTS NEEDED

- Microwave oven
- Midi horizontal gel electrophoresis system
- Orbital shaker
- UV trans illuminator
- Gel documentation system



Gel loading

MATERIALS

- Agarose
- 1X TBE (Tris Borate EDTA) buffer
- 6X single loading dye
- DNA ladder (100bp)
- Ethidium bromide (EtBr)

❖ Gel documentation system

❖ Wear appropriate personal protective equipments; lab coat, safety glasses and gloves.

Gel Documentation System

PROCEDURE

- To prepare 100 ml of 1.5% gel solution, measure 1.5 g of agarose into 250 ml conical flask and add 100 ml 1x TBE buffer.
- Microwave the mixture until agarose is dissolved and solution is clear
- Cool down the solution to approx. 55°C under room temperature or by keeping it under tap water.
- Prepare gel tray by sealing ends with tape or other custom-made dam. Place comb in end notch of the tray and position the comb vertically such that the teeth are about 1-2 mm above the surface of the tray.
- Place the gel tray onto tissue paper and pour the gel solution to about 5.0 mm depth. Make sure that no bubbles are trapped underneath the comb.
- Allow to stand for 20-30 min for polymerisation.
- Remove gently the combs, tape or dam from the gel, place gel-tray in the electrophoresis chamber.
- Prepare samples for electrophoreses, add 4µl of 6X single loading dye for every 20µl of PCR reaction.
- Mix well and load 8-12µl of DNA sample per well.
- Load 5µl of DNA marker in border wells.
- Electrophoresis at 80-100 volts until blue dye has migrated an appropriate distance, depending on the size of product to be visualized.
- Turn off power pack and transfer gel onto UV transilluminator or gel documentation system to view DNA samples or take photo.
- Gels should be dried in the dedicated tray located in the fume hood before discarding.

Ethidium Bromide (Health Hazards)

Acute

- Material harmful by all routes of entry; inhalation, ingestion, or skin absorption.
- Material causes eye and skin irritation and is irritating to mucous membranes and upper respiratory tract.

Chronic

- This agent intercalates DNA strands and was mutagenic in a number of test systems
- The chemical, physical, and toxicological properties have not been thoroughly investigated in humans.

A. GEL ELECTROPHORESIS ON BIOANALYZER

Bioanalyzer DNA kits contain chips and reagents designed for sizing and analysis of DNA fragments. Each DNA chip contains an interconnected set of microchannels that is used for separation of nucleic acid fragments based on their size as they are driven through it electrophoretically.

EQUIPMENTS NEEDED

- Agilent 2100 Bioanalyzer
- Chip priming station
- Vortex mixer
- Microcentrifuge

MATERIALS

Agilent DNA 1000 Kit

- DNA Chips
- Electrode Cleaner
- Syringe Kit
- (yellow) DNA 1000 Ladder
- (green) DNA 1000 Markers 15/1500 bp
- (blue) DNA Dye Concentrate
- (red) DNA Gel Matrix

PROCEDURE

a. Preparing the Gel-Dye Mix

- Allow DNA dye concentrate (blue) and DNA gel matrix (red) to equilibrate to room temperature for 30 min.
- Vortex DNA dye concentrate (blue) and add 25µl of the dye to a DNA gel matrix vial (red).
- Vortex solution well and spin down. Transfer to spin filter.
- Centrifuge at 2240 g ± 20 % for 15 min. Protect the solution from light. Store at 4°C.

b. Loading the Gel-Dye Mix

- Allow the gel-dye mix equilibrate to room temperature for 30 min before use.
- Put a new DNA chip on the chip priming station.
- Pipette 9.0 µl of gel-dye mix in the well marked.
- Make sure that the plunger is positioned at 1 ml and then close the chip priming station.
- Press plunger until it is held by the clip.
- Wait for exactly 60 s then release clip.
- Wait for 5s. Slowly pull back plunger to 1ml position.
- Open the chip priming station and pipette 9.0µl of gel-dye mix in the wells marked.

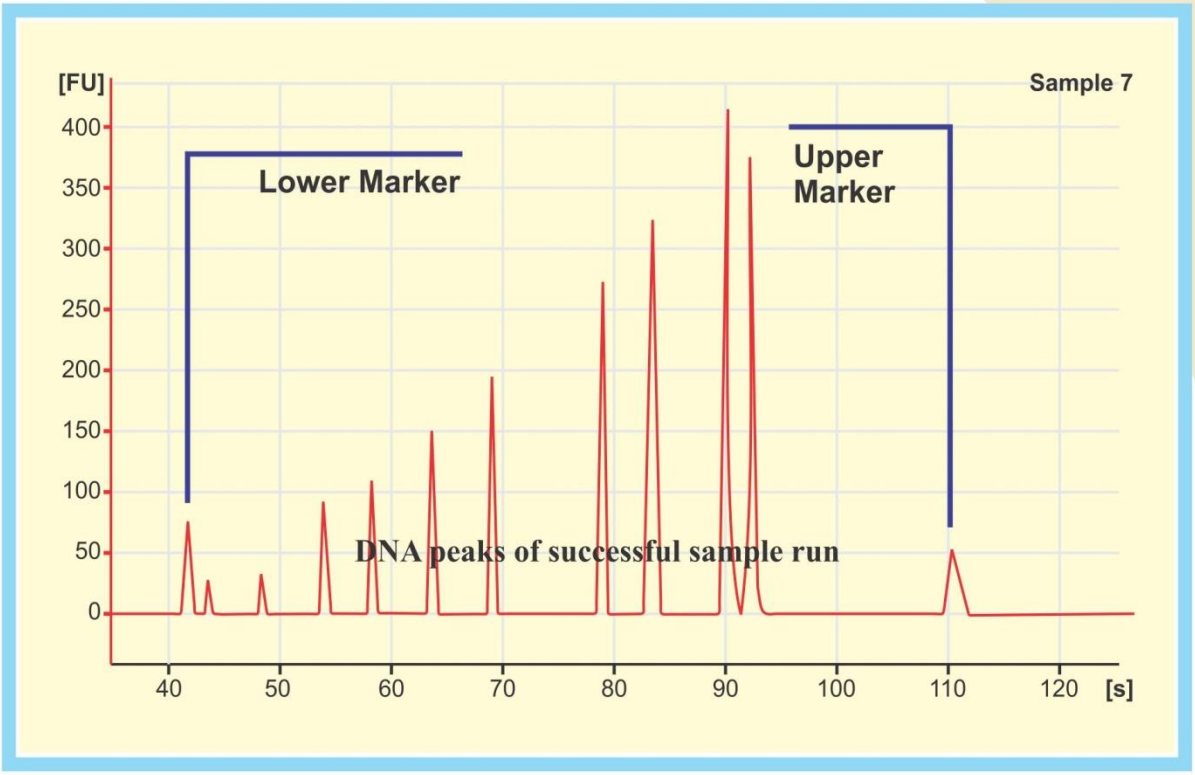
c. Loading the Markers

- Pipette 5µl of marker (green) in all 12 sample wells and ladder well. Do not leave any wells empty.

d. Loading the Ladder and the Samples

- Pipette 1 µl of DNA ladder (yellow) in the well marked.
- In each of the 12 sample wells pipette 1 µl of sample (used wells) or 1µl of deionized water (unused wells).
- Put the chip horizontally in the adapter and vortex for 1 min at the indicated setting (2400 rpm).
- Run the chip in the Agilent 2100 Bioanalyzer within 5 min.

Agilent 2100 expert software will be used for graphical analysis.



1. PROTEIN BASED TESTING

- Protein detection methods are based mainly on immunoassays
- Immunoassays use antibodies as test reagent
- Antibodies are proteins produced in response to foreign substances (antigens) and specifically bind to the antigens

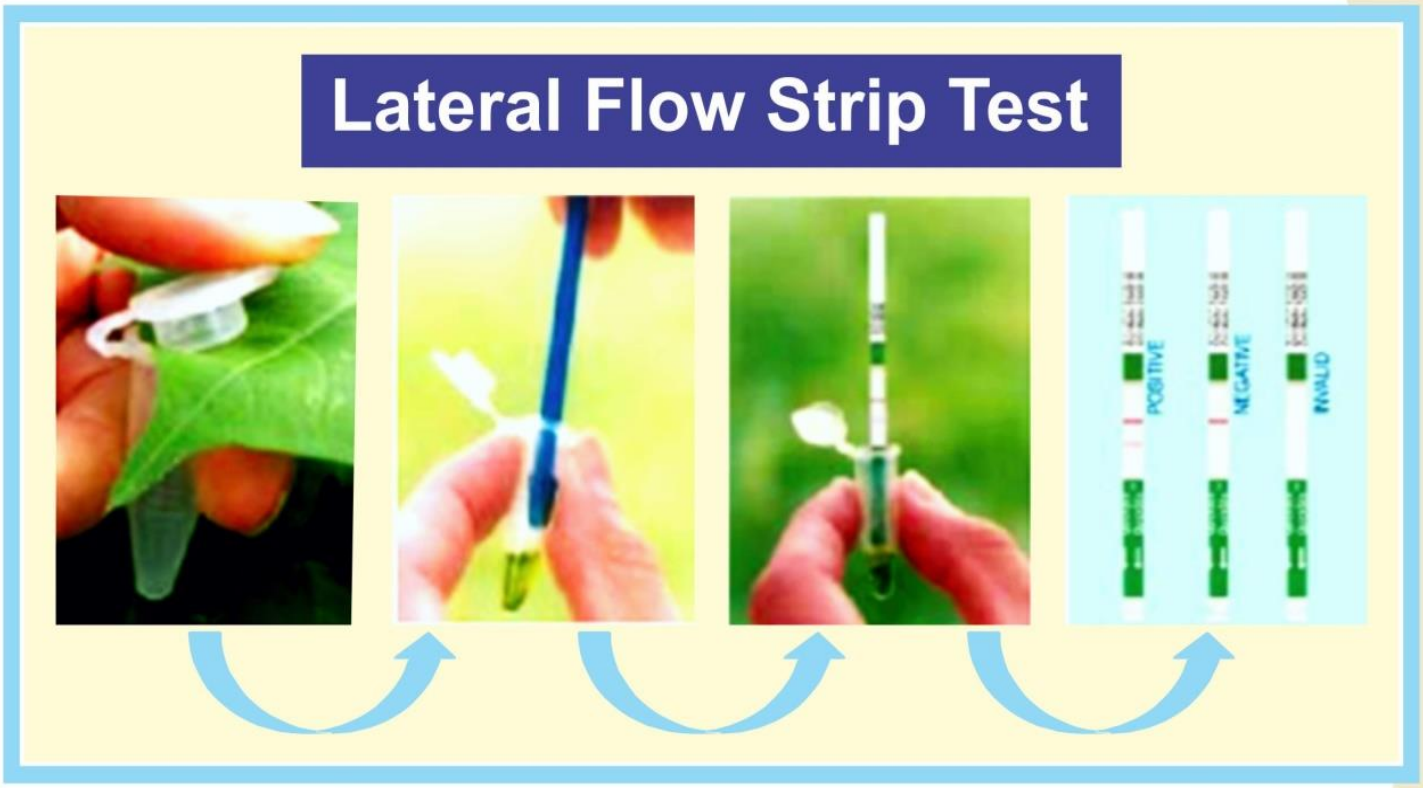
2.1. Lateral Flow Strip Test

The most commonly used immuno- or antibody-based test for GMO detection is the strip test (also called a lateral flow device or dipstick).

Strip tests are thin strips comprised of a nitrocellulose membrane covered by a sample pad on one end and a wicking pad on the other end. This test provides plus minus assay for specific protein through ELISA (enzyme-linked immunosorbent assay). A variety of strip kits are available for different transgenes.

Qualitative Lateral flow strip test procedure

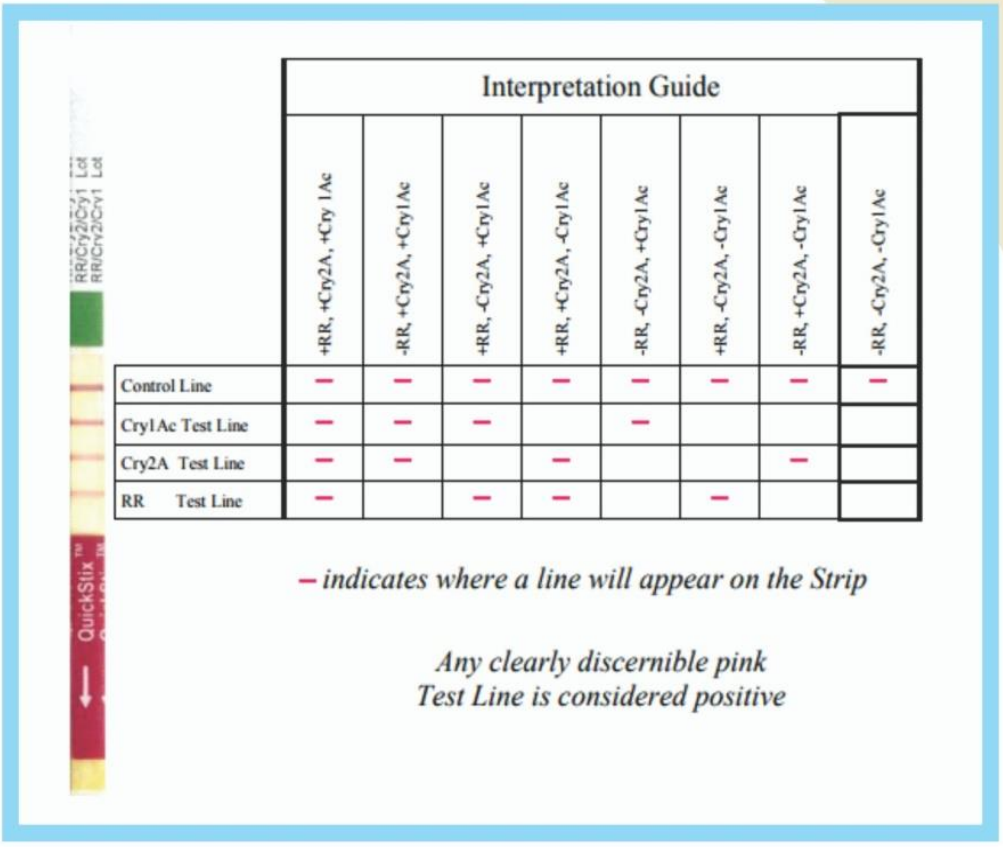
1. Snap two circular tissue punches by closing the cap
2. Grind the tissue by rotating the pestle against the sides of the tube until the leaf tissue is well ground
3. Place the strip into the extraction tube, Allow the strip to develop for 10 minutes before making final assay interpretations



Interpretation of strip test results

1. The sample is considered positive for the GMO protein of interest if the test and control lines are visible.
2. Sample is considered negative for the GMO protein if only the control line is visible.
3. Absence of both lines indicates that the test is invalid and should be repeated.

See following figure for interpretation of the results.



2.2. Quantitative ELISA Test

Enzyme Linked Immuno Sorbent Assay (ELISA) tests for the presence of the specific protein that the genetically modified DNA produces in the plant. ELISA procedures use antibodies that react with specific proteins produced by the GMO. The principle of ELISA is to detect antigen A, purified antibody specific for antigen A is linked chemically to an enzyme. The samples to be tested are coated onto the surface of plastic wells to which they bind. The labelled antibody is then added to the wells under conditions where nonspecific binding is prevented, so that only binding to antigen A causes the labelled antibody to be retained on the surface. Unbound labelled antibody is removed from all wells by washing, and bound antibody is detected by an enzyme-dependent colour-change reaction, which can be measured photometrically or recognised by naked eye.

ELISA procedure of Cotton leaf

This procedure describes for the detection and quantification of Bt endotoxin residues in cotton leaf tissues. The plant found positive for Cry1Ac through lateral flow strip test will be tested for quantification of Cry1Ac endotoxins. A young fully expanded main stem leaf (node3-4) will be used for quantitative ELISA.

EQUIPMENTS NEEDED

- Centrifuge
- Vortex
- Physical balance
- Timer/Stop watch
- Microplate or ELISA strip reader
- Automated plate washer

Wear appropriate personal protective equipments; lab coat and gloves

MATERIALS

Consumable chemicals

- Bt-Cry1Ab/Cry1Ac detection kit
- Disposable pestles
- 1.5 ml microcentrifuge tubes
- Fresh and clean leaf tissue samples

PROCEDURE

Sample preparation

- Take two leaf punch samples (approximately 10mg each) by snapping the tube cap of the sample-extracting device down on the leaf.
- Insert the pestle into the tube and grind the tissue by rotating the pestle against the sides of the tube with a twisting motion.
- Continue the process until the tissue is well ground.
- Add 0.5ml of the 1X Sample Extraction/Dilution buffer to the tube.
- Repeat the grinding step to mix the tissue in Extraction/Dilution buffer. Allow the debris to settle down in each tube for few minutes before proceeding.

PROCEDURE

- Add 100 µl of Negative Control, 100 µL of each Calibrator, and 100 µl of each sample extract to their respective wells according to layout.
- Thoroughly mix the contents of the wells by moving the strip holder in a rapid circular motion on the bench top for a full 20-30 seconds. Cover the wells to prevent evaporation and incubate at ambient temperature for 45 minutes
- Wash the plate 3-4 times after incubation with wash buffer.
- Add 100 µL of Cry1Ac-Enzyme Conjugate to each well, incubate at ambient temperature for 30 minutes.
- Again wash the plate 3-4 times after incubation with wash buffer.
- Add 100 µL of Substrate to each well. Thoroughly mix the contents of the wells. Cover the wells and incubate for 15 minutes at ambient temperature.
- Add 100 µL of Stop Solution to each well and mix thoroughly. This will turn the well contents yellow.

NOTE: Read the plate within 30 minutes of the addition of Stop Solution.

Schedule of Three days Training Course on "Detection, Identification & Quantification of Bt Cotton"

SR. #	ACTIVITY	OFFICER CONCERNED	TIMINGS
1	Registration of Participants	Seminar Room, ABRI	08:00-09:00
2	Inaugural Session	Miss Erum Yasmeen	09:00-09:30
3	Introduction of ABRI	Director ABRI	09:30-10.00
4	Departmental visit	Dr. Sajid-Ur-Rahman (Botanist Cyto.)	10:00-10.15
5	Strip Test (Lecture and Practical)	Miss Shakra Jamil (Botanist Biotech.)	10:15-11:00
TEA BREAK			11:00-11:30
6	DNA Isolation (Lecture)	Miss Shakra Jamil (Botanist Biotech.)	11:30-11:45
7	DNA Isolation (Practical)	Miss Shakra Jamil (Botanist Biotech.)	11:45-01:00
LUNCH AND PRAYER BREAK			01:00-2:00
8	DNA Isolation (Practical) continued	Miss Shakra Jamil (Botanist Biotech.)	02:00-04:00
DAY – 2			
9	DNA Quantification	Miss Shakra Jamil (Botanist Biotech.)	08:30-09:30
10	PCR (Lecture)	Dr. Sajid-Ur-Rahman (Botanist Cyto.)	09:30-10.00
11	PCR (Practical)	Miss Shakra Jamil (Botanist Biotech.)	10:00-11:00
TEA BREAK			11:00-11:30
12	Gel Electrophoresis (Lecture)	Mr. Rahil Shahzad (ARO)	11:30-12:00
13	Gel Electrophoresis (Practical)	Miss Shakra Jamil & Rahil Shahzad	12:00-1:00
LUNCH AND PRAYER BREAK			01:00-2:00
14	Gel documentation (Practical)	Miss Shakra Jamil (Botanist Biotech.)	02:00-03:30
15	Group Discussion	Seminar Room ABRI	03:30-04:00
DAY – 3			
16	ELISA (Lecture)	Miss Shakra Jamil (Botanist Biotech.)	08:30-09:00
17	ELISA (Practical)	Miss Shakra Jamil (Botanist Biotech.)	09:00-11:00
TEA BREAK			11:00-11:30
18	ELISA (Practical) continued	Miss Shakra Jamil (Botanist Biotech.)	11:30-12:30
19	Juma Prayer Break	Central Mosque, AARI	12:30-01:30
20	Lunch Break	Seminar Room, ABRI	01:30-02:00
21	Group Discussion	Seminar Room, ABRI	02:00-02:30
Certificate Distribution and Feed back			02:30-04:00