Laboratory Manual for Detection of Genetically Modified Foods and Crops in Tanzania

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For further information, please contact:
Permanent Secretary,
Vice President's Office,
P. O. Box 5380,
Dar es Salaam.
TANZANIA.
Phone: +255-22-2113983
Fax: +255-22-2125297
E-mail: biosafety@vpdoe.go.tz

PREFACE

Modern biotechnology is opening new frontiers in many fields in this era of globalization, however, its development and application has been associated with both opportunities and concerns. Therefore, in order to tap the benefits arising from modern biotechnology, it is of priority to institute biosafety measures that would ensure its safe transfer, handling and use.

In relation to this, Tanzania has already put in place biosafety framework and legal instruments as a response to this monumental challenge. One of the tools for facilitating enforcement of the biosafety legal regime includes administrative and technical manuals to contribute in the management of safety, development, use including transboundary movements of GMOs that may negatively affect environment, biodiversity as well as human and animal health. Similarly, this operational manual for GMO testing aims to set standard procedures (protocols) to be used by laboratories primarily for enforcement purposes as well as research and training.

The manual outlines the procedures and steps to be followed in laboratory testing/analysis of GMOs and products thereof. It anticipated the manual would be revised periodically to accommodate emerging issues as need arises. Therefore, suggestion for improvement from stakeholders would be highly appreciated.

ACKNOWLEDGEMENT

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Ruth H. Mollel
Permanent Secretary
VICE PRESIDENT'S OFFICE

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1.0 BACKGROUND

Across the globe, there are constantly changing regulatory, industry and consumer demands regarding Genetically Modified Organisms (GMOs). Therefore, testing for Genetically Modified Organisms (GMOs) has become a necessity in light of the requirements of the existing legal framework which put in place measures to protect environment, human and animal health, biodiversity as well as socio-economic, cultural and ethical concerns.

Two different categories of analytical methods are commonly used to detect GMOs. One is Polymerase Chain Reaction (PCR) which is based on the detection of genetic material (DNA). It is most versatile for detection of GMOs and therefore useful in many applications. The alternative is Enzyme-Linked Immunosorbent Assay (ELISA) which detects foreign protein(s) which most GMOs contain as a result of the insertion of engineered gene(s). As compared to PCR, ELISA is more restricted in its applicability but can be very useful in certain raw commodities.

1.1 DNA Extraction from Plant Materials

Various DNA extraction procedures for isolating genomic DNA from various plant sources have been described, including the salt extraction method and the cetyltrimethyl ammonium bromide (CTAB) method and its modifications. Most methods require the use of liquid nitrogen or freeze-drying (lyophilization) of the tissue for the initial grinding. However, in remote parts of Tanzania where facilities are not available alternative methods described below can be used. After grinding the tissues in various extraction buffers, DNA is extracted with phenol-chloroform, or the extract is dialyzed against EDTA and a buffered Tris-HCl solution. After extraction, the aqueous phase is concentrated, either by ethanol or isopropanol precipitation.

1.2 Screening For Non Specific GMO using Polymerase Chain Reaction (PCR)

The PCR concept is based on amplification of fragments of DNA common in most GMO events. The majority of GM plants have been transformed with constructs containing:-

i) Promoter from Cauliflower Mosaic Virus (CaMV) 35S promoter (P-35S) and/or the terminator from CaMV (T-35S); or

ii) Terminator from the *Agrobacterium tumefaciens* nopaline synthase gene known as T-Nos.

The former is most represented in general screening laboratory protocols.

1.3 Specific GMO Identification Using PCR

At some point during implementation of biosafety regulations in Tanzania the actual, specific type of GMO will have to be identified. This is due to the fact that some GMOs in the world have been banned for human consumption (e.g. Star link maize), while others have been allowed (e.g. MON 810, Liberty).

Although both Star Link and MON 810 have been modified using the bacterial BT gene, there is variation on both the landing site of the transgene on the maize genome (different events) and also the crystal structure of the BT protein that will be formed (CRY1 vs CRY 9). Such differences are used as PCR amplification targets to discriminate the events.

Variable primers will be used many including DNA sequences of the host genome at the landing site.

1.4 Rapid GMO Detection kits based on Protein Reaction

A number of hand held GMO detection kits available commercially are suitable for the Tanzania situation, this is because of the big distances between laboratories and inspection sites such as border posts . The scientific basis for the activity of these hand held detection kits is an immune (antibody - antigen reaction) reaction to some specific proteins expected in the GMO products.

Among the earliest to be developed are the **‘Lateral Strips**’ and ELISA kits that have been in the market for the last 5 years and can be procured even from within Tanzania.
Some limitations of these kits include:

i) Inability to detect low GMO concentrations (some have a 5% w/w limit).

ii) Strips and Kits are not available for all known GMOs but limited to only few GMOs mostly those based on expression of BT proteins in GMO product.

iii) Being based on expression in specific parts of plants, GMO seeds whose plant GMO product is only expressed on leaves will mean the seeds will test negative hence pass undetected.

2.0 DNA EXTRACTION PROTOCOLS

2.1 Protocol for Extraction of DNA from Grains

This protocol has been developed at the Department of Molecular Biology and Biotechnology, University of Dar es Salaam and it has two main possible approaches:

i) A rapid approach to be carried out in Tanzanian laboratories having tissue dismembrator or related equipment such as plant shredder/pulverizer; and

ii) A modified approach for laboratories with minimal equipment and without a tissue dismembrator/plant shredder.

2.1.1 Protocol for DNA extraction from Grain using a Tissue Shredder/ Dismembrator

Seed/grain is milled to a coarse powder and weighed to 0.1g and then subjected through the following procedure:

To each sample, 0.1g of glass beads or quartz sand is added and then the mixture suspended in 800µl of extraction buffer. The sample is then homogenised for 1 minute using a tissue shredder for 2 minutes (set at 200rpm or equivalent) followed by the following steps:-

i) 60µl of 20% SDS is added followed by vortexing for 10 seconds.

ii) The samples are incubated at 60 degree centigrade for about 20 minutes during which the samples are mixed gently by inversion after every 6 minutes.

iii) 600µl of Phenol:Chloroform:Isoamyl alcohol (25:24:1) is added and samples incubated for about 10 minutes.

iv) Samples are mixed by vortexing for 10s and centrifuged for 2 minutes at 9000g at room temperature.

v) The aqueous phase is transferred to a fresh vial and mixed with one volume of Chloroform:isoamyl alcohol (24:1), vortexed for about 20s and again centrifuged for 6 min at 9000g at room temperature

vi) To precipitate the DNA, the aqueous phase is transferred to a fresh tube and gently mixed with an equal volume of ice-cold isopropanol before being stored on ice for about 20 minutes.

vii) The samples are then centrifuged at 14000g at 4 degree centigrade for 15 minutes to pellet the DNA.

viii) The DNA pellet is washed with 300µl of 70% ethanol (without dislodging) and centrifuged again for 5 minutes at 14000g at room temperature. The ethanol is then removed by aspiration, and the DNA pellets dried through exposure to air for 10 minutes before the pellets are suspended in 40µl of TE buffer and stored at minus 20^0C until further use.

Extraction buffer

200 mM Tris-HCl (pH 8.0), 200 mM NaCl, 25 mM EDTA, 0.5% SDS].

2.1.2 Protocol for DNA extraction from Grain using a vortex machine

This is a more laborious procedure for a simple laboratory with only a vortex machine, a centrifuge and water bath and a freezer. The steps to be followed are the following:

i) To each sample, 0.15g of glass beads (quartz sand) is added and the mixture suspended in 800µl of extraction buffer and incubated in a water bath at 60^oC for 15 minutes.

ii) The sample is then vortexed vigorously for 6 minutes [during which a 5 seconds pause is made after every 1 minute]. 60µl of 20% SDS is added followed by vortexing for 10 seconds and the samples incubated at 60^oC for about 30 minutes during which the samples are mixed gently by inversion after every 6 minutes. 600µl of Phenol:Chloroform:Isoamyl alcohol (25:24:1) is added and samples incubated for about 10 minutes.

iii) Samples are mixed by vortexing for 10 seconds and centrifuged for 2 minutes at 9000g at room temperature (25-30^{o}C).The aqueous phase are transferred to a fresh vial and mixed with one volume of Chloroform:isoamyl alcohol (24:^{1}), vortexed for about 20 seconds and again centrifuged for 6 minutes at 14000 rpm at room temperature.

iv) To precipitate the DNA, the aqueous phase is transferred to a fresh tube and gently mixed with an equal volume of ice-cold isopropanol before being stored on ice for about 20 minutes and then centrifuged at 14000g at 4^{o}C for 15 minutes to pellet the DNA.

v) The DNA pellet is washed with 300µl of 70% ethanol (without dislodging pellet) and centrifuged again for 5 minutes at 14000g at room temperature. The ethanol is then removed by aspiration before the DNA pellets are dried through exposure to air for 10 minutes and then suspended in 40µl of TE buffer and stored at minus 20 degree centigrade until use.

Extraction buffer

200 mM Tris-HCl (pH 8.0), 200 mM NaCl, 25 mM EDTA, 0.5% SDS.[Whereby; Sodium dodecyl sulphate (SDS), Tris EDTA (TE) and Ethylenediamine tetra-acetic acid (EDTA)]

2.2 Protocol for DNA Extraction from Soft, Small Plant Seeds

This protocol has been modified from that first described by Kang *et al.,* (1998). DNA from seeds of variable sizes including small seeds of tomato, Chinese, cabbage and sesame; and larger seeds such as maize, rice and wheat can be extracted using this protocol.

The protocol entails the following steps:

i) Remove the seed coat and cut the seed in half.

ii) Place the half seed containing the storage tissue (cotyledon) in a microcentrifuge tube (size 1.5 ml).

iii) Add 400 μl of the Extraction buffer containing proteinase K (50 μg), and incubated at 37^0C for 1 h.

iv) Grind the seed in the buffer with a glass rod.

v) Add 400 μl of CTAB solution (2%).

vi) Gently extract using chloroform:isoamyl alcohol (24:1) with 5% phenol.

vii) Centrifuge at 10,000 g in microcentrifuge at 4 ^{0}C for 10 min and transfer the supernatant to new tubes.

viii) Add 2 to 3/3 v/v isopropanol and incubate the tube at room temperature for 10 min to precipitate DNA.

ix) Centrifuge at 10,000 g for 5 min, remove supernatant, and ish the DNA pellet with 70% Ethanol, air dry, and re-suspend in 50 μl of TE buffer.

x) Remove RNA by adding 1 μl of RNase (10 mg/ml).

Buffer Composition

i) *Extraction buffer composition:* 200 mM Tris-HCl (pH 8.0), 200 mM NaCl, 25 mM EDTA, 0.5% SDS, and Proteinase K 50μg.

ii) *CTAB (cetyltrimethylammonium bromide) solution:* 2% CTAB(w/v), 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4M NaCl, 1% PVP (polyvinylpyrrolidone) Mr 40,000.

2.3 Protocol for extraction of DNA from Dry Leaves/Poorly Stored Leaves

This protocol has been adopted from that described by Jobes et al., (1995) and utilizes PVP to bind the plant phenolic compounds, as well as using a high molar concentration of sodium chloride to inhibit co-precipitation of the polysaccharides and DNA, and concluding with removal of RNA by selective precipitation with lithium chloride.

i) To each ground leaf sample, add 1 ml extraction buffer and incubate the sample for 1 h at 55°C with occasional swirling.

ii) Add Sodium Dodecyl Sulphate (SDS) to each tube (final concentration 1.5%). Mix gently and incubate for 1 h at 55°C with occasional swirling.

iii) Centrifuge for 10 min at 8000g. Transfer the supernatant to a new tube and add 1/3 volume of 5 M potassium acetate. Mix gently and incubate for 30 min at -20°C.

iv) Centrifuge for 10 min at 10,000g. Transfer the supernatant to a new tube and add 2/3 volume of isopropanol. Mix gently and incubate at -20°C overnight.

v) Centrifuge for 10 min at 14,000g and carefully pour off the supernatant.

vi) Dissolve the pellet in autoclaved deionized water. Add 0.5 volume of 5 M NaCl and mix well. Add two volumes of cold absolute ethanol and incubate for 30 min at -20°C.

vii) Centrifuge at 14,000g for 10 min. Dissolve the pellet in autoclaved deionized water. Precipitate RNA with 1/3 volume of cold 8 M LiCl (final concentration 2 M) and incubate at -20°C for at least 1 h.

viii) Recover RNA by centrifugation for 15 min and carefully transfer supernatant to a new tube. Precipitate DNA by adding 0.6 volume of isopropanol and incubate for 1 h at -20°C.

ix) Centrifuge for 10 min at 14,000g and aspirate. Wash the pellet with 70% ethanol and centrifuge for 5 min. Pour off ethanol and briefly dry the pellet by exposure to air (room temperature for 15 minutes).

x) Dissolve the pellet in 20-50 µl TE buffer and store at -20 until used.

Buffer Compositions

i) *Extraction buffer*: 200 mM Tris-HCl (pH 8.0), 200 mM NaCl, 25 mM EDTA, 0.5% SDS and Proteinase K 50µg.

ii) *T.E. buffer*: 10 mM Tris-HCl, 1 mM EDTA (pH 8.0).

2.4 Protocol for Rapid-Plant DNA Extraction Using a Kit [QuickExtract™]

The QuickExtract™ Plant DNA Extraction Solution can be used to rapidly and efficiently extract PCR-ready genomic DNA from most plant samples using a simple 1-tube protocol that takes only 8 minutes. Most leafy plants are acceptable for DNA extraction using the QuickExtract™ Plant Solution, including Cabbage, Soybeans, Spinach, and Pepper leaves.

The method involves addition of the QuickExtract™ Plant solution to the sample and perform two sequential heating steps;

(i) Add QuickExtract™ Extraction solution to a plant sample
(ii) Heat at 65^{o}C for 8 minutes
(iii) Heat at 98^{o}C for 2 minutes

A small aliquot of the sample mix is then used as a template for the PCR or qPCR reactions.
The QuickExtract™ Plant method allows for the inexpensive processing of one to hundreds of samples simultaneously, without centrifugation, spin columns or use of any toxic organic solvent. The procedure is fully compatible with robotic automation, provides a PCR-ready sample.

Kit-in Tanzania: Obtainable From NY Group (attn O777-486 289)

3.0 ANALYSIS OF EXTRACTED DNA USING AGAROSE GEL ELECTROPHORESIS

3.1 Introduction

Agarose gel electrophoresis is the most common method of preliminary analysis of DNA in molecular biology. It addresses two important questions that are foremost; (i) Whether DNA has been extracted at the completion of an extraction procedure, or at the end of a PCR amplification and (ii) what is the size of the DNA fragments in the sample being studied as determined by comparison with DNA fragments of known sizes (molecular weight markers).

Electrophoresis is a procedure by which charged DNA molecules migrate in an electric field, the rate of migration being determined by the size of the molecules and their electric charges.

The gel is a complex network of fibrils, and the pore size of the gel can be controlled by the way the gel is prepared. The nucleic acid molecules migrate through the pores of the gel at rates depending on their molecular weight and shape. Small or compact molecules migrate more rapidly than large or those with irregular morphologies. Concentration of agarose used is usually determined by size of the DNA fragments expected in the sample. During analysis of large fragments such as genomic DNA only a small amount of agarose is used in the gel (0.5%) whereas analysis of a small fragment such as a 300bp fragment would warrant the use of 2 % agarose (refer to Sambrok et al., 1983).

After defined period of time of migration, the locations of the DNA molecules in the gel are assessed by making the DNA molecules fluorescent and observing the gel with UV radiation.

3.2 Protocol for Laboratory Agarose Gel Electrophoresis

a) Gel preparation to make 1% agarose gel (1g in 100ml):

i) Weigh out 0.5 g of agarose powder in a 125 ml Erlenmeyer flask

ii) Add 50 ml of 1x TBE buffer and swirl to suspend the agarose powder in the liquid.

iii) Place the suspension in a microwave oven[1] or another heater and proceed to heat to boiling point with regular swirling.

iv) During heating strive to break the bubbles overflowing before they reach the neck of the flask

v) Boil the solution until all of the suspensions disappear and a transparent gel is formed.

vi) Add 5μl of stock Ethidium bromide[2] (final concentration 1 μg/ml) and mix well and pour the gel.

vii) Remove your gloves and dispose them carefully.

b) Casting the gel

i) Assemble the gel casting unit as per instructions specified for model.

ii) Place the combs into appropriate slots so that the sample wells are near the cathode (black). DNA will migrate towards the anode(red) during electrophoresis.

iii) Pour the cooled molten gel (60 oC)

iv) Allow 20-40 min. for the gel to solidify at room temperature.

v) Carefully remove the comb from the solidified agar. It helps to wet the gel area around the comb before trying to remove the comb.

vi) Carefully remove the gel casting gates.

vii) Submerge the gel beneath 2 to 6 mm of 1x electrophoresis buffer.

DNA sample preparation before loading into casted Agarose gel

i) Mix the sample with the loading mixture as follows:

- 5 μl DNA (extracted genomic DNA)

[1] A microwave oven works very well as an alternative to a hot plate. Stop the oven every 30 seconds and swirl the flask gently until the gel is clear and transparent indicating that all the suspensions of agarose are molten.

[2] Care is needed during handling Ethidium bromide. It is carcinogenic therefore tips as well as gloves used should be changed and disposed of carefully.

- 1 μl loading buffer (standard 6X or another used according to manufacturer instruction)

ii) Load the mixture into the respective wells beneath buffer using the standard pipette after removing the combs.

iii) Load 2-4 μl of a DNA step ladder (molecular weight marker- According to manufactures protocol) 4. Carefully place the lid on the gel casting cell to avoid disturbing the sample

iv) Map clearly all the loaded wells

v) Connect the power supply unit to the cell l and run the gel for about 45 minutes at a voltage range of 80-90 V. Check the extent of migration from time to time.

c) *Visualizing the gel*

i) Switch off the power unit

ii) Put on a pair of hand gloves and a pair of protective eye glasses

iii) Remove the gel from the tray. Be careful!!

iv) Place the gel on the UV documentation tank / transilluminator and visualize the DNA bands

Materials

i) DNA isolated from samples being tested for GMOs

ii) Molecular weight markers Standard DNA with known sizes(l100bp, Lambda Hind III etc)

iii) Electrophoresis buffer (1 x TBE)

iv) Sample loading dye: 6X

v) Agarose (with ethidium bromide) ***(Be careful! Ethidium Bromide is Carcinogenic, always wear gloves).***

vi) Automatic Micro pipettors:

vii) Clean micropipette tips

TBE Buffer composition : Tris Borate EDTA (108g Tris base, 55g Boric acid, 20ml 0.5M EDTA pH 8 in 1000ml sterile distilled water)

4.0 POLYMERASE CHAIN REACTION (PCR) ANALYSIS

4.1 Introduction to PCR Technique

Since the discovery of PCR reaction in mid 1980s by Kerry Mullis (received the Nobel Prize in 1993), PCR has become the tool for biotechnologists worldwide including Tanzania. Basically, PCR is a simple technique for making an exponential number of copies of a portion of DNA in just a few hours.

There are three basic steps in PCR that are repeated over and over again.

Step 1: Denaturation

In this step, double-stranded DNA molecules are heated to a temperature of 94^{o}C, which separates or denatures them so that they become single-stranded. These single strands of DNA then become the templates for the new DNA strands that are made.

Step 2: Annealing

The Taq DNA polymerase must have something to hook onto to start building a new strand of DNA. A primer is used to start the process. Primers are short (10 to 30 base pairs long) pieces of single stranded DNA that will anneal, or stick, to the separated DNA template strand. Scientists use specific primer sets that flank the portion of the DNA they are interested in amplifying. In order for the primer to anneal to the template strand, the temperature must be lowered to a temperature that will allow a double stranded complex to form to start making a new strand.

Step 3: Extension

During this step of the cycle, the Taq DNA polymerase will extend the primer by bringing in complementary nucleotides as it moves along the template strand. The Taq DNA polymerase works best at temperatures between 72 to 75^{o}C. All four nucleotides are added to the reaction mixture so that the Taq DNA polymerase builds a new strand complementary to the template strand.

These three steps are repeated over and over again, the newly made strands are separated from the template strands by heating. The new strands then go on to serve as template strands for new strands to be made in each new cycle. This is called amplification. The reaction may be kept going for 20 to 50 cycles.

4.1.1 PCR requirements

Apart from the machine (DNA thermocycler or PCR machine), the scientist conducting GM detection in Tanzania requires the following:

i) a DNA sample; Also known as a template, containing the fragment to be copied (amplified). This template must be visible by Agarose electrophoresis
ii) Primers; with nucleotide sequences specific for the site to be amplified
iii) DNA polymerase (originally Taq but nowadays from various other sources and not necessarily Thermus bacteria
iv) deoxyribonucleotides (dNTPs) that serve as the building blocks (v) Magnesium Chloride (MgCl2) that serve as a catalyst for the PCR reaction (vi) A buffer that maintains correct ionic concentration and pH of reaction at around pH 9.0.

4.1.2 PCR Application in GMO analysis

For this purpose, PCR can be applied to achieve three main goals

i) Screening for GMOs without determination of specific events present
ii) Specific Detection of Specific GMO events using specific primers
iii) Determination of Quantity of GMO present in a sample (using Quantitative or real time PCR)

However in the scope of this Manual, it is only protocols and descriptions of the first two (qualitative) PCR analyses are provided. The first protocol describes the application of PCR for general screening. The most common fragment of DNA targeted during non specific screening of GMOs is the P35S promoter.

4.2 Protocol for Screening Detection of GMOs targeting the P35S promoter

Step 1: Template dilution

Dilution of Template DNA samples to around 20-50ng/ml concentration [in Tanzanian laboratories where DNA quantification equipments is available]. For the laboratories where there is no DNA quantification equipment, a mere visualization on Agarose gel may suffice. whereby samples appearing as very shiny bands may mean 75-150ng/ml and may be used as template undiluted or diluted 5-10X fold.

Step 2: Preparation of a PCR Mix

Preparation of a PCR mix having the following composition

20-50ng/ml Template DNA
2.5U/25ml DNA polymerase
10mM Tris-HCL PCR buffer
(1.5mM-2,5mM) $MgCl_2$
200*u*M dNTP (dATP, dCTP, dGTP, dTTP)
0.5-1*u*M Oligonucleotide Primers CR4, CF3 below;

Primer Sequences
CF3 5'- CCA CGT CTT CAA AGC AAG TGG-3'
CR4 5'- GGA AGT TCA TTT CAT TTG GAG AGG A-3'

Step 3: PCR Reaction

The above PCR mix is then put in PCR machine (thermocycler) set at the following PCR conditions: Initial denaturation is 95°C for 10 minutes followed by 50 cycles of denaturation at 95°C for 25 s, annealing at 62°C for 30 s and extension at 72°C for 45 seconds. Final extension is 72°C for 7 min. The expected PCR product is 200bp.

Step 4: Analysis of PCR Products

The PCR products are then checked using Agarose Gel electrophoresis as described under Section 3.

4.3 PCR Protocols for Amplification of Specific Fragments of GMOs

This involves selecting specific primers for the specific GMO to be detected. The scientist uses these primers with the isolated DNA from the sample being analyzed using specific PCR conditions dictated by the type of primers

These specific PCRs vary according to GMO and GMO but all of the them are preceded by a control PCR involving the amplification of a known gene (housekeeping gene) already present in the test plant sample (e.g. the invertase gene in maize). This control PCR acts as a positive control for the rest of the parameters including quality of genomic DNA, the polymerase and the thermocycler itself.

Invertase gene (endogenous control)
A fragment 226 bp from the maize gene invertase is targeted using the following primers;
Forward ivr1-1 CCG CTG TAT CAC AAG GGC TGG TAC C
Reverse ivr1-2 GGA GCC CGT GTA GAG CAT GAC GAT C

Conditions for the Control invertase PCR
Initial denaturation is 95°C for 5 minutes followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 57 ^{o} C for 40 s and extension at 72^{o}C for 1 min. Final extension is 72 ^{o} C for 3 min. -The expected PCR product is 221bp.

4.3.1 Protocol for Screening BT 11 GMO Event

Step 1: Template Dilution

Dilution of Template DNA samples to around 20-50ng/ml concentration [in laboratories where DNA quantification equipments are not available, a mere visualization on Agarose gel whereby samples appearing as very shiny bands need to be diluted 5X may suffice.

Step 2: Preparation of a PCR Mix

The mixture should contain 20-50ng/ml Template DNA ; 2.5U/25ml DNA polymerase (Taq polymerase or other), 10mM Tris-HCL PCR buffer;(1.5mM-2,5mM) MgCl / 200uM dNTP (dATP, dCTP, dGTP, dTTP) 0.5-1uM Oligonucleotide Primers

Specific Primers

The primer pair IV01-cry is used to amplify a fragment of DNA specific to the maize alcohol dehydrogenase No.6 intron sequence and the Bt gene fragment *cryIA (b)* using the following primer pair:

Forward: IV01 GGT ACA GTA CAC ACA CAT GTA T *adh1*-1S

Reverse: Cry IA: GAT GTT TGG GTT GTT GTC CAT *cryIA (b)*

Step 3: PCR Reaction

The PCR Mix in vials is then put in into the DNA thermocycler set at the following conditions: Initial denaturation is 95°C for 5 minutes followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 60◦C for 40 s and extension at 72◦C for 1 min. Final extension is 72◦C for 3 min.

Step 4: Analysis of PCR Products

This is carried out as described under Section 3 using an agarose of 1.5-20%.

4.3.2 Protocol for Screening Event 176 GMO event

Step 1: Template Dilution

Dilution of Template DNA samples to around 20-50ng/ml concentration

Step 2 Preparation of a PCR Mix

The mixture should contain 20-50ng/ml Template DNA ; 2.5U/25ml DNA polymerase (Taq polymerase or other), 10mM Tris-HCL PCR buffer;(1.5mM-

2,5mM) MgCl2 / 200μM dNTP (dATP, dCTP, dGTP, dTTP) 0.5-1μM Oligonucleotide Primers

Specific primers

The primer pair CDPK-cry is used to amplify a segment from the maize pollen-specific calcium-dependent protein kinase (CDPK) promoter (forward primer) and part of the Bt gene *cryIA (b)* using the following primers

- Forward: CDPK 5' (CTC TCG CCT TCC ATG TCC GT) '3
- Reverse: *cryIA (b)* 5'(GGT CAG GTC CAG GTC CAT GT) '3

Step 3: PCR Reaction

Initial denaturation is 95°C for 5 minutes followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 63°C for 40 s and extension at 72 °C for 1 min. Final extension is 72 °C for 3 min.

Step 4: Analysis of PCR Products

This is carried out as described under Section 3 using an agarose of 1.5%. The expected PCR product is 211bp.

4.3.3 Protocol for Screening MON 810 GMO Event

Step 1: Template Dilution

Dilution of Template DNA samples to around 20-50ng/ml concentration.

Step 2: Preparation of a PCR Mix

The mixture should contain 20-50ng/ml Template DNA ; 2.5U/25ml DNA polymerase (Taq polymerase or other), 10mM Tris-HCL PCR buffer;(1.5mM-2,5mM) MgCl / 200μM dNTP (dATP, dCTP, dGTP, dTTP) 0.5-1μM Oligonucleotide Primers

Specific Primers

HS01-cry primer combination is used to amplify a fragment of DNA combining maize *Heat Shock protein Hsp70* No.1 intron sequence and part of *cryIA (b)* structure gene in MON810

- Forward: HS01 5' (AGT TTC CTT TTT GTT GCT CTC CT) '3 *hsp70*
- Reverse: Cry1a 5' (GAT GTT TGG GTT GTT GTC CAT) '3 *cryIA(b)*

Step 3: PCR Reaction

The PCR Mix in vials is then put in into the DNA thermocycler set at the following conditions: Initial denaturation is 95°C for 5 minutes followed by 40 cycles of denaturation at 95°C for 20s, annealing at 60◦C for 40 s and extension at 72◦C for 1 min. Final extension is 72◦C for 3 min.

Step 4: Analysis of PCR Products

Is carried out as described under Section 3 using an agarose of 1.5-20%. Expected PCR product is 194bp.

4.3.4 Protocol for Screening LIBERTY GMO Event

Step 1: Template Dilution

Dilution of Template DNA samples to around 20-50ng/ml concentration.

Step 2: Preparation of a PCR Mix

The mixture should contain 20-50ng/ml Template DNA ; 2.5U/25ml DNA polymerase (Taq polymerase or other), 10mM Tris-HCL PCR buffer;(1.5mM-2,5mM) MgCl / 200uM dNTP (dATP, dCTP, dGTP, dTTP) 0.5-1uM Oligonucleotide Primers

Specific Primers

Primers targeted a DNA fragment from the Cauliflower Mosaic Virus Promoter (CAMV) and the structural gene *pat* for herbicide tolerance expected in Liberty GM maize

- Forward: 5' (CCT TCG CAA GAC CCT TCC TCT ATA) 3 CaMV
- Reverse 5' (AGA TCA TCA ATC CAC TCT TGT GGT G) 3 *pat*

Step 3: PCR Reaction

The PCR Mix in vials is then put in into the DNA thermocycler set at the following conditions: Initial denaturation is 95°C for 5 minutes followed by 40 cycles of denaturation at 95°C for 20s, annealing at 60◦C for 40 s and extension at 72◦C for 1 min. Final extension is 72◦C for 3 min.

Step 4: Analysis of PCR Products

This is carried out as described under Section 3 using an agarose of 1.5%. The expected PCR product is 437 bp.

4.3.5 Summary of Specific Primers Used for Specific Identification of GMOs

Trade name	Characteristic	Inserted gene		
		Promoter	**Structure**	**Terminator**
Event 176 (Novartis)	ECB (GA)	1) P-PEPC P-CDPK 2) P-35S 3) Bacterial	1) Two synthetic, truncated *crylA(b)* *2) bar* *3) bla*	19, T-35S
Bt11 (Novartis)	ECB GA	1) P-35S with IVS6-int 2) P-35S with IVS2-int	*1)* Synthetic *crylA(b)* 2) Synthetic *bar*	1) nos 3 2) nos 3
MON810 (Monsanto)	ECB	1) P-35S with *hsp70*-int.	1) Synthetic *crylA(b)*	1) Nos 3
Liberty (AgrEvo)	GA	1) P-35S 2) Bacterial	1) Synthetic *bar* 2) *bla* (incomplete)	1) T-35S 2) bacterial

5.0 ANALYSIS FOR PCR PRODUCTS/SEQUENCING

PCR products are purified using any available specially made clean up kits e.g. Qiagen, Montage® PCR Cleanup Kits according to manufacturer's instructions. In laboratories where these are not affordable, 5mM Potassium acetate may be used in order to remove primer dimers, primers, nucleotides, and extraneous bands.

5.1 DNA base sequencing

Although many scientific publications make conclusions on GM status following getting PCR amplicons of the right size, confirmatory test should involve sequencing of these PCR products and a BLAST analysis.

After PCR amplification critical cases should be submitted to commercial sequencing facilities accessible from Tanzania these include; International Livestock Research Institute (ILRI), Nairobi or MACROGEN, Korea where more than 20 samples are transported by DHL and the customer pays only the sequencing charges of 5$ per sample and another 2$ per sample if did not clean up the PCR products

5.2 Alignment of Sequence Results

To align (compare) DNA sequence received, existing DNA databases freely available are used eg an online nucleotide-nucleotide BLAST (Basic Local Alignment Search Tool) algorithm (Blast n) is carried out. The BLAST is carried using a free program at a public DNA database (http://www.ncbi.nlm.nih.gov/BLAST/) hosted by the National Centre for Biotechnology Information (NCBI) Bethesda, USA, among other free internet services.

6.0 PROTEIN BASED GMO DETECTION KITS

6.1 Introduction

During the last 5 years an increasing number of hand operated GMO detection kits have been available commercially. These are based on immuno-reaction to a protein present in the GMO product. Among the earliest to be develop were the Strip and ELISA kits that have been in the market for the last 5 years. The kit usually takes about 5~10 min to complete a test run and therefore is suitable for a routine inspection at the Tanzanian borders.

The Strip kit contains an antibody, which is specific to Bt protein expressed by the *cryIA(b)* gene. Many GMOs developed for insect pest resistance eg Maize MON810, Bt11, and Event176 are all inserted with a *cryIA(b)* gene(1). All these will react to show a positive reaction on Strip kit detection. Some limitations can be noted for use of these:

i) They cannot detect low GMO concentrations (some have a 5% w/w limit).

ii) Kits are not available for most GMO events. Kits are mostly available for events based on BT toxin and a few other proteins

iii) They detect transgenic proteins not transgenic DNA, so a GMO seed that usually expresses its Gene product on its leaves will pass the GMO test as negative due to lack of the GM protein.

6.2 Methods of detection

Step 1

Most hand held kits contain an extraction buffer in a small well on which you would put a small amount of the test sample and grind or mix. Before addition of test sample however, most kits would advise you to start by putting a positive control sample protein that is usually provided.

Step 2

Insert a strip containing antibody to the GMO protein. A visible positive result will be seen by an appearance of a colour line or dot. This assures you that the kit is working when you start with the positive control sample and

Step 3

Test your own sample. Most of these test strips will take about 10-15 minutes from start to finish.

7.0 REFERENCES FOR FURTHER READING

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