NATIONAL BIOSAFETY AUTHORITY

GUIDELINES FOR TESTING OF GENETICALLY MODIFIED ORGANISMS IN CERTIFIED LABORATORIES

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FOREWORD

Genetically Modified Organisms (GMOs) contain DNA sequences which are distinct from those in the conventional organisms. They carry a synthetic DNA construct made up of a gene conferring a novel trait and gene regulatory elements; promoters and terminators which control gene expression. Polymerase chain reaction (PCR) is a recognized standard method for testing GMO to meet government and research requirements worldwide. If performed correctly, PCR tests give precise and accurate result with regard to content of interest. PCR can be used to amplify these sequences, enabling detection of the organism's DNA even when present at low levels. Presence of these sequences indicates that the sample either contains DNA derived from a GMO or it contains DNA from a naturally occurring organism that has the regulatory sequences.

These guidelines allow detection of specific GM DNA markers (CaMV35S promoter and Nos terminator) and endogenous markers (lectin and zein) found in raw materials derived from GM soya and maize. It is also suitable for screening some processed foods, animal feeds and ingredients. The guideline proposes a simple, cost effective, GMO screening method that enables the simultaneous detection of DNA targets associated with the presence of GM soya and maize. It also allows detection of CaMV, a potential cause of false positives when carrying out the CaMV 35S promoter screen.

GMO testing Laboratories that will be engaged by the Authority will be required to use these guidelines and protocols in the testing of samples submitted to them.

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DEFINITION OF TERMS

CaMV: Cauliflower Mosaic Virus

DNA: Deoxy-ribonucleic acid. This molecule comprises strings of the four bases (G, A, T, C) forming genes.

dNTP: deoxy-nucleotide triphosphates. An abbreviation for any of the four bases forming DNA.

PCR: Polymerase Chain Reaction – a method of amplifying a single DNA fragment to produce millions of copies, which can be detected.

Primer: A short oligonucleotide designed to anneal to specific regions of DNA in order to facilitate the PCR. Primers are designed to complement regions of DNA bounding the gene of interest.

SDW: Sterile distilled water of molecular biology grade.

***Taq* polymerase:** A specific, heat-stable DNA polymerase used to replicate DNA targets during PCR.

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CHAPTER ONE
INTRODUCTION

1.1 Background of NBA

The National Biosafety Authority (NBA) is a state corporation in Kenya mandated to ensure safety of human and animal health and provide adequate protection of the environment from harmful effects that may result from genetically modified organisms (GMOs).

The Authority was established pursuant to the provisions of the Biosafety Act, 2009 to regulate all activities involving GMOs in food, feed, research, industry, trade and environmental release and it fulfills its mandate by ensuring and assuring safe development, transfer, handling and use of GMOs in Kenya.

NBA has made great strides in establishing strong Biosafety framework in Kenya by developing and publishing the implementing Biosafety Regulations. These regulations laid down a clear procedure on handling GMOs whether plants, animals or microorganisms. NBA is the National Focal Point for the Cartagena Protocol on Biosafety to the Convention on Biological Diversity (CBD) and is mandated to implement the provisions of the Cartagena Protocol on all Biosafety matters pertaining to GMOs.

1.2 Vision Statement

A World-class Biosafety Agency

1.3 Mission Statement

To ensure and assure safe development, transfer, handling and use of genetically modified organisms (GMOs) in Kenya.

1.4 Our Core Values

a) Integrity
b) Professionalism
c) Transparency
d) Accountability

1.5 Our Objectives

a) To facilitate responsible research and minimize risks that may be posed by genetically modified organisms;
b) To ensure adequate level of protection in the development, transfer, handling and use of genetically modified organisms that may have an adverse effect on the health of the people and the environment; and
c) To establish a transparent, science-based and predictable process for reviewing and making decisions on the development, transfer, handling and use of genetically modified organisms and related activities.

1.6 Our Core Functions

The Biosafety Act no.2 of 2009 lists the functions of NBA as follows:

a) Consider and determine applications for approval for the development, transfer, handling and use of genetically modified organisms, and related activities in accordance with the provisions of the Biosafety Act;
b) Co-ordinate, monitor and assess activities relating to the safe development, transfer, handling and use of genetically modified organisms in order to ensure that such activities do not have adverse effect on human health and the environment;
c) Co-ordinate research and surveys in matters relating to the safe development, transfer, handling and use of genetically modified organisms, and to collect, collate and disseminate information about the findings of such research, investigation or survey;
d) Identify national requirements for manpower development and capacity building in biosafety;
e) Advise the Government on legislative and other measures relating to the safe development, transfer, handling and use of genetically modified organisms;
f) Promote awareness and education among the general public in matters relating to biosafety; and
g) Establish and maintain a Biosafety clearing house (BCH) to serve as a means through which information is made available to facilitate exchange of scientific, technical, environmental and legal information on, and experience with, living modified organisms;
h) To exercise and perform all other functions and powers conferred on by the Act.

CHAPTER TWO

2.0 SCOPE AND OBJECTIVES

2.1 Scope

These guidelines describe the procedures for the detection of specific GM DNA markers (CaMV35S promoter and Nos terminator) and endogenous markers (lectin and zein) found in raw materials derived from GM soya and maize. They are also suitable for screening some processed foods, animal feeds and ingredients.

2.2 Objectives of the manual

These guidelines propose a simple, cost effective, GMO screening method enabling the simultaneous detection of DNA targets associated with the presence of GM soya and maize. They also allow detection of CaMV, a potential cause of false positives when carrying out the CaMV 35S promoter screen. They cannot be used for GMO quantification purposes.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 PRINCIPLE OF THE METHOD

The polymerase chain reaction (PCR) is used to detect DNA sequences in living organisms and in materials derived from living organisms. It relies on the binding of single-stranded DNA primers to a specific DNA target sequence and the copying of this target in the presence of excess amounts of DNA subunits (nucleotides) and a DNA polymerase (Taq). Multiple cycles at specific temperatures result in the million-fold copying of the target sequence. Size separation and detection of the amplification products is performed an appropriate percentage of Agarose gel with Ethidium bromide. The size of the amplification products are then compared to sizes of amplification products produced from reference materials.

3.2 MATERIALS AND EQUIPMENT

3.2.1 Chemicals

All reagents should be of a suitable purity defined for molecular biology analysis (e.g. Sigma molecular biology products). The water used should be sterile molecular biology grade.

3.2.2 Solutions, standards and reference materials

Solutions should be prepared in a laminar flow cabinet. The cabinet should be decontaminated using UV irradiation. Latex gloves should be worn throughout the procedure.

3.2.3 QIAGEN® Multiplex PCR kit

2x QIAGEN Multiplex PCR Mastermix, containing: Hot StarTaq® DNA Polymerase Multiplex PCR Buffer (containing 6 mM $MgCl_2$) Store at -15°C to -22°C for up to 12 months. Catalogue no. 206143

3.2.4 Primer Mastermix Solution

Obtain primers with the sequences found in Table 1.

TABLE 1: Primer specifications

CaMV 35S promoter	35S-CF3 for	CCA CGT CTT CAA AGC AAG TGG
	35S-CR4 rev	TCC TCT CCA AAT GAA ATG AAC TTC C
Nos terminator	HA-NOS 118 for	GCA TGA CGT TAT TTA TGA GAT GGG
	HA-NOS 118 rev	GAC ACC GCG CGC GAT AAT TTA TCC
Soya lectin gene	STLM-1	AAC CGG TAG CGT TGC CAG
	STLM-2	AGC CCA TCT GCA AGC CTT T
Maize zein gene	ZET-M1	TGT TAG GCG TCA TCA TCT GTG
	ZET-M2	TGC AGC AAC TGT TGG CCT TAC
CaMV	CaMV1156F	AAG CAA AGA CCC TTC GGA GT
	CaMV1659R	CCT TTA GTT GGC TCG AGT AAT CA

Dilute the primers specified in Table 1 (10 in total), using sterile water according to the manufacturer's instructions, to produce a primer concentration of 100µM, thoroughly agitate until dissolved. The solution may be stored at this point at -15°C to -22°C for up to 1 year.

Using a suitable pipette, dilute 20µl of each 100µM primer solution in a labelled sterile 0.5ml Eppendorf using 80µl of sterile water to give a working primer solution of 20µM. Using a suitable pipette, take 5 µl of each 20 µM primer solution and add to a single 0.5ml tube. Then add 50 µl of sterile water to give a total volume of 100 µl. This solution is 1 µM with respect to each primer and is known as the primer mastermix. All the primer solutions at 100µM, 20 µM and the primer mastermix solution can be stored in a dedicated PCR freezer at -15°C to -22°C for up to 6 months.

3.2.5 PCR Mastermix

A PCR mastermix is prepared for the analysis of a batch of several samples. Remove aliquots of each reagent from the freezer and allow to thaw in the laminar flow cabinet then place the tubes on ice. Prepare the mastermix using the reagents and volumes detailed in the table below and scale up according to the number of samples, positive and negative controls. Add the reagents to a sterile 1.5ml tube and mix thoroughly by gentle pipette aspiration prior to use.

Reagents	Initial Concentration	Final Concentration in PCR reaction (15µl reaction vol.)	Equivalent in a single reaction (µl)
QIAGEN multiplex PCR Mastermix	2X	1X	7.5
Primer mastermix	1µM for each primer	100nM	1.5
DNA			2.0
Sterile water	-	-	4
Total volume			15.0

Note: the total volume recommended for the PCR reaction is 15µl (including the DNA). This volume can be reduced or increased if required.

3.2.6 Plastic ware

All equipment required to be autoclaved is sterilised using the following conditions: 121°C for 15 min at 1.0 Bar.

The following items are sterilised by autoclaving;

2ml Eppendorf tubes, 1.5 ml Eppendorf tubes, and 0.5 ml Eppendorf tubes

The following items are UV sterilised for 5 minutes using a UV light source in a laminar flow cabinet; PCR tube and caps strips (eight reactions each), PCR tube storage block for twenty four tubes

3.2.7 Equipment

Thermocycler, Laminar flow hood, Centrifuge, Micro-centrifuge, Vortex Mixer, -30°C Freezers, 4°C Refrigerator, Ice maker, Shakers, Transilluminator, Sets of precision pipettes (including P10, P20, P100, P200, P1000), Sterile filter pipette tips.

3.3 METODOLOGY

3.3.1 Preparation of sample DNA

Samples are extracted using an appropriate method for the extraction of DNA from food. Standard CTAB extraction, kits by Promega, Tepnel, R-Biopharm or QIAGEN would be suitable. Dilute the extracted DNA to 1/5 (10 µl DNA extract plus 40 µl sterile water) and to 1/25 (10 µl of 1/5 dilution plus 40 µl sterile water). Alternatively if you can quantify the DNA, dilute the DNA to about 50ng/µl and 10ng/ µl.

3.3.2 Amplification of target sequence

UV sterilise all equipment and empty tubes in the PCR laminar flow cabinet. Make up PCR mastermix as outlined in Mastermix preparation Using a suitable pipette, add 13µl aliquots of PCR mastermix to PCR tube strips contained in a UV sterilised PCR tube set-up block. Using a suitable pipette, add 2µl of each diluted DNA (1/5 and 1/25 dilutions) to PCR . Use 2 µl of sterile water for PCR negative control. Fit PCR strip caps to tube strips and secure using capping tool if necessary. Transfer PCR tubes to the thermocycler laboratory. **In thermocycler laboratory** Set the PCR programme and leave until the program has finished (approximately 3 hours or overnight).

	Temperature	**Time**
GMO Multiplex Assay	95^{o}C	15 min
	95^{o}C	25 sec
	62ºC	30 sec **40 cycles**
	72ºC	45sec
	72ºC	7 min
	4ºC	HOLD

After the PCR programme is complete, remove tubes from thermocycler and proceed with electrophoresis. PCR products can be stored at 1ºC to 6ºC for up to 2 days or alternatively, at between –15ºC and –22ºC for up to three months.

Note: Do not remove PCR products from the thermocycler laboratory.

3.3.3 Detection of PCR product

Agarose gel electrophoresis is a method used to separate DNA molecules based on their size. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field. Shorter molecules move faster and migrate further than larger ones. The DNA fragments are visualized by staining with ethidium bromide. This fluorescent dye intercalates between bases of DNA and fluoresces under UV light.

Prepare the gel tray by sealing the edges with the adhesive tape and place the gel comb in position. Prepare 2% agarose gel by adding 0.8g of agarose to 40 ml of 1X TAE buffer in a conical flask. Dissolve the agarose by heating in a microwave oven. The temperature of the solution is brought down to ~60°C 1 µl of ethidium bromide is added into the solution and mixed well. **Note:** GelRed could be used in place of ethidium bromide since it is non-carcinogenic. The gel is slowly poured onto a gel casting tray and allowed to solidify. After solidification, the comb and the adhesive tape are carefully removed. The gel tray is kept inside the electrophoresis tank and 1X TAE buffer is added to a level that immerses the gel.

10 µl of each sample is mixed with 3 µl of sample loading dye and loaded into well. The molecular weight ladder is loaded into to the first well, followed by negative control, positive control and samples. The power pack is connected to gel tank and electrophoresis is carried out at 100V for 30 min. At the end of the run, the power supply is disconnected. The gel is removed from the electrophoresis tank, placed inside a gel documentation system with UV transilluminator and observed.

The PCR products from the reference DNA (containing GM soya, GM maize and CaMV) must also be run on each gel containing samples.

Primer set	**Theoretical size (bp)**
CaMV	104
CaMV 35S promoter	123
NOS terminator	118
lectin	80
zein	68

3.4 QUALITY CONTROL

3.4.1 PCR Negative Controls

A PCR negative must be amplified at the same time as a batch of samples. For a PCR negative, 2µl sterile Milli-Q water replaces the sample DNA extract, when setting up the PCR. It should not give any amplification products apart from primer dimers at about 50bp.

Note: A negative control supplied by the manufacturer can be used as a PCR negative control.

3.4.2 Extraction Negative Control

An extraction negative (no sample added) should be extracted with each batch of samples. It should not give any amplification products apart from primer dimers at about 50bp.

3.4.3 PCR Positive Control

PCR positive control containing a mixture of DNA from Roundup Ready soya, GM maize and CaMV. The DNA should produce PCR amplification products for the CaMV 35S promoter, Nos terminator, CaMV, lectin and zein targets.

CHAPTER FOUR

REFERENCES

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