UNEP

CBD

Convention on Biological Diversity

Distr.
GENERAL

CBD/CP/MOP/9/8/Add.1
13 September 2018

ORIGINAL: ENGLISH

CONFERENCE OF THE PARTIES TO THE CONVENTION ON BIOLOGICAL DIVERSITY SERVING AS THE MEETING OF THE PARTIES TO THE CARTAGENA PROTOCOL ON BIOSAFETY
Ninth meeting
Sharm El-Sheikh, Egypt, 17-29 November 2018
Item 16 of the provisional agenda*

DRAFT TRAINING MANUAL ON DETECTION AND IDENTIFICATION OF LIVING MODIFIED ORGANISMS

Note by the Executive Secretary

INTRODUCTION

1. In decision CP-VIII/16, the Conference of the Parties serving as the meeting of the Parties to the Cartagena Protocol on Biosafety requested the Executive Secretary to continue working on a draft training manual on detection and identification of living modified organisms, in an expeditious manner, in collaboration with the Network of Laboratories for the Detection and Identification of Living Modified Organisms, and make a draft version available, in all official languages, for consideration by the Conference of the Parties serving as the meeting of the Parties to the Cartagena Protocol at its ninth meeting with a view to its possible approval before its official final publication.

2. The resulting draft training manual is annexed hereto.

* CBD/CP/MOP/9/1.

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DRAFT

Training Manual on the Detection and Identification of Living Modified Organisms in the Context of the Cartagena Protocol on Biosafety

Convention on Biological Diversity

This manual was produced by the Secretariat of the Convention on Biological Diversity in collaboration with the Network for the Detection and Identification of Living Modified Organisms established under the Cartagena Protocol.

Module 1:

Overview of Biosafety and the Cartagena Protocol on Biosafety

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Introduction to biosafety and the Cartagena Protocol on Biosafety

History of the Protocol

The United Nations Conference on Environment and Development, also known as the "Earth Summit", held in Rio de Janeiro in 1992 marked a significant achievement in the overall policy of the United Nations on the environment. Several documents resulting from that meeting formed the basis of international law on biosafety, including Agenda 21, the Rio Declaration on Environment and Development and the United Nations Convention on Biological Diversity.

Agenda 21 is a comprehensive programme for action in social and economic areas and for conserving and managing natural resources. Chapter 16 of the programme addresses the "Environmentally sound management of biotechnology" by recognizing that modern biotechnology can make a significant contribution to enhancing food security, health and environmental protection.

The Rio Declaration on Environment and Development is a series of principles defining the rights and responsibilities of States. Principle 15 allows countries to take precautionary action to prevent environmental degradation where there are threats, but no conclusive evidence, of serious or irreversible damage (see box below).

Principle 15 of the Rio Declaration on Environment and Development

"In order to protect the environment, the precautionary approach shall be widely applied by States according to their capabilities. Where there are threats of serious or irreversible damage, lack of full scientific certainty shall not be used as a reason for postponing cost-effective measures to prevent environmental degradation."

Source: UNCED (1992b).

The Convention on Biological Diversity (CBD) was inspired by the global community's growing commitment to sustainable development. It represents a step forward in the conservation of biological diversity, the sustainable use of its components, and the fair and equitable sharing of benefits arising from the use of genetic resources. The CBD addresses access to biotechnology and the sharing of its benefits in Articles 16 ("Access to and Transfer of Technology") and 19 ("Handling of Biotechnology and Distribution of its Benefits"). The issue of safety in biotechnology is addressed in Articles 8(g) and 19(3).

Specifically, Article 8(g) calls on Parties to the CBD to establish or maintain means to regulate, manage or control the risks associated with the use and release of living modified organisms (LMOs) resulting from biotechnology which are likely to have adverse impacts on the conservation and sustainable use of biological diversity.

Article 8(g) of the Convention on Biological Diversity: In situ Conservation

"Each Contracting Party shall, as far as possible and as appropriate:

Establish or maintain means to regulate, manage or control the risks associated with the use and release of living modified organisms resulting from biotechnology which are likely to have adverse environmental impacts that could affect the conservation and sustainable use of biological diversity, taking also into account the risks to human health".

Source: Convention on Biological Diversity (1992).

Furthermore, Article 19(3) calls upon Parties to consider the need for and modalities of a protocol for the safe transfer, handling and use of LMOs resulting from biotechnology that may have adverse effect on the conservation and sustainable use of biological diversity.

Article 19(3) of the Convention on Biological Diversity: Handling of Biotechnology and Distribution of its Benefits

"The Parties shall consider the need for and modalities of a protocol setting out appropriate procedures, including, in particular, advance informed agreement, in the field of the safe transfer, handling and use of any living modified organism resulting from biotechnology that may have adverse effect on the conservation and sustainable use of biological diversity."

Source: Convention on Biological Diversity (1992).

Taking these provisions into account, the Conference of the Parties to the Convention on Biological Diversity decided, at its second meeting, to develop a protocol on biosafety, specifically focusing on the transboundary movement of LMOs that may have adverse effects on the conservation and sustainable use of biological diversity, taking into account human health.

As a preliminary tool to serve as interim guidance for biosafety, a set of International Technical Guidelines for Safety in Biotechnology was drafted by the United Nations Environment Programme (UNEP) and adopted by the Global Consultation of Government-designated Experts in Cairo, Egypt, in December 1995.

In 1996, the Conference of the Parties for the Convention on Biological Diversity established an Open-ended Ad Hoc Working Group on Biosafety to develop a draft protocol. This Working Group met six times between 1996 and 1999 and, at the conclusion of its last meeting, a draft protocol was submitted for consideration by the Conference of the Parties at an extraordinary meeting in February 1999, in Cartagena, Colombia. The Conference of the Parties was not able to finalize its work in Cartagena. As a result, the Conference of the Parties suspended its first extraordinary meeting and agreed to reconvene as soon as possible.

The Conference of the Parties reconvened and adopted the Cartagena Protocol on Biosafety on 29 January 2000 in Montreal, Canada. The Protocol entered into force on 11 September 2003 upon ratification by the fiftieth Party. As of March 2018, 171 Parties have acceded/ratified the Protocol.

What is biosafety?

In its broadest sense, the term biosafety refers to the 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 (hereinafter "the Protocol"),[1] the term biosafety refers to safety procedures aimed at regulating, managing or controlling the risks associated with the use and release of LMOs resulting from biotechnology which are likely to have adverse environmental impacts that could affect the conservation and sustainable use of biological diversity, taking also into account risks to human health. Biosafety is a multidisciplinary field that includes but is not limited to biology, ecology, microbiology, molecular biology, animal and plant pathology, entomology, agriculture and medicine as well as legal and socioeconomic considerations, and public awareness.

What are living modified organisms?

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

An LMO is therefore an organism that contains a novel combination of genetic material and results from (i) in vitro modification of nucleic acid (DNA or RNA) molecules; or (ii) cell fusion between organisms of different taxonomic families. In either case, for an organism to be considered an LMO, the techniques used in its development should be ones "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 (e.g. by insertion, modification or deletion of genes or other nucleic acid sequences) in all types of organisms, such as plants, animals, microbes and viruses.

Objective and scope of the Protocol

The objective of the Protocol is "to contribute to ensuring an adequate level of protection in the field of the safe transfer, handling and use of living modified organisms resulting from modern biotechnology that may have adverse effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health, and specifically focusing on transboundary movements".

The Protocol establishes rules and procedures for the safe handling, transfer, and use of LMOs. The Protocol focuses on the transboundary movement of LMOs destined for introduction into the environment and those intended for use directly as food, feed or for processing. The protocol seeks to protect biological diversity, taking into account human health, from the potential risks posed by living modified organisms resulting from modern biotechnology (UNEP, 2006).

[1] The text of the Cartagena Protocol on Biosafety is available at http://bch.cbd.int/protocol/text/.

All LMOs that may have adverse effects to biodiversity or human health are within the scope of the Protocol. Nevertheless, some types of LMOs may be excluded from some provisions, as indicated below:

Scope of the Cartagena Protocol on Biosafety

► ***LMOs subject to the provisions of the Protocol***

All LMOs [that] may have adverse effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health (Article 4).

► ***LMOs excluded from the Protocol's provisions on transboundary movements***

LMOs that are pharmaceuticals for humans that are addressed by other international organizations or agreements (Article 5).

Source: IUCN (2003).

Living modified organisms for intentional introduction into the environment - advance informed agreement (AIA)

The advance informed agreement (AIA) procedure defines mandatory procedures to be applied to the first transboundary movement of an LMO for intentional introduction into the environment. LMOs intended for direct use as food, feed, or for processing are subject to a different procedure, as outlined in the next section.

The AIA procedure, as outlined in figure 1, begins with the Party of export or the exporter notifying the Party of import of the proposed transboundary movement of an LMO for intentional introduction into the environment. The notification must contain at a minimum the information specified in Annex I of the Protocol including, among other things, contact details of the exporter and importer, name and identity of the LMO and its intended use, as well as a risk assessment report consistent with Annex III of the Protocol.

The Party of import has 90 days to acknowledge the receipt of the notification, and 270 days to communicate its decision to the notifier and the Biosafety Clearing-House (BCH).[2] In its decision, the Party of import may approve[3] or prohibit the import of the LMO, request further information or extend the decision period for a defined amount of time. If the Party of import does not communicate its decision within 270 days, it should not be understood that consent was given.

Application of the advance informed agreement (AIA) procedure

► ***LMOs subject to AIA provisions***

LMOs intended for intentional introduction into the environment (Article 7(1)).

► ***LMOs excluded from the Protocol's AIA provisions***

• LMOs in transit (Article 6(1)).

[2] Unless Article 10, paragraph 2(b) applies.

[3] A decision that approves the use of an LMO may be done with or without conditions. If there are conditions, the decision must set out the reasons for the conditions.

- LMOs destined for contained use in the Party of import (Article 6(2)).

- LMOs intended for direct use as food or feed, or for processing (LMOs-FFP) (Article 7(2)).

- LMOs identified by the meeting of the Parties to the Protocol as being not likely to have adverse impacts (Article 7(4)).

Source: IUCN (2003).

Living modified organisms for direct use as food, feed, or for processing (LMOs-FFP)

According to Article 11 of the Protocol, as per figure 1, a Party that makes a final decision regarding domestic use, including placing on the market, of an LMO that may be subject to transboundary movement for direct use as food or feed, or for processing shall submit to the BCH the information specified in Annex II of the Protocol, within fifteen days. This information includes, among other things, the name and identity of the LMO and its approved uses, as well as a risk assessment report consistent with Annex III of the Protocol (see Article 11, paragraph 1).

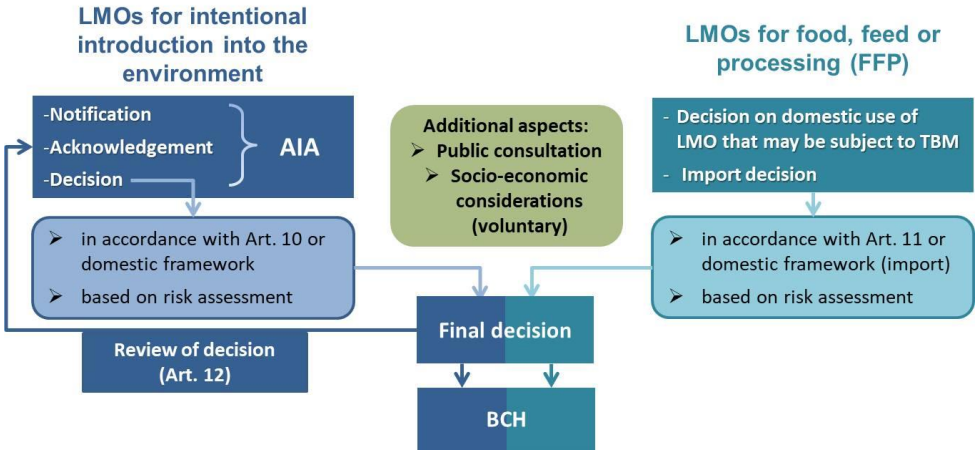

Figure 1: schematic representation of the steps involved under the Protocol for the decision-making regarding LMOs for intentional introduction into the environment and LMOs destined for food, feed and processing.

Competent national authorities

Each Party designates one or more competent national authority that will perform the administrative functions required by the Protocol and is authorized to take decisions on the LMOs for which they are designated.

Detection and identification in the context of the Cartagena Protocol

With the enactment of regulatory frameworks that outline countries' approaches to the implementation of the Cartagena Protocol, detection and identification of LMOs becomes a vital element in contributing to the implementations of the objectives of the Protocol, with respect to the safe transfer, handling and use of living modified organisms resulting from modern biotechnology that may have adverse effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health, and specifically focusing on transboundary movements.

Detection and identification of LMOs provides a science-based tool to facilitate the application of countries' regulatory frameworks in various areas due to the cross-cutting nature of detection and identification. The successful implementation of such tools can facilitate the application of several Articles of the Cartagena Protocol on Biosafety, such as Article 15 and 16 on risk assessment and risk management and Article 17 on unintentional transboundary movements and emergency measures, among others, as shown in figure 2.

With the adoption of the Strategic Plan for implementation of the Protocol for the period 2011-2020[4] by the Conference of the Parties serving as the meeting of the Parties to the Cartagena Protocol on Biosafety as per decision BS-V/16, several outcomes were set out that are relevant to LMO sampling, detection and identification, specifically:

- Easy to use and reliable technical tools for the detection of unauthorized LMOs are developed and made available (under focal area 1, operational objective 1.6);

- Guidance developed to assist Parties to detect and take measures to respond to unintentional releases of living modified organisms (under focal area 1, operational objective 1.8);

- Personnel are trained and equipped for sampling, detection and identification of LMOs (under focal area 2, operational objective 2.3).

The successful implementation of these operational objectives will therefore have a direct impact on the application of several articles of the Cartagena Protocol on Biosafety, as represented in Figure 2.

Figure 2: Articles of the Cartagena Protocol on Biosafety whose implementation is influenced by Parties' ability to detect and identify LMOs.

For example, under Article 17 on unintentional transboundary movements and emergency measures, the availability of tools for the detection and identification of LMOs would be useful to monitor for the presence of LMOs that have been released and therefore may lead, to an unintentional transboundary movement.

[4] Available at http://bch.cbd.int/protocol/issues/cpb_stplan.shtml.

Likewise, under Article 25 on illegal transboundary movements which provides that Parties must adopt measures aimed at preventing and, if appropriate, penalizing transboundary movements of LMOs carried out in contravention of their domestic measures to implement the Protocol, the ability to sample and analyse the shipments for the presence of unauthorized LMOs is facilitated by the use of common LMO detection and identification methods.

With respect to Article 18, which addresses the handling, transport, packaging and identification of LMOs, including the use of documentation accompanying shipments to identify whether or no they may contain LMOs. Detection and identification methods can be used as a means of confirming whether or not the accompanying documentation reflects the actual content of the shipment as stated.

Example: National regulatory contexts

Detection and identification therefore serves as a cornerstone in facilitating Parties' capacity to make informed decisions on the handling, transport and use of LMOs with a view to implementing the Protocol effectively. Examples of areas within national regulatory frameworks where the availability of tools for the detection and identification of LMOs have proven useful are listed below:

(a) Ensuring traceability and compliance with national regulations in terms of LMO labelling

Traceability facilitates the checking of the origins of consignments and their contents. While proper documentation can assist in this process the use of molecular detection and identification techniques allow for a more accurate and science-based verification of the contents of a shipment. Furthermore, it also facilitates the implementation of regulations that relate to labelling. For example, in the European Union (EU), labelling is not required for products containing materials which contain, consist of or are produced from LMOs in a proportion no higher than 0.9% provided that this presence is adventitious or technically unavoidable. Molecular detection and identification techniques are the only way to make such determinations.

(b) Compliance with legislation on handling, transport, packaging and identification of LMOs

New Zealand, for example, under its Hazardous Substances and New Organisms (HSNO) Act, has not approved any LM seeds for release into the environment. Therefore, imported seeds of species that have GM varieties commercialized overseas, including *Zea mays* (maize and sweetcorn), *Glycine max* (soybean), *Brassica napus* var. *oleifera* (oilseed rape), and *Medicago sativa* (lucerne/alfafa) are sampled and tested for the presence of unapproved LM seeds and the presence of an LM seed testing certificate that declares a non-LM status before the seeds can be cleared for entry into New Zealand.[5]

(c) Monitoring

An example of the need for the detection and identification of LMOs for monitoring is through research carried out to investigate for the presence of transgenes in maize landrace populations in Mexico. In 1998 Mexico established a moratorium on the field testing and commercial release of LM maize in order to protect local landraces and wild relatives from the risk of gene flow of transgenes since it is a centre of origin for maize. As a result, several monitoring studies, based on the use of molecular techniques to test for the presence of transgenes, have taken place to examine whether or not gene flow has occurred.

[5] New Zealand submission, "Actual cases of unintentional transboundary movement and case studies related to existing mechanisms for emergency measures", August 2015, http://bch.cbd.int/database/attachment/?id=15643 (response in part to notification 2015-002 of 7 January 2015, Ref. No.: SCBD/BS/MPM/DA/84222).

The Biosafety Clearing-House

The Biosafety Clearing-House (BCH; http://bch.cbd.int) is a mechanism set up under the Cartagena Protocol on Biosafety to facilitate the exchange of information on LMOs and assist countries that are Parties to the Protocol to better comply with their obligations.

The BCH provides open and easy access to a variety of scientific, technical, environmental, legal and capacity-building information provided in all 6 official languages of the United Nations.

The BCH contains the information that must be provided by Parties to the Protocol, such as decisions on release or import of LMOs, risk assessments, competent national authorities, and national laws.

Governments that are not Parties to the Protocol are also encouraged to contribute information to the BCH, and in fact a large number of the decisions regarding LMOs have been registered in the BCH by non-Party governments.

The records of country decisions, risk assessments, LMOs, donor and recipient organisms, and genetic elements are cross-referenced in a way that facilitates data retrieval. For instance, while looking at an LMO record, all the records for the decisions that reference that specific LMO can be easily accessed and retrieved. In addition, links to available detection methods from external websites such as CropLife and the EU Reference Laboratory for GM Food and Feed, are also accessible through the LMO records, as shown in figure 3.

Figure 3: Example of the availability of links to detection methods within LMO records in the BCH.

The BCH also contains other relevant information and resources, including information on national contacts, capacity-building, a roster of government-nominated biosafety experts, and links to other websites, publications and databases through the Biosafety Information Resource Centre (BIRC).

Other provisions under the Protocol

In addition to the provisions above, the Protocol also requires the Parties to the Protocol, consistent with their international obligations, to consult the public during the decision-making process regarding LMOs (Article 23); make the results of such decisions available to the public (Article 23) and allow the decision-making process to take into account socioeconomic considerations arising from the impact of the LMOs on the conservation and sustainable use of biodiversity (Article 26).

Other international biosafety-related bodies

Several other international bodies and organizations carry out activities that are relevant to the trade and environmental aspects of LMOs. A brief overview of these bodies is provided below.

Codex Alimentarius Commission

The Codex Alimentarius Commission (CAC; www.codexalimentarius.net) is a subsidiary body of the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) established in 1961-63 to protect the health of consumers and ensure fair practices in food trade. It currently has 188 members.

Codex Alimentarius is a compilation of standards, codes of practice, guidelines and recommendations on food safety prepared by the Commission. There are several CODEX standards that are relevant to the detection and identification of foods that contain transgenes, such as the "CODEX General Guidelines on Sampling" and the "Guidelines on Performance Criteria and Validation of Methods for Detection, Identification and Quantification of Specific DNA Sequences and Specific Proteins in Foods".

Food and Agriculture Organization of the United Nations (FAO)

FAO (www.fao.org) also carries out activities on biosafety and biosecurity. Among these, the FAO Working Group on Biosafety is responsible for two of FAO's Priority Areas for Interdisciplinary Action (PAIAs), namely "Biosecurity for Agriculture" and "Food Production and Biotechnology Applications in Agriculture, Fisheries and Forestry".

Organisation for Economic Co-operation and Development (OECD)

The OECD (www.oecd.org) provides a setting where governments compare policy experiences, seek answers to common problems, identify good practice and coordinate domestic and international policies.

Of relevance to the detection and identification of LMOs, the OECD Working Group on Harmonisation of Regulatory Oversight in Biotechnology developed a standard system for the naming of commercialized LMOs. This is through the introduction of unique identifiers which are nine-digit codes given to each LM plants that are approved for commercial use.

World Trade Organization (WTO)

The WTO (www.wto.org) is an international organization responsible for establishing the rules of trade between nations. It has a number of agreements that affect the trade of LMOs. One such agreement is the international treaty of "Agreement on the Application of Sanitary and Phytosanitary Measures", also known as the SPS Agreement.

The SPS Agreement concerns the application of sanitary and phytosanitary measures for food safety and animal and plant health regulations and may apply to LMOs. Article 5 of the SPS Agreement is of interest in the context of this training material since it addresses risk assessment and the determination of the appropriate level of sanitary or phytosanitary protection. Article 3 of the SPS Agreement recognizes the standards, guidelines and recommendations set by International Plant Protection Convention (IPPC), World Organisation for Animal Health (OIE) and Codex Alimentarius Commission.

Other WTO agreements, such as the Technical Barriers to Trade (TBT) Agreement, Agreement on Trade-Related Aspects of Intellectual Property Rights (TRIPs) and the General Agreement on Tariffs and Trade (GATT) may also apply to LMOs.

International Seed Testing Association (ISTA)

The ISTA (www.seedtest.org) was founded in 1924 during the 4th International Seed Testing Congress held in Cambridge, United Kingdom. ISTA membership consists of member laboratories and sampling entities, personal members, associate members and industry members from more than 75 countries/distinct economies around the world. The primary purpose of the Association is to develop, adopt and publish standard procedures for sampling and testing seeds, and to promote uniform application of these procedures for evaluation of seeds moving in international trade.

Bilateral, regional and multilateral agreements

In addition to international treaties and standards, countries may engage in bilateral, regional and multilateral agreements, such as free-trade agreements (FTAs), provided they are consistent with the objective of the Protocol and do not result in a lower level of protection than that provided for by the protocol. Such agreements could also be used to undertake shared responsibilities in assessing risks to facilitate decisions on LMOs.[6]

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[6] According WTO (at http://www.wto.org/english/tratop_e/region_e/region_e.htm), the overall number of Regional Trade Agreements (RTAs) in force has been increasingly steadily, a trend likely to be strengthened by the many RTAs currently under negotiations. Of these RTAs, Free Trade Agreements (FTAs) and partial scope agreements account for 90%, while customs unions account for 10 %. The Regional Trade Agreements Information System (RTA-IS), at http://rtais.wto.org/UI/PublicMaintainRTAHome.aspx, contains information on those agreements that have either been notified, or for which an early announcement has been made, to the WTO.

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Module 2

Overview of the Detection and Identification of Living Modified Organisms

Contents of this module

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Pipeline for the development of LM Crops

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- Comparison between protein- and DNA-based methods for LMO detection

Considerations for national strategies towards the detection and identification of LMOs
- Challenges in LMO detection and new technology developments

Overview of techniques used in modern biotechnology

Introduction

Historically, agricultural crop cultivars have been produced through the domestication of wild crop relatives through a continuous process of selection and controlled breeding by humans, for the development of more productive, better adapted, or pest resistant crops as well as crops that produce improved or alternative products than previous ancestral lines. Such changes involve, for example, the cross breeding of sexually compatible plants of the same species or closely related species resulting in the introduction and/ or culling of traits, and their associated genes. In recent decades, advances in breeding technologies have made it possible to produce, not only crosses between plants that are sexually compatible, but also between plants that are considered as naturally cross sterile. Examples of techniques used in such cases are embryo-rescue techniques, in vitro/in vivo embryo cultivation, ovary and ovule cultures, in vitro pollination and in vitro fertilization. In addition, mutational changes could be induced, for instance, through seed irradiation.

Over the past four or five decades, many of the limitations of conventional breeding, e.g. hybridization and selection methods including the ability to target and introduce single selected traits rather than evaluating randomly recombining genomes have been overcome with modern biotechnology.

These drawbacks may be alleviated through the use of modern biotechnology techniques. Under the Cartagena Protocol, modern biotechnology means the application of:

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

(b) 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.

The use of modern biotechnology techniques results in the production of living modified organisms (LMOs), which are also referred to as genetically modified organisms (GMOs) by other bodies and legislations. Other terms used for this process are genetic modification, genetic engineering, recombinant DNA and DNA manipulation. It should be noted that; the Cartagena Protocol emphasizes the “living” nature of the organism, and some of its provisions also apply to LMOs intended for direct use as food or feed, or for processing.

In the context of agricultural crops, the term LMO terms refer to plants in which foreign genetic material has been stably introduced into a host genome using modern biotechnology techniques. This genetic material may have originated from a different species, giving rise to a “transgenic” plant. Alternatively, if the genetic material originated from a plant of the same or other sexually compatible species, then this gives rise to a “cisgenic” plant. In either case, the introduction of a modification may result in the formation of a gene product, i.e. a protein. In some other modified plants, the desired effect may, for example, be the silencing of the expression of an endogenously produced protein or to confer antisense RNA mediated virus resistance in plants through gene silencing mediated by RNA. Some of the most common traits that are introduced into crops using modern biotechnology include: herbicide tolerance, male sterility/fertility restoration, Bt-derived insect resistance, virus resistance, fungal resistance and modification of nutrient biosynthesis.

Overview of commonly used methods for genetic modification

LMOs are most commonly developed through the use of in vitro nucleic acid techniques by inserting, deleting or modifying a gene or DNA/RNA sequence in a recipient or parental organism.

The terms genetic modification, genetic engineering, recombinant DNA and DNA manipulation are terms that apply to the direct modification of an organism's genes. Consequently, the terms genetically modified organism (GMO) as well as genetically engineered organism are often used interchangeably with LMO. As indicated above, the Cartagena Protocol emphasizes the "living" nature of the organism, and some of its provisions also apply to LMOs intended for direct use as food or feed, or for processing.

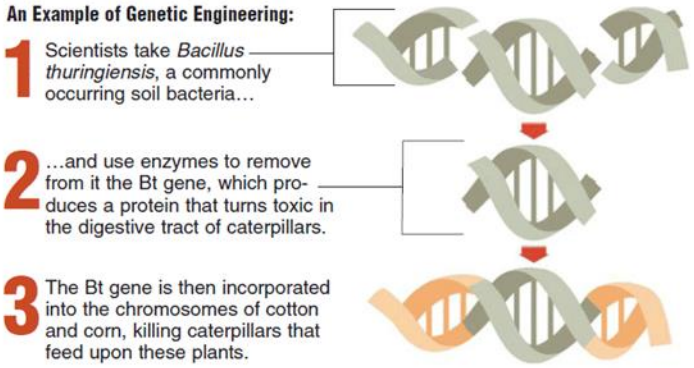

Figure 4: In vitro nucleic acid techniques. Source: North Carolina State University, College of Agriculture and Life Sciences (website).

Production of LMOs through genetic modification is a multistage process that can be achieved through a variety of methodologies. Below is a summarized description of the principal steps that are commonly used in the development of LM plants:[7]

- Once a gene of interest has been identified and isolated from a donor organism, it is manipulated in the laboratory in order for it to be inserted effectively into the intended recipient organism. The manipulation may, for example, include changes to the nucleotide sequence so as to enhance or modulate the expression of the gene once it has been inserted into the intended recipient organism.

- The gene(s) of interest, is placed in between a "promoter sequence" and a "terminator sequence" which are needed for the proper expression and functioning of the gene(s) of interest in an orderly manner. This combination of genetic elements is known as a "transformation cassette",[8] as shown in figure 5. Different promoter sequences control gene expression in different ways depending on their structure and/or cellular signals. For example, some promoters allow for the continuous expression of the gene (these are known as "constitutive promoters"), while others switch the expression of the gene on or off in different organs and/or developmental stages of the organism or in response to stimuli or other external influences. Some promoters may be "tissue specific",

[7] Adapted from IUCN (2003).

[8] A transformation cassette comprises a group of DNA sequences (e.g. parts of a vector and one or more of the following: a promoter, a coding sequence of a gene, a terminator, other regulatory sequences), which are physically linked and often originated from different donor organisms. The transformation cassette is integrated into the genome of a recipient organism through methods of modern biotechnology to produce an LMO. A transformation cassette may also be called "expression cassette" (mainly when a specific expression pattern is aimed at), "DNA cassette" or "gene construct".

meaning that they are able to switch on and regulate gene expression only in a few cells or in specific tissues of the organism.

- A "marker gene" is sometimes incorporated into the transformation cassette to help identify and/or select cells or individuals in which the transformation cassette(s) was successfully introduced. Marker genes may, in some cases, be removed from the LMOs at a later stage.

- Finally, the transformation cassette is normally incorporated into a larger DNA molecule known as a vector.[9] The purpose of the vector is to assist the transfer of the transformation cassette into the recipient organism.

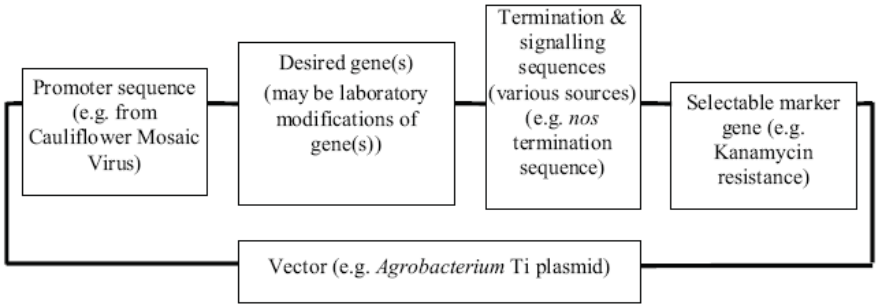

Figure 5: Scheme of a transformation cassette and vector. Transformation cassettes that are currently being developed may include multiple elements – for example, several desired genes accompanied by their respective promoter and termination sequences. Source: IUCN (2003).

The transformation cassettes are integrated into the genome of the recipient organism through a process known as transformation, as outlined in figure 6. This can be carried out through different methods such as infection using, for example, *Agrobacterium tumefaciens*, particle bombardment, protoplast transformation or microinjection.

Transformed cells are then selected, for example, with the help of a marker gene, and regenerated into complete organisms through further steps such as cell culture and breeding techniques. The resulting organisms are further characterized in order to identify those that continue to express the desired phenotype of the recipient organism while also containing the desired transgene(s)[10] or modification. During the course of the characterization process, many experimental LMOs are discarded since they do not exhibit the desired trait(s). Only a few independent LMO lines, designated as "events", of one transformation experiment may reach the commercialization stage.

[9] In the context of genetic modification, a vector is an organism (e.g., virus) or a DNA molecule (e.g., plasmid, nucleic acid cassettes) or an organism (e.g. a virus) used to assist the transfer of genetic material from a donor organism to a recipient organism.

[10] A nucleic acid sequence in an LMO that results from the application of modern biotechnology, as described in Article 3, paragraph (i) a, of the Cartagena Protocol.

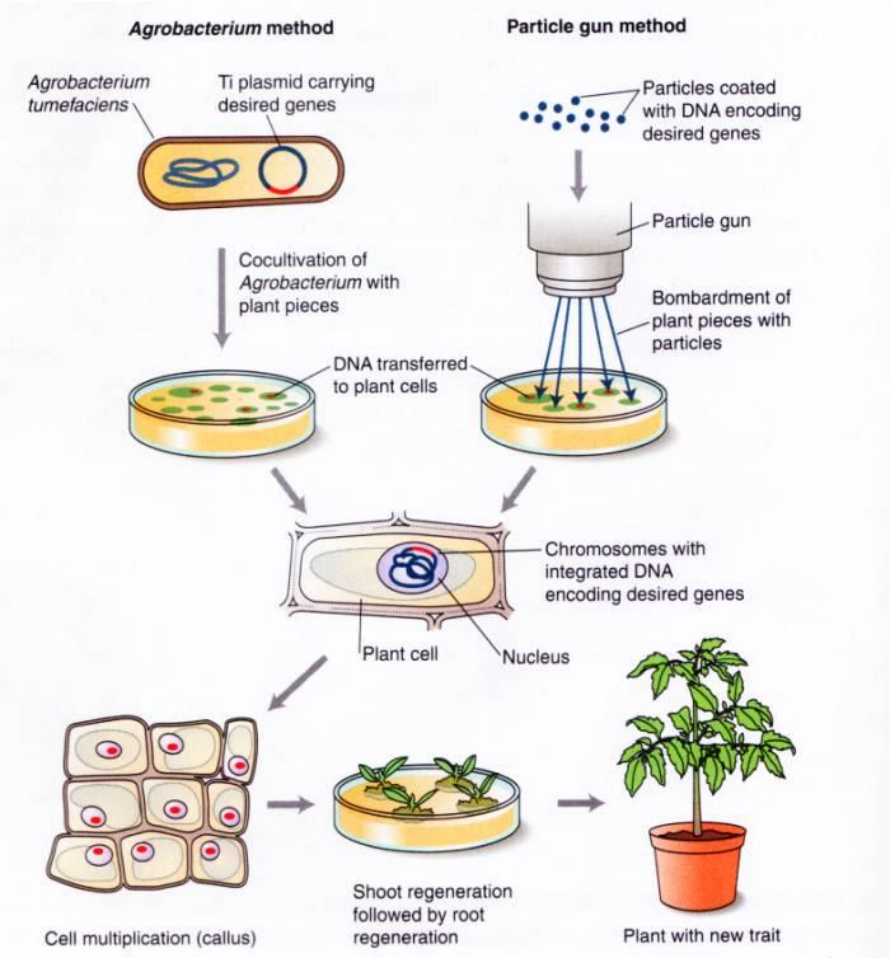

Figure 6: Genetic modification of plants. Source: Mirkov (2003).

LMOs can also be produced through cell fusion where cells from two different organisms that do not belong to the same taxonomic family are fused resulting in an organism that contains the genetic information from both parental cells. The resulting LMO may contain the complete genomes of the parental organisms or parts of their genomes. Cell fusion can be applied to bacterial, fungal, plant or animal cells using a variety of techniques to promote fusion. To date, no commercial LMOs have ever been produced using this technique.

Pipeline for the development of LMOs

The pipeline for the development of an LMO and its subsequent commercialization and release into the environment is a long, multi-step process that follows the research and development process, in order to obtain an LMO with the desired traits, as represented in figure 7.

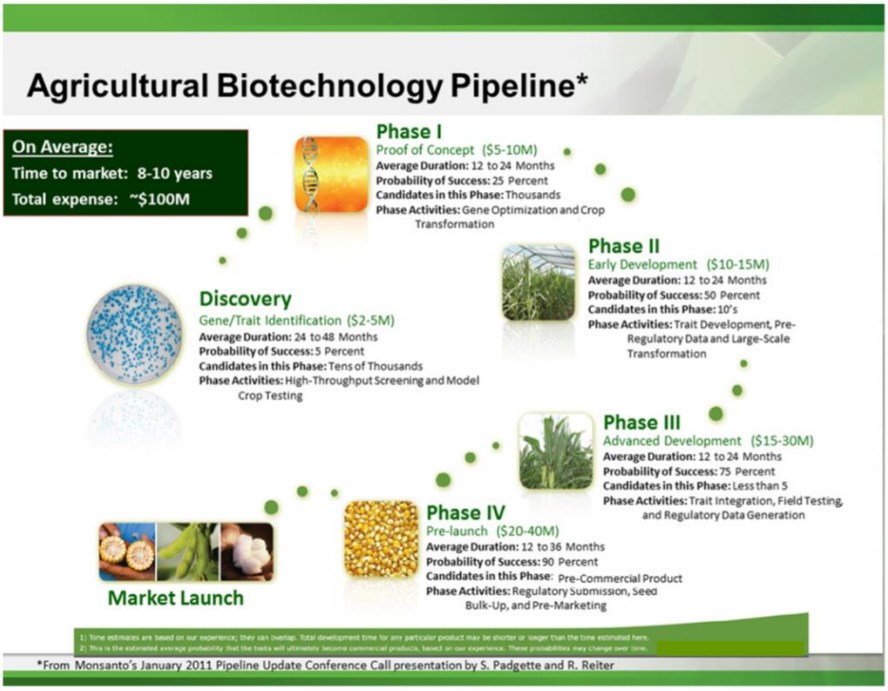

Figure 7: Example of a commercial pipeline process to develop transgenic events, which includes several phases to market launch. Source: http://pubs.acs.org/doi/pdf/10.1021/jf400685y.

The first of these steps is the identification of a desired trait and the isolation of the genetic information that is responsible for imparting this trait in the organism.

This is followed by further research and development in order to optimize the genetic construct that will be used to transform the recipient organism. This first phase includes the testing of the optimal promoter and terminator sequences as well as the stability of the construct.

Once the recipient organism is transformed, the selection of the biologically relevant and higher performing events takes place. In the case of developing LM plants this includes testing the events under greenhouses and field testing conditions. Introgression breeding can be used to develop the best performing plant varieties that will eventually be commercialized. Once the optimal plant variety is selected, the production of seed for distribution can take place.

The final stages of the LMO development pipeline consist of conducting regulatory studies or analyses in order to provide scientific evidence on the biosafety measures that may be required for adequate handling and responsible use of the LMO. This involves generating the data required to obtain regulatory approval of the LMO. The type of data that is required varies from country to country, but normally includes information on the stability of the genetic construct, nutritional equivalence as compared to the parental organism, allergenicity testing and risk assessment studies.[11] Once completed the necessary data is submitted to the relevant competent national authorities that are responsible for regulating the import and/or cultivation of LMOs, in order to obtain approval.

The first engineered organism that was considered an LMO, produced in 1978, using recombinant DNA techniques was a strain of *Escherichia coli* that produced biologically active human somatostatin. It was

[11] Please refer to information depicted in Annexes I, II and III to the Cartagena Protocol.

produced using recombinant DNA techniques. Subsequently, other proteins, such as insulin, were produced at a commercial scale using similar methods.

In 1996, the first genetically modified seeds of the FLAVR SAVR™ Tomato were authorized in the United States for use as in commercial agricultural applications, as were E176 maize and Roundup Ready™ soy. According to the International Service for the Acquisition of Agri-biotech Applications (ISAAA), the worldwide area cultivated with LM crops has been growing steadily since 1996, and in 2016, the cultivation of LM crops accounted for 185.1 million hectares.

The most common LMOs currently being cultivated are LM canola, maize, cotton and soybean. These crops have been modified to exhibit herbicide tolerance and/or insect resistance. Research has shown that the amount of time it takes to develop and commercialize LM plants carrying new traits in each of these crops is on average 11.7, 12.0, 12.7 and 16.3 years, respectively. The introduction of new traits, including agronomic and quality traits, into other crops, such as potato and rice, among others, is taking place at faster rates, as shown in figure 8.

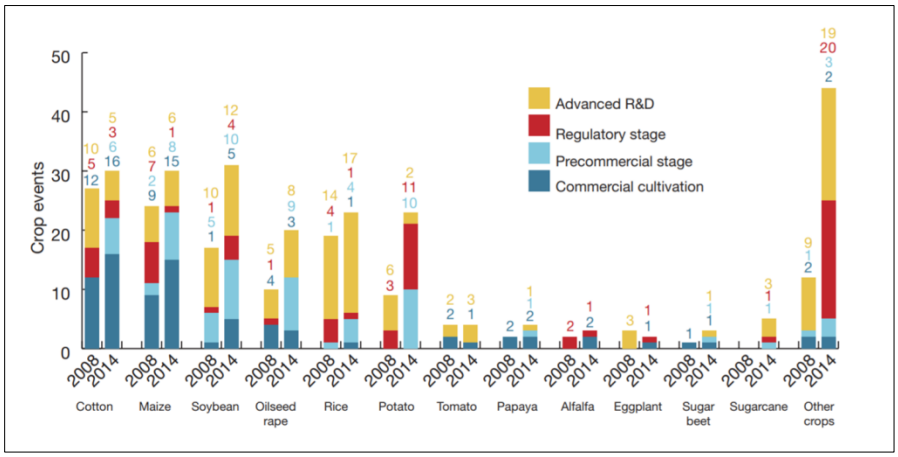

Figure 8: GM crop events in the market and at the precommercial, regulatory and advanced R&D stages in 2008 and 2014, illustrated by crop.
Source: https://sites.biochem.umass.edu/vierlinglab/files/2015/04/2016nbt.3449Status-of-GMO-crops.pdf.

Among the agronomic traits which have recently been introduced are tolerance to the herbicide dicamba, as found in MON-877Ø8-9 soybean as well as tolerance to abiotic stress and increased yield, as observed in DroughtGard™ maize, MON-8746Ø-4 and MON-87403-1 which is maize modified to obtain increased ear biomass. Furthermore, the introduction of quality traits has also seen a rise in the rate of development. Examples of such crops include the Arctic™ apple OKA-NBØØ1-8 and OKA-NBØØ2-9 which were modified to resist to enzymatic browning, as well as pineapple which was modified for altered colour and reduced ripening FDP-ØØ114-5.

Beyond the LMOs that have been developed for agricultural crop applications, there are also several LM animals that have been developed for commercial, and pharmaceutical purposes. For example, in 2009, a goat that produces a recombinant human antithrombin was the first LM animal to be approved for commercial production of a pharmaceutical. In addition, the AquAdvantage® salmon, which was modified to grow to market size in half the normal amount of time, has been produced and its commercialization has been recently approved in the United States (2015) and in Canada (2016).

Module 2

Overview of available detection and identification methodologies[12]

Many methodologies and techniques to detect, identify and quantify LMOs have been published. They range from fast and cost-effective, such as the lateral flow assay and endpoint polymerase chain reaction (PCR), to those that can be more complex, such as quantitative real-time PCR and whole genome sequencing.

When planning and setting up a laboratory for the detection and identification of LMOs choices can be made regarding which methods and protocols will be adopted. For service-oriented laboratories, particularly those serving regulatory authorities, the selection of methods is guided by, among other considerations, the country's specific regulatory requirements in accordance with national biosafety laws. The methodologies may range from qualitative methods that are used to detect for the presence of LMOs in general to those that allow for the identification of individual LMOs as well as quantitative tests that provide information on the quantity of LMOs present in a sample.

Either the DNA or the protein molecules can be targeted for the detection of such LMOs. Both approaches have advantages and disadvantages and the adoption of one over the other, or both, depends largely on the available expertise, infrastructure, capacity to handle samples, laboratory equipment, required assay sensitivity and regulatory requirements.

Below is a brief overview of some of the most commonly used methodologies for the detection, identification and quantification of LMOs, their strengths and limitations.

[12] Text adapted from Technical tools and guidance written by Chris Viljoen, Sarah Agapito-Tenfen, and Gretta Abou-Sleymane.

Table 1. Summary of available testing options for the detection and identification of LMOs, including the advantages and drawbacks of each method.

Analysis	Test	Advantages	Disadvantages
Protein based methods	Strip test	Because the test is rapid and can be performed onsite, this method is very useful as an initial screen for the presence of LMO in seed and grain.	Lower sensitivity (limit of detection typically 0.1-1% m/m)
			Testing may not be performed with laboratory controls; operator error resulting in inaccurate test results may be an issue.
		May be used for quantification using a subsampling approach for seed and grain	Not appropriate for degraded samples where proteins may have become denatured
			Expressed protein levels may vary between different commercial LMO cultivars and different parts of the same LMO
	ELISA	Higher sensitivity (limit of detection 0.01-0.1% m/m)	Not appropriate for degraded samples where proteins may have become denatured
		May be used for quantification when measured against a known protein standard	Expressed protein levels may vary between different commercial LMO cultivars and different parts of the same LMO
			Must be performed in a laboratory
DNA based methods	PCR	Higher sensitivity and specificity (approximate limit of detection 0.01% cp/cp)	Most commonly used PCR techniques must be performed in a laboratory
		Capable of detecting most LMOs	
		Allows definitive quantification (approximate limit of quantification 0.1% cp/cp)	
		Effective with broad range of sample types	
		Industry standard used worldwide in surveillance and testing laboratories	

Protein-based methods for LMO detection

LMO-specific proteins (i.e. those encoded by the inserted genes) can be detected using immunoassays which are based on antibody recognition of an epitope specific to the transgenic protein. The method of protein testing is either in the form of a lateral flow strip test, a micro-titre plate format such as the enzyme-linked immunosorbent assay (ELISA) or a gel electrophoresis protein immunoblot, also known as western blot. Protein-based detection methods require the use of antibodies that specifically bind to the transgenic protein. Since the process of antibody production is extremely complex and costly, detection using these methods typically relies on the availability of commercial antibodies.

Protein detection using lateral flow strip tests, ELISA or western blot is performed through a simple procedure of extracting total crude proteins from a sample by adding water or buffer followed by sample homogenization.

For lateral flow strip testing, a strip is placed in the crude protein extract and a positive result is indicated by the appearance of a test line due to the antibody recognition of the transgenic protein. The advantages of this qualitative method are as follows: it is very simple to perform, requires little technical expertise or

equipment and can be performed at the point of sampling. Electronic devices have also been developed for a semi-quantitative interpretation of the result. They can also be used when applying the subsampling approach to provide quantitative results. Lateral flow assays can be used to test samples for multiple LMO traits, using strips that are designed to detect multiple proteins, or combs that are composed of multiple strips.

The ELISA based approach to LMO detection follows the same principle as lateral flow strips, and as such it also involves a crude protein extraction step. However, the antibody is used to pre-coat the internal surface of a micro-titre plate. Following a series of steps which allow the target protein to bind to the antibody, the cellular debris, including other proteins, are removed from the plate through a series of wash steps. The bound protein is detected using a colorimetric assay that can be evaluated through visual inspection or using an optical plate reader. This method produces a qualitative result if read through visual inspection. However, a quantitative result can be obtained if the necessary protein standards and calibration curves are included on the plate and an optical plate reader is used to evaluate the intensity of the colorimetric reaction resulting from antibody recognition of the target protein in the sample. The advantages of LMO testing using ELISA are that a qualitative or quantitative result can be produced relatively easily.

For western blotting, the extracted proteins are separated according to their size by gel electrophoresis. The proteins are then transferred from the gel to a membrane for the detection of the target protein. This step usually involves two antibodies: first a primary antibody that is specific to the target protein followed by a secondary antibody, which is linked to a reporter molecule that binds to the primary antibody. After the excess antibody is removed from the membrane, the secondary antibody is typically visualized by colorimetric, chemiluminescent or fluorescent methods performed by either colouring the membrane itself or by exposing it to a light sensitive film, such as X-ray film. Once the membrane or film is developed, the presence of the transgenic protein is indicated as a distinct band on the membrane or film. The advantage of this method is that it is sensitive and may detect different isoforms of the target protein.

A general disadvantage of all protein-based methods is that their sensitivity is dependent on the binding affinity between the antibody and the target protein. Furthermore, the level of expression of the protein in different parts of the LMO may vary which may lead to misleading results depending on which part of the plant is being tested. Protein-based methods may also be affected by cross-reactivity between the antibody and native forms of the same protein that may be present in the organism, or by similar proteins expressed by other LMOs. Finally, such methods would not be appropriate for degraded samples where proteins may have become denatured and are not capable of identifying LMO event.

DNA-based methodologies for LMO detection

DNA-based methodologies for LMO detection and identification rely primarily on the use of the polymerase chain reaction (PCR) and related molecular techniques. PCR is a method that utilizes synthetic DNA oligonucleotides, so-called “primers”, to replicate or “amplify” targeted regions of an inserted DNA sequence that is present in the LMO. The amplified product can then be visualized to determine whether or not DNA originating from an LMO is present in a sample.

DNA extraction and purification is required prior to PCR. The choice of extraction method depends on the type of LMO being analysed since some methods are more suitable than others for particular types of LM materials, for example leaves versus oleaginous seeds. Following DNA extraction and purification, target sequences are amplified using primers that are designed to specifically bind to the target sequence during the PCR. The resulting PCR product can either be detected during the amplification process (real-time PCR) or after the PCR is completed (end-point PCR).

Visualizing the presence of the target sequence following an end-point PCR is done through gel electrophoresis. This process separates the amplified DNA according to its size by allowing it to migrate

through a gel matrix under the influence of an electric current. The amplified DNA fragment that corresponds to amplicons of the DNA inserted into the LMO can be visualized using a dye that binds to double stranded DNA and fluoresces under ultraviolet light.

Real-time PCR (RT-PCR) technology allows for the detection of the amplified target sequence during the PCR amplification process, by using either a fluorescent DNA binding dye or a third oligonucleotide that is fluorescence-tagged and is called probe. The DNA binding dye fluoresces when it intercalates within double stranded DNA molecules. As PCR progresses the level of fluorescence increases proportionately with the amount of newly synthesized DNA. Therefore, such dyes simply detect the amount of PCR product generated during the amplification, but they do not discriminate between specific and non-specific amplification. A melting curve analysis can be performed in order to identify non-specific amplicons; however, it can become more labour-intensive to optimize a PCR targeting a single specific region within an LMO.

In contrast, fluorescent probes can be used to verify that a specific target sequence is amplified during the PCR process. If real-time PCR technology is used in conjunction with the necessary standards, the quantity of LMO in a sample can be determined.

An advantage of PCR based methods is that they can be used to screen a sample for the presence of LMOs by using primers that target genetic elements that are commonly found in a number of different LMOs. Depending on the combination of primers used, PCR detection can specifically target certain genetic elements, construct-specific or event-specific. The differences between each type of PCR target region is represented in Figure 9.

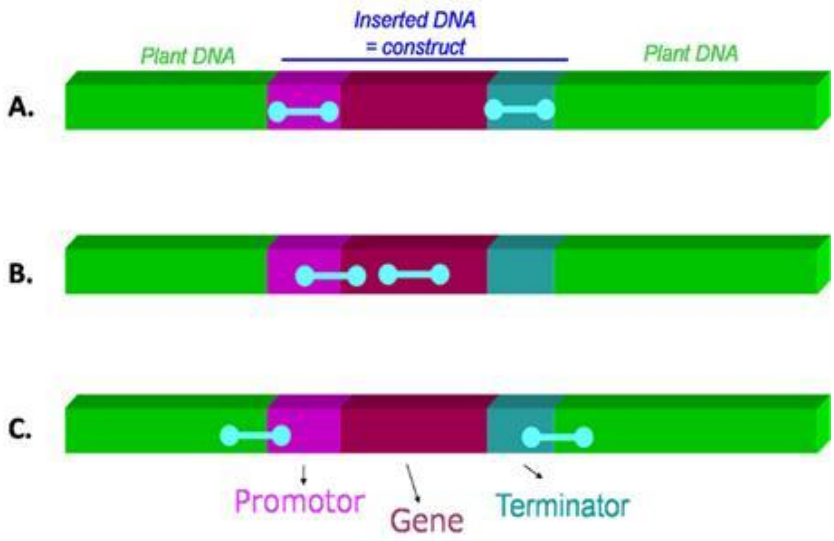

Figure 9: A construct (inserted DNA) commonly consists of several genetic elements such as a promoter, a gene and a terminator. PCR can be designed to target different regions (a) common regulatory elements (such as promoters, terminators) (b) construct-specific (junction between two genetic elements within the construct) (c) event-specific (junction between the inserted construct and the plant genome). The turquoise dumbbells represent the region targeted by the PCR, whereby dots mark the start and end of the sequence to which primers bind (probes are not shown in this figure). Source: National Institute of Biology.

PCR is sensitive, specific and versatile, and can be used to simultaneously screen a sample for LMO content, detect specific genetic elements or LMO events present and, when used in a real-time PCR

platform, quantify the amount of LMO present in the sample. Multiplex PCR and matrix[13] approaches can also be used for multi-target detection. A disadvantage of the most commonly used PCR-based approaches, however, is that it requires specialized expertise and equipment.

Comparison between protein- and DNA-based methods for LMO detection

Both protein- and DNA-based approaches to LMO detection are useful as each one serves different purposes.

Protein-based methods, such as lateral flow strip assays and ELISA, are simple, time efficient (several minutes to a few hours), and convenient for a small number of samples. The lateral flow assay is useful for LMO testing at the point of sampling. However, protein-based detection of LMOs requires a different test for the detection of individual LMO traits and cannot be used to simultaneously distinguish between different LM events that may be present in a single sample. In contrast, the more commonly used DNA based approaches, while more specific and sensitive, may require more steps and time (several hours to days) and rely on specialized laboratory equipment and expertise.

Protein-based methods are only suitable for detecting LMOs that are designed to produce a protein from the DNA inserted. However, they are not suitable for detecting inserted genetic elements that do not produce a protein, such as regulatory sequences or LMOs that were designed, for example, to silence the expression of target genes through RNAi.

Protein-based methods rely on the specificity of the antigen-antibody interaction. Therefore, any changes in the tertiary structure of the target protein renders the method ineffective. Such conformational changes are sometimes introduced during sample processing if the samples are subjected to heat and/or chemical treatment. The detection capability of protein-based methods is also affected by the expression level of the target protein which can vary between different parts of the LMO or different stages of its life cycle and can be influenced by external factors such as climate and soil conditions. In addition, some LMOs have been specifically designed to express a transgenic protein in a specific tissue and may not be necessarily present in the part of the organism being tested.

In contrast, DNA is more stable as compared to proteins, so detection continues to be possible even if the sample has been exposed to heat and/or mild chemical treatments. DNA is also present in all cells and therefore any part of the organism can be used for testing. Furthermore, PCR methods are more versatile than protein methods, and PCR can be used to screen a sample for the presence of several potential LMOs simultaneously with relative ease. PCR can also be applied qualitatively, to detect specific genetic elements or events and identify individual LMOs. It can also be applied quantitatively, to determine the quantity of a particular LMO event in a sample.

Considerations for national strategies towards the detection and identification of LMOs

A Party that is considering establishing provisions for the detection and identification of LMOs in their system may need to take into account several factors First, assessing their existing capacities in terms of infrastructure, technical expertise, the availability of equipment needed to perform the testing procedures, as well as considerations on how the implementation of such measures may affect inter-nation trade in grains. Establishing the effective communication channels among the relevant authorities also facilitates the efficient implantation of relevant provisions. A possible strategy for the efficient sampling, detection

[13] JRC GMO-Matrix application, http://gmo-crl.jrc.ec.europa.eu/jrcgmomatrix/.

and identification of LMOs may be initiated by the country by defining each of the following parameters should they wish to establish the scope and procedure of LMO testing within the country:

1. The national implementation of a system with objectives and sampling targets;

   Possible targets may include the environmental monitoring and sampling at points of entry for the monitoring of transboundary movements of authorized LMOs and/or the detection and control of unauthorized LMOs.

2. The parameters that may be monitored and controlled;

   Possible parameters include maintaining a suitable documentation system that traces the transboundary movement of material that may contain LMOs, and if established in the regulation, and relevant for the decision-making process, quantify the amounts present. Depending on the intended control level, the required analytical capability established, may range from qualitative (presence/absence) or quantitative (e.g. if certain thresholds require different measures.). The resources (financial, human) that can be made available in the foreseeable future are also an important consideration.

   Besides the resources required for laboratory testing capacities, eventually, countries may also wish to develop adequate capacities to sample LMOs, including training of their laboratory technicians, and border control officers and field inspectors.[14] Adequate sampling is critical for the detection pipeline, in that it determines the quality of the results and the overall outcomes of the system to monitor, detect and/or identify LMOs. Taking the importance of sampling into account, where resources are limited, efforts may be more efficiently allocated if they are focused on sound field sampling combined with basic yet reliable, qualitative laboratory capacities.

Challenges in LMO detection and new technology developments

There are several challenges that need to be considered when applying available methodologies to LMO detection. Below is a brief overview of some of the key considerations.

Availability of validated methods

It is important that criteria to test the performance of the methods is harmonized internationally to make results comparable throughout the world, taking into account the availability of resources in different countries, for example, as has been specified by Codex CAC/GL 74-2010 and other international standards such as ISO 21569, ISO 21570, ISO 21571, ISO 24276. The development of harmonized performance criteria was aimed at simplifying and increasing the accessibility to LMO detection technologies in countries with less capacity and fewer resources. Thus, a significant challenge for LMO detection is to ensure that methods are validated and meet the necessary minimum performance criteria for quality control purposes.

Implementation of a quality system

An important consideration in the application of LMO detection is that standardized operating procedures (SOPs) are used. This means that the LMO detection laboratory is required to develop and maintain the necessary quality control measures, such as those that have been set out by ISO 17025, to ensure the reliability, sensitivity and reproducibility of the methods in use.

[14] Training for border control officers and field inspectors is currently available through the BCH through the "Course on the Cartagena Protocol on Biosafety for Customs/Border Officials" available at https://scbd.unssc.org/course/view.php?id=10.

Module 2

Access to detection methods and reference material

While methods for the detection of most commercialized plant LMOs are accessible through several online platforms, such as the BCH database, challenges in detecting some LMOs, particularly unapproved LMOs, are posed due to the difficulty faced by laboratories in accessing sequence data of the genetic elements that in order to facilitate the design of specific detection systems. Some countries require developers of LMO to provide a detection method during the authorization process; however, this is not always the case.

Reference materials are used to verify detection methods. They are also used as a positive control. Reference materials for most commercial plant LMOs are available from the American Oil Chemists' Society (AOCS) or the Institute for Reference Materials and Measurements (IRMM). However, certain reference materials may not be available for some LMOs or detection laboratories may not have easy and cost-effective access to reference material for the specific LMO event in question in order to be able to verify the detection method that is being used in their laboratory.

Gaps in national biosafety frameworks

In the light of the challenges faced with access to detection methods and reference material, the Conference of the Parties serving as the meeting of the Parties to the Cartagena Protocol, in its decision BS-VII/10, encouraged Parties and other Governments, without prejudice to Article 21 on confidential information, to ensure that, for regulatory purposes, the information provided by a notifier at the time of notification includes all the information necessary to detect and identify the living modified organism, including information that allows for its unique identification and where reference materials may be obtained.

Novel approaches for simultaneous detection of multiple LMOs

The increase in LMO research and commercialization is resulting in a continuous increase in the number of LM events that laboratories may need to detect as required by their competent national authority. In addition, the potential for the presence of LM events that are not authorized in one country but approved in another may be increasing, and detection laboratories are faced with the challenge of having to potentially detect multiple LMOs that are present in a single sample, some of which may be approved, while others may have not yet been authorized or are illegal, according to the Party's regulatory context. One approach to address this challenge is to use a suitable screening strategy such as following a matrix approach in order to simultaneously detect multiple genetic elements that are commonly used in an attempt to widen the screening capabilities. When used in conjunction with bioinformatics software, the matrix results can be used to identify the potential LMOs that are present in the sample. This approach can be customized to include as many LMOs as the laboratory is required to detect, and it is primarily applicable to PCR-based methods that can be multiplexed in order to be able to simultaneously detect and identify multiple LMOs.

Emerging technologies for LMO development and detection methods

New and emerging technologies for developing LMOs are proving to be a challenge for the detection and identification of the LMOs. For example, new gene silencing technologies include the use of double stranded RNA (dsRNA) molecules to confer desirable traits to LMOs. In such cases, a transgenic protein is not produced, and therefore only DNA-based detection methods can be used. Furthermore, other LMOs are being developed through emerging synthetic biology techniques where the resulting LMOs may include a genetic modification that may not be readily detected or resemble naturally occurring genetic elements.

On the other hand, the continuous development of new technologies makes LMO detection more readily accessible, especially in countries with fewer resources. For example, digital PCR isothermal nucleic acid amplification and other chip-based technologies will soon enable the routine detection of LMOs in the field with portable devices. While many countries may consider the development of a framework for LMO detection to be a burden, the technology may also be applied to other purposes such as human, plant and animal pathology, among other uses.

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Module 3

Sample Preparation and Extraction

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Introduction

Analytical testing of LMOs using scientifically validated methods are carried out following a stepwise process in private, commercial and government testing laboratories; agricultural fields, shipping and storage vessels and facilities, among other places. Since there are different levels of competence and technology required for sampling and tests in different contexts, analysts need to evaluate their sampling and testing processes within the scope of their customer's needs in order to ensure standardized testing and laboratory criteria are not only reproducible and technically sound with defined patterns and known nomenclatures, but also cost-effective with respect to the products that are sampled and tested.

Samples are submitted to the laboratory in their raw form; therefore, certain manipulations have to take place in order to prepare the sample for analysis. This involves the preparation and extraction of the analyte from the laboratory sample. The validity of procedures for subsampling in the laboratory, sample preparation and extraction are important in obtaining reliable results regarding the presence of LMOs in a sample. Therefore, appropriate procedures have to be followed in order to ensure that the sample being analysed is homogeneous and representative of the original sample.

Sample management

Laboratories that are involved in the detection and identification of LMOs need to have procedures in place for sample management, including the steps to follow for sample submission, receipt, labelling, storage, laboratory sample preparation and destruction, including an outline of the procedures for documenting each step. Suitable standards for these procedures can be found in ISO 6498, ISO 24276 and ISO 17025.

Sampling plan and procedure

Many sampling plan standards are available for commercial agricultural products. In many cases, they are also applicable to LMOs. Some are produced from regulatory authorities. Securing a representative sample requires that equal portions are taken from evenly distributed parts of the quantity of leaves, stems, fruits, seeds, grains or screenings to be sampled. As batches are sampled, each portion should be examined. If it appears to lack of uniformity, the portions should be retained as separate samples or combined to form individual-container samples to determine the apparent heterogeneity. When the portions are uniform, they can be combined to form a composite sample. Included with published methods are provisions for the collection and documentation of traceable records to ensure the preservation of the samples' integrity and minimizing contamination. Below is a description of several steps to consider for sample management.

Sample submission

All samples accepted by the laboratory should be accompanied by an official laboratory form requesting an examination in either hardcopy or electronic format as specified by the laboratory. These forms, as a minimum, should include the following information:

1. Data about the person from the relevant competent national authority that is submitting the information. This data would include their name, location of employment and contact information. This information is used by the laboratory in order to direct them to the correct contact person in the event the laboratory has any questions that may come up during sample processing. It also provides information regarding who to send the final report to and payment procedures. This section must also include the submitter's signature.
2. Information about the sampling plan.

3. A description of each sample submitted to the laboratory is provided by the submitter, including information on the type of sample, for example if it is food, feed or seed as well as its weight and composition. Information on the point and time of collection of the sample should also be included.
4. An indication of what the target organism may be and what type of analysis is requested.
5. Information how the samples were delivered to the laboratory, including as appropriate, the name of the person who delivered the sample and/ or shipping labels if the sample arrived by mail.
6. Any required regulatory permit information (such as phytosanitary or LMO movement permits)

Once the documentation is checked and verified for completeness, the laboratory personnel should inventory the sealed sample packages and compare the number and descriptions of the samples received with the information in the official laboratory form to ensure that all samples are present. If there is a discrepancy in the number or description of the samples, the submitter should be contacted to clarify and reconcile the discrepancy. Any changes to the submission information should be documented in laboratory case file.

Furthermore, the condition of the sample packaging should be evaluated. If the sample packaging has been compromised or damaged during shipping, for example a tear in the packaging that resulted in the cross-contamination of samples, then this must be recorded in the case file. If the compromised packaging affects the suitability of a sample to be tested, then laboratory personnel should contact the submitter to inform them. This information should also be recorded in the case file.

Following the verification of documentation, a verification of the sample size (see section on *Considerations for sample size*) must be made. The sample is weighed and a note is made if the weight of the sample is in accordance with the specifications set out by the laboratory. If the sample size is too small, this will be documented in the laboratory case file and the submitter will be informed, according to laboratory policy, whether or not this sample will be processed. If the sample size is larger, than the specifications set out by the laboratory, then a representative mass reduction (see Box 1) may need to be carried out in accordance with laboratory policy. In either case, a note would have to be issued in the laboratory report.

Box 1: Mass reduction

Mass reduction is the part of the sample preparation procedure to reduce the mass of a laboratory sample through subsampling (ISO 6498). This would need to be carried out if the laboratory sample that was submitted for analysis is bigger than the sample size required by the laboratory.

There are several techniques that can be used to reduce the mass of the sample in such a way that the resulting subsample that will be carried forward during processing continues to be representative of the whole laboratory sample. These include:

1. Pre-grinding: if the laboratory sample is composed of lumps, or if the individual unit of the sample are large, for example large seeds, such as coconuts, and fruits, or below ground plant parts tubers such as potato, Cassava, sweet potato or sugar beets. In such cases the whole sample should be pre-ground before mass reduction.

2. Subsampling: Several methods can be used to reduce the size of the laboratory sample:

a. Fractional shovelling

b. Spoon method

c. Long pile method

d. Coning and quartering

e. Splitters and dividers

Adapted from JRC's "Guidelines for sample preparation procedures in GMO analysis"

Once these checks for documentation, sample condition and sample size have been carried out the samples should be registered within the laboratory's information management system.

A sequentially generated laboratory number is assigned to the case upon receipt of the first official laboratory form requesting an examination. Any supplemental sample submission(s) should be assigned the same laboratory number as the original submission. These laboratory file numbers are unique identifiers and can be placed on all documents relating to a case. Furthermore, each individual sample that is submitted for a case should be assigned a consecutive and unique item number, for example using a sequential numbering system or a barcode label. If a subsample and/or multiple test portions of a sample are created during processing, a sub-item number should be generated using the sample's unique numeric sequence separated by a hyphen (e.g., 1-1, 1-2, etc.).

Box 2: Sequentially generated numbering

When selecting a numbering system for samples it would be useful to follow a system that is intuitive and meaningful, for example: "*LYYYY###*", where

• L - is the laboratory acronym. This would be particularly useful if the laboratory storage space is shared and the samples need to be identified as belonging to the LMO/ GMO testing laboratory.

• YYYY - are the four digits of the calendar year.

• ### - is a sequentially assigned three-to-five-digit number, beginning with the number one (001) assigned to the first case submitted in the calendar year

Once the review is complete, the laboratory personnel that received the samples and carried out the check should sign or initial the documentation in the designated area and mark the date the samples were received in the case file. The samples can now be placed in storage until further processing.

Considerations for sample size

The minimum size of any given sample depends on the statistical confidence required, the lowest concentration of LMO that is to be detected and on the sample type and matrix which is usually defined by number of seeds, kernels, grains or by weight. Below is a table recommending appropriate laboratory sample sizes based on the type of matrix.

Table 2. Recommended laboratory sample sizes according to the type of matrix. Source: Adapted from the JRC's "Guidelines for sample preparation procedures in GMO analysis"

Products	Recommended laboratory sample size
Leaves	10 different leaves
Seeds	Mass equivalent of 3000 kernels
Commodity grains	Mass equivalent of 10000 grains

For seeds and commodity grains, the recommended laboratory sample size corresponds to the mass equivalent of a certain number of kernels or grains. Table 3 provides information on the mean mass of 1000 kernels of various plant species from which the appropriate laboratory sample size can be extrapolated.

Table 3. Data concerning the mean mass of 1000 kernels for different plant species. Source: Adapted from the JRC's "Guidelines for sample preparation procedures in GMO analysis"

Plant species	Mean mass of 1,000 kernels (in g)
Barley *(Hordeum vulgare)*	37
Linseed *(Linum usitatissimum)*	6
Maize *(Zea mays)*	285
Millet *(various species)*	23
Oat *(Avena sativa)*	32
Rapeseed *(Brassica napus)*	4
Rice *(Oryza sativa)*	27
Rye *(Secale cereale)*	30
Soybean *(Glycine max)*	200
Sugar beet *(Beta vulgaris)*	11
Sunflower *(Helianthus annuus)*	100
Tomato *(Solanum lycopersicum)*	4
Wheat *(Triticum aestivum)*	37

It should be noted that, for each plant species, there may be many varieties that are encountered in the laboratory. Each of these varieties may have different-sized kernels; therefore, a variety with larger kernels (compared to the average values of kernels) may contain fewer than 1,000 kernels in a mean sample mass.

If seeds/grains are tested the procedure should follow a test plan and taking subsamples should be considered, depending on the mass of the kernel and the targeted limit of detection. A working sample can be divided into one or more subsamples, if the mass of the subsample is large then it might be necessary to split the subsample before grinding and then to recombine the ground seeds before DNA extraction. DNA is then extracted from each subsample and tested independently by PCR.

In certain cases where samples that have been submitted to the laboratory with a mass equivalent that is lower than the recommended laboratory sample size or a sample with larger kernels, it would be good practice, on a case-by-case basis, as appropriate, to check the number of kernels that are present in those samples. This can be done by weighing the mass of 100 kernels and extrapolating an estimate of the number of seeds present based on the mass of whole sample.

In addition to establishing minimum size requirements of the laboratory sample, it is also advisable to fix a maximum size. This will facilitate the need to perform a mass reduction step on large laboratory samples. Maximum numbers should not be fixed in a single unit for all samples but should be adapted to the kind of matrix analysed.

For leaves it is recommended to use the size mentioned in table 4, from which a representative part of the total leaves (approximately 1 cm^2 of each leaf) must be taken and these are pulverized by the addition of liquid nitrogen.

Table 4. Minimum number of leaves for testing different species.

Plant species	Minimum number of leaves
Maize (*Zea mays*)	50
Soybean (*Glycine max*)	50
Sugar beet (*Beta vulgaris*)	50
Wheat (*Triticum aestivum*)	75
Alfalfa (*Medicago sativa*)	50 stems
Cotton (*Gussypium hirsutum*)	75

Sample storage

Storage of the samples prior to and during sample preparation, as well as after its analysis should be performed under appropriate conditions (e.g. at room temperature, refrigerated, frozen). It is the responsibility of laboratory personnel to ensure, insofar as possible and reasonable, that samples do not experience loss, cross-contamination, or deleterious change while in the laboratory.

While seed and grain samples may be stored at room temperature without any negative effect on the sample's quality, it is important to ensure that the original moisture content of the sample along with the environmental conditions in the laboratory are appropriate to do so. For example, if the room environment is warm and humid this may lead to the growth of mould in the sample, leading to a deleterious change in the quality of the sample. To prevent insect damage seed and grain in long term storage requires temperatures low enough to prevent insect reproduction. Therefore, samples that may experience a deleterious change without refrigeration should be placed in a refrigerator or freezer as soon as possible until such time that they will be prepared for analysis. Furthermore, in the case of seed samples, it might be necessary to store them in a dry environment and care must be taken that there is no accidental release of the seeds into the environment. In this case it is advisable to homogenize the sample as soon as possible to eliminate the risk of an accidental release, especially if it is necessary to grow the seed out to perform the required test

Sample homogenization

In general, the whole laboratory sample is used to obtain a homogenized test sample for the analysis, if the size of the sample conforms to the laboratory's requirements. Homogenization is required for two reasons:

1. To achieve sufficient efficiency of analyte extraction; and,

2. To ensure homogeneity and an equal representation of LMO-derived particles in the test samples.

The aim of homogenization is particle size reduction. Size reduction permits a wider distribution of each kernel in the sample, making it more likely that a small subsample will contain the target. Furthermore, homogenization improves DNA and protein extraction. Homogenization is the step with the highest risk of error and contamination.

The risk of error arises because, as indicated in Tables 1 and 2 above, a laboratory sample can range from 100 g – 3 kg in mass, depending on the plant species. Whereas the resulting test potion that will be used to carry out the analysis can range from 200 mg – 10 g in mass. This represents a 200-to-10,000-fold difference in mass and potentially the largest possible source of error. Therefore, a specific procedure should be followed during the sample homogenization to manage and reduce the likelihood of error.

The contamination risk that occurs during the homogenization procedure is largely due to the formation of a fine dust that often appears during the grinding step. This dust could contaminate subsequent laboratory samples, if appropriate laboratory practices are not in place. Therefore, all the sample preparation steps should be done under stringent conditions to avoid cross-contamination while minimizing the degradation of the target analyte, be it DNA or proteins, in the test sample.

The homogenization of samples must be carried out in a room or area dedicated primarily to that purpose following strict procedures for sample processing and the subsequent cleaning of surfaces and equipment. This procedure is outlined in further detail in the next section.

Sample preparation procedure

The choice of method used to carry out the particle size reduction also depends on the physical and chemical composition of the material, its quality and the laboratory's capacity. Particle size reduction may be achieved with mills, homogenizers, immersion blenders, coffee grinders, or other suitable equivalent device, depending on the size and the structure of the laboratory sample, as indicated in the table below.

Table 5. Various commonly encountered sample matrices and possible equipment / method used for their homogenization. Source: Adapted from Žel et al. Extraction of DNA from different sample types - a practical approach for GMO testing.

Species	Matrix	Possible homogenization method
Soybean	Grain / seed	Retsch ZM200 rotor mill or a coffee grinder
	Leaves	Small pieces from different leaves, combined and homogenized as one sample with FASTprep instrument or manually crushed with LN_2
Maize	Grain / seed	Retsch ZM200 rotor mill or a coffee grinder
	Leaves	Small pieces from different leaves, combined and homogenized as one sample with FASTprep instrument or manually crushed with LN_2
Rapeseed	Grain / seed	Retsch GM200 knife mill + LN_2 or coffee grinder + LN_2
	Leaves	Small pieces from different leaves, combined and homogenized as one sample with FASTprep instrument or manually crushed with LN_2
Flax	Grain / seed	Retsch GM200 knife mill + LN_2 or coffee grinder + LN_2
	Leaves	Retsch GM200 knife mill + LN_2
Rice	Grain	Retsch ZM200 rotor mill or a coffee grinder
Potato	Tuber	Bioreba
Tomato	Fruit	Bioreba
	Leaves	Small pieces from different leaves, combined and homogenized as one sample with FASTprep instrument or manually crushed with LN_2
Wheat	Grain	Retsch ZM200 rotor mill or a coffee grinder
Sunflower	Grain	Retsch ZM200 rotor mill or a coffee grinder

However, grinding using a mill with an integrated sieve gives you control and uniformity over particle size. In the case of difficult samples, such as fatty samples, that may be prone to degradation during the homogenization step, the use of successive grinding steps with sieves of decreasing mesh sizes or repeated grinding with the same mesh size sieve may be appropriate. Furthermore, difficult samples can also be treated with liquid nitrogen before grinding. This serves to reduce the generation of heat that is

produced during the grinding process, thus reducing the chances of analyte degradation and maintaining sample integrity.

Other samples such as large fruits, tubers or seeds, it may be necessary to take a subsample of equal size from each individual item in a lot. These are then combined and processed as appropriate in order to obtain the test sample. For example, if a lot contains 1,000 sugar beets, then a 1-centimetre cubed subsample from each beet is taken and ground up in order to obtain the test sample. This is more practical than grinding 1000 whole sugar beets and since the beets are not homogenous in size they would not contribute equally to the test sample.

After grinding, the test sample should be thoroughly mixed, or blended, to obtain a very homogeneous analytical sample. This is carried out by placing the homogenized sample in a disposable plastic bag or other suitable container, that is, at a maximum, half full and inverting the contents of the container at least 20 times.

Once the sample is homogeneous, following grinding and mixing, if required, the test portion is obtained through "grab sampling". The grab sampling must be carried out immediately after the mixing procedure in order to minimize gravitational segregation of the homogenized particles, which may affect the homogeneity of the sample. For each sample, two 200 mg test portions are taken from the prepared sample. For some matrices a greater amount may be required. A back-up aliquot of the prepared sample is also preserved in the event that a reanalysis of the sample is needed in the future. If the backup aliquot is ever used, the homogeneity of the sample would have to be re-established using the bag mixing technique.

All samples taken during the grab sampling procedure must then be stored in an environment that is conducive to the maintenance of the sample's integrity. The most appropriate storage depends highly on the sample type and the length of time that the sample will be stored for. For example, the two test portions can be stored at 4^{o}C for a few days until they are further processed. On the other hand, the backup aliquot, which is normally kept for longer periods of time (e.g. three months), would need to be stored at -20^{o}C for the period time specified before it is either used or destroyed.

Once the test portions are successfully processed and analysed the resulting DNA, or protein and back up aliquot will have to be appropriately labelled and stored for the length of time specified by the laboratory. Once the time has elapsed it would be authorized to destroy the samples. Any sample destruction would have to be appropriately documented in the sample case file. This includes documenting the date of destruction and the identity of the person who destroyed the sample.

Module 3

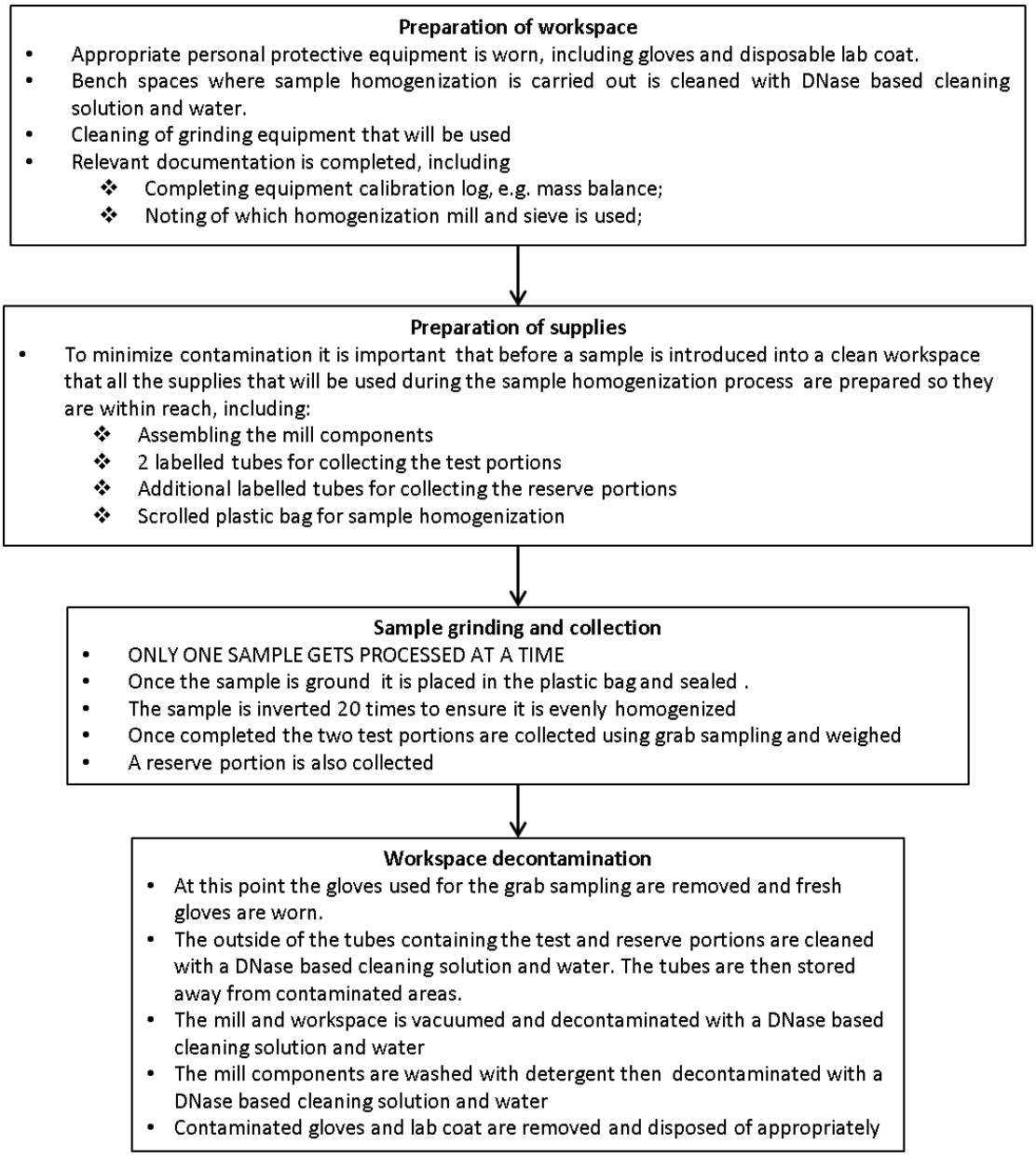

Figure 10: Step-by-step guide on practical, hands-on procedures that can be followed in order to successfully homogenize a sample.

Considerations for quality assurance and quality control during sample homogenization

The application of stringent quality assurance and quality control (QA/QC) procedures during the sample homogenization step is vital since, as previously indicated, it is the step with the highest contamination risk and the quality of the homogenization will affect the efficiency of downstream processes. Suitable QA/QC management procedures are outlined in ISO 17025. In addition to the measures described above that can be put in place during sample preparation, the following considerations should also be kept in mind:

Maintaining homogenized particle size: Ensuring that the particles produced during the homogenization step are of a consistent and specific size can be done by using a grinder with an integrated sieve. However, with time, the size of the openings within the sieve can increase. Routinely calibrating and inspecting the sieve should be done to ensure that at least 95% of particles produced during the homogenization procedure are at the correct size.

Carry over: To validate whether or not the decontamination procedure that has been adopted by your laboratory is adequate in ensuring that the surfaces and equipment are analytically clean and free of material that can be carried over into the next sample, a test can be performed to validate the procedures. This involves either:

1. Processing an LMO sample followed by a non-LMO sample then analytically testing for the presence of any traces of LMO material in the sample; or

2. Processing a sample of species A (for example corn) followed by a sample of species B (for example soybean) then analytically testing for the presence of traces of species A, in this case corn, in the test portion of species B, the soybean.

Representativeness of test portion: As previously indicated, a laboratory sample can range from 100 g – 3 kg in mass, depending on the species, whereas the resulting test potion that will be used to carry out the analysis can range from 200 mg – 1 g in mass. Due to this large difference in size between the laboratory sample and the test portion, validation tests must be carried to ensure the representativeness of the test portion. Similarly to the approach provided above, this involves either:

1. Processing a sample that has been spiked with 1 LMO grain in 10,000 non-LMO grains of the same species; or

2. Processing a sample that has been spiked with one grain from species A (for example corn) in 10,000 grains of species B (for example soybean). Grains from species A and B should be of similar size.

In both cases six test portions are tested for the presence of the spiked grain. The testing procedure is considered representative if all six test portions test positive for the presence of the spiked grain. If not, then possible ways forward may include either increasing the test portion size or decreasing the particle size of the homogenized samples.

Sample DNA extraction

The objective of nucleic acid extraction methods is to obtain purified nucleic acids from various sources with the aim of conducting specific analyses using, for example, the polymerase chain reaction (PCR). The quality and purity of the extracted nucleic acids are critical factors when it comes to PCR analysis. Therefore, suitable extraction methods should be applied in order to obtain highly purified nucleic acids free from any residues of the extraction reagents and other cellular content, such as proteins, polysaccharides, lipids and RNA, that may inhibit the performance of a PCR analysis. Examples of such inhibiting contaminants are listed in table 6.

Table 6. Examples of PCR inhibitors.

Inhibitor	Inhibiting concentration
SDS	> 0.005%
Phenol	> 0.2%
Ethanol	> 1%
Isopropanol	> 1%
Sodium acetate	> 5 mM
Sodium chloride	> 25 mM
EDTA	> 0.5 mM
Haemoglobin	> 1 mg/ml
Heparin	> 0.15 IU/ml
Urea	> 20 mM
Reaction mixture	> 15%

In order to avoid false negative results arising from the presence of PCR inhibitors in the sample, it is recommended to perform a control experiment to test for the presence of PCR inhibition using a plant or species-specific PCR analysis.

A number of methods exist for the extraction and purification of nucleic acids therefore the choice of the most suitable technique can be based on the following criteria:

- Target nucleic acid
- Source organism
- Starting material (tissue, leaf, seed, processed material, etc.)
- Desired results (yield, purity, purification time required, etc.)
- Downstream application (PCR, cloning, labelling, blotting, RT-PCR, cDNA synthesis, etc.)

The principles of some of the most common methodologies that are currently used for the extraction and purification of nucleic acids for the purposes of LMO detection are described below. Guidance and specific extraction methods are given in ISO 21571.

Extraction methods

The extraction of nucleic acids from biological material serves to achieve 3 basic goals: (i) lysis; (ii) inactivation of cellular nucleases; and (iii) separation of the desired nucleic acid from cellular debris.

The ideal lysis procedure must be rigorous enough to disrupt the complex starting material and cellular structures, yet gentle enough to preserve the target nucleic acid.

Some common lysis techniques include:

- Mechanical disruption (e.g. grinding, hypotonic lysis)
- Chemical treatment (e.g. detergent lysis, chaotropic agents, thiol reduction)
- Enzymatic digestion (e.g. proteinase K)

These techniques can be combined to achieve the desired results, for example, by using a single solution that contains detergents to solubilize cellular membranes and strong chaotropic salts to inactivate intracellular enzymes. One such solution is cetyltrimethylammonium bromide (CTAB), an ionic detergent that is often used for the extraction and purification of DNA from plants and plant-derived foodstuff. It is particularly suitable for the elimination of polysaccharides and polyphenolic compounds that would otherwise affect DNA purity and quality.

The CTAB extraction method has been widely applied in the field of plant molecular genetics and has already been tested in validation trials for LMO detection. Several variations of this method have been developed and adapted to a wide range of raw and processed matrices.

The principles of this method make use of the chaotropic properties of CTAB to disrupt cellular membranes and form insoluble complexes with nucleic acids in a low-salt environment. Furthermore, under these conditions, polysaccharides, phenolic compounds and other contaminants remain in the supernatant and can be washed away during downstream purification techniques. The theory and steps of the CTAB method are further detailed below.

Lysis using the CTAB method

The first step of any DNA extraction procedure is the disruption of the cell wall and cellular membranes, including the nuclear and organelle membranes. All biological membranes have a common overall structure comprising of a continuous phospholipid bilayer embedded with integral membrane proteins that are held together by non-covalent interactions, as shown in Figure 11.

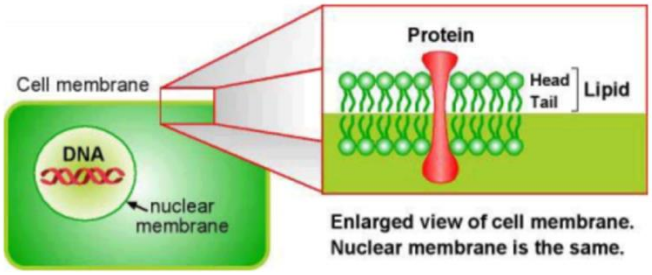

Figure 11: Simplified representation of cellular membranes. Source: JRC User Manual "The Analysis of Food Samples for the Presence of Genetically Modified Organisms".

In the CTAB method, disruption of the cellular membrane is achieved by treating the homogenized sample with an extraction buffer containing CTAB. The polar nature of the CTAB component of the extraction buffer makes it an ideal chaotropic agent and allows it to lyse and bind to the membrane components such as phospholipids, lipoproteins, polysaccharides and inhibits their co-precipitation with the DNA. A schematic representation of the mechanism of lipid solubilization using a detergent is shown in Figure 12.

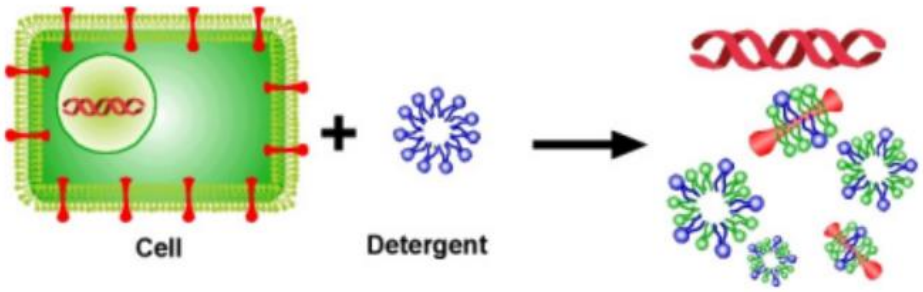

Figure 12: Schematic representation of the disruption of the cellular membrane and extraction of genomic DNA. Source: JRC User Manual "The Analysis of Food Samples for the Presence of Genetically Modified Organisms".

Additional components of the extraction buffer include sodium chloride, EDTA and Tris/HCl, each of which serves a specific function to facilitate the extraction process.

- Sodium chloride facilitates the formation of an insoluble complex between CTAB and nucleic acids.
- EDTA, which is a chelating agent that binds magnesium ions, among others. Magnesium ion is a DNase cofactor therefore the presence of EDTA reduces the amount of bioavailable magnesium ions and, therefore, minimizes the activity of any DNase.
- Tris/HCl maintains the pH-buffering capacity of the extraction buffer since extreme fluctuations in pH leads to DNA damage.

It is important to note that at this stage of the extraction process, nucleic acids are highly susceptible to degradation, as such the time elapsed between sample homogenization and addition of the CTAB buffer should be minimized.

Purification methods

Various methods are available for the purification of the target nucleic acids from cellular debris once the cell membranes have been lysed. This step focuses on the removal of contaminants such as polysaccharides, phenolic compounds, lipids and proteins. The elimination of such contaminants is particularly important due to their capacity to inhibit downstream enzymatic reactions, including PCR reactions (see table 6). The purification step makes use of a combination of techniques that have been developed to employ the biochemical properties of the target nucleic acids as compared to the contaminants. Such techniques include:

1. Solvent extraction and nucleic acid precipitation

Solvent extraction is often used to eliminate contaminants from nucleic acids. For example, a combination of phenol and chloroform is frequently used to remove proteins. Subsequently, precipitation of the nucleic acids with isopropanol or ethanol is carried out. If the amount of target nucleic acid is low, then an inert carrier, such as glycogen, can be added to the mixture to increase precipitation efficiency. Other nucleic acid precipitation methods include selective precipitation using high salt concentrations, also known as "salting out", or precipitation of proteins using changes in pH.

IN THE LAB: Phenol: chloroform extraction

This is also known as an organic extraction. The cell lysis step is carried out using an aqueous extraction buffer, whereas the phenol: chloroform mixture is organic and therefore immiscible with aqueous solutions. Alternatively a mixture of chloroform:octanol (24:1) can also be used in order to avoid the handling of phenol. The addition of the phenol-chloroform to the mixture of lysed cells allows for the partitioning of contaminants such as polysaccharides, phenolic compounds, lipids and proteins into the organic phase therefore facilitating the purification of the DNA, which remains in the aqueous phase.

To separate the two phases from each other the sample is centrifuged, resulting in the formation of distinct aqueous and organic phases. Due to the difference in density between the two phases, the aqueous phase will normally form the upper layer. However, under certain circumstances the phases may be inverted if the density of the aqueous phase increases due to altered salt concentrations that are greater than 0.5 M. Furthermore, the nucleic acid may partition into the organic phase if the pH of the aqueous solution has not been adequately equilibrated to a value of pH 7.8 - 8.0.

If needed, the phenol: chloroform extraction step can be performed several times in order to increase DNA purity. In addition, to increase DNA recovery, the organic phase can be back-extracted with an aqueous solution that is then added to the previous extract. It is also common that following the completion of a phenol: chloroform extraction that one final extraction step is carried out using chloroform: isoamyl alcohol mixture. This is done in order to remove any residual phenol, which may be an inhibitor to PCR as stated in table 4 above. Once the nucleic acid complex has been purified, the DNA can be precipitated.

In this final stage, the nucleic acid is liberated from the detergent complex. For this purpose, the aqueous solution is first treated with a precipitation solution comprised of a mixture of CTAB and NaCl at elevated concentrations, greater than 0.8M. The salt is needed to facilitate the formation of the nucleic acid precipitate. Sodium acetate may be preferred over NaCl due to its buffering capacity.

Following the NaCl treatment, 95% alcohol is added to the sample mixture. Under these conditions, the detergent, which is more soluble in alcohol than in water, can be washed away, while the nucleic acid will precipitate out of solution. In some protocols NaCl is incorporated at the beginning of the extraction process with the extraction buffer therefore it is only necessary to add cold isopropanol (-20^{o}C) to initiate the precipitation process.

The nucleic acid precipitate is then collected using high speed centrifugation which forces it to collect at the bottom of the sample tube. The supernatant is aspirated, leaving a pellet of crude nucleic acids. The successive treatments, or washes, with 70% ethanol allows for additional purification of the nucleic acid from any residual salts. The nucleic acids can then be resuspended into solution by adding water or other appropriate buffers and is ready to use in downstream applications.

2. Chromatography

Different chromatographic methods are based on a number of separation techniques as outlined below:

- *Gel filtration:* exploits the molecular sieving properties of porous gel particles. A matrix with a defined pore size allows smaller molecules to enter the pores by diffusion, whereas bigger molecules are excluded from the pores and eluted at the void volume. Thus, molecules are eluted in order of decreasing molecular size.
- *Ion exchange:* is a technique that utilizes an electrostatic interaction between a target molecule and a functional group on the column matrix. Nucleic acids, which are highly negatively charged,

linear polyanions, would interact with a positively charged ion exchange column and can subsequently be eluted with simple salt buffers.

- *Adsorption chromatography*: utilizes the properties of solutes in a mobile liquid or gaseous phase to selectively adsorb onto the surface of a stationary solid phase. Nucleic acids, for example, adsorb selectively onto silica or glass in the presence of certain salts while other biological molecules do not. A low salt buffer or water can then be used to elute the nucleic acids, producing a sample that may be used directly in downstream applications.
- *Affinity binding:* utilizes the specific biochemical binding interactions between molecules. A specific ligand is bound to a solid phase support. Any molecules in the mobile phase that have specific binding affinity to the ligand become bound to it. After other sample components are washed away, the bound molecule is eluted from the support.

IN THE LAB: Spin column-based extraction

Spin column-based extraction involves the use of any of several commercially available columns that contain a membrane that is impregnated with silica to facilitate the nucleic acid purification process. Under the right conditions the silica membrane selectively attracts, or "binds" the nucleic acids in the cell lysate through electrostatic interactions that allow for their adsorption onto the silica. The remaining contaminants in the cell lysate, such as polysaccharides, phenolic compounds, lipids and proteins are washed through the column.

The purification of nucleic acids using spin column-based extraction consists for several distinct steps following cellular lysis. While components of the buffers and solutions used are different based on the choice of commercial kit, they all serve similar purposes at any particular step.

1. Binding: a "binding buffer" is added to the cellular lysate to equilibrate it such that the conditions, such as pH or salt concentrations, are optimal for the binding of the nucleic acids to the silica membrane in an efficient manner. The sample mixture is then passed through the silica membrane using a centrifuge, which forces the mixture through the column. Nucleic acids are bound to the membrane while any remaining solution, or "flow through" is discarded.

2. Washing: subsequent to the binding step a wash buffer is passed through the membrane in order to remove any residual contaminants that may have inadvertently bound to the silica. This may be done two or three times in order to ensure that the maximum amount of contaminant(s) has been removed from the membrane.

3. Elution: Water or another appropriate elution buffer is added to the membrane which results in the nucleic acids separating from the membrane and resuspending into solution. The final eluate containing the nucleic acid is centrifuged off the membrane and can be used for downstream applications.

3. Centrifugation

Selective centrifugation is a powerful purification method. For example, ultracentrifugation in self-forming CsCl gradients at high g-forces has long been used for plasmid purification. Frequently, centrifugation is combined with another method. An example of this is spin column chromatography that combines gel filtration and centrifugation to purify DNA or RNA from smaller contaminants (salts, nucleotides, etc.), for buffer exchange, or for size selection. Some procedures combine selective adsorption on a chromatographic matrix (see above paragraph "Chromatography") with centrifugal elution to selectively purify one type of nucleic acid.

4. Magnetic separation[15]

In recent years, more and more purification methods have combined affinity binding with magnetic separation. For instance, streptavidin-coated magnetic particles linked to biotin-labelled oligo(dT) tag can be used to purify mRNA as a result of the tag's affinity with the mRNA the poly(A) tails. The particle complex is then removed from the solution with a magnet, leaving behind any unbound contaminants. This solid phase technique simplifies nucleic acid purification since it can replace several steps of centrifugation, organic extraction and phase separation with a single, rapid magnetic based separation step.

Considerations for quality assurance and quality control during sample extraction

Quality assurance and quality control procedures have to be applied during the sample extraction process. This includes the use of controls in order to ensure that the extraction was carried out efficiently and to monitor for contamination. Table 7 outlines the two common extraction controls that need to be present during the extraction procedure.

Table 7. Common controls that can be used to monitor reagent and environmental conditions during DNA extraction.

Name	Description	Purpose	Expected result
Extraction blank control	Control in which the sample is substituted by nucleic acid-free water and that follows all the extraction steps	Monitor contamination during the extraction procedure and in the DNA extraction reagent	Negative
Positive extraction control	Control sample positive for the target analyte (it can be certified reference material)	Monitor the extraction and PCR amplification of a sample containing the target analyte	Positive

DNA quantification

Following the extraction and purification of DNA from a sample it is useful to get an assessment of the nucleic acid concentration which is an important factor when it comes to the PCR test and limit of detection which can be achieved (maximum load of DNA mass per PCR, genome size etc.). There are several methods that can be used to measure nucleic acid concentrations. The theory and practical application of some of the more commonly used methods is presented below.

Spectrophotometric quantification

All molecules absorb radiant energy at a specific wavelength, including nucleic acids, which absorb radiant energy in the ultraviolet range. Therefore, nucleic acid concentration can be quantified directly in aqueous solutions by using a spectrophotometer to measure the amount of ultraviolet light absorbed by the sample. The nucleic acid concentration can then be calculated using the Beer-Lambert law, which describes a linear relationship between absorbance and the concentration of the target macromolecule using the following equation:

$$\mathbf{A = OD = \varepsilon lc}$$

[15] http://www.nature.com/nature/journal/v340/n6236/abs/340733a0.html http://cmr.asm.org/content/7/1/43.full.pdf.

where:

A:	Absorbance
OD:	Optical density
ε:	Molar extinction coefficient
c:	Concentration
l:	Cuvette path length

The maximum absorbance of DNA and RNA solutions is at a wavelength of 260 nm. Therefore, their concentration is determined by measuring absorbance by the sample at 260 nm against a blank. It is important to mention the fact that spectrophotometry cannot be used to distinguish between the concentration of DNA or RNA present in a solution. If the sample has been well purified and is without significant amounts of contaminants, such as proteins, carbohydrates or phenols, the spectrophotometric measurement of the amount of ultraviolet irradiation absorbed by the nucleic acids is simple and accurate. For this method, aqueous buffers with low ion concentrations, such as TE buffer, are ideal.

However, contaminants may continue to be present in spite of the purification process, which will influence the accuracy of the quantification process. Interference by such contaminants can be recognized by the calculation of a "ratio" as follows:

- Proteins, like nucleic acids, absorb light in the ultraviolet range with a maximum absorbance at 280nm. Since, nucleic acid solutions partially absorb light at 280 nm, and protein solutions partially absorb light at 260 nm, the ratio between the readings at 260 nm and 280 nm (A_{260}/A_{280}) can be used to provide an estimate of the purity of the nucleic acid solution. Well-purified DNA and RNA solutions have A_{260}/A_{280} values of 1.8 and 2.0 respectively. If there is protein contamination the A_{260}/A_{280} ratio will be significantly lower.
- Similarly, absorption at 230 nm indicates the presence of contaminants such as carbohydrates, peptides, phenols or aromatic compounds. Well-purified samples should have an A_{260}/A_{230} ratio of approximately 2.2.
- An absorbance at 325 nm can be used to indicate the presence of debris in the solution or that the cuvette itself is dirty.

Spectrophotometers

A spectrophotometer is a piece of laboratory equipment that transmits light through a solution to determine the concentration of a target solute. The apparatus operates on the basis of a simple principle in which light of a known wavelength is passed through a cuvette containing a sample and the amount of light energy transmitted is measured with a photocell on the other side of the cuvette.

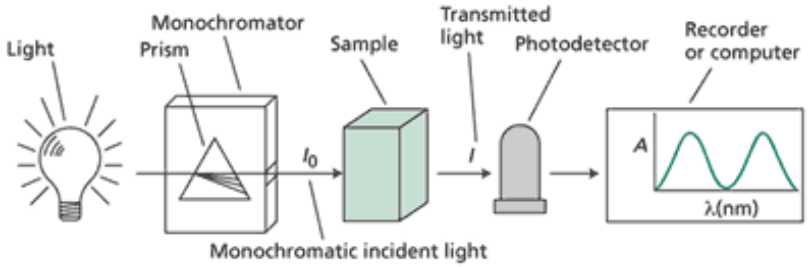

Figure 13: Schematic representation of the components of a spectrophotometer http://6e.plantphys.net/topic07.01.html.

As shown in Figure 13, the design of the single beam spectrophotometer involves a light source, a prism, a sample holder and a photocell. Connected to each are the appropriate electrical or mechanical systems to control illumination intensity, desired wavelength and for the conversion of energy received at the photocell into a voltage fluctuation. The voltage fluctuation is then displayed on a meter scale or is recorded via connection to a computer for analysis.

IN THE LAB: Spectrophotometric determination of DNA concentration

Choice of the cuvette

The amount of nucleic acid solution needed to measure absorbance depends on the cuvette's size and capacity, which can range from 1 ml to microvolume cuvettes with a capacity of 5 to 70 µl. The choice of cuvette should therefore be based on the sample concentration range, dilution factor and available sample volume.

Spectrophotometer calibration

Prior to analysing any samples, the spectrophotometer needs to be calibrated in order for the machine's software to take into account the presence of background absorbance that may result from the elution buffer. The following steps provide a general description of the steps to follow during the calibration process; however, they may vary slightly depending on the specific machine being used.

- Select the correct cell path length based on the choice of cuvette being used.
- Set the target molecule; dsDNA, ssDNA or RNA
- Measure a solution blank, which consists of water or an appropriate buffer solution, to set the calibration reference. It is important that the solution blank is renewed periodically.
- Measure a known amount of pure nucleic acid in order to verify the reliability of the solution blank.

Measurement of an unknown sample.

Depending on the capacity of the cuvette used, a specific amount of the DNA extract is used to evaluate the solution's concentration, for example if the cuvette has a capacity lower than 0.2 ml then 5 µl of DNA extract is diluted in 195 µl of water to measure absorbance. After calibrating the spectrophotometer and adding the nucleic acid solution to the cuvette, it is capped, the solution mixed, and the absorbance measured. In order to reduce pipetting errors, the measurement should be repeated at least twice and at least 5 µl of the DNA solution should always be added to the cuvette.

It is recommended that A_{260} readings lower than 0.02 or between 1 and 1.5 (depending on the instrument used) are disregarded due to the possibility of a high margin of error.

The concentration c of a specific nucleic acid present in a solution is calculated using the following equations:

- Single-stranded DNA: $c(\text{pmol}/\mu\text{l}) = A_{260}/0.027$
- Double-stranded DNA: $c(\text{pmol}/\mu\text{l}) = A_{260}/0.02$
- Single-stranded RNA: $c(\text{pmol}/\mu\text{l}) = A_{260}/0.025$
- Oligonucleotide: $c(\text{pmol}/\mu\text{l}) = A_{260}*100/1.5NA+0.71NC+1.2NG+0.84NT$

For example, the absorbance readings of highly purified calf thymus DNA suspended in 1x TNE buffer assuming that the reference DNA is dsDNA, with an A_{260} = 1, the concentration of DNA was nominally 25µg/ml.

For a 10 mm path length cuvette and a 260 nm wavelength, an absorption A = 1 corresponds to approximately 50 µg/ml of dsDNA, approximately 37 µg/ml of ssDNA, 40 µg/ml of RNA or approximately 30 µg/ml of oligonucleotides.

Spectrophotometry is a method that continues to be commonly used in many laboratories due to the relative ease with which the process is performed. Furthermore, it also provides an estimate of DNA purity through the A_{260}/A_{280} measurement.

On the other hand, one drawback of the use of spectrophotometry lies in the need for a relatively large volume of DNA sample in order to take an accurate measurement. In addition, the signal obtained from a spectrophotometer is more susceptible to contaminants, such as proteins and RNA, which may lead to an overestimation of DNA concentration.

Technological advances in spectrophotometric instruments have led to the development of microvolume spectrophotometers, such as the NanoDrop™. Such instruments require a sample size of 1–2µL and do away with the need for a cuvette.

Fluorometric quantification

Fluorometric quantification of DNA concentration relies on the use of dyes that selectively bind to DNA. Upon binding, the dye undergoes a conformational change leading to the emission of fluorescent energy, which can be detected by the appropriate equipment. Examples of such dyes include ethidium bromide, SYBR® Green, Hoechst (bis-benzimide) dyes and PicoGreen®. The amount of fluorescence emitted by these dyes is directly proportional to the concentration of DNA present in a sample. Therefore, concentration can be estimated by comparing the intensity of the fluorescence emitted by the sample with a series of concentration standards using, for example, a mass ladder on a gel or using a calibration curve established using a fluorometer, as shown in Figure 14.

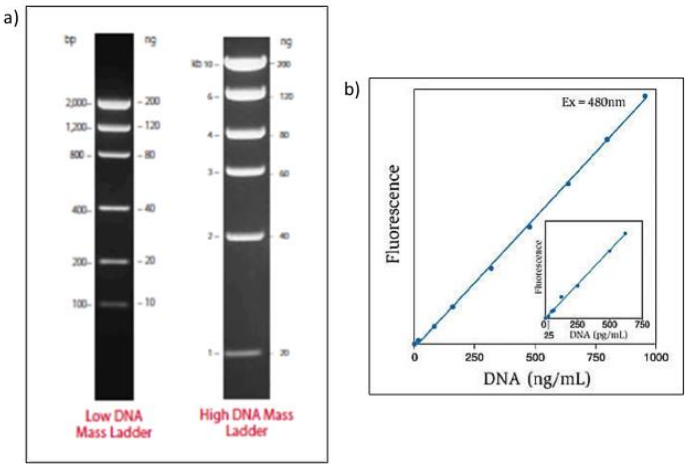

Figure 14: (a) DNA mass ladders consist of equimolar mixtures of DNA fragments for the estimation of the mass of unknown DNA samples on gels. Source: https://www.thermofisher.com/ca/en/home/life-science/dna-rna-purification-analysis/nucleic-acid-gel-electrophoresis/dna-ladders/dna-size-markers-mass-ladders.html. (b) Calibration curve showing the linear quantification of calf thymus DNA at concentrations ranging from 25pg/ml to 1000ng/ml

using the PicoGreen® dsDNA quantitation reagent. Source: https://www.thermofisher.com/order/catalog/product/P7589.

The advantages of using fluorometric DNA quantification methods over the use of absorbance are due to the selectivity of the dyes in binding to DNA to the exclusion of contaminants that may be present in the DNA solution. This selectivity reduces the interference that can be observed from proteins and carbohydrates that may be present in the DNA extract, therefore leading to a misestimation of the actual concentration of DNA. The use of DNA-specific dyes also allows fluorometric DNA quantification methods to distinguish between DNA and RNA. Furthermore, the high affinity of the dyes to DNA and the ability to measure signals from low DNA concentrations makes fluorometric DNA quantification methods more sensitive than spectrophotometric methods and therefore lower concentrations of DNA can be measured with greater accuracy.

However, some drawbacks include the relatively greater expense of using fluorometric DNA quantification methods due to the need to purchase the dyes in order to perform the test as well as the potential health risks associated with the possible misuse of some of the fluorometric dyes.

IN THE LAB: Verification of DNA integrity

In the case of DNA quantification using spectrophotometry or fluorometry, it is advisable that, once the DNA has been quantified, its integrity be verified by amplifying a species-specific reference gene using real-time PCR. This is because measurements such as A_{260}/A_{230} and A_{260}/A_{280} relationships are not indicators of the quality of the DNA. Furthermore, the amplification of a reference gene also demonstrates the presence or absence of PCR inhibitors.

Quantification using gel electrophoresis

Electrophoresis through an agarose gel is another possible method of estimating the concentration and quality of extracted DNA (see section 4 for more information on Gel Electrophoresis). This method relies on the use of DNA mass ladders which DNA fragments that are of known quantity. To estimate the quantity of DNA that is present in a sample a comparison is made between the intensity of the fluorescence emitted from the band in a sample to a band in the DNA mass ladder. This can be carried out by any number of available gel analysis software.

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Module 4

Techniques for detection and identification

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References

Introduction

A number of methodologies and techniques are available to detect, identify and quantify living modified organisms (LMOs). These methodologies generally target one of two components, specifically, the proteins that are expressed by the LMO or the DNA insert that was transferred into the organism's genome.

When planning and setting up a laboratory for the detection and identification of LMOs a choice must be made regarding which methodologies and protocols will be adopted. The techniques that are commonly used to target either of these components, as indicated in table 1, range from those that are fast and more cost-effective, such as lateral flow tests and endpoint PCR, to those that can be more complex, such as quantitative real-time PCR. Furthermore, the methodologies range from qualitative methods to detect the presence of LMOs, to tests that identify individual LMOs, to quantitative tests that measure the percentage of LMOs present in a sample. All of these methodologies have their advantages and drawbacks that need to be taken into account when selecting which of the methodologies should be adopted by the laboratory.

In addition, for service-oriented laboratories, particularly those servicing regulatory authorities, the selection of methods is guided by, among other things, the country's specific regulatory requirements in accordance with national biosafety laws, and should include an assessment of the nature of the goods that would be commonly under investigation as well as the available technical capacity within each individual laboratory to successfully preform the methods of choice.

This module presents an overview of the theoretical and practical aspects of some of the more commonly used methodologies for the detection, identification and quantification of LMOs, including their strengths and limitations.

Protein-based methods

Introduction

One approach for the detection and identification of LMOs is through the immunological detection of the protein that has been expressed as a result of the insertion of the gene during transformation. The principal behind this technique is the use of antibodies as test reagents. Antibodies are immune system specific proteins that selectively bind to the substance that elicited their production, otherwise known as an antigen.

Antibody production

Antibodies are made by injecting the protein of interest, such as CP4 EPSPS, the protein that confers resistance to the herbicide glyphosate, into animals such as rabbits and mice. The animal's immune system recognizes the substance as "foreign" and responds by producing antibodies against it. The antibodies are then purified, conjugated to a detection label, and then used as a reagent to detect the protein of interest.

Therefore, a prerequisite for the use of immunological detection methods is that antibodies that are specific to the transgenic protein of interest are available. While antibodies can be powerful tools in the detection and identification of LMOs there are some considerations that need to be taken into account that may affect the success of immunological testing, such as:

- A genetic modification does not always lead to the production of a new protein, for example LM plants have been developed using RNAi.
- Certain proteins may be under the control of selective promoters and may, therefore, only be expressed in specific parts of the LMO or during specific stages of physiological development.
- The expression levels of transgenic proteins in plants can be variable, even when strong constitutive promoters are used to drive expression the expression levels may be too low for immunological testing to be successful. This can be particularly challenging when attempting to detect a small number of LMO seeds in a bulk sample.
- LMOs might express insoluble proteins that are hard to tackle/target with easy-to-use immunological methods
- All immunoassays are based on the specific binding of antibody to antigen therefore the sample should not be significantly compromised by exposure to harsh environmental conditions or degraded in order for the integrity of the antigen to be maintained. This might demand specific storage conditions of the sample.

There are several testing applications that are based on immunological testing, specifically, lateral flow strip tests and the enzyme-linked immunosorbent assay (ELISA). In the following sections an explanation of each of these testing methods will be provided as well as their applications in GMO detection.

Lateral flow assay

A lateral flow assay is a simple and easy-to-use detection method that is based on the use of immunochromatography to confirm the presence or absence of a specific analyte. The assay is available in the form of a test strip impregnated with antibodies. These antibodies recognize and bind to a specific antigen that is the target of the testing. The test strips are composed of the following components, as shown in figure 15:

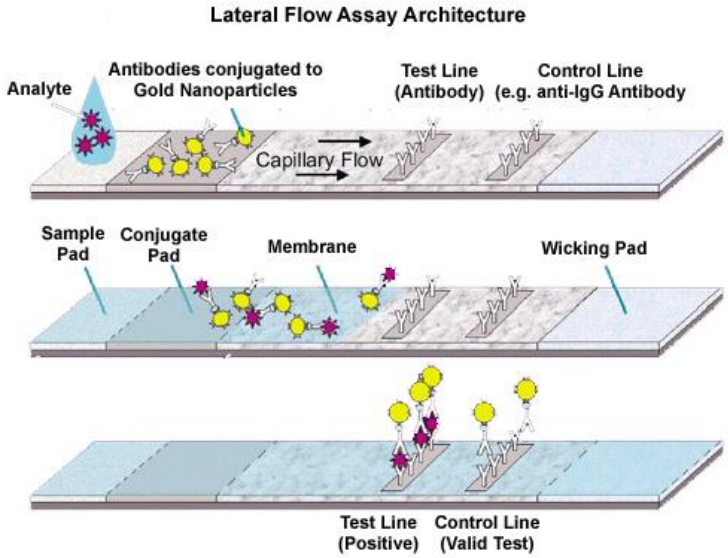

Figure 15: Schematic diagram of the structure and components of a lateral flow assay test strip. Source: http://www.cytodiagnostics.com/store/pc/Lateral-Flow-Immunoassays-d6.htm.

1. Sample pad: This is the section of the test strip to which the liquid test sample is applied. It is made of a porous material that facilitates the transport of the test sample, via capillary action, upwards through the test strip.

2. Conjugate pad: This area contains a secondary antibody that is specific to the target analyte which is being tested for in the sample. The secondary antibodies are labelled, or conjugated, with a reporter molecule, such as an enzyme, nanoparticles or fluorescence dyes. As the sample passes through this section of the strip the antibodies recognize and bind to the target analyte and continue flowing through the test strip as a combined sample-antibody complex.

3. Reaction membrane: This section of the strip contains the test and control lines of the assay and is where the result of the test is displayed.

    a. The test line contains an immobilized primary antibody that is also analyte specific and therefore has the capacity to capture the analyte as the sample-antibody complex passes over the test strip. This forms an analyte "sandwich" that results in the activation of the reporter molecule leading to a visual colour change at the test line in the presence of the target analyte.

    b. The control line is present to confirm that the reaction conditions and components of the test strip are functioning as expected. It contains immobilized antibodies that bind the secondary antibody whether or not the analyte is bound to it. When the two antibodies bind, the reporter molecule is activated, leading to a visual colour change that should take place with every test strip.

4. Wicking pad: This portion of the test strip contains additional absorbent material that acts as a sink to facilitate the movement of the sample through the test strip and maintain capillary flow.

In the context of LMO testing using the lateral flow assay, the test strip is placed in a crude protein extract of the seed or plant part and a positive result is indicated by the appearance of a test line due to the antibody recognition of the transgenic protein as well as the presence of a control line which indicates that the test is functioning as expected. The absence of a control line renders the test invalid even if the test line displays a colour change, as indicated in figure 16.

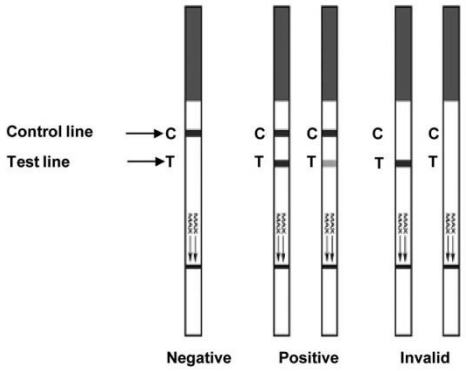

Figure 16: Representative results of a lateral flow assay. Source: Adapted from https://www.spandidos-publications.com/ijmm/30/5/1041.

The advantages of this qualitative method are that it is simple to perform, requires little technical expertise or equipment and can be performed at the site of sampling. Electronic devices have also been developed that allow a semi-quantitative interpretation of the result.

IN THE LAB

To perform a lateral flow test on a bulk sample of seeds:

1. For seed samples, place the sample into a clean, dry grinding jar and grind with a blender on high speed for about 30 s or until all the grains are ground.

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 ground 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 ImmunoStrip®

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.

ELISA technique

The enzyme-linked immunosorbent assay, ELISA, is another type of immunological testing procedure that is based on the use of the specific interaction that takes place between antibodies and their antigens, which in the case of the detection of LMOs, are the proteins synthesized by the newly introduced genes.

There are three different forms of the ELISA assay, as shown in figure 17, each of which is based on the same basic principal of immobilizing the antigen, either using an antibody or directly to a solid surface, followed by the use of an enzyme-linked antibody to assess for the presence of the target antigen through the enzyme's catalysis of a substrate to a detectable signal, typically a colour change. Examples of such enzymes include horseradish peroxidase or alkaline phosphatase.

Direct ELISA

In a direct ELISA the antigen, which is contained in a crude protein extract, is bound directly to a solid surface, typically a polystyrene multi-well plate. Detection of the antigen is achieved by a single antibody, which is linked to the reporter enzyme.

Indirect ELISA

Like a direct ELISA, the antigen is also bound to a solid surface during an indirect ELISA. However, rather than using a single antibody to detect the antigen, two antibodies are used. The first, primary, antibody is specific to the antigen and it is not labelled with a reporter enzyme. After incubation with the antigen the excess primary antibody is washed away and a secondary antibody is added to the sample well. This antibody is directed against the primary antibody and it is conjugated to the reporter enzyme.

Sandwich/ capture assay

In this version of the ELISA assay the antigen is not bound to the solid surface, rather an antibody, known as a capture antibody, is immobilized. When the sample is added, the capture antibody binds the antigen. Unbound components of the sample are removed by washing. Subsequently, a primary antibody, which is specific to a second epitope of the target antigen, is added to the sample. This antibody may be directly conjugated to the reporter enzyme (direct detection) or a pair of unlabelled primary and conjugated secondary antibodies (indirect detection) may be used to carry out the detection step, as shown in figure 17. This version of the ELISA assay is the one most commonly used in detection and identification of LMOs.

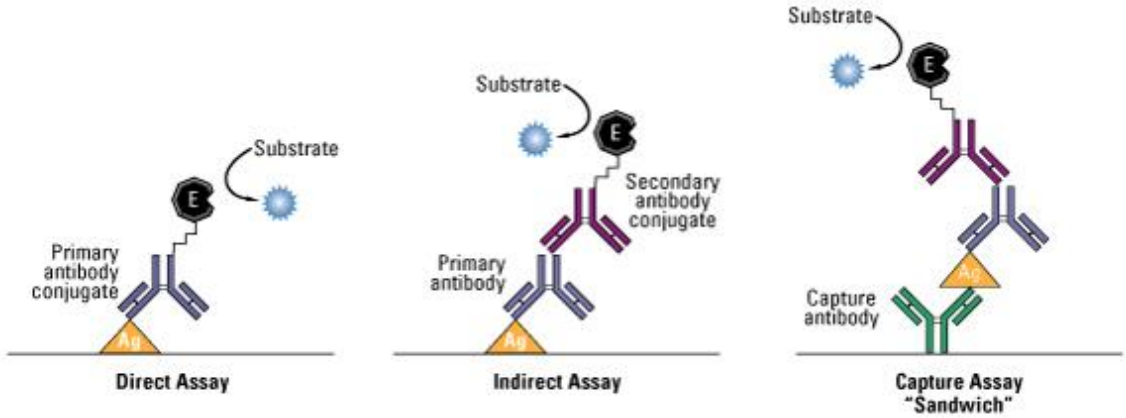

Figure 17: Schematic diagram of the three types of commonly used ELISA assays. Source: https://www.thermofisher.com/ca/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-elisa.html.

Given the nature of the ELISA test it is possible to quantify the amount of antigen present in a sample since the intensity of the colour change observed within the sample is directly proportional to the concentration of the antigen, as indicated in figure 18.

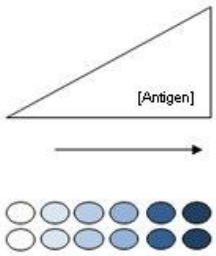

Figure 18: Schematic representation of the correlation between signal intensity and antigen concentration. Source: https://www.bio-rad-antibodies.com/elisa-types-direct-indirect-sandwich-competition-elisa-formats.html.

Therefore, concentration can be estimated by comparing the intensity of the colour changes produced by the sample with those of a series of concentration standards. The intensity of the colour changes in sample and concentration standards can be quantified using a microplate reader. ELISA tests can detect the presence of LMOs at concentrations ranging from 0.1% to 5% of the crude extract. The sensitivity of ELISA tests varies depending on the quality of the antigen/ antibody interaction and the integrity of the protein in the crude extract.

Availability of protein-based methods

In the context of LMO detection, ELISA has been widely applied to evaluate the expression level of the protein(s) synthesized by the newly introduced gene(s) at the experimental stage. Information regarding production and use of specific antibodies can be therefore found in many articles describing the developments of transgenic organisms. However, these antibodies re generally made available as part of a kit or integrated into a lateral flow strip[16] rather than as raw antibodies.

Advantages and limitations of protein-based methods

Protein based analysis can be a quick and easy tool that can be used to detect and identify LMO traits. They may also be more affordable compared to DNA based detection methods. Their ease of use also means that they can be used at points of sampling at borders and in the field as well as being used in the laboratory. There are also some limitations to their usefulness.:

Antibody sensitivity

The sensitivity of the antibodies used in protein-based methods is dependent on the binding affinity between the antibody. This factor affects the sensitivity of the assay. Detection of the protein can be compromised by a number of factors such as degradation due to excessive sample processing.

Antibody specificity and cross-reactivity

Antibodies can also bind an unintended antigen if it is structurally similar to the antigen contained within the protein of interest therefore leading to a false positive signal. Therefore, the LMO event cannot be identified. This can be controlled for in the design of the kits that are commercially available.

Restriction to LMOs with traits/modifications for protein expression

Only LMOs expressing (detectable amounts of) protein as the result of the genetic modification can be detected with protein-based detection methods.

DNA-based methods

Introduction

The polymerase chain reaction (PCR) is an in vitro molecular biology technique used to enzymatically amplify a specific region of DNA. It was invented by K. Mullis et al. in 1985 and has since revolutionized molecular biology and molecular medicine. It forms the cornerstone for many common experimental procedures such as DNA cloning, detecting and identifying genes for diagnostic and forensic purposes,

[16] A number of commercially available ELISA and lateral flow assay kits are available from companies such as EnviroLogix, AgDia and Romer.

and for the investigation of gene expression patterns. More recently, PCR has been applied to a number of new and emerging fields such as investigating the authenticity of foodstuff, the presence of genetically modified DNA and the presence of microbiological contamination.

In the following sections the nature of DNA, including its structure and in vivo replication, will be described. This will be followed by an explanation of the theoretical and practical principles of PCR and its applications in LMO detection.

Nature of DNA

Components of DNA

The basic building blocks of DNA are known as nucleotides. A nucleotide is made up of three different chemical components:

1. A phosphate group
2. A pentose sugar molecule, known as deoxyribose
3. One of 4 nitrogenous bases: adenine and guanine, which have double ring structures, known as purines, and, cytosine and thymine which have single ring structures, known as pyrimidines.

When the base binds with the sugar, they form a nucleoside: adenosine, guanosine, cytidine and thymidine. A nucleotide is formed when a purine or pyrimidine base is bound to the pentose ring by an N-glycosylic bond and the phosphate group is bound to the 5' carbon atom of the sugar by a diesteric bond, as shown in figure 19.

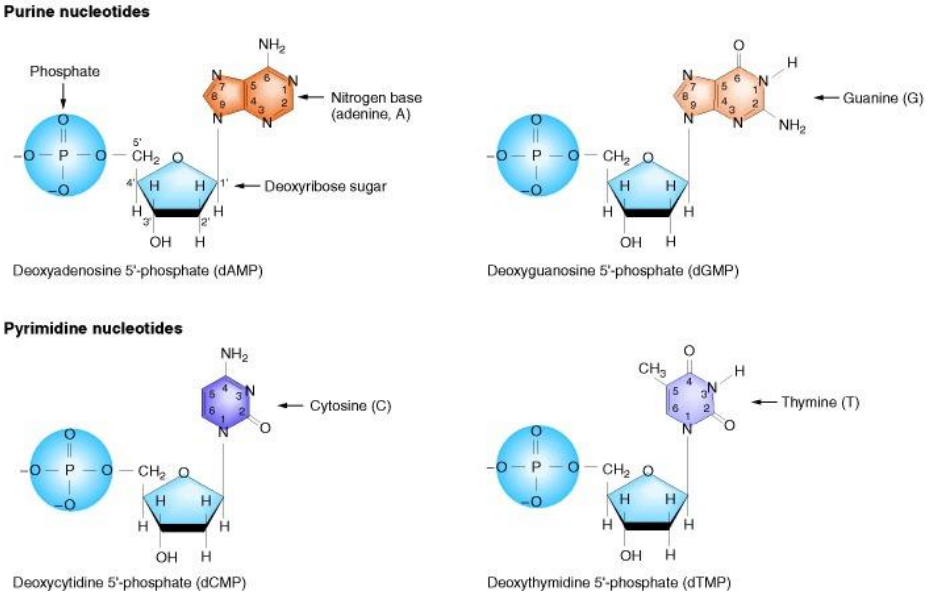

Figure 19: Chemical structure of the four nucleotides that form the building blocks of DNA. Source: Griffiths A.J.F., Gelbart W.M., Miller J.H., et al. Modern Genetic Analysis. New York: W. H. Freeman; 1999. The Nature of DNA. Available from: https://www.ncbi.nlm.nih.gov/books/NBK21261/.

DNA structure

A strand of DNA is formed by linking a series of nucleotides together. This is achieved through formation of a phosphodiester bond between the hydroxyl group on the third carbon atom of the deoxyribose sugar molecule and the phosphate group of the subsequent nucleotide. Such a series of linked phosphate and deoxyribose sugar moieties form what is known as a sugar-phosphate backbone. Furthermore, the specific order in which nucleotides are added gives strands of DNA directionality, that is referred to as 5'→3'.

Once a single strand of DNA is made it will pair up with another strand through the bases that are attached to the sugar-phosphate backbone. Bases pair up with each other in a very specific manner to form what is known as a "base pair" due to the formation of hydrogen bonds. Adenine will always pair with thymine through two hydrogen bonds while cytosine will always pair with guanine through three hydrogen bonds. This complementarity between the bases results in the formation of two complementary DNA strands that arrange themselves in an antiparallel orientation into a double helix, i.e. one strand runs from 5'→3' while the complementary strand runs in the 3´→5 direction. Under physiological conditions, a double-stranded DNA helix is more stable than a single-stranded DNA helix since the bases form a hydrophobic core within the double helix while the sugar-phosphate backbone forms an external hydrophilic layer.

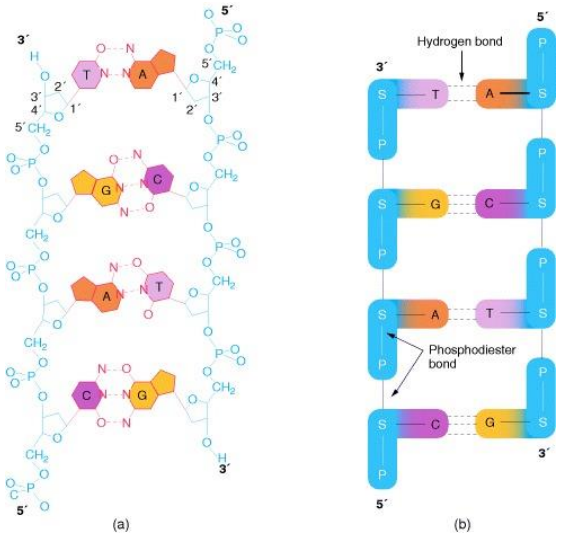

Figure 20: A schematic representation of (a) the base pairing between the bases of complementary DNA stands and (b) the antiparallel orientation of the DNA strands within a double helix. Source: Griffiths A.J.F., Gelbart W.M., Miller J.H., et al. Modern Genetic Analysis. New York: W.H. Freeman; 1999. The Nature of DNA. Available from: https://www.ncbi.nlm.nih.gov/books/NBK21261/.

DNA replication

The aim of DNA replication is to synthesize a second, identical copy, of the existing DNA double helix with the view to preserving and propagating the genetic information. During DNA replication, the double helix unwinds, with each single strand becoming a template for the synthesis of a new, complementary strand. Each of the newly synthesized DNA helices consists of one parent strand and daughter strand. Hence, DNA replication is semi-conservative.

Several enzymes are required in the DNA replication process, as indicated in figure 21.

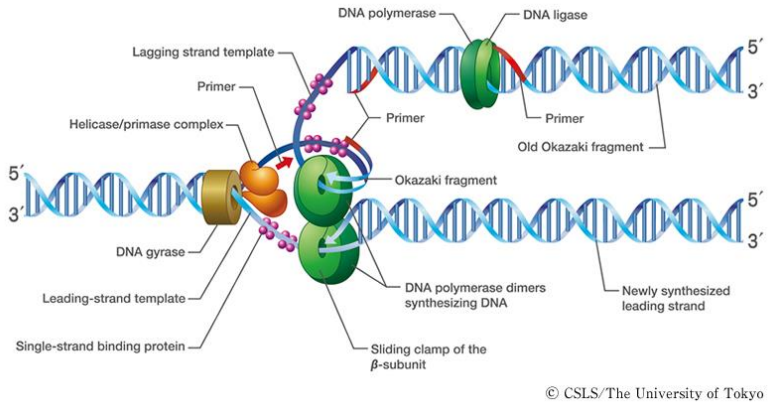

Figure 21: Schematic representation of the enzymes involved in DNA replication at the replication fork. Source: http://csls-text3.c.u-tokyo.ac.jp/active/07_04.html.

The first set of enzymes, topoisomerase/ DNA gyrase and helicase, are responsible for unwinding the DNA double helix in order to make it possible for the other enzymes of the replication machinery to access each of the single strand templates of DNA. Furthermore, the two enzymes help minimize any supercoiling of the DNA by nicking and re-joining single strand of the double helix. Single-stranded binding proteins are also recruited to the replication fork as they play an important role in maintaining the stability of the replication fork. Furthermore, single-stranded DNA is very unstable, so single-stranded binding proteins protect it from degradation.

Since the two strands of the DNA helix are arranged in an antiparallel orientation the synthesis of the daughter strand based on the 3'→5' template, also known as the leading strand, is different than that of the 5'→3' template, otherwise known as the lagging strand since DNA can only be synthesized from 5'→3'.

Synthesis of the leading strand proceeds directly, from 5'→3', as the helix unwinds. However, DNA polymerase cannot start synthesizing *ex novo* on a bare single stranded template. It needs a primer with a free 3'OH group onto which it can attach a dNTP. This is achieved by the enzyme primase, which is a part of an aggregate of proteins called the primosome. This enzyme synthesizes a small RNA primer on the leading DNA strand from which DNA polymerase can begin its synthesis. Once it is recruited to the template, DNA polymerase continues synthesizing the daughter strand using the parent strand as a template. This involves the recruitment of the appropriate free dNTP to its complementary base on the template. The DNA polymerase then catalyses the formation of the 5'→3' phosphodiester bond between the nucleotides. Once DNA synthesis is complete the RNA primers are removed by and the gaps are filled in by a DNA polymerase.

As stated above, DNA can only be synthesized from 5' → 3'. Accordingly, the synthesis of the lagging strand proceeds in a discontinuous manner and takes place in spurts of short 100 base pair fragments of DNA. These short fragments are known as Okazaki fragments, as shown in figure 22. The Okazaki fragments are then joined together with the enzyme DNA ligase following the removal of the primers.

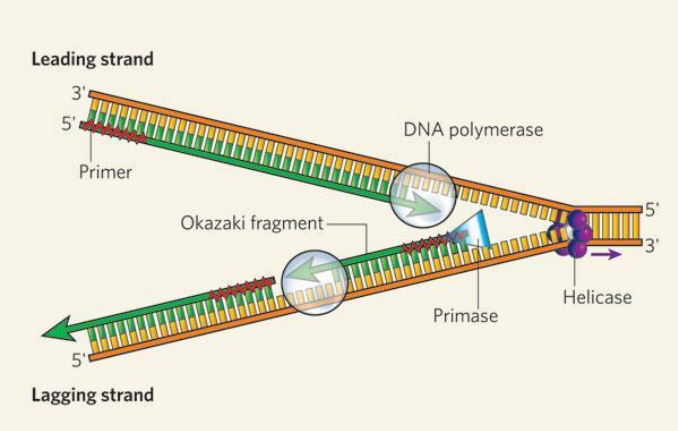

Figure 22: Schematic diagram of DNA replication of the leading strand and the lagging strand, including Okazaki fragments. Source: http://www.nature.com/scitable/content/dna-replication-of-the-leading-and-lagging-14668888.

Principles of PCR

PCR is based on the mechanism of DNA replication in vivo whereby a double stranded DNA helix is unwound to form two single stranded DNA molecules that can act as templates which are then duplicated and rewound. To successfully perform a PCR the conditions in the reaction tube need to mirror the conditions that are present in vivo. This includes the addition of a set of critical reagents and components for the PCR and exposing these components to a series of very specific temperature conditions that are needed to drive the reaction forward.

Critical reagents and components of PCR

Target DNA

This is the extracted DNA that contains the sequence of DNA that is targeted for amplification. The target sequence can be anywhere from less than 100 to a few thousand bases in length. The mass of DNA template that is typically added to a PCR is between 0.05-1.0 µg. In principle, a target sequence can be amplified using PCR if at least one intact copy of the sequence is present in the reaction mixture. However, the higher the number of target copies available the greater the probability of a successful amplification. Furthermore, a higher amount of DNA may compensate for the presence of any damage in the template DNA which may inhibit PCR and allow for the detection of single copies of target sequence.

Primers

Primers are a pair of short oligonucleotides that are specifically designed to have a high level of complementarity to the regions flanking the segment of DNA that is targeted for amplification. Generally, primers are between 18-24 nucleotides in length with a melting temperature between 55-65^{o}C. Primers are usually added to a PCR at a concentration of 1 µM. The presence of primers at higher concentrations can result in mispriming, primer-dimer formation or amplification of non-target sequences. Conversely, PCR efficiency is reduced if primers are available in limiting concentrations.

Melting temperature

The temperature at which half of the double stranded DNA has dissociated into single stranded DNA is known as the melting temperature (Tm).

Tm, and by extension the denaturation process, can be influenced by a number of reaction conditions. For example, at low salt concentrations, high pH and in the presence of organic solvents such as formaldehyde, the melting temperature Tm decreases.

Furthermore, the concentration of G/C, which share 3 hydrogen bonds, and T/A, which share 2 hydrogen bonds, can also affect the Tm. Therefore, the Tm of DNA containing an elevated G/C content is higher compared to that of T/A rich DNA. For example, the *Serratia marecescens* genome has a G/C content of approximately 60% and a Tm of approximately 94^{o}C, whereas the *Pneumococcus* genome has a G/C content of approximately 40% and Tm of approximately 85°C.

An estimate of melting temperature can be calculated using the following equation:

$$Tm = 2(A+T) + 4(G+C)$$

where A, T, G, C are the number of purinic and pyrimidinic bases in the primer.

One of the more critical parameters for a successful PCR is the use of well-designed primers. The primer sequence determines several things such as the position and length of the product, its melting temperature and ultimately the yield. A poorly designed primer can result in little or no product due to non-specific amplification and/or primer-dimer formation, which can become competitive enough to suppress product formation. Ideally a primer will have a near random mix of nucleotides, 45-55% GC content and be approximately 20 bases long with a Tm in the 55-65°C range. Below is an overview of some of the considerations that contribute to good primer design.

Primer length

The length of a primer contributes to its specificity, temperature and annealing time. Primers that are between 18-24 bases long are extremely sequence-specific, provided that the annealing temperature is optimal. A general rule-of-thumb is to use an annealing temperature that is 5°C lower than the melting temperature. Optimal annealing temperature should be practically determined using a temperature gradient PCR experiment. Primer length is also proportional to annealing efficiency, with longer primers resulting in less efficient annealing which would lead to fewer templates that are primed at each step and ultimately a decrease in yield.

Melting temperature (Tm)

It is important to keep in mind that both PCR primers should be designed to have a similar Tm. If there is a mismatch in Tm, a reduction in efficiency or a failure of the amplification will be observed.

Specificity

Primers must be designed such that they are complementary to a unique region within the template DNA. Primers that contain running stretches of the same nucleotide, such as poly dG, or repeating motifs would have a higher rate of nonspecific priming.

Inter/ Intra-primer homology

Primers need to be designed with no intra-primer homology, meaning that no regions within the primer should be complementary to each other. This is in order to avoid the formation of secondary structures such as “hairpins”, which will interfere with the primer annealing to the template. Similarly, inter-primer

homology, meaning the presence of complementary regions between the two primers, should also be avoided. This may lead to primer dimer formation and therefore a reduction in yield.

GC content

Primers should be designed to contain between 45-55% G and C bases. Stretches of G/C bases should be avoided in order to reduce the likelihood of non-specific annealing. Similarly, stretches of A/T bases should also be avoided, as these will "breathe" and open up stretches of the primer-template complex, leading to reduced amplification efficiency.

3'-end sequence

The 3' end of a primer plays an important role in ensuring specific binding. It is therefore often recommended to include G or C residues at the 3' end of primers, which is known as a "GC clamp". This helps ensure correct binding at the 3' end, due to the stronger hydrogen bonding between G and C residues as well as minimizing any primer "breathing" that might occur.

DNA polymerase

The DNA polymerase elongates the primers to replicate the targeted DNA sequence. It should have an optimal working temperature of about 70^{o}C and be thermostable.

History and properties of DNA polymerases

When the PCR method was first described by Saiki et al., 1985 the polymerase of choice was the Klenow fragment of DNA polymerase I from *Escherichia coli*. This enzyme, however, denatures at temperatures lower than those required to melt the DNA template. Thus, in earlier PCR experiments, fresh polymerase had to be added to the reaction after each cycle. The discovery and use of thermostable DNA polymerases has facilitated the use of PCR because it was no longer necessary to add fresh polymerase to the reaction after every denaturation step.

The first thermostable DNA polymerase used was the Taq polymerase, which was isolated from the bacterium *Thermus aquaticus* that lives in the hot springs of Yellowstone National Park, USA. Even though this enzyme is probably the most widely used in PCR applications, several other DNA polymerases with varying properties are commercially available. Some of these properties include:

- **Processivity:** This is the average number of nucleotides, which a polymerase incorporates into DNA before detaching itself from the template. Taq, for example, synthesizes DNA at a rate of 35-100 nucleotides/sec.

- **5'- 3' exonuclease activity:** This property of polymerases allows it to remove nucleotides ahead of the growing chain. Both Taq, and AmpliTaq® possess a 5'-3' exonuclease activity.

- **3'-5' exonuclease activity:** This allows for proof reading by the DNA polymerase whereby nucleotide mismatches are replaced until the correct base is added. However, the 3'-5' exonuclease activity can cause primer degradation. Therefore, such enzymes, which include Vent^{TM}, DeepVent^{TM}, Pfu and UITma^{TM}, should only be added after the reaction has started, or alternatively, chemically modified primers should be used.

- **Hot-start activation:** Polymerases with hot-start properties, such as AmpliTaqGold^{TM}, are inactive at room temperature, and can only be activated following an incubation period at 94°C. This provides an increase in specificity, sensitivity and yield.

Deoxyribonucleoside triphosphates

Free deoxyribonucleoside triphosphates (dNTPs) are required for DNA synthesis, as they are the building blocks for generating the new strands of DNA. The working concentration of each dNTP is between 20-200 μM. Each of the four dNTPs should be used at equal concentrations in order to minimize misincorporation errors. High-purity dNTPs are supplied by several manufacturers either as four individual stocks or as a mixture. The pH of dNTPs stock solutions should be adjusted to 7.0-7.5 to ensure that the pH of the final reaction does not fall below 7.1; however, many dNTP stock solutions are now supplied with pH already adjusted.

Reaction buffers and $MgCl_2$

Successful PCR requires the use of a suitable buffer in addition to the reagents directly involved in DNA synthesis. The buffer composition depends on the type and characteristics of the polymerase being used and most commonly contain (a) 10 mM Tris, pH 8.3; and (b) 50 mM KCl. Most suppliers provide a 10x buffer for use with the respective polymerase.

In addition to the buffer components, the presence of divalent cations, in the form of magnesium chloride ($MgCl_2$), is critical. The magnesium ions function to:

- form a soluble complex with dNTPs which is essential for their incorporation
- stimulate polymerase activity
- stabilize the interaction of the primer to the template DNA

Low magnesium ion concentrations may lead to low/no yields whereas a high magnesium ion concentrations may lead to the accumulation of non-specific products due to mispriming. $MgCl_2$ concentration in the final reaction mixture is usually between 0.5-5.0 mM, and the optimum concentration is determined empirically. Furthermore, it is important to avoid the presence of contaminants such as chelating agents like EDTA or negatively charged ionic groups such as phosphates since these will alter the concentration of magnesium ions in the reaction. Multiplex PCR requires a specific reaction buffer that is optimized to amplify multiple target sequences in one single reaction.

Other additives and stabilizers

Current literature includes discussions on various PCR buffers and supplements, such as DMSO, PEG 6000, formamide, glycerol, spermidine, BSA and non-ionic detergents which can be used to increase PCR specificity or efficiency. Certain DNA polymerases will indeed reach their optimum level of activity only in the presence of such supplements.

IN THE LAB: Preparation of a PCR master mix

The essential components of a PCR are water, reaction buffer, thermostable DNA polymerase, primers, dNTPs, magnesium ions and, finally the template DNA.

When preparing several identical PCRs a single master mix is assembled rather than preparing several individual reactions. This involves mixing a sufficiently scaled up volume of each of the reagents, except the template DNA, in a single tube. The master mix is then aliquoted into individual tubes and the template DNA is added. The advantages of using a master mix include:

- More uniform quantity and quality of reagents present in each individual PCR
- Fewer pipetting transfers reduces risk of contamination of stock solutions

- More accurate volume transfer due to larger volumes being pipetted
- Fewer pipetting transfers reduce the amount of time needed to prepare a PCR

PCR procedure

The PCR technique consists of a series of three steps that are carried out under different temperature conditions, which together comprise a single "cycle". These three steps, as shown in figure 23, are (a) DNA denaturation, (b) primer annealing / hybridization, and (c) primer extension.

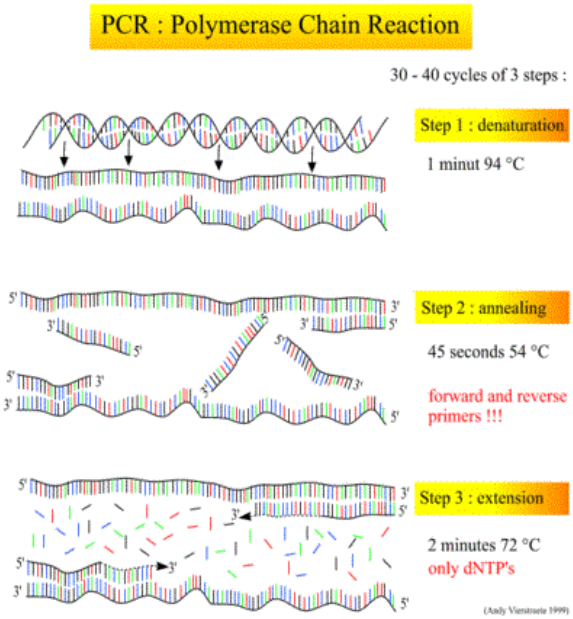

Figure 23: The steps of a PCR amplification Source: Picture: Andy Vierstraete, 1999. http://users.ugent.be/~avierstr/principles/pcr.html.

- ***DNA denaturation***

The purpose of the denaturation step is to separate any double stranded DNA into two single stranded templates from which DNA synthesis can be based. The two complementary strands are separated as a result of increasing the reaction temperature up to 93-96°C, for a short period of time. This results in the breaking of the hydrogen bonds between the base pairs and the "melting" of any double stranded DNA. The reaction is complete when the entire double stranded DNA becomes single stranded. Furthermore, during this step all enzymatic reactions stop due to the elevated temperature conditions.

- ***Primer annealing***

The primer annealing step allows the primers to bond to its complementary sequence in preparation for the DNA amplification step. It takes place at temperatures around 55-65°C or within 3-5^{o}C of the primer pair's average Tm. At these reduced temperatures primers are freely flowing in solution and hydrogen bonds are being formed and broken between the bases of the single-stranded primer and the melted template. The primers will form the most stable hydrogen bonds when they are hybridized to a template sequence that is complementary to their own. It is, therefore, important to carry out this step at the right temperature since it needs to be low enough to allow for the hybridization of the primer to the template

DNA but high enough to discourage non-specific priming. Once the primer/template hybrid is formed the DNA polymerase will attach to it and initiate primer extension.

- ***Primer extension***

The primer extension step is when the thermostable DNA polymerase synthesizes the new DNA strand by extending the primers across the target sequence using the dNTPs as building blocks. The optimal working temperature for most polymerases is approximately 72°C.

At such temperatures, primers that have successfully annealed to their complementary sequence and have been extended by a few bases possess stronger ionic attraction to the template, which reduces the probability that the nascent strand dehybridizes from the template before extension is complete. However, primers that have misprimed will be released from the templates due to the higher temperatures and therefore the primer will not be extended.

The length of time for the primer extension steps can be increased if the target region of DNA is particularly long; however, for the majority of PCR experiments an extension time of one minute is sufficient for complete extension.

The final product of the primer extension step is multiple copies of the target DNA fragment whose ends are defined by the 5' termini of the primers and length is defined by the distance between the primers on the DNA template.

IN THE LAB

As previously illustrated, PCR is a widely used diagnostic tool. However, due to the highly sensitive nature of this procedure, it is highly prone to contamination by trace amounts of DNA that could serve as templates therefore resulting in amplification of contaminating DNA. Thus, it is critical to perform PCR amplifications under stringent conditions in order to minimize the likelihood of contamination. PCR contamination can originate from several sources such as:

- Laboratory benches, equipment and pipetting devices, which can be contaminated by previous DNA preparations, or by purified restriction fragments.
- Cross-contamination between samples.
- Products from previous PCR amplifications, also known as carry over contamination.

Below are some standard procedures that should be implemented in diagnostic laboratories in order to establish and maintain a clean environment for any PCR-based assay system. Additional information is provided in the chapter on QA/QC.

Physical prevention methods

Providing physically separate working areas with dedicated equipment reduces the risk of contamination. Strict compliance with decontamination requirements is the most important prerequisite to minimize the probability of false-positive results. Laboratory facilities should contain physically separated working areas as follows:

1. *Sample preparation area:* Sample grinding, homogenization and weighing of the required starting material for DNA extraction, are performed in a dedicated sample preparation area.
2. *DNA extraction area:* DNA extraction is performed in a separated area with dedicated pipettes and equipment.

3. *PCR set-up area:* This "clean" room is devoted to the procedures related to the preparation of the PCR reaction.
4. *Post-PCR area:* The amplification of the target DNA sequence is carried out in this area as well as the detection and analysis of PCR products.

Biochemical prevention methods

One common biochemical prevention method is through the use of Uracil-DNA Glycosylase (UNG). This method's mechanism of action involves substituting dUTP for dTTP during PCR amplification, to produce uracil-containing DNA. Subsequent PCR reaction mixtures are then treated with UNG prior to PCR amplification. This leads to the release of free uracil from uracil-containing DNA and degradation of the contaminating uracil-containing DNA during the initial denaturation step of PCR due to the elevated temperatures and alkaline conditions

For this method to function as a method of contamination prevention, it requires that all PCR-reactions in the laboratory be carried out with dUTP instead of dTTP. However, using uracil-containing DNA may affect downstream applications such as reduced activity of some restriction enzymes and protein-binding or DNA- protein interaction studies.

PCR instrumentation

Two major advances have allowed the PCR process to be automated:

- The use of thermostable DNA polymerases, which resist denaturation and thus inactivation at high temperatures. Therefore, an initial aliquot of polymerase can last throughout numerous PCR cycles.
- The development of thermocyclers, or PCR machines, which could be programmed to alter their temperatures rapidly and in an automated manner. There are several thermocycler designs in use, for example: heating and cooling by fluids, heating by electrical resistance and cooling by fluids and heating by electric resistance and cooling by semiconductors.

A typical thermocycler temperature cycling profile for a three-step PCR is shown in Figure 24.

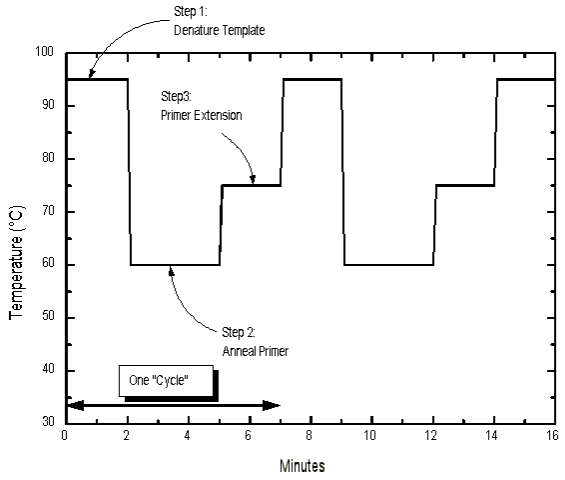

Figure 24: PCR temperature cycling profile.

Exponential amplification

After each PCR cycle, the newly synthesized segments of DNA can serve as templates during subsequent cycles leading to an exponential increase in the number of copies of the target sequence. The number of copies obtained can be expressed by the following equation:

$(2^n-2n)x$ where: x: Template copy number

n: Number of cycles

2n: Products of undefined length obtained after the first and second cycles

The equation takes into account the fact that the products of the first successful cycle of amplification are heterogeneously sized DNA molecules, with lengths that may exceed the distance between the binding sites of the two primers. In the second cycle, these molecules generate DNA strands of defined length that will accumulate in an exponential fashion during later rounds of amplification and will form the dominant products of the reaction, as indicated in figure 25.

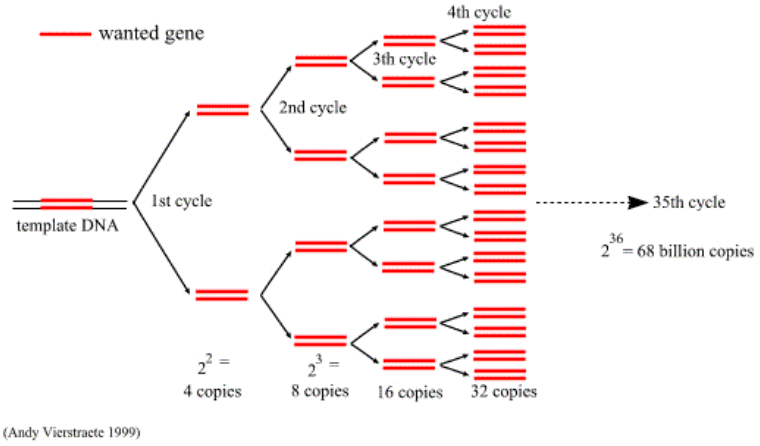

Figure 25: The exponential amplification of DNA in PCR. Source: Picture: Andy Vierstraete, 1999. http://users.ugent.be/~avierstr/principles/pcr.html.

Therefore, after 20 PCR cycles there will potentially be a 220-fold amplification of the target sequence, assuming there is 100% amplification efficiency during each cycle. The efficiency of a PCR will vary from template to template and based on the degree to which the PCR has been optimized.

Cycle number and plateau effect

The number of amplification cycles necessary to produce enough target DNA so that it can be visualized using an agarose gel depends largely on the starting concentration of the template DNA. For example, in order to amplify a starting concentration of 50 target molecules 40-45 cycles are recommended, whereas 25-30 cycles are enough to amplify a starting concentration of $3x10^5$ molecules to the same concentration.

This non-proportionality is due to the so-called “plateau effect”, which is the attenuation of the exponential rate of product accumulation during the later cycles of PCR. This may be caused by reagent degradation, reagent depletion, byproduct inhibition, competition for reagents by non-specific products and competition for primer binding due to the re-annealing of the product.

If the desired product is not obtained in 30 cycles, a small sample of the amplified product could be used as a template in a new reaction mixture and re-amplified for 20-30 cycles rather than extending the PCR to more cycles. In some cases where the template concentration is limiting, this re-amplification can produce an acceptable concentration of product, whereas extension of cycling to 40 times or more would not and may also increase the likelihood of non-specific amplification.

IN THE LAB: Template DNA and cycle number

The selection of the ideal number of PCR cycles for the successful amplification of the target region depends on the number of template DNA copies available.

The concentration of DNA that is usually added to PCR should ideally be approximately 10-50 ng/µl. However, the number of copies of the target sequence that is available within this amount of DNA depends on the complexity of the DNA sample. A comparison of the genome size of various plant species that are frequently used in plant transformation and the corresponding number of genome copies in a defined amount of DNA are given in table 8.

For example, in a 4kb plasmid containing a 1kb insert that is targeted for PCR, 25% of the input DNA is the target of interest. Conversely, a 1kb gene in the maize genome, which is $5x10^9$ bp in size, represents approximately 0.00002% of the input DNA. Therefore, approximately 1,000,000 times more maize genomic DNA is required to maintain the same number of target copies per reaction.

For optimal results, at least 100 copies of the target sequence should be available in the starting template to obtain a signal within 25-30 cycles.

In cases where the template DNA contains less than 100 copies of the target sequence more than 30 PCR cycles would be needed in order to detect a signal by gel electrophoresis. As previously discussed, this should be avoided, and alternate methods should be employed.

Table 8. Comparison of genome size of some plant species and corresponding genome copies in defined amount of DNA.

Sample	Genome size	Genome copies in 1 µg DNA	Genome copies in 1 ng DNA
Maize	5×10^9 bp	1.85×10^5	185
Soybean	1.55×10^9 bp	5.98×10^5	598
Tobacco	3.8×10^9 bp	2.43×10^5	245
Rice	4×10^8 bp	2.31×10^6	2310

Example experimental procedure - Conventional PCR

Note: The following methods are provided as a sample experimental procedures and other options for the detection of LMO-specific targets in maize are available.

The following protocols are examples of end-point PCR-based methods that allow for the screening of LMOs, by targeting the 35S promoter and the nopaline synthase (nos) terminator, for the detection of specific LMOs (for example GTS 40-3-2, Roundup Ready® soybean, MON810 maize and Bt-11 maize).

The following methods allow only a qualitative result with indication of presence/absence of the target sequence in the sample by analysis of electrophoresis results.

Equipment

- Micropipettes
- Thermocycler
- Microcentrifuge
- Vortex mixer
- Rack for reaction tubes
- 0.2 ml PCR reaction tubes
- 1.5 ml microcentrifuge tubes
- Separate sterile room with UV hood

REMARK

All equipment should be DNA-free and where possible, sterilized prior to use.

In order to avoid contamination, barrier pipette tips protected against possible aerosol formation should be used.

Reagents

- dATP
- dCTP
- dGTP
- dTTP
- 10x PCR buffer (usually delivered from the same supplier as the Taq DNA polymerase)
- 25 mM MgCl2
- Taq DNA polymerase 5 U/µL
- Upstream and downstream oligonucleotides (i.e. forward and reverse primers)
- Nuclease-free water

4 mM dNTP stock solution

- dNTPs might be supplied in pre-mixed stocks - containing dATP, dCTP, dGTP, dTTP in equal concentration - or separated in individual concentrated stocks. If individual stocks are used, dissolve each dNTP in sterile deionized water, to obtain a final 4 mM dNTP stock solution.
- Divide in aliquots and store at -20°C, dNTPs are stable for several months.

20 µM primer solutions

Primer oligonucleotides are generally supplied in lyophilized form and should be diluted to a final concentration of 20 µM.

- Prepare 20 µM primer solution according to the supplier's instructions.

- 1 µM = 1 pmol/µL so 20 µM = 20 pmol/µL

- X nmol primer + 10X µL sterile water = 100 pmol/µL = 100 µM
- Incubate 5 min at 65°C, shake and incubate for another 3 min at 65°C
- To prepare a 1:5 dilution: Prepare 1 microcentrifuge tube with 400 µL sterile water and add 100 µL of the primer solution (100 µM) Final concentration: 20 µM

- Divide into small aliquots and store at -20°C. The aliquots stored at -20°C are stable for at least 6 months; the lyophilized primers are stable at -20°C for up to three years unless otherwise stated by the manufacturer.

10x PCR buffer

- Usually the 10x PCR buffer is provided together with the *Taq* DNA polymerase and is ready to use. The buffer should be mixed and briefly centrifuged prior to use.
- Aliquots are stored at -20°C and are stable for several months.

25 mM $MgCl_2$ solution

"PCR grade" $MgCl_2$ solution is generally supplied together with the *Taq* DNA polymerase and is ready to use. The solution should be mixed (vortex) before each use and briefly centrifuged (destruction of the concentration gradient which can be formed in the case of a prolonged conservation). Store at -20°C.

Nuclease-free water aliquots

Sterile nuclease-free, deionized water aliquots are prepared for the master mix and for the dilution of the DNA. For each series of analyses, a new aliquot should be used.

Taxon-specific PCR: soybean-lectin

The identification of soybean DNA is performed targeting the lectin gene.

The PCR with the primers GMO3/GMO4 determines if amplifiable soybean DNA is present in the sample.

Characteristics of primers GM03 and GM04

	GM03
Sequence	GCCCTCTACTCCACCCCCATCC
Length	22
Mol. weight (g/mol)	6471.6
Melting point (G/C) based on a [Na^+] of 50 mM	65.1
	GM04
Sequence	GCCCATCTGCAAGCCTTTTTGTG
Length	23
Mol. weight (g/mol)	6981.1

Melting point (G/C) based on a [Na^+] of 50 mM	59.6

Controls

It is important to always include controls at every PCR. Negative controls are designed to check if the PCR reagents are contaminated with DNA. Positive controls with characterized samples are also critical in determining the efficiency and specificity of PCR.

The following controls must be introduced in analysis performed with PCR:

- Positive DNA target control: pure DNA, isolated from the conventional soybean
- Negative DNA target control: pure DNA, isolated from another species, not containing the *lectin* gene
- No-template control (NTC): negative control of the master mix, in which water is used instead of DNA

Master mix preparation

The necessary reagents for a series of 10 samples (including positive/negative/no-template controls) are mixed together according to the instruction given in table 9. The following procedure applies to a sample containing 20 µL of GMO3/GMO4 master mix and 5 µL of DNA solution. All solutions are stored on ice during the preparation of the master mix.

Table 9. GM03/GM04 master mix

	Final concentration	**Master mix for one sample**	**Master mix for 10 samples**
Sterile, deionized water		10.3 µL	103 µL
10x PCR Buffer	1x	2.5 µL	25 µL
25 mM $MgCl_2$	1.5 mM	1.5 µL	25 µL
4 mM dNTPs	0.8 mM	5 µL	50 µL
20 µM oligonucleotide GM03	0.2 µM	0.25 µL	2.5 µL
20 µM oligonucleotide GM04	0.2 µM	0.25 µL	2.5 µL
5 U/µL Taq DNA polymerase	1 U/rcn	0.2 µL	2 µL
TOTAL		**20 µL**	**200 µL**

- Prepare a 1.5 ml microcentrifuge tube
- Add the reagents following the order given in table 9
- Mix gently the GMO3/GMO4 master mix by pipetting and centrifuge briefly
- Divide the master mix into aliquots of 20 µL in 0.2 ml PCR reaction tubes
- Add 5 µL of the DNA solution to the previous aliquots (note: dilute the DNA at 10-20 ng/µL)
- Shake gently and centrifuge briefly
- Place the PCR reaction tubes in the thermocycler

PCR programme

Stage	Temperature	Time
Activation/initial denaturation	95°C	10 min
Denaturation	95°C	30 sec
Annealing	60°C	30sec
Extension	72°C	1 min
Denaturing, annealing & extension		
Number of cycles	35	
Final extension	72°C	3 min
	4^{o}C	∞

Following amplification, the samples are centrifuged briefly and put on ice.

* **Note**: The use of a different thermocycler model or brand leads to the same results provided that PCR programmes are adapted and validated accordingly

Analysis of PCR products

After amplification of the target sequence, the PCR products are analysed by agarose gel electrophoresis in the presence of ethidium bromide. 8 µL of a PCR reaction is mixed with 2 µL of loading buffer; samples are then loaded onto the agarose gel. Migration is performed at 100 V over a period of 1 hour. Size markers (15 µL of 100 bp ladder) are electrophoresed in adjacent wells of the gel to allow accurate size determination of the amplicons. After the run, UV light allows visualization of the amplified DNA on the gel. The gel may be photographed to provide a permanent record of the result of the experiment.

Interpretation of the results

The primer pair GM03/GM04 for the detection of the native *lectin* gene is used as a system's control check; the presence of a lectin-specific band at **118 bp** confirms that the extracted DNA is of appropriate amplifiable quality.

The positive control will amplify a band at 118 bp. The negative control and the no-template control should not provide any band.

If the positive/negative controls do not give the expected results, the PCR analysis of the selected samples is not valid.

If the controls give the expected results and the sample shows absence of the 118 bp band, in this sample no amplifiable soybean DNA is present. It should be noted that this as well as other protocols in this chapter are qualitative methods, therefore allowing only a qualitative (yes/no) result.

Taxon-specific PCR: maize-invertase

The identification of maize DNA is performed targeting the invertase gene.

The PCR with the primers IVR1-F/IVR1-R determines if maize DNA of suitable amplification quality is present in the sample.

Characteristics of primers IVR1-F and IVR1-R

IVR1-F	
Sequence	CCGCTGTATCACAAGGGCTGGTACC
Length	25
Mol. weight (g/mol)	7643
Melting point (G/C) based on a [Na^+] of 50 mM	63.2
IVR1-R	
Sequence	GGAGCCCGTGTAGAGCATGACGATC
Length	25
Mol. weight (g/mol)	7732
Melting point (G/C) based on a [Na^+] of 50 mM	62.8

Controls

- Positive DNA target control: pure DNA, isolated from the conventional maize
- Negative DNA target control: pure DNA, isolated from a species other than maize not containing the invertase gene
- No-template control (NTC): negative control of the master mix, in which water is used instead of DNA

Master mix preparation

The necessary reagents for a series of 10 samples (including positives/negative/no-template controls) are mixed together as indicated in table 10.

The following procedure applies to a sample containing 20 µL of IVR1-F/IVR1-R master mix and 5 µL of DNA solution. All solutions are stored on ice during preparation of the master mix.

Table 10. IVR1-F/IVR1-R master mix

Reagents	Final concentration	Master mix for one sample	Master mix for 10 samples
Sterile, deionized water		12.05 µL	120.5 µL
10x PCR buffer	1x	2.5 µL	25 µL
25 mM $MgCl_2$	1.5 mM	1.5 µL	15 µL
4 mM dNTPs	0.4 mM	2.5 µL	25 µL
20 µM oligonucleotide IVR1-F	0.5 µM	0.63 µL	6.3 µL
20 µM oligonucleotide IVR1-R	0.5 µM	0.63 µL	6.3 µL
5 U/µL Taq DNA polymerase	1 U/rcn	0.20 µL	2 µL
TOTAL		**20 µL**	**200 µL**

- Prepare a 1.5 ml microcentrifuge tube
- Add the reagents following the order given in table 10
- Mix gently the IVR1-F/IVR1-R master mix by pipetting and centrifuge briefly
- Divide the master mix into aliquots of 20 µL in 0.2 ml PCR reaction tubes

- Add 5 µL of the DNA solution to the previous aliquots (note: dilute the DNA at 10-20 ng/µL)
- Shake gently and centrifuge briefly
- Place the PCR reaction tubes in the thermocycler

***PCR programme* (IVR1-F/IVR1-R)**

Stage	Temperature	Time
Initial denaturation	95˚C	12 min
Denaturation	95 ˚C	30 sec
Annealing	64 ˚C	30 sec
Extension	72 °C	1 min
Number of cycles	35	
Final extension	72˚C	10 min
	4˚C	∞

Following amplification, the samples are centrifuged briefly and put on ice.

Analysis of PCR products

After amplification of the DNA, the PCR products are analysed using agarose gel electrophoresis with ethidium bromide. 8 µL of the solution is mixed with 2 µL of loading buffer. The solution mixture is then loaded onto an agarose gel. Migration should take place at 100 V over a period of 1 hour. Size markers (15 µL of 100 bp ladder) are electrophoresed in adjacent wells of the gel to allow accurate size determination of the amplicons. After the run, ultraviolet transillumination (UV) allows visualization of the DNA in the gel. The gel may be photographed to provide a permanent record of the result of the experiment.

Interpretation of the results

The primer pair IVR1-F/IVR1-R is used for the detection of the native maize invertase gene as a control check on the amplification quality of the extracted DNA. If the extracted DNA is of sufficient amplification quality an invertase-specific band at approximately **225 bp** will be observed on the gel.

The positive control should also amplify showing a band size of 225 bp.

The negative control and the no-template should not provide any band.

If the positive/negative controls do not give the expected results, the PCR analysis of the selected samples is not valid.

If the controls give the expected results and the sample shows absence of the 225 bp band, then, provided that the DNA is not inhibited, in this sample, no amplifiable maize DNA is present.

Examples of screening methods for the detection of genetically modified plants

Note: The following sample experimental procedures should be considered basic examples of LMO analysis using the conventional PCR approach. It is recommend that pertinent sources and literature are periodically reviewed in order to acquire information on more recently developed and validated protocols (For example as is available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx and https://croplife.org/plant-biotechnology/regulatory-2/detection-methods/).

In general, genes are under the regulation of promoters and terminators. The most widely used sequences for the regulation of a transgene are the 35S promoter (derived from the *CaMV*) and the *nos* terminator (derived from *Agrobacterium tumefaciens).* The detection of one of these regulatory sequences in the soybean and/or maize containing sample under examination may indicates presence of an LMO.

In GTS 40-3-2 Roundup Ready® soybean as well as in Bt-11, the identification of both the 35S promoter and the *nos* terminator is possible, whereas only the 35S promoter is present in the MON810 maize line.

It should be noted that the targeted sequences are also found in nature, for example the 35S promoter sequence originates from the cauliflower mosaic virus, which is found widely in brassicas and can lead to a false positive test result. Secondary analyses can be applied in order to rule out presence of the virus. Secondary analysis to confirm the results obtained can also be achieved by employing primers that target an alternative sequence; this is particularly suitable for confirmation of screening test results. A positive identification of the specific target sequence may be confirmed by an appropriate method other than size determination of the PCR product, e.g. hybridization of the PCR product with specific probes, carrying out restriction analyses of the PCR product, sequencing of the PCR product or other equivalent confirmation.

Detection of the *CaMV* 35S promoter

***Characteristics of primers* p35S-cf3 *and* p35S-cr4**

	p35S-cf3
Sequence	CCACGTCTTCAAAGCAAGTGG
Length	21
Mol. weight (g/mol)	6414.5
Melting point (G/C) based on a [Na^+] of 50 mM	57.4
	p35S-cr4
Sequence	TCCTCTCCAAATGAAATGAACTTCC
Length	25
Mol. weight (g/mol)	7544.2
Melting point (G/C) based on a [Na^+] of 50 mM	56.3

Controls

- Positive control: DNA from reference material (e.g. maize 0.5% GM)
- Negative control: DNA from reference material (e.g. maize 0% GM)
- No-template control (NTC): negative control of the master mix, in which water is used instead of DNA

Master mix preparation

The necessary reagents for a series of 10 samples (including positive/negative/no-template controls) are mixed together according to the instructions given in table 11.

The following procedure applies to a sample containing 20 µL of p35S-cf3/p35S-cr4 master mix and 5 µL of DNA solution. All solutions are stored on ice during the preparation of the master mix.

Table 11. p35S-cf3/p35S-cr4 master mix

Reagents	Final concentration	Volume for one	Volume for 10 samples
Sterile, deionized water		13.94 µL	139.4 µL
10x PCR buffer	1x	2.5 µL	25 µL
25 mM $MgCl_2$	1.5 mM	1.5 µL	1.5 µL
4 mM dNTPs	0.64 mM	0.4 µL	4 µL
20 µM oligonucleotide 35s-cf3	0.6 µM	0.75 µL	7.5 µL
20 µM oligonucleotide 35S-cr4	0.6 µM	0.75 µL	7.5 µL
5 U/µL Taq DNA polymerase	0.8 U/rcn	0.16 µL	1.6 µL
TOTAL VOLUME		**20** µL	**200** µL

- Prepare a 1.5 ml microcentrifuge tube
- Add the reagents following the order given in table 11
- Mix gently the p35S-cf3/p35S-cr4 master mix by pipetting and centrifuge briefly
- Divide the master mix into aliquots of 20 µL in 0.2 ml PCR reaction tubes
- Add 5 µL of the DNA solution to the previous aliquots
- Shake gently and centrifuge briefly
- Place the PCR reaction tubes in the thermocycler

PCR programme **(p35S-cf3/p35S-cr4)**

Stage	Temperature	Time
Initial denaturation	95°C	10 min
Denaturation	95°C	25 sec
Annealing	62°C	30 sec
Extension	72°C	45 sec
Number of cycles	50	
Final extension	72°C	7 min
	4^oC	∞

Following amplification, the samples are centrifuged briefly and put on ice.

Analysis of PCR products

After amplification of the target sequence, the PCR products are analysed by agarose gel electrophoresis with ethidium bromide. 8 µL of the solution is mixed with 2 µL of loading buffer; the solution is then loaded onto the agarose gel. Migration should take place at 100 V over a period of 1 hour. Size markers (15 µL of 100 bp ladder) are electrophoresed in adjacent wells of the gel to allow accurate size determination of the amplicons. After the run, ultraviolet transillumination allows visualization of the DNA in the gel. The gel may be photographed to provide a permanent record of the result of the experiment.

Interpretation of the results

The primer pair p35S-cf3/p35S-cr4 is used for detection of the CaMV 35S promoter, yielding a **123 bp** fragment. This promoter regulates the gene expression of many transgenic plants such as GTS 40-3-2 Roundup Ready® soybean and maize line Bt-11.

The positive control will amplify showing a band at 123 bp. The negative control and the no-template control should not give any band. If the positive/negative controls do not give the expected results, the PCR analysis of the selected samples is not valid.

If the controls give the expected results and the sample gives a band at 123 bp, it can be claimed that in this sample, genetically modified DNA is present.

Detection of the *nos* terminator

***Characteristics of primers* HA-*nos* 118-f and HA-*nos* 118-r**

	HA-*nos* 118-f
Sequence	GCATGACGTTATTTATGAGATGGG
Length	24
Mol. weight (g/mol)	7462.8
Melting point (G/C) based on a [Na^+] of 50 mM	56.2
	HA-*nos* 118-r
Sequence	GACACCGCGCGCGATAATTTATCC
Length	24
Mol. weight (g/mol)	7296.9
Melting point (G/C) based on a [Na^+] of 50 mM	61.2

Controls

- Positive DNA target control: DNA from reference material (e.g. RRS 0.5% GM)
- Negative DNA target control: DNA from reference material (e.g. soybean 0% GM)
- No-template control (NTC): negative control of the master mix, in which water is used instead of DNA

Master mix preparation

The necessary reagents for a series of 10 samples (including positive/negative/no-template controls) are mixed together according to the instructions given in table 12.

The following procedure applies to a sample containing 20 µL of HA-*nos*118-f/HA-*nos*118-r master mix and 5 µL of DNA solution. All solutions are stored on ice during the preparation of the master mix.

Table 12. HA-*nos*118-f/HA-*nos*118-r master mix

Reagents	Final concentration	Volume for one	Volume for 10 samples
Sterile, deionized water		10.34 µL	103.4 µL
10x PCR buffer	1x	2.5 µL	25 µL
25 mM $MgCl_2$	1.5 mM	1.5 µL	15 µL
4 mM dNTPs	0.64 mM	4 µL	40 µL
20 µM oligonucleotide HA-nos118f	0.6 µM	0.75 µL	7.5 µL
20 µM oligonucleotide HA-nos118r	0.6 µM	0.75 µL	7.5 µL
5 U/µL Taq DNA polymerase	0.8 U/rcn	0.16 µL	1.6 µL
TOTAL VOLUME		**20** µL	**200** µL

- Prepare a 1.5 ml microcentrifuge tube
- Add the reagents following the order given in table 12
- Mix gently the HA-*nos*118-f/HA-*nos*118-r master mix by pipetting and centrifuge briefly
- Divide the master mix into aliquots of 20 µL in 0.2 ml PCR reaction tubes
- Add 5 µL of the DNA solution to the previous aliquots
- Shake gently and centrifuge briefly
- Place the PCR reaction tubes in the thermocycler

PCR programme (HA-nos118-f/HA-nos118-r)

Stage	Temperature	Time
Initial denaturation	95°C	10 min
Denaturation	95°C	25 sec
Annealing	62°C	30 sec
Extension	72°C	45 sec
Number of cycles	50	
Final extension	72°C	7 min
	4°C	∞

Following amplification, the samples are centrifuged briefly and put on ice.

Analysis of PCR products

Following amplification, the PCR products are analysed using agarose gel electrophoresis with ethidium bromide. 8 µL of the solution is mixed with 2 µL of loading buffer; the solution is then loaded onto an agarose gel. Migration should take place at 100 V over a period of 1 hour. Size markers (15 µL of 100 bp ladder) are electrophoresed in adjacent wells of the gel to allow accurate size determination of the amplicons. After the run, ultraviolet transillumination allows visualization of the DNA in the gel. The gel may be photographed to provide a permanent record of the result of the experiment.

Interpretation of the results

The primer pair HA-*nos*118-f/HA-*nos*118-r is used for detection of the *nos* terminator, yielding a **118 bp** fragment. This terminator is present in the Roundup Ready® soybean and other lines of transgenic plants (e.g. Maize line Bt-11).

The positive control will amplify showing a band at 118 bp.

The negative control and the no-template should not give any band.

If the positive/negative controls do not give the expected results, the PCR analysis of the selected samples is not valid.

If the controls give the expected results and the sample gives a band at 118 bp, this means that in this sample modified DNA is present.

Specific detection of GTS 40-3-2 Roundup Ready® soybean by end-point PCR

This is a construct-specific method for the detection of genetically modified glyphosate resistant GTS 40-3-2 (Roundup Ready®) soy beans in raw/processed materials by amplification of a 172 bp single copy sequence representing the junction region between the CaMV 35S promoter and the *Petunia hybrida* chloroplast targeting signal preceding the *Agrobacterium* EPSPS sequence.

Characteristics of primers

P35S-af2	
Sequence	TGATGTGATATCTCCACTGACG
Length	22
Mol. weight (g/mol)	6725,4
Melting point (G/C) based on a [Na^+] of 50 mM	53
petu-ar1	
Sequence	TGTATCCCTTGAGCCATGTTGT
Length	22
Mol. weight (g/mol)	6707,4
Melting point (G/C) based on a [Na^+] of 50 mM	53

Controls

- Positive DNA target control: DNA from reference material (e.g. RRS 0.1% GM)
- Negative DNA target control: DNA from reference material (e.g. soybean 0% GM)
- No-template control (NTC): negative control of the master mix, in which water is used instead of DNA

Master mix preparation

The necessary reagents for one sample (including positive/negative/no-template controls) are mixed together according to the instructions given in table 13.

The following procedure applies to a sample containing 24 µL of P35S-af2/petu-ar1 master mix and 1 µL of DNA solution. All solutions are stored in ice during the preparation of the master mix.

Table 13. P35S-af2/petu-ar1 master mix

Reagents	Final concentration	Volume for one	Volume for 10 samples
Sterile, deionized water		10.4 µL	104 µL
10x PCR buffer	1x	2.5 µL	25 µL
25 mM $MgCl_2$	1.5 mM	1.5 µL	15 µL
4 mM dNTPs	0.8 mM	5 µL	50 µL
20 µM oligonucleotide 35s-f2	0.2 µM	0.25 µL	2.5 µL
20 µM oligonucleotide petu-r1	0.2 µM	0.25 µL	2.5 µL
5 U/µL Taq DNA polymerase	0.5 U/rcn	0.1 µL	1 µL
TOTAL VOLUME		**20** µL	**200** µL

- Prepare a 1.5 ml microcentrifuge tube
- Add the reagents following the order given in table 13
- Mix gently the P35S-af2/petu-ar1 master mix by pipetting and centrifuge briefly
- Divide the master mix into aliquots of 20 µL in 0.2 ml PCR reaction tubes
- Add 5 µL of the DNA solution to the previous aliquots
- Shake gently and centrifuge briefly
- Place the PCR reaction tubes in the thermocycler

PCR programme **(P35S-af2/petu-ar1)**

Stage	Temperature	Time
Initial denaturation	95 °C	10 min
Denaturation	95 °C	30 sec
Annealing	60 °C	30 sec
Extension	72 °C	25 sec
Number of cycles	35	
Final extension	72 °C	3 min
	4 °C	∞

Following amplification, the samples are centrifuged briefly and put on ice.

Analysis of PCR products

Following amplification, the PCR products are analysed using agarose gel electrophoresis with ethidium bromide. 8 µL of the solution is mixed with 2 µL of loading buffer; the solution is then loaded onto an agarose gel. Migration should take place at 100 V over a period of 1 hour. Size markers (15 µL of 100 bp ladder) are electrophoresed in adjacent wells of the gel to allow accurate size determination of the amplicons. After the run, ultraviolet transillumination allows visualization of the DNA in the gel. The gel may be photographed to provide a permanent record of the result of the experiment.

Interpretation of the results

The primer pair P35S-af2/petu-ar1 is used for detection of the construct present in Roundup Ready® soybean, yielding a **172 bp** fragment.

This construct is present in GTS 40-3-2 Roundup Ready® soybean and stacked events of this GM crop.

The positive control will amplify showing a band at 172 bp.

The negative control and the no-template should not give any band.

If the positive/negative controls do not give the expected results, the PCR analysis of the selected samples is not valid.

If the controls give the expected results and the sample gives a band at 172 bp, this means that in this sample GTS 40-3-2 Roundup Ready® soybean DNA is present.

Specific detection of Bt-11 by end-point PCR

This is a construct-specific method for the detection of genetically modified *Bacillus thuringiensis* toxin-producing Bt-11 maize (Syngenta, former Novartis) in raw materials by PCR amplification of the junction region of single copy sequence elements originating from the maize *adh* 1S-Intron2 (IVS2) and the *pat* gene from *Streptomyces viridochromogenes*.

Characteristics of primers

IVS2-2	
Sequence	CTGGGAGGCCAAGGTATCTAAT
Length	22
Mol. weight (g/mol)	6799,5
Melting point (G/C) based on a [Na^+] of 50 mM	54,8
PAT-B	
Sequence	GCTGCTGTAGCTGGCCTAATCT
Length	22
Mol. weight (g/mol)	6717,4
Melting point (G/C) based on a [Na^+] of 50 mM	56,7

Controls

- Positive DNA target control: DNA from reference material (Bt-11 1% GM)
- Negative DNA target control: DNA from reference material (maize 0% GM)
- No-template control (NTC): negative control of the master mix, in which water is used instead of DNA

Master mix preparation

The necessary reagents for a series of 10 samples (including positive/negative/no-template controls) are mixed together according to table 14.

The following procedure applies to a sample containing 20 µL of IVS2-2/PAT-B master mix and 5 µL of DNA solution. All solutions are stored in ice during the preparation of the master mix.

Table 14. IVS2-2/PAT-B master mix

Reagents	Final concentration	Volume for one	Volume for 10 samples
Sterile, deionized water		11.55 µL	115.5 µL
10x PCR buffer	1x	2.5 µL	25 µL
25 mM $MgCl_2$	2 mM	2 µL	20 µL
4 mM dNTPs	0.4 mM	2.5 µL	25 µL
20 µM oligonucleotide IVS2-2	0.5 µM	0.63 µL	6.25 µL
20 µM oligonucleotide PAT-B	0.5 µM	0.63 µL	6.25 µL
5 U/µL Taq DNA polymerase	1 U/rcn	0,2 µL	2 µL
TOTAL VOLUME		**20** µL	**200** µL

- Prepare a 1.5 ml microcentrifuge tube
- Add the reagents following the order given in table 14
- Mix gently the IVS2-2/PAT-B master mix by pipetting and centrifuge briefly
- Divide the master mix into aliquots of 20 µL in 0.2 ml PCR reaction tubes
- Add 5 µL of the DNA solution to the previous aliquots
- Shake gently and centrifuge briefly
- Place the PCR reaction tubes in the thermocycler

***PCR programme* (IVS2-2/PAT-B)**

Stage	Temperature	Time
Initial denaturation	95°C	12 min
Denaturation	95°C	30 sec
Annealing	64°C	30 sec
Extension	72°C	30 sec
Number of cycles	40	
Final extension	72°C	10 min
	4°C	∞

Following amplification, the samples are centrifuged briefly and put on ice.

Analysis of PCR products

Following amplification, the PCR products are analysed using agarose gel electrophoresis with ethidium bromide. 8 µL of the solution is mixed with 2 µL of loading buffer; the solution is then loaded onto an agarose gel. Migration should take place at 100 V over a period of 1 hour. Size markers (15 µL of 100 bp ladder) are electrophoresed in adjacent wells of the gel to allow accurate size determination of the amplicons. After the run, ultraviolet transillumination allows visualization of the DNA in the gel. The gel may be photographed to provide a permanent record of the result of the experiment.

Interpretation of the results

The primer pair IVS2-2/PAT-B is used for detection of the construct present in Bt-11 maize yielding a **189 bp** fragment.

This construct is present in Bt-11 and stacked events of this GM crop.

The positive control should amplify a band of 189 bp.

The negative control and the no-template should not give any band.

If the positive/negative controls do not give the expected results, the PCR analysis of the selected samples is not valid.

If the controls give the expected results and the sample gives a band at 189 bp, this means that in this sample Bt-11 DNA is present.

Specific detection of MON810 maize by end-point PCR

This is an event-specific method for the detection of genetically modified insect-protected MON 810 maize in raw materials by amplification of the single copy DNA integration-border region of the genomic sequence and the inserted sequence element originating from the CaMV 35S promoter as a result of in vitro recombination.

Characteristics of primers

	VW01
Sequence	TCGAAGGACGAAGGACTCTAACG
Length	23
Mol. weight (g/mol)	7106,7
Melting point (G/C) based on a [Na^+] of 50 mM	57,1
	VW03
Sequence	TCCATCTTTGGGACCACTGTCG
Length	22
Mol. weight (g/mol)	6677,4
Melting point (G/C) based on a [Na^+] of 50 mM	56,7

Controls

- Positive DNA target control: DNA from reference material with a certain percentage of GM material.
- Negative DNA target control: DNA from reference material (maize 0% GM)
- No-template control (NTC): negative control of the master mix, in which water is used instead of DNA

Master mix preparation

The necessary reagents for one sample are mixed together according to table 15.

The following procedure applies to a sample containing 20 µL of VW01/VW03 master mix and 5 µL of DNA solution. All solutions are stored in ice during the preparation of the master mix.

Table 15. VW01/VW03 master mix

Reagents	Final concentration	Volume for one	Volume for 10 samples
Sterile, deionized water		11.55 µL	115.5 µL
10x PCR buffer	1x	2.5 µL	25 µL
25 mM $MgCl_2$	2 mM	2 µL	20 µL
4 mM dNTPs	0.4 mM	2.5 µL	25 µL
20 µM oligonucleotide VW01	0.5 µM	0.63 µL	6.25 µL
20 µM oligonucleotide VW03	0.5 µM	0.63 µL	6.25 µL
5 U/µL Taq DNA polymerase	1 U/rcn	0.2 µL	2 µL
TOTAL VOLUME		**20** µL	**200** µL

- Prepare a 1.5 ml microcentrifuge tube
- Add the reagents following the order given in table 15
- Mix gently the VW01/VW03 master mix by pipetting and centrifuge briefly
- Divide the master mix into aliquots of 20 µL in 0.2 ml PCR reaction tubes
- Add 5 µL of the DNA solution to the previous aliquots
- Shake gently and centrifuge briefly
- Place the PCR reaction tubes in the thermocycler

***PCR programme* (VW01/VW03)**

Stage	Temperature	Time
Initial denaturation	95°C	12 min
Denaturation	95°C	30 sec
Annealing	64°C	30 sec
Extension	72°C	30 sec
Number of cycles	40	
Final extension	72°C	10 min
	4°C	∞

Following amplification, the samples are centrifuged briefly and put on ice.

Analysis of PCR products

Following amplification, the PCR products are analysed using agarose gel electrophoresis with ethidium bromide. 8 µL of the solution is mixed with 2 µL of loading buffer; the solution is then loaded onto an agarose gel. Migration should take place at 100 V over a period of 1 hour. Size markers (15 µL of 100 bp ladder) are electrophoresed in adjacent wells of the gel to allow accurate size determination of the amplicons. After the run, ultraviolet transillumination allows visualization of the DNA in the gel. The gel may be photographed to provide a permanent record of the result of the experiment.

Interpretation of the results

The primer pair VW01/VW03 is used for detection of the construct present in MON810 maize yielding a

170 bp fragment.

This construct is present in MON810 and stacked events of this GM crop.

The positive control should amplify a band of 170 bp.

The negative control and the no-template should not give any band.

If the positive/negative controls do not give the expected results, the PCR analysis of the selected samples is not valid.

If the controls give the expected results and the sample gives a band of 170 bp, this means that in this sample MON810 DNA is present.

Specialized PCR

In addition to the amplification of a target DNA sequence by the typical PCR procedures already described, several specialized variations of PCR have been developed for specific applications. Below is a non-exhaustive list of some of the more common PCR techniques that may be used within a diagnostic laboratory.

Multiplex PCR

Standard PCR usually involves the use of one primer pair at a time to amplify a specific target sequence. Multiplex PCR, on the other hand, uses multiple primer pairs to amplify several target sequences simultaneously, as represented in figure 26.

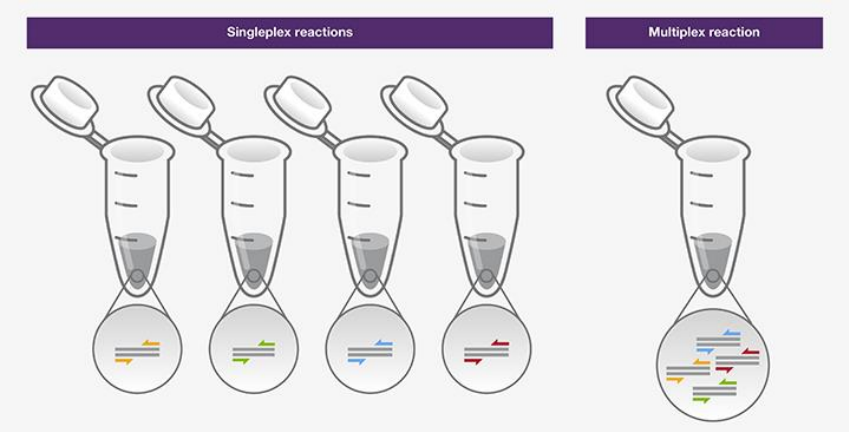

Figure 26: Schematic representation of multiplex PCR. Source: https://www.thermofisher.com/ca/en/home/life-science/cloning/cloning-learning-center/invitrogen-school-of-molecular-biology/pcr-education/pcr-reagents-enzymes/pcr-methods.html.

For multiplex PCR amplification, primers are to be designed to have similar annealing temperatures. Furthermore, the lengths of amplified products should be similar since large differences in the lengths of the target DNAs will favour the amplification of the shorter target over the longer one, resulting in differential yields of amplified products. In addition, multiplex PCR buffers tend to require the addition of

additives in order to decrease the likelihood of competition among amplicons and the discrimination against longer DNA fragments during the amplification process.

Multiplex PCR is advantageous in that it can greatly reduce time and cost of analysis. However, the presence of several primers in a single reaction tube could cause problems, such as the increased mispriming, amplification of nonspecific PCR products, primer dimers, and the preferential amplification of shorter DNA fragments.

Real-time PCR

In end-point PCR, as described above, the PCR products are typically analysed at the end of the PCR run. However, with advances in PCR technology, it is possible to monitor and gather information throughout the process of PCR product formation in real time. This technology was made possible by the development of thermocyclers that combine the function of conventional temperature cycling and the ability to detect fluorescent emissions. Real-time PCR, also known as quantitative PCR (qPCR), is carried out in the presence of dyes that emit fluorescence in proportion to the amount of DNA as it increases with each PCR cycle. By recording the amount of fluorescence emitted at the end of each cycle, it is possible to monitor the PCR reaction during its exponential phase and to therefore quantify ration/proportion of target sequence to taxon-specific sequence in the starting material.

The advantages of using real-time PCR include:

1. The ability to collect data during the exponential amplification phase of the reaction as opposed to just at the plateau end-point. This allows for accurate quantification.
2. Minimal to no post-PCR processing is needed when carrying out real-time PCR, which minimizes the risk of contamination.
3. The real-time PCR method is highly sensitive and detection is possible down to 1-10 copies of template DNA.

The specificity of a real-time PCR method depends on (i) the chemistry used to generate and monitor the amplification reaction and (ii) the instrument used to monitor the signal. Various chemistries have been developed for this purpose such as intercalating dyes and hybridization probes. The details of two of the most commonly used chemistries are described below.

SYBR® Green

SYBR® Green is an intercalating dye that binds to the minor groove of double stranded DNA. When bound to DNA the intensity of the fluorescent emissions increases and can therefore be detected by the fluorimeter contained within the thermocycler (figure 27). The more double stranded amplicons are produced following each PCR cycle, the stronger the SYBR® Green dye signal will be. However, it should be noted that the length of the double stranded amplicon also affects signal strength, meaning that a longer product will generate a stronger signal than a shorter one. Therefore, quantification of the presence of a LMO might be hindered if the LMO-specific amplicon varies in length to the chosen taxon-specific amplicon.

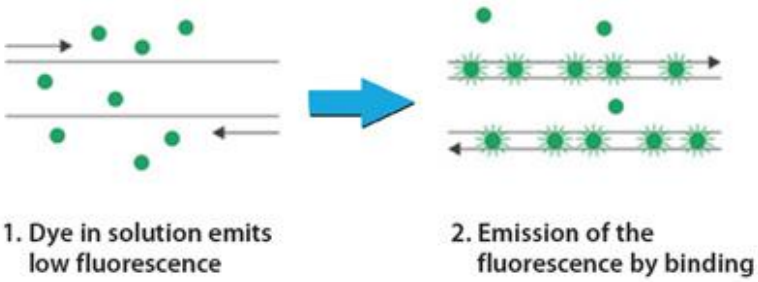

Figure 27: Schematic diagram of the mode of action of SYBR® Green, an intercalating dye that binds to the minor groove of double stranded DNA. Source http://www.sigmaaldrich.com/technical-documents/protocols/biology/sybr-green-qpcr.html.

SYBR® Green can be used to monitor the amplification of PCR products of any double-stranded DNA regardless of its sequence. This, however, can prove disadvantageous since it means that the dye can intercalate with non-specific DNA sequences and primer dimers and thus give false positive signals. However, it is possible to correct for the presence of fluorescence signal that are due to non-specific DNAs and to primer dimers on some devices by performing a melting curve analysis. This is done after the final stage of PCR whereby the products are slowly melted and fluorescence data collected. Since every double stranded DNA has a specific melting temperature, it is possible to quantify the components having different melting temperatures in one single reaction mix, and therefore to eliminate the non-specific components from the quantification.

Taqman® assay

The Taqman® assay, also known as "fluorogenic 5' nuclease chemistry", uses a different approach that includes the use of a probe that binds to a specific DNA sequence located between the two PCR primers. The probe is labelled with a fluorescent dye at the 5' end and a quencher at the 3' end.

The Taqman® assay makes use of two specific principals:

- The 5' nuclease activity of Taq polymerase: in addition to being able to elongate the primers for the replication of the targeted DNA sequence, Taq also possesses a 5'→3' nuclease activity, which allows it to remove any double stranded DNA that is downstream and may impede the synthesis of the new strand.

- Fluorescent resonance energy transfer (FRET) is an energy transfer phenomenon that occurs when two fluorophore molecules are in close proximity, typically within 10–100 Å. Specifically, a donor fluorophore in an excited energy state transfers its excitation energy to an acceptor fluorophore that is in close proximity, where the absorption spectrum of the acceptor overlaps with the fluorescence emission spectrum of the donor, as shown in figure 28. The acceptor fluorophore can either quench the light emitted by the donor or emit light of longer wavelength.

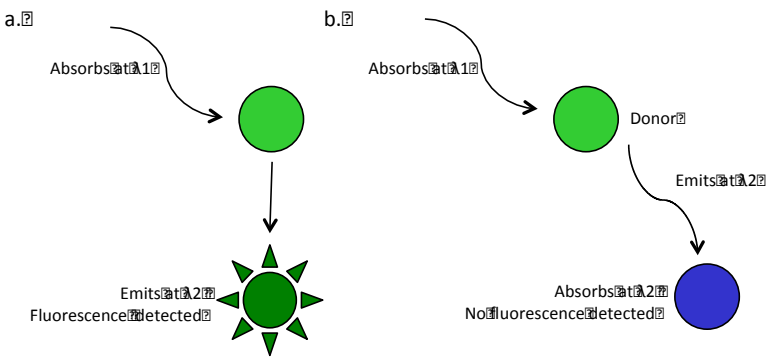

Figure 28: Schematic diagram representing the transfer of energy from a donor fluorophore to an acceptor fluorophore.

Based on these two principals, the Taqman® assay therefore involves the design of a probe, which is typically a 20-30 base long oligonucleotide, with a melting temperature that is approximately 10°C higher than that of the primers. The probe contains a reporter fluorescent dye at the 5' end and a quenching dye at the 3' end (Figure 29). The probe cannot be extended like a primer due to the presence of the quencher that blocks extension.

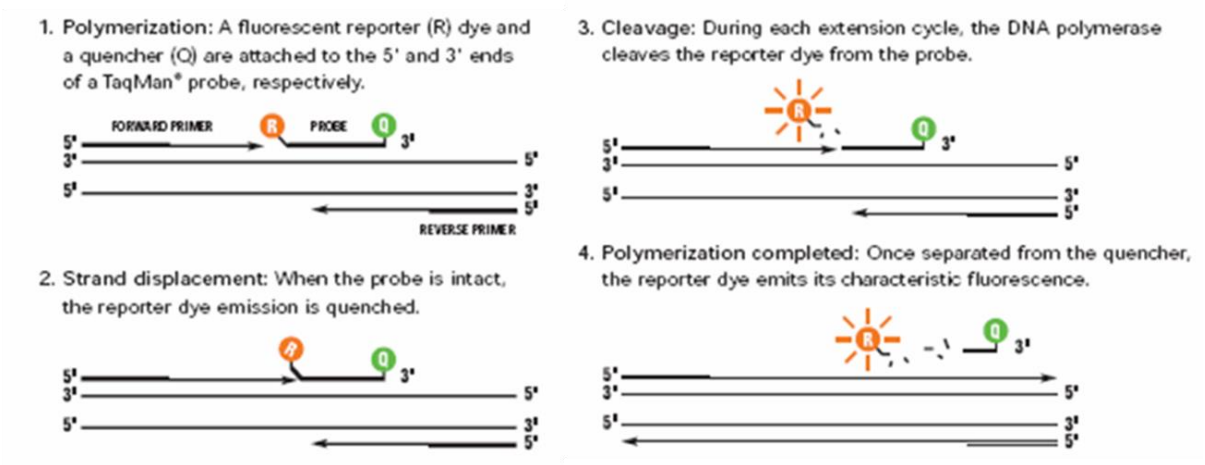

Figure 29: Scheme of TaqMan® Gene Expression Assay Based Real-time PCR. Source: Wang et al. 2006 (https://bmcgenomics.biomedcentral.com/articles/10.1186/1471-2164-7-59).

During the PCR reaction the probe specifically anneals to the target DNA between the forward and reverse primer sites. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence as a result of the FRET phenomenon. During the amplification phase of PCR, the 5' nuclease activity of Taq DNA Polymerase degrades the probe as it extends the newly synthesized strand. This results in an increase in fluorescence as amplification proceeds. Accumulation of PCR product is detected by monitoring the increase in fluorescence of the reporter dye. This process occurs during every cycle and does not interfere with the exponential accumulation of product.

The advantages of the Taqman® assay include increased specificity due to the specific hybridization of the probe to the target DNA. Furthermore, probes can be labelled with different, distinguishable reporter dyes, which allows for the multiplexing of a number of different reactions in one reaction tube. However,

a drawback of the Taqman® assay is that the design and synthesis of different probes is required for each target sequence, which can prove to be costly and time consuming.

Special classes of hybridization probes

With advances in real-time PCR analysis, several novel designs and different types of hybridization probes have been developed and are available for real-time PCR analysis. Below is a description of other types of hybridization probes that can be used.

MGB probes

A minor groove binder (MGB) is a tripeptide that binds to the minor groove of DNA with high affinity. When attached to a probe the MGB stabilizes annealing by folding into the minor groove of the DNA duplex created between the probe and the target sequence. This increased stability means that MGB probes can be designed to be very short, typically between 13–20 bases. MGB probes have several advantages for quantitative PCR, especially for multiplex assays. Improved spectral performance allows for greater precision and consistency between individual assays and the greater hybridization specificity enables enhanced target discrimination. Furthermore, the smaller probe can make it easier to design assays by providing more scope for fitting probes within shorter target regions. Amplicon size can be reduced to a minimum by using shorter MGB probes. MGB can be used with the Taqman® assay; the MGB group is attached at the 3' end along with the quencher dye. They can also be used in Eclipse probes where the MGB is conjugated to the 5’ end of the probe with the quencher and the fluorophore is on the 3’ end. The unhybridized probe exists as a random coil that keeps the fluorophore and quencher in close proximity. Once the probe anneals to its target sequence, with added stability from the MGB, the fluorophore and quencher are sufficiently far apart that fluorescence is detected, as shown in figure 30.

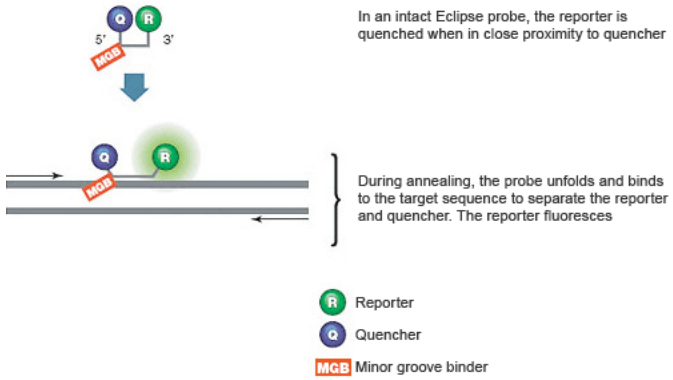

Figure 30. Schematic diagram of the mechanism of action of eclipse probes. Source: http://www.bio-rad.com/en-ca/applications-technologies/introduction-pcr-primer-probe-chemistries.

FRET hybridization probes

FRET hybridization probes are designed as a pair of which one probe is labelled with the donor fluorophore and the other with an acceptor fluorophore. FRET hybridization probes are designed to hybridize to adjacent regions of the template DNA, to within 1-5 nucleotides apart. If both probes hybridize, the two fluorophores are brought close together resulting in FRET taking place and a signal being released by the acceptor fluorophore, as shown in figure 31.

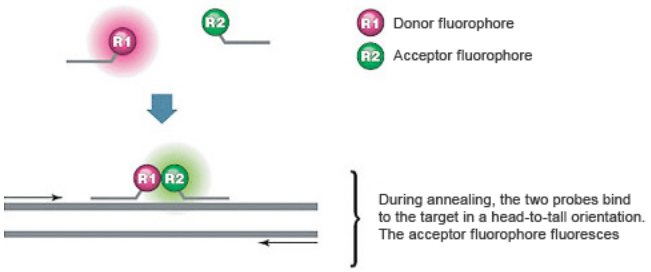

Figure 31: Schematic diagram of the mechanism of action of FRET hybridization probes. Source: http://www.bio-rad.com/en-ca/applications-technologies/introduction-pcr-primer-probe-chemistries.

Molecular beacons

Molecular beacons are DNA probes designed to contain a stem-loop structure. The loop sequence is complementary to the specific target of the probe and the stem sequences are designed to be complementary to each other, as shown in figure 32. The 5' and 3' ends of the probe are covalently bound to a fluorophore and a quencher. When the stem-loop structure is closed the fluorophore and the quencher are close together. In this case, all photons emitted by the fluorophore are absorbed by the quencher. In the presence of a complementary sequence, the probe unfolds and hybridizes to the target. The fluorophore is displaced from the quencher, and fluorescence is detected.

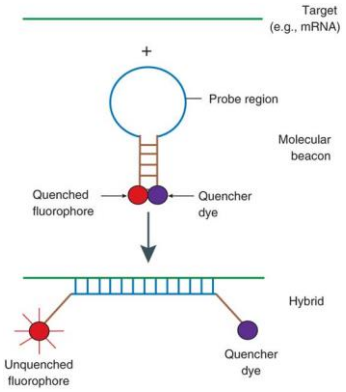

Figure 32: Principle of operation of molecular beacons. The probe sequence in the loop binds spontaneously to the target DNA at physiological temperatures, separates a terminally linked pair of fluorophore and quencher, and restores the fluorescence of the quenched fluorophore. Source: http://www.nature.com/nprot/journal/v1/n3/fig_tab/nprot.2006.242_F1.html.

Scorpion probes

A scorpion probe consists of a specific probe sequence with a stem-loop structure. A fluorophore is attached to the 5' end giving a fluorescent signal that is quenched in the stem-loop configuration by a moiety joined to the 3' end. The stem-loop is linked to the 5' end of a primer, as shown in figure 33. Therefore, unlike molecular beacons, TaqMan, MGB or FRET assays, scorpion assays do not require separate primers and probes. After the extension of the primer moiety within the scorpion probe, a portion of the probe sequence, which was designed to be complementary to a sequence within the target DNA, binds to its complement. This hybridization event opens the hairpin loop in such a way that fluorescence is no longer quenched and an increase in signal is observed. A PCR blocker is placed between the primer

and the stem sequence preventing read-through of the hairpin loop during extension of the opposite strand.

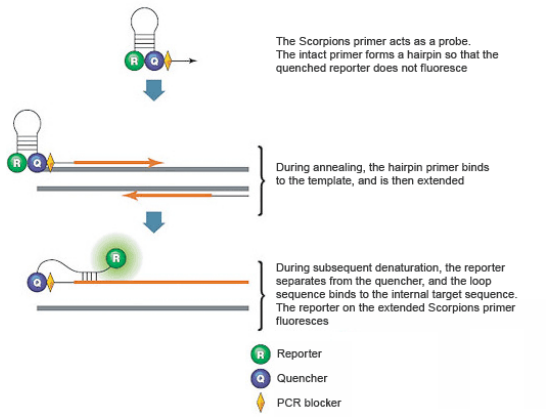

Figure 33: Scorpions PCR primers contain a sequence complementary to an internal portion of the target sequence. Source: http://www.bio-rad.com/en-ca/applications-technologies/introduction-pcr-primer-probe-chemistries.

Analysis of PCR products

Introduction

Once PCR amplification has been completed subsequent post-PCR processing steps have to be taken in order to visualize the resulting amplification products. There are 2 main methods that can be used to do so, as described below.

Intercalating dyes and gel electrophoresis

Electrophoresis through an agarose gel is a standard method used to separate, identify and purify DNA fragments following PCR.

The term electrophoresis describes the migration of charged macromolecules under the influence of an electric field. This results in the separation of macromolecules, in this case DNA, on the basis of their charge. The properties of a macromolecule determine how rapidly an electric field can move it through a gelatinous medium, such as an agarose gel, as outlined in figure 34.

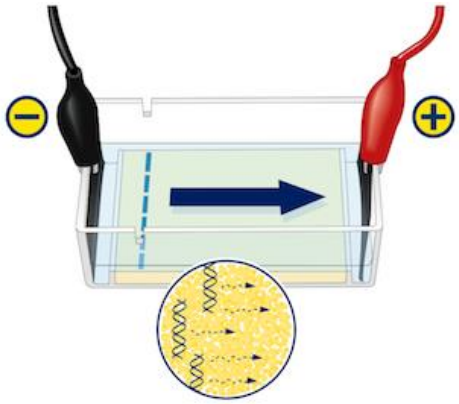

Figure 34: Schematic diagram of an electrophoresis set-up. Source: http://www.ncbe.reading.ac.uk/MATERIALS/Electrophoresis%20and%20DNA/electrophoresis.html.

Electrophoresis is performed by mixing the DNA sample with a loading dye and applying it to an agarose gel that is submerged in a buffer. An electrical current is applied across the gel which results in the DNA molecules being repelled from the negative electrode, the cathode, while being simultaneously attracted to the positive electrode, the anode. The frictional force of the agarose gel acts as a "molecular sieve" as the DNA is forced to move through the pores within the gel. The rate of the DNA fragment's migration through the gel depends on the following factors:

- the strength of the electric field
- the ionic strength and temperature of the buffer in which the molecules are moving
- the relative hydrophobicity of the samples
- the size and shape of the molecules

Since the first three factors care consistent for all the samples that are migrating on the same gel, it is therefore the size of the DNA fragment that determines its rate of migration. Shorter fragments will migrate faster than longer ones. The position of the bands on the gel can be compared to a ladder, which consists of DNA fragments of various known sizes, this allows for the determination of the size of the DNA fragment of interest.

Once the electrophoresis is completed the location of DNA within the gel can be visualized by staining the gel with an intercalating dye. These are molecules that specifically target double stranded DNA and insert themselves within the base pairs. In doing so, the ability of the dye to fluoresce is greatly increased and the location of the DNA can be visualized when the gel is exposed to ultraviolet light. Ethidium bromide is a commonly used intercalating dye that is used to stain agarose gels. It can either be mixed into the molten gel before it is allowed to set or the gel can be soaked in an ethidium bromide solution after the completion of electrophoresis. Another commonly used dye is SYBR® Green which works in a similar fashion as ethidium bromide. It is advantageous over ethidium bromide in that it does not pose a carcinogenic or teratogenic health risk; however, it is more costly.

IN THE LAB: Components of agarose gel electrophoresis

Agarose

Agarose, a natural colloid extracted from seaweed, is a linear polysaccharide made up of alternating units of galactose and 3,6- anhydrogalactose. Agarose gels have large "pore" sizes and are used primarily to separate large molecules with a molecular mass greater than 200kDa. They are processed relatively quickly, but with limited resolution since the bands formed in the agarose gels tend to be fuzzy/diffuse and spread apart. The gels are made by suspending dry powdered agarose in an aqueous buffer, then boiling the mixture until the agarose melts into a clear solution. The solution is then poured onto a gel-tray and allowed to cool to room temperature to form a solid gel. Upon hardening, the agarose forms a matrix whose density is determined by its concentration. The concentration of the agarose gel affects the rate at which DNA migrates through it. In horizontal gels, agarose is usually used at concentrations between 0.7% and 3%, as indicated in table 16.

Table 16. Recommended agarose gel concentration for resolving linear DNA molecules

Agarose Concentration (%)	DNA size range (bp)
0.75	10,000 - 15,000
1	500 - 10,000
1.25	300 - 5,000
1.5	200 - 4,000
2	100 - 2,500
2.5	50 - 1,000

Electrophoresis buffer

The electrophoretic mobility of DNA is affected by the composition and ionic strength of the electrophoresis buffer in which the agarose gel is submerged. In the absence of ions, electrical conductance is minimal and DNA migrates slowly, if at all. In a buffer of high ionic strength electrical conductance is very efficient. Several buffers are available for electrophoresis of native double-stranded DNA. These contain EDTA and Tris-acetate; Tris-borate; or Trisphosphate at a concentration of approximately 50 mM. Electrophoresis buffers are usually prepared as concentrated solutions and stored at room temperature.

DNA molecular weight ladder

Since migration distance depends on the size of the DNA fragment, a molecular weight ladder, which consists of mixtures of DNA fragments of varying known sizes, should be loaded into wells on both the right and left sides of the gel to facilitate the estimation of the size of the unknown DNA fragments on gels.

Loading buffer

Before loading DNA samples into the wells of an agarose gel, they must first be mixed with a loading buffer, which usually comprised of water, sucrose, and a dye, such as xylene cyanole, bromophenol blue, or bromocresol green. The loading buffer serves three purposes: 1) increases the density of the sample ensuring that the DNA drops evenly into the well; 2) adds colour to the sample, thereby simplifying the loading process; and 3) imparts a dye to the sample that, in an electric field, moves toward the anode at a predictable rate therefore providing a visual indication of how far along the gel migration has progressed.

Example experimental procedure – Agarose gel electrophoresis

<u>Caution</u>: Ethidium bromide is a powerful mutagen/carcinogen and is moderately toxic. Gloves should always be worn when handling solutions and gels containing ethidium bromide.

Equipment

- Horizontal electrophoresis unit with power supply
- Microwave oven or heating stirrer
- Micropipettes
- 1.5 ml reaction tubes
- Balance capable of 0.1 g measurements
- Spatulas
- Rack for reaction tubes
- Glassware grade A
- Transilluminator (UV wavelength: ~312 nm)
- Instruments for documentation like a digital camera based image acquisition system

Reagents

- TBE Tris/Boric acid/EDTA buffer (10x)
- Deionized water
- Agarose, suitable for DNA electrophoresis
- Tris[hydroxymethyl] aminomethane (Tris) (CAS 77-86-1)
- Boric acid (CAS 10043-35-3)
- Na2EDTA (CAS 139-33-3)
- Ethidium bromide (CAS 1239-45-8)
- Sucrose (CAS 57-50-1)
- Xylene cyanole FF (CAS 2650-17-1)
- DNA markers: Lambda DNA EcoRI/HindIII digested (or other similar suitable marker) or 100bp DNA ladder

10x TBE buffer (1 litre)

Prepare 10x TBE buffer according to the instructions below or buy a ready-to-use solution.

Tris [hydroxymethyl] aminomethane (Tris)	54.0 g
Boric acid	27.5 g
Na_2EDTA	7.44 g

- Mix reagent to deionized water to obtain a 1 litre solution at pH 8.3
- Store at room temperature

6x loading buffer (10 ml)

Prepare 6x loading buffer according to the instructions below or buy a ready-to-use solution.

Xylene cyanole FF	0.025 g
Sucrose	4 g

- Add sucrose and xylene cyanole FF to deionized water to obtain 10 ml of solution.
- Mix the solution, autoclave and store at 4°C.

Procedure

- Seal the edges of a clean, dry plastic gel-tray either with tape or other means. Position the appropriate comb so that complete wells are formed when the agarose solution is added.
- Dilute 10x TBE buffer to prepare the appropriate amount of 0.5x TBE buffer to fill the electrophoresis tank and to prepare the gel.
- Weigh powdered agarose depending on the dimensions of the amplicon and add it to an appropriate amount of 0.5x TBE buffer in an Erlenmeyer flask with a loose-fitting cap (usually 150 ml gel solution for a 15 x 15 cm gel-tray and 100 ml gel for a 15 x 10 cm gel-tray).
- Heat the slurry in a microwave oven or in a boiling water bath until the agarose dissolves (check the volume of the solution after heating).
- Cool the mixture to 50 - 60°C and add ethidium bromide (from a stock solution of 10 mg/ml) to a final concentration of 0.2 µg/ml and mix thoroughly.
- Pour the solution into the gel-tray and allow the gel to set. The amount of gel used should correspond to a depth of approximately 3 - 5 mm.
- After the gel is completely set, carefully remove the comb and the tape and place the gel in the electrophoresis tank.
- Add enough 0.5 x TBE buffer to the electrophoresis unit to cover the gel to a depth of about 2 - 5 mm.

Prepare samples and marker for genomic DNA as follows:

sample		***marker***	
water	3 µl	water	6 µl
loading buffer	2 µl	loading buffer	2 µl
sample	5 µl	DNA *Eco*RI / *Hind*III	2 µl
	10 µl		10 µl

Prepare samples and marker for PCR products as follows:

sample		***marker***	
loading buffer	2 µl	100 bp DNA ladder	15 µl
sample	8 µl		
	10 µl		

- Load 10 µl of each sample into consecutive wells and the appropriate DNA marker into the first and last lane.
- Close the lid of the gel tank and attach the electrical leads so that the DNA will migrate toward the anode and apply a voltage of 90-100 V/15 cm.
- Run the gel until the xylene cyanole has migrated the appropriate distance through the gel (~ 40 - 60 minutes).
- Turn off the current; remove the leads and the lid from the gel tank. Place the gel on a UV lightbox and photograph the gel.
- Discard the gel into the provided ethidium bromide solid waste bin.

Fluorescent labelling

Fluorescent labelling can be used in a number of ways to analyse the products of PCR amplification. One method of fluorescent labelling involves the use of primers that have been labelled with a fluorescent dye. This leads to the amplification product having a label directly attached to it during PCR amplification. Visualization of such PCR products requires the use of specialized equipment, such as capillary electrophoresis in order to complete the visualization process and the gathering of data.

In addition, as previously described, fluorescent labelling is the cornerstone of real-time PCR based techniques whereby PCR is carried out in the presence of dyes that emit fluorescence in proportion to the amount of DNA as it increases with each PCR cycle. By recording the amount of fluorescence emitted at the end of each cycle, it is possible to monitor the PCR reaction during its exponential phase. This can be performed by using intercalating dyes such as SYBR® Green or using fluorescently labelled probes, as described in the previous section. This type of visualization technique is advantageous in comparison to gel electrophoresis since there is minimal post-PCR processing involved in order to obtain data. This reduces labour and material costs as well as minimizes the risk of contamination within the laboratory because there is no manipulation of the amplified PCR product in preparation for the visualization technique, such as mixing with loading buffer or placement onto a gel.

LMO detection using PCR

Introduction

The various tools and methodologies that are available for the detection and identification of LMOs can be used to test samples for the presence of LMOs. There are a number of ways that these tools can be used in the context of each country's national framework. Specifically, there are two main types of LMO detection categories. The first is qualitative detection which is used to indicate whether or not LMOs are present in the sample. Following this type of screening further qualitative testing may need to be undertaken if a sample is found to be positive for the presence of LMO in order to fulfil legislative requirements that may be in force. These requirements vary from country to country and may include further testing to determine the specific identity of the LMO events that may be present in the sample and/or measuring the quantity of each LMO event present in the sample. Existing ISO standards provide valuable general guidance and recommendations for reliable detection and identification of LMOs using PCR (ISO 24276, ISO 21569, ISO 21570, ISO 21571). General requirements and guidance concerning effective PCR-based LMO screening strategies are provided by an European Technical Specification (CEN/TS 16577).

Qualitative detection

Qualitative detection methods can be used as an initial screening and identification step to investigate whether or not a sample contains LMO-specific DNA. Qualitative analysis could therefore be performed on products sampled from any point along the supply chain. If the qualitative screening indicates the presence of LMOs, subsequent testing can be carried out to determine the specific identity of LMOs that may be present in the sample, or further quantitative testing may be performed, as further in this chapter.

Qualitative testing is carried out in a methodical manner in order to maximize the amount of information obtained from the smallest number of reactions, in an attempt to minimize cost and time dedicated to this step. There are four main categories of target amplicons that are used during the screening and identification process (Figure 35):

1. **Element-specific** – This type of testing can be used to target any of the commonly used genetic elements that are known to be present in the various commercialized LMOs such as promoters, terminators or transgenes.

2. **Construct-specific** – This amplifies regions within the transgene construct, spanning the junctions between different genetic elements. Depending on the primer pair being used, this type of testing can be used to narrow down the pool of potential LMOs that may be present in a sample.

3. **Event-specific** – This testing method is made up of primer pairs that overlap the junction between the inserted construct and the genomic DNA and is used to identify individual LMOs.

4. **Taxon-specific** – This targets endogenous genes within the genome of the recipient organism and is used to confirm whether or not DNA that is of sufficient quality and quantity is available for amplification in PCR-reactions. Such a reaction also confirms the species of origin.

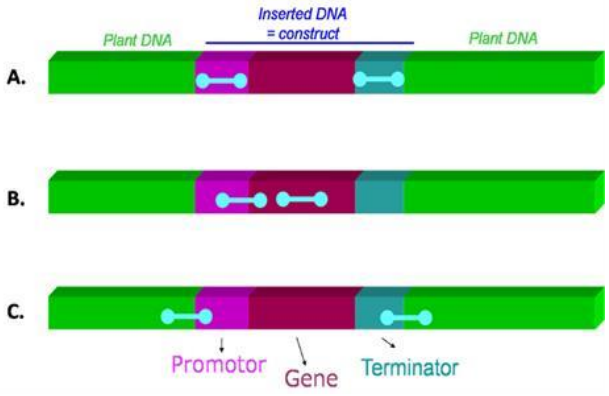

Figure 35: A construct (inserted DNA) commonly consists of several genetic elements such as a promoter, a gene and a terminator. PCR can be designed to target different regions (a) common regulatory elements (such as promoters, terminators) (b) construct-specific (junction between two genetic elements within the construct) (c) event-specific (junction between the inserted construct and the plant genome). The turquoise dumbbells represent the region targeted by the PCR, whereby dots mark the start and end of the sequence to which primers bind (probes are not shown in this figure). Source: National Institute of Biology.

Screening methods

When analysing a sample for the presence of LMOs in a sample, laboratories generally perform an initial "screening" step. Once a sample is received and DNA has been extracted the initial screening step involves amplifying the DNA with a taxon-specific target region and a set of element or construct-specific amplicons in order to detect target sequences that are commonly present in known LM events.

As previously described the taxon-specific amplification is used to confirm that DNA from a specific species is present and whether or not it is of sufficient quality to be amplified using PCR. This is important since it ensures that any negative result from subsequent element-specific tests can be attributed to the absence of the target LM sequence.

Amplification with a set of element or construct-specific primers allows for the detection of sequences that are commonly present in known LM events. Some of the most commonly targeted elements for the

initial screening step include p35S, tNOS, EPSPS, bar and pat. Additional screening targets can be added if it is appropriate in the context of a specific country's national legislation.

If amplification is detected using an element or construct-specific method, this indicates that DNA from an LMO is present in the sample.

Identification methods

The further narrowing down of the possible identity of the LMOs present in a sample can be carried out depending on the combination of elements that gave a positive signal during the screening test. The pattern of positive elements can be compared to the patterns that would be expected from LMOs of a known identity and, therefore, the identity of the LMOs that may be present in a sample can be narrowed down to a few possible choices. This is known as the matrix approach. Several such reference matrices exist, such as the Waiblinger table and the GMOseek matrix, among others.[17] There are two different types of commonly used matrices for the application of PCR-based screening strategies using the matrix approach.

1. LMO method matrix – which is defined as a relational presentation, for example a table, of symbols or numbers, where the genetic elements and genetic constructs that are detected by a defined PCR method and the corresponding LMOs are tabulated. The symbols ("+" or "-", or numbers) indicate whether or not the target sequence is detectable by the specified method in a given LMO.

2. LMO target matrix – tabulates only information about the presence of genetic elements or genetic constructs in LMOs. The entered symbols or numbers indicate the presence or absence of the target sequence and copy number, if available, in a given LMO event. In contrast to LMO method matrices, LMO target matrices are independent from a particular detection method.

Once the identity of the LMO is determined, or narrowed down to a few possible choices, further identification methods can be carried out, using event-specific methods, to conclusively establish identity, as shown in the table, below.

Table 17. Example-Application of the matrix approach to establish possible identities of LMOs present in a sample. In the examples given in this table, assuming that the samples are not mixed, samples 1 and 3 would need further testing using event-specific targets to specifically identify which of the possible LMOs listed is actually present in the sample.

Sample	**Result (+/ -)**						**Possible LMO(s) present**
	Plant specific	p35S	tNOS	EPSPS	bar	pat	
1	Soybean	+				+	ACS-GMØØ5-3 or ACS-GMØØ6-4
2	Soybean	+	+			+	SYN-ØØØH2-5
3	Maize	+	+	+			MON-ØØ6Ø3-6 or MON-88Ø17-3 or MON-87427-7
4	Maize	+	+		+		ACS-ZMØØ4-3

As powerful as the matrix approach is, it does have some limitations:

[17] Links to publicly available matrices can be found in chapter 5.

1. Mixed samples: samples obtained for testing may contain more than one LMO either from the same or different species. While the screening method could give some indication as to the possible LMOs that may be present, the pool of potential LMOs can still be quite vast and several event-specific tests would still need to be carried out, which poses a burden on testing laboratories.

2. Stacked events: A stacked event is an LM event that results from cross-breeding of two or more different LMOs and therefore carries more than one transgene in its genome. The location of the transgenes in the stacked event are normally determined by the original single events. The matrix approach cannot be used to distinguish between a mixed sample and a stacked event.

3. Complex LMOs: As LMOs develop, novel genetic elements, that are not part of the screening method being employed, may be used to build the construct. Such an LMO may therefore not be detected using these methods.

Quantitative analysis

Quantitative analysis involves the analytical determination of the relative percentage of LMO content in a sample by normalizing the amount of the LMO-specific sequences against the amount of a taxon-specific gene, such as lectin for soybean and invertase, or zein for maize. The resulting percentage of LMO content is therefore expressed as:

$$\text{Percentage LMO content} = \frac{\text{LM-DNA}}{\text{Reference-DNA}} \times 100\%$$

Such a determination can be carried out using conventional PCR based analysis. However, a major drawback of conventional PCR is the lack of accurate quantitative information due to amplification efficiency. If the reaction efficiency for each amplification cycle remained constant, the concentration of DNA following PCR would be directly proportional to the amount of initial DNA target. However, the amplification efficiency of PCR varies among different reactions, as well as in subsequent cycles in a single reaction, in particular, in the later cycles of the PCR, where the amplification products are formed in a non-exponential fashion at an unknown reaction rate.

DNA quantification based on conventional PCR would rely on end-point measurements, in order to achieve maximum sensitivity, when the amplification reaches the maximum product yield (i.e. the "plateau phase"). At this stage the reaction has gone beyond the exponential phase primarily due to depletion of reagents and the gradual thermal inactivation of the polymerase used. The resulting correlation between the final product concentration and number of initial target molecules is therefore limited. To overcome these limitations, real-time PCR can be used to address the problems of establishing a relationship between the initial concentration of target DNA and the amount of PCR product generated by amplification.

Principles of quantification with real-time PCR

The relative LMO content of a sample can be quantified and expressed as the number of target DNA sequences per target taxon-specific sequences, as outlined above. The amount of taxon-specific sequences involves measuring the amount of DNA sequences of an endogenous reference gene, which is used as a "normalizer". When choosing such reference genes the following considerations should be kept in mind:

a) species specificity

b) a single copy of the gene per haploid genome,
c) be stably represented in different lines of the same species
d) has an amplification efficiency that is equivalent to the LMO traits being analysed by ensuring good primer and probe design.

Similarly, with regards to measuring the amount of LM-DNA in a sample certain considerations have to be taken into account when selecting a target site in order to facilitate the interpretation of the results and avoid misestimating the amount of DNA present:

a) *Event ploidy:* It is possible that the LM event has a different ploidy as compared to the wild type genome, for example tetraploid instead of diploid. This can be empirically evaded by using reference materials that are consistent with the sample, for example using a maize flour reference material to quantify maize flour. Furthermore, quantification standards that do not originate from certified reference materials, such as cloned DNA sequences or genomic DNA mixtures, can be calibrated against certified reference materials in order to correct for molecular discrepancies in quantification.

b) *Event zygosity:* The LM trait could be either homozygous or heterozygous. Event zygosity can also be corrected by following the precautions as those listed under event ploidy.

c) *Event copy number:* The number of insertions per haploid genome of a construct which could have been inserted as a single copy per haploid genome or more. This can be taken into account by designing the primer-probe system that overlaps the junction between the inserted construct and the plant genome. Since border sequences are unique this event-specific detection system will not amplify multiple insertions of the same construct during quantification.

As a result of these possible sources of error it is widely accepted to report such results by expressing the percentage LMO in terms of haploid genomes. Furthermore, such sources of discrepancy should be taken into account when a method is developed and validated, since the limit of detection (LOD) and the limit of quantification (LOQ) are influenced by the real number of copies being quantified.

Designing a real-time LMO quantification experiment

When designing a real-time PCR amplification the following components must be included:

- A first set of primers/probes that were designed to amplify an LMO-specific target DNA sequence.

- A second set of primers/probes that were designed amplify an endogenous, species-specific reference sequence, for use as a “normalizer” when calculating the relative percentage of LMO content in a sample.

- A set of serial dilutions of DNA from an appropriate positive control amplified by the primers/probes that were designed to amplify both the LMO-specific target DNA sequence and the endogenous, species-specific reference sequence in order to create a standard curve. Standard curves should include at least four different concentration points. Each point of the standard curve, and the sample, should be loaded at least in triplicate. These standard curves are used to

determine the amount of LMO-specific target DNA sequence and the endogenous, species-specific reference sequence in unknown samples.

- A no-template control that is amplified by the primers/probes that were designed to amplify both the LMO-specific target DNA sequence and the endogenous, species-specific reference sequence.

Depending on the chemistry and the equipment available for carrying out the experiment, it may be possible to amplify both the LMO-specific target DNA sequence and the endogenous, species-specific reference sequence as a multiplexed reaction. This is made possible due to the availability of several reporter dyes that have different maximal emission wavelengths, which are used to synthesize TaqMan® probes, as shown in figure 36. This allows the detection of amplification products from multiple targets in the same tube at the same time.

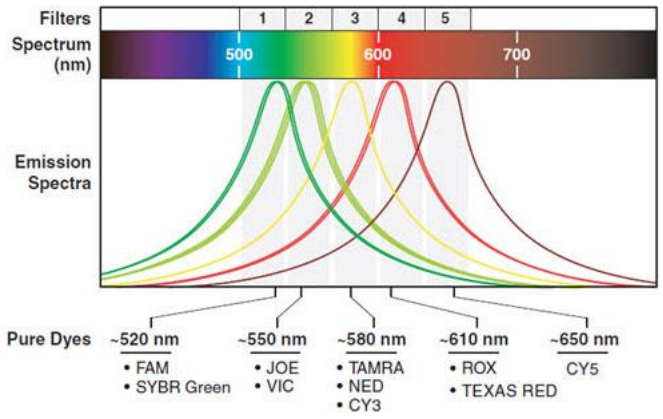

Figure 36: The emission spectra of various dyes that are commonly used in real-time PCR. Source: https://www.thermofisher.com/ca/en/home/technical-resources/technical-reference-library/real-time-digital-PCR-instruments-support-center/7500-real-time-pcr-systems-support/7500-real-time-pcr-systems-support-getting-started.html.

Multiplexed reactions are advantageous in that they save time since fewer reactions would have to be set up per sample. This in turn also leads to a reduction in the likelihood that pipetting errors occur during the PCR set-up. In addition, there would also be a reduction in costs due to the use of less reagents and the possibility of being able to place more samples on a single PCR run. Multiplexing also reduces the amount variability between measurements of the amplification results of the LMO-specific target DNA sequence and the endogenous, species-specific reference sequence since both amplifications occur in the same tube under identical conditions. However, it should be noted that multiplexed reactions may result in decreased detection sensitivity with respect to the reaction's limit of quantification due to interference between the two reactions and their variable consumption of reagents.

Interpretation and analysis data produced by real-time PCR

As a real-time PCR experiment proceeds, the instrument measures fluorescence at the end of each cycle. The data is compiled into an amplification plot which displays cycle number on the x-axis against fluorescence on the y-axis. The plot is usually graphed on a semi-logarithmic scale.

The amplification plot makes it possible to distinguish between the three different phases that occur during PCR.

1. An initial "lag" phase, which takes place between cycles 1-18, shows minor fluctuations in fluorescence. This corresponds to background signal.

2. This is followed by an exponential phase, which takes place between cycles 18-28, when the accumulation of amplified product is significantly higher than the background signal. The cycle number at which this occurs is known as the "cycle quantification" (C_q value) or "threshold cycle" (C_t value). When using real-time PCR for quantification data is collected during this point. This significantly enhances the accuracy of quantification since there is a direct correlation between the starting amount of template and the cycle number at which amplification starts to become exponential. The higher the initial amount of template DNA, the sooner accumulated product is detected in the PCR process, and the lower the C_T value is.

3. The final stage in when the plots tend to reach a "plateau". This corresponds to the end-point of the PCR amplification.

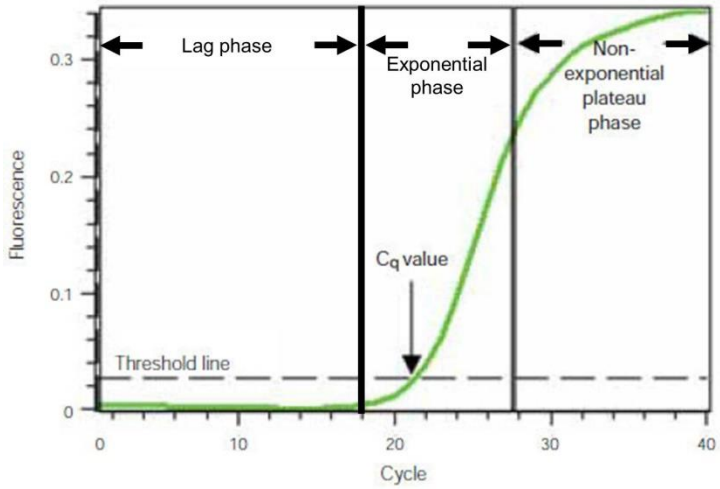

Figure 37: A real-time PCR amplification plot displaying baseline-subtracted fluorescence against the number of PCR cycles. Source: Adapted from http://www.bio-rad.com/en-ca/applications-technologies/what-real-time-pcr-qpcr.

In practice, the choice of positioning the threshold line, which is used to determine C_t values, is often up to the operator, therefore representing one of the subjective elements in real-time PCR. Data produced by real-time indetermination displays the amplification plots produced in each well on a single overlapping graph, as indicated in figure 37. In such a plot the threshold line should be placed above any baseline activity and within the exponential increase phase, which appears linear in the log transformation representation of the data.

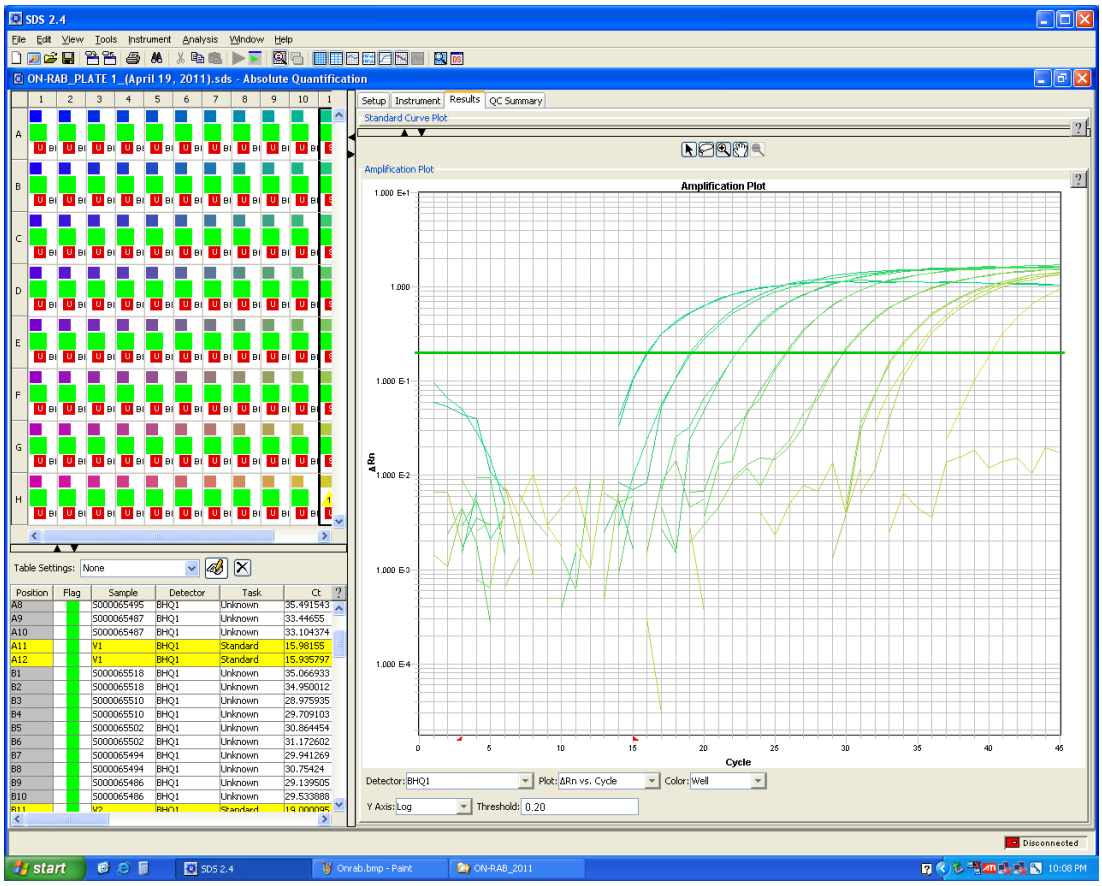

Figure 38: Amplification plot from a real-time PCR experiment.

Calculating LMO content

The LMO content of a sample can be determined in two different ways:

<u>Standard curve method</u>

Standard curves for each of the certified reference material that was amplified by the primers/probes that were designed to amplify both the LMO-specific target DNA sequence and the endogenous, species-specific reference sequence are plotted as shown in figure 39.

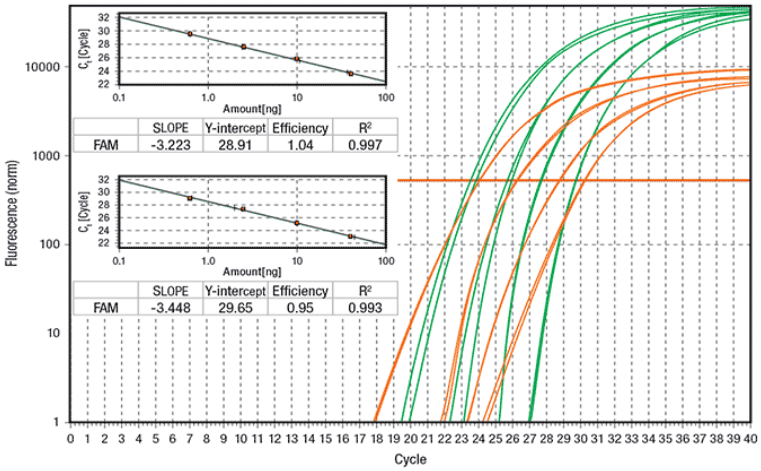

Figure 39: Schematic representation of the amplification plots of a set of four 4-fold serial dilutions of a reference material to produce a standard curve of the endogenous reference gene and the event-specific amplification. Data represents four replicates for each DNA dilution. Source: http://www.sigmaaldrich.com/technical-documents/articles/biology/roche/kapa-multiplex-rtpcr.html.

Using these standard curves, the amount of LMO-specific target DNA and the endogenous genomic DNA can be calculated by obtaining the C_t values from the unknown samples and using the appropriate standard curve to determine the amount of DNA.

The resulting percentage of LMO content is therefore calculated as the ratio between the GM target sequence amount and the reference gene sequence amount, using the formula:

$$\text{Percentage LMO content} = \frac{\text{LM-DNA}}{\text{Reference-DNA}} \times 100\%$$

It is necessary that the C_t values of the samples fall within the upper and lower limits of both standard curves. Outliers must be excluded since they are prone to quantification errors.

Comparative C_t method

This method, also known as $\Delta\Delta C_t$, compares the differences in C_t values of the unknown samples and those of the certified reference materials, which have been normalized against the C_t values of the endogenous genomic DNA.

The first step in this procedure is to calculate the first ΔC_t for each of the unknown samples and the endogenous genomic DNA, as shown in the table below:

Unknown sample		Certified reference material		ΔC_t	
Average C_t value LMO-specific target	Average C_t value endogenous genomic DNA	Average C_t value LMO-specific target	Average C_t value endogenous genomic DNA	Unknown Sample	Certified reference material
	20		18	25-20 = 5	22-18 = 2
25		22			

The $\Delta\Delta C_t$ values are then calculated as follows:

$$\Delta\Delta C_t = (\Delta C_t \text{ unknown sample}) - (\Delta C_t \text{ certified reference material})$$

$$\Delta\Delta C_t = 5\text{-}2 = 3$$

The final result of this method is presented as the fold change of LMO-specific target DNA in the unknown sample relative to the certified reference material, normalized to the endogenous genomic DNA. Therefore, in order to determine the fold change between the unknown sample and the certified reference material the following calculation is used:

$$2^{-\Delta\Delta Ct}$$

$2^{-3} = 0.125$ fold difference between the unknown sample and the certified reference material

For this method to be successful, the amplification efficiencies of both the LMO-specific target DNA and the endogenous genomic DNA reactions should be similar. A method for determining this is to establish how the ΔC_t varies with template dilution. If the efficiencies of the two amplicons are approximately equal, a plot of template DNA dilution versus ΔC_t would display a line that is almost horizontal. If amplification efficiencies are unequal then the standard curve method should be used for quantification.

Availability of DNA-based methods

In the context of LMO detection, PCR has been widely applied and information regarding method development and can be therefore be found in many literature articles and databases (see module 5) In addition, methods have been made available as kits which are available for many screening methods and some commercial events.

Advantages and Limitations of DNA-based methods

PCR-based analysis is not a quick, cheap or easy tool but can be widely used to detect and identify LMOs. The cost of real-time PCR machines, and more important, their maintenance and the availability of supplies can be a significant factor in whether they are suitable for a particular Party to employ. PCR cannot be deployed at elevators, in the field and at remote seed production locations, it is generally restricted to use in the laboratory.

The sensitivity of PCR is not usually dependant on the event being detected, and this makes PCR an excellent screening choice. However, for events that are not well described, such as those developed by those other than the major commercial plant trait developers, sequence and method information can be difficult to find. For LMOs that are not plants, such as yeasts and bacteria, there is no public database available for such methods.

As new events and types of construct are developed, screening methods become more complex, as developers have moved away in plants from the 35S and nos DNA elements used in the early years of LMO technology.

Example experimental procedures – Real-time PCR

Note: This sample experimental procedure should be considered basic examples of LMO analysis using the real-time PCR approach. It is recommend that pertinent sources and literature are periodically reviewed in order to acquire information on more recently developed and validated protocols (For example as is available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx and https://croplife.org/plant-biotechnology/regulatory-2/detection-methods/).

The following protocols are real-time PCR-based methods for the quantification of the specific LM event MON810 by single real-time PCR and for the detection of 35S promoter and *nos* terminator by multiplex real-time PCR (qualitative analysis). It should be noted that both experiments should be preceded by an inhibition run to test the amplifiability of the extracted DNA.

Real-time for quantitative analysis

Real-time PCR will be used to amplify an endogenous reference target DNA sequence that is unique to maize (taxon-specific), plus a DNA target sequence that indicates the presence of the genetically modified crop.
The assays can encompass two independent PCR systems, or happen in the same well as a multiplex reaction. Each target DNA has specific DNA primers and dye-labelled probes. One PCR system detects a LMO-specific target DNA sequence, the other is an endogenous reference system designed to serve as a quantitative reference that detects GM and non-GM maize.

Real-time for qualitative analysis

As previously stated, being widespread in many LMO laboratories, Real-time is used also for qualitative purposes. The following duplex real-time PCR is an example of screening method putting together the detection of 35S promoter and nos-terminator. The two reactions will take place in the same well, this being possible because the specific probes are labelled with different dyes, allowing the two amplifications to be monitored separately.

Multiplex element-specific method for the detection of *P35S* and T-nos by real-time PCR

This section describes the protocol for a duplex real-time PCR screening method for the detection of modified plants.

Target DNA sequences from cauliflower mosaic virus 35S promoter (P35S) and nos-terminator from *Agrobacterium tumefaciens* (T-nos) are amplified. The duplex real-time PCR method uses primer and probe sequences that have already been published for the individual ("single") detection of both target sequences. Combined with a reference gene and using reference standard material, the method can be used to semi-quantitatively estimate the amount of LM plants in an unknown sample.

Materials and equipment

- ABI PRISM® 7500 Sequence Detection System (Applied Biosystems)
- 96-well reaction plates
- Optical caps/adhesion covers
- Micropipettes
- Racks for reaction tubes
- 0.5 mL and 2 mL DNase free reaction tubes
- Standard bench top centrifuge with rotor or standard microfuge fit for 0.5 mL reaction tubes, centrifuge for 96-well reaction plates

- Vortex mixer
- 1.5 ml microcentrifuge tubes

Characteristics of primers for T-nos

Primer forward: 180-F	
Sequence	CATGTAATGCATGACGTTATTTATG
Length	25
Mol. weight (g/mol)	7686.1
Melting point (G/C) based on a [Na^+] of 50 mM	51.1

Probe: TM-180YY	
Sequence	YY-ATGGGTTTTTATGATTAGAGTCCCGCAA-BHQ1
Length	28

Primer reverse: 180-R	
Sequence	TTGTTTTCTATCGCGTATTAAATGT
Length	25
Mol. weight (g/mol)	7643.1
Melting point (G/C) based on a [Na^+] of 50 mM	49.5

Characteristics of primers for P 35S

Primer forward: 35S-FTM	
Sequence	GCCTCTGCCGACAGTGGT
Length	18
Mol. weight (g/mol)	5491.6
Melting point (G/C) based on a [Na^+] of 50 mM	54.9
Probe: 35S-TMP-FAM	
Sequence	FAM-CAAAGATGGACCCCCACCCACG-BHQ1
Length	22
Primer reverse: 35S-RTM	
Sequence	AAGACGTGGTTGGAACGTCTTC
Length	22
Mol. weight (g/mol)	6790.5
Melting point (G/C) based on a [Na^+] of 50 mM	54.8

Controls

Each test series shall include the controls as stated in this manual. If the controls do not yield the expected results, the test results shall be rejected and the analysis shall be repeated.

Experimental procedure

1. Thaw, mix and centrifuge the components needed for the run. Keep thawed reagents on ice.

In a 2 mL tube on ice, add the components in the order mentioned in table 18 (except DNA) to prepare the reaction mix.

Table 18. Reaction mix for real-time PCR (ABI 7500)

Reagents*	**Final concentration**	**Volume per reaction (µL)**
TaqMan® universal PCR Master Mix (2x)	1X	12.5
Primer 35S-F (2µM)	0.1 µmol/L	1.25
Primer 35S-R (2µM)	0.1 µmol/L	1.25
Probe 35S-TMP FAM (2µM)	0.1 µmol/L	1.25
Primer 180-F (20 µM)	1.0 µmol/L	1.25
Primer 180-R (20 µM)	1.0 µmol/L	1.25
Probe TM-180 YY (4 µM)	0.2 µmol/L	1.25
DNA-extract	Samples: about 50 000 cp maize DNA per reaction	5
TOTAL REACTION VOLUME		**25**

*Waiblinger et al. 2008 (DOI 10.1007/s00217-007-0748-z)

2. Mix well and centrifuge briefly.
3. Label one 0.5 mL reaction tube for each DNA sample to be tested.
4. Add into each reaction tube the amount of reaction mix needed for 3.3 repetitions (66 µL). Add into each tube the proper amount of DNA for 3.3 repetitions (16.5 µL DNA). The additional 0.3 repetition included will ensure adequate volume when loading the samples. Vortex for approximately 10 sec. each tube. This step is of mandatory importance to reduce to a minimum the variability between the replicates of each sample.
5. Spin down in a microcentrifuge. Aliquot 25 µL in each well according to the chosen plate set-up loading order.
6. Place an optical cover on the reaction plate and briefly centrifuge the plate.
7. Place the reaction plate into the ABI real-time PCR equipment.
8. Programme the real-time equipment.
9. Set up the plate layout. A sample layout is indicated in figure 40.

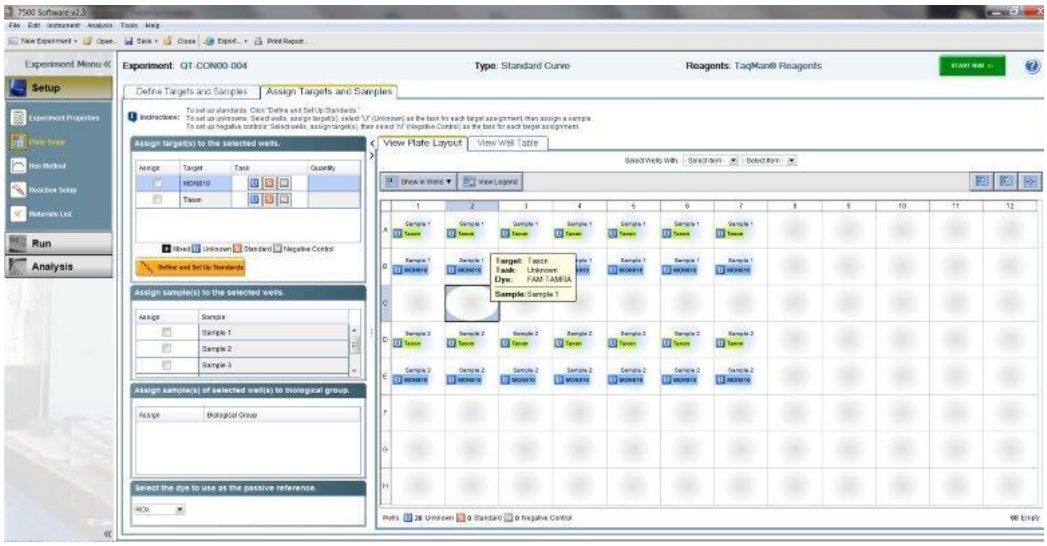

Figure 40. Software layout.

10. Choose the number of cycles, the reaction volume and the details of each reaction step (table 19).
11. Start the run.

Table 19. Amplification conditions

Stage	Temperature	Time	No cycle
Decontamination (UNG)	50°C	2 min	1
Activation/initial denaturation	95°C	10 min	1
Denaturation	95°C	15 sec	
Annealing & extension	60°C	60 sec	
Denaturing, annealing & extension			45

Data analysis and interpretation of results

Subsequent to the real-time PCR run, the data are evaluated using the following procedure:

- Set the threshold: display the amplification curves in logarithmic mode, place the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR)**.**
- Set the baseline: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest Ct = 25 → baseline Ct = 25 – 3 = 22).
- Save the settings and export the data.

This being a qualitative method, we only need to verify that the amplification took place. Adding reference amplification to the plate we could get some semi-quantitative information.

Protocol for a construct-specific method for the quantitation of MON810 by Real-Time PCR

The following describes a method for the detection and quantitation of a taxon-specific maize gene (maize starch synthase IIb: *zSSIIb*) and of the specific DNA construct junction region between the intron sequence of maize heat shock protein 70 gene and synthetic *cryIA(b)* gene derived from *Bacillus thuringiensis* present in the genetically modified (GM) maize MON810. The method is based on real-time PCR using plasmid as a reference material in order to quantify the relative amount of MON810 using a conversion factor (Cf) that is the ratio of copy numbers between construct-specific and taxon-specific DNA sequences in the representative genuine MON810 seeds.

Equipment and reagents

- ABI PRISM® 7500 Sequence Detection System (Applied Biosystems)
- 96-well reaction plates
- Optical caps/adhesion covers
- Micropipettes
- Racks for reaction tubes
- 0.5 mL and 2 mL DNase free reaction tubes
- Real-time PCR detection system and appropriate analysis software
- Standard bench top centrifuge with rotor or standard microfuge fit for 0.5 mL reaction tubes, centrifuge for 96-well reaction plates
- Vortex mixer
- 1.5 ml microcentrifuge tubes.

Characteristics of primers for the MON810 specific system (QT-CON-00-004)

Primer forward: M810 2-5'	
Sequence	TCGAAGGACGAAGGACTCTAACG
Length	22
Mol. weight (g/mol)	6692.4
Melting point (G/C) based on a [Na^+] of 50 mM	54.8
LMO target probe: M810-Taq	
Sequence	FAM-AGATACCAAGCGGCCATGGACAACAA-TAMRA
Length	26
Primer reverse: M810 2-3'	
Sequence	GGATGCACTCGTTGATGTTTG
Length	21
Mol. weight (g/mol)	7106.3
Melting point (G/C) based on a [Na^+] of 50 mM	52.4

Characteristics of primers for the taxon-specific system (QT-CON-00-004)

	SSIIb1-5'
Sequence	CTCCCAATCCTTTGACATCTGC
Length	22
Mol. weight (g/mol)	7204.3
Melting point (G/C) based on a [Na^+] of 50 mM	54.8
	Taxon probe: SSIIb1-Taq
Sequence	FAM-AGCAAAGTCAGAGCGCTGCAATGCA-TAMRA
Length	25
	SSIIb1-3'
Sequence	TCGATTTCTCTCTTGGTGACAGG
Length	23
Mol. weight (g/mol)	7659.6
Melting point (G/C) based on a [Na^+] of 50 mM	55.3

Standard curve

The calibration curve method has been used for quantitation of copy numbers in extracted DNA from unknown test sample DNA extracts. Separate calibration curves with each primer/probe system are generated in the same analytical amplification run. The calibration curves are composed of five concentrations including 20, 125, 1500, 20000, 250000 copies of DNA of plasmid pMul5. At each of the five calibration points, triplicate measurements are performed. Triplicate reactions using an appropriate dilution of the DNA extracted from the unknown sample were also measured in the ABI PRISM® 7500 SDS (Applied Biosystems) in the same analytical run.

The *C*q values determined for the calibration points in the *zSSIIb* or MON810 construct-specific target, respectively, are plotted against the logarithm of the copy number of plasmid DNA of pMul5 to establish a calibration curve. The copy numbers measured for the test sample DNA are obtained by interpolation from the standard curves. For the determination of the amount of MON810 in the test sample, the copy number of the MON810 construct is divided by the copy number of the *zSSIIb* gene and the construct-specific Cf of MON810, multiplied by 100 to obtain the percentage.

Controls

Each test series shall include the controls as stated earlier. If the controls do not yield the expected results, the test results shall be rejected and the analysis shall be repeated.

As a positive control/calibration reference material, at least two alternatives are available, as follows.

- High quality, pure genomic DNA extracted from maize kernel may be used if the quantity of DNA is known, on the basis of calculating copy numbers of the target sequence from the genome size of MON810 maize.
- A plasmid containing the target sequence(s) may be added in different concentrations with known copy numbers.

Master mix preparation

1. Thaw, mix and centrifuge the components needed for the run. Keep thawed reagents on ice.

2. In a 2 mL tube on ice, add the components in the order mentioned in the table (except DNA) to prepare the reaction mix. Please prepare only the reaction mix needed for the run. Mix well and centrifuge briefly.
3. Label one 0.5 mL reaction tube for each DNA sample to be tested.
4. Add into each reaction tube the amount of reaction mix needed for 3.3 repetitions (66 µL).
5. Add into each tube the proper amount of DNA for 3.3 repetitions (16.5 µL DNA). The additional 0.3 repetition included will ensure adequate volume when loading the samples. Vortex for approximately 10 sec. each tube. This step is of mandatory importance to reduce to a minimum the variability between the replicates of each sample.
6. Spin down in a microcentrifuge. Aliquot 25 µL in each well according to the chosen plate set-up loading order.
7. Place an optical cover on the reaction plate and briefly centrifuge the plate.
8. Place the reaction plate into the ABI real-time PCR equipment.
9. Programme the real-time equipment
10. Set up the plate layout.

The length of the SSIIb PCR product is 151 bp; the length of the MON 810 PCR product is 113 bp.

Table 20. PCR reaction set-up

GM target		**Taxon target**	
Reagent	Final concentration	Reagent	Final concentration
TaqMan® Universal PCR Master	1x	TaqMan® Universal PCR Master	1x
Primer Fw	0.50 µmol/L	Primer Fw	0.50 µmol/L
Primer Rev	0.50 µmol/L	Primer Rev	0.50 µmol/L
Probe	0.20 µmol/L	Probe	0.20 µmol/L
Template DNA	50 ng	Template DNA	50 ng
TOTAL VOLUME	**25 µL**	**TOTAL VOLUME**	**25 µL**

Table 21. Reaction conditions

Stage	**Time**	**Temperature °C**	**No cycles**
Pre-PCR: decontamination	2 min	50°C	1
Pre-PCR: activation of DNA polymerase and denaturation of template DNA	10 min	95°C	1
Step 1 Denaturation	30 sec	95°C	
Step 2 Annealing and elongation	60 sec	59°C	
Denaturing, annealing & elongation			40

Plate set-up

Table 22. Plate set-up.

	1	2	3	4	5	6	7	8	9	10	11	12
a	*S1*	*S1*	*S1*	*S2*	*S2*	*S2*	*S3*	*S3*	*S3*	*S4*	*S4*	*S4*
b	*S5*	*S5*	*S5*	*U1*	*U1*	*U1*	*U2*	*U2*	*U2*	*U3*	*U3*	*U3*
c	*U4*	*U4*	*U4*	*U5*	*U5*	*U5*	*U6*	*U6*	*U6*	*U7*	*U7*	*U7*
d	*U8*	*U8*	*U8*	*U9*	*U9*	*U9*	*U10*	*U10*	*U10*	*C0*	*C0*	*C0*
	Upper half: MON810 maize specific system											
	Lower half: Reference specific system											
e	*S1*	*S1*	*S1*	*S2*	*S2*	*S2*	*S3*	*S3*	*S3*	*S4*	*S4*	*S4*
f	*S5*	*S5*	*S5*	*U1*	*U1*	*U1*	*U2*	*U2*	*U2*	*U3*	*U3*	*U3*
g	*U4*	*U4*	*U4*	*U5*	*U5*	*U5*	*U6*	*U6*	*U6*	*U7*	*U7*	*U7*
h	*U8*	*U8*	*U8*	*U9*	*U9*	*U9*	*U10*	*U10*	*U10*	*C0*	*C0*	*C0*

Data analysis and interpretation of results

Subsequent to the real-time PCR run, the data are evaluated using the following procedure:

- Set the threshold: display the amplification curves in logarithmic mode, place the threshold line in the area where the amplification profiles are parallel (exponential phase of PCR)**.**
- Set the baseline: determine the cycle number at which the threshold line crosses the first amplification curve and set the baseline three cycles before that value (e.g. earliest C_q = 25 → baseline C_q = 25 – 3 = 22).
- Save the settings and export the data on an excel file Opening the exported results file in Microsoft®Excel.
- Calculate the C_q average of each group of replicate to calculate the ΔC_q values.

For each sample, %LMO is calculated by analysing the sample's ΔC_q, comparing it to the set of log (% LMO) and ΔC_q values obtained from the concentration standards set.

Inhibition run

Preparation of DNA dilution series

The inhibition run preparation starts by bringing the extracted DNA to a level corresponding to the highest DNA concentration intended to be used in the subsequent PCR method, the so-called "undiluted" sample. From this first sample, a fourfold dilution series of each DNA extract is prepared (1:4, 1:16, 1:64, and 1:256).

40 µl of each dilution should be prepared as follows:

- Label tubes with the number of the corresponding DNA extract plus the dilution rate from the working dilution. In the table below, DNA extract number 1 is taken as example.
- Distribute appropriate volumes of dilution buffer, i.e. TlowE buffer, in labelled tubes (see table below, column named "TlowE buffer")
- In the tube labelled 1 (1:4) add 10 µl of the working dilution 1 and mix by pipetting at least 20 times or vortex for at least 3 seconds.
- In the tube labelled 1 (1:16) add 10 µl of the 1 (1:4) diluted sample and mix by pipetting at least 20 times or vortex for at least 3 seconds.
- Proceed in this way to prepare the dilution series described in the table below.

DNA dilutions	Starting DNA	Dilution factor	Vol. DNA (µl)	TlowE buffer (µl)	Total (µl)
1 (1:4)	Working Dilution	4	10	30	40
1 (1:16)	1 (1:4)	4	10	30	40
1 (1:64)	1 (1:16)	4	10	30	40
1 (1:256)	1 (1:64)	4	10	30	40

The test is conducted at least with the taxon-specific reference system. To assess the presence of inhibitors, the C_q values of the diluted samples are plotted against the logarithm of the dilution factor and an equation is calculated by linear regression. Three criteria have to be met: the slope of the regression line should be within -3.6 and -3.1, the linearity should be above 0.98, and the C_q value for the "undiluted" sample extrapolated from the linear regression is compared with the measured C_q for the same sample. The difference (ΔC_q), average between the measured C_q and the extrapolated C_q value, should be within 0.5. Therefore, one of the criteria, as defined in the "EU Minimum Performance Requirements for Analytical Methods of GMO Testing", is dedicated to the evaluation of the expected vs. measured DNA content in the most concentrated sample which can, in case of inhibition, result in underestimation (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

Limitations of DNA-based methods

While DNA analysis is a very powerful tool in the field of LMO detection and identification, there continue to be some limitations posed on this diagnostic tool. Below is a brief discussion of some of the most common limitations faced by laboratories when using DNA based methods.

DNA sequence information

Sequence information is necessary to facilitate the development of detection protocols in order to be able to design primers that target the genetic elements that have been inserted in the LMO. However, such information is considered confidential business information and is not always readily available to regulatory laboratories. While the regulatory frameworks of some countries require notifiers to include a detection method in their notification, it is still necessary to validate such methods and the availability of sequence information would facilitate this process.

Certified reference materials

Certified reference materials (CRMs) are necessary for the validation of testing procedures in laboratories as well as in their day to day operations for ensuring that quality control measures meet the necessary standards. While some CRMs that correspond to commercially available LMOs may be available for purchase, they are not always available for locally developed LMOs or LMOs that are unauthorized. Some laboratories have also turned to using reference materials in the form of plasmid DNA fragments or by assembling DNA fragments that serve to mimic the junctions of transgenic elements in a given LMO. However, this can result in an overestimation of the LMO concentration present in a case sample due to the increased PCR efficiency of amplifying purified plasmid DNA. While it is possible for individual laboratories to generate the necessary CRMs for them to perform their testing, this can prove to be laborious and costly. It may be worthwhile for regulatory systems to consider requiring LMO developers to provide reference material to the regulatory laboratory performing LMO detection for each LMO being notified to the regulatory authorities in order to overcome this limitation.

Degraded samples

DNA degradation can occur if samples are exposed to an unfavorable environment prior to its extraction, such as high heat or humidity. Care must therefore be taken during sample collection and sample storage to ensure that the integrity of the DNA within the sample is maintained. The risk of DNA degradation is also higher in samples that may have been processed which may lead to fragmentation of the DNA.

Sample inhibition

Inhibition can occur if inhibitors that have been co-extracted with the DNA from the sample. Looking for inhibition is important because it provides information on the efficiency of the reaction. If an LMO is present in very low concentration and the extracted DNA contains inhibitors, the analysis could give a negative result even if the target DNA is present, meaning a false negative result. Inhibition tests can therefore be carried out by preparing a set of serial dilutions of the extracted DNA.

Stacked events

A stacked LMO event is when a single LMO contains two or more transgenic traits. This can be obtained through the cross breeding of two or more LM plants with individual traits, the transformation of a novel LMO with a multi-gene transformation cassette or with multiple transformation cassettes with individual

genes, or the retransformation of an LMO. Such LMOs pose a challenge in their detection and identification because it is virtually impossible to differentiate between samples that contain a mixture of single events and samples that contain DNA from a stacked event. This may prove problematic since in some countries the appropriate approvals may have been obtained for a given single event but not for a stacked event containing that same single event.

Novel technologies for LMO detection

Introduction

While real-time PCR represents the most commonly used method for the detection and identification of LMOs, with advances in DNA detection technologies, new methods have been adapted for use in the field of LMO detection. These methods are being applied to address some of the limitations that traditional methodologies have not been able to address, particularly with the advances in LMO development. Below is a discussion of some of the more popular methods that are being implemented in LMO detection laboratories.

Digital PCR

Digital PCR (dPCR) is based on the same principals of Taqman® chemistry; however, prior to PCR amplification the reaction components are partitioned in such a way that they are broken up into several thousand droplets. During the partitioning process the sample DNA, that has been diluted down to a limiting quantity, is randomly distributed into individual partitions, such that each partition which would contain anywhere from zero to a few copies of sample DNA, as shown in figure 41.

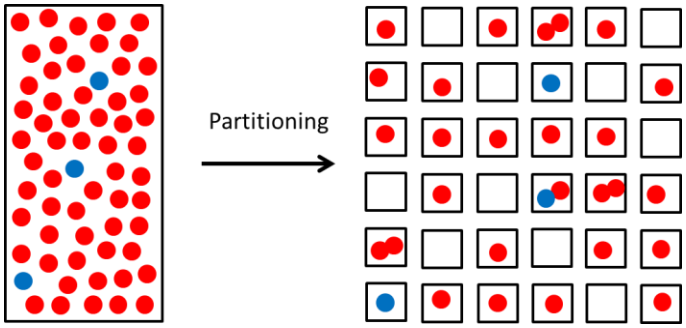

Figure 41: Schematic representation of the partitioning process that is carried out for dPCR.

The partitioning process varies depending on the choice of instrumentation used. There are two main methods:

1. Droplet based dPCR: This method involves separating a sample into thousands of nanolitre sized droplets using a droplet generator instrument.

2. Array based dPCR: This involves partitioning the sample on a reaction plate that contains several thousand reactions “through-holes” each of which can accommodate nanolitre sized reaction volumes

Each partition also contains all the necessary PCR reaction components therefore once they are exposed to traditional PCR reaction conditions any DNA that may be present in a partition will be amplified. Results of the amplification are measured either at the end point of the PCR or in real time, depending on

the instrument being used, by detecting the presence of a fluorescence signal that would have been released by the Taqman® probe.

This method of PCR is advantageous in that it allows for easier detection of low concentrations of target DNA that may be present in a sample. This is because traditional real-time PCR provides a single data point from each DNA sample. Any signal produced from low concentrations of target DNA that may be present in the sample will be drowned out by the signal produced from the more abundant DNA that is present or will not produce a sufficient signal that meets threshold requirements. However, with dPCR each partition serves as an individual amplification that provides a unique data point for the DNA that is present in the partition. Therefore, a strong signal is obtained from target DNA that is present in low concentrations and occupies a single partition. This results in a more accurate representation of the nature of the whole DNA sample.

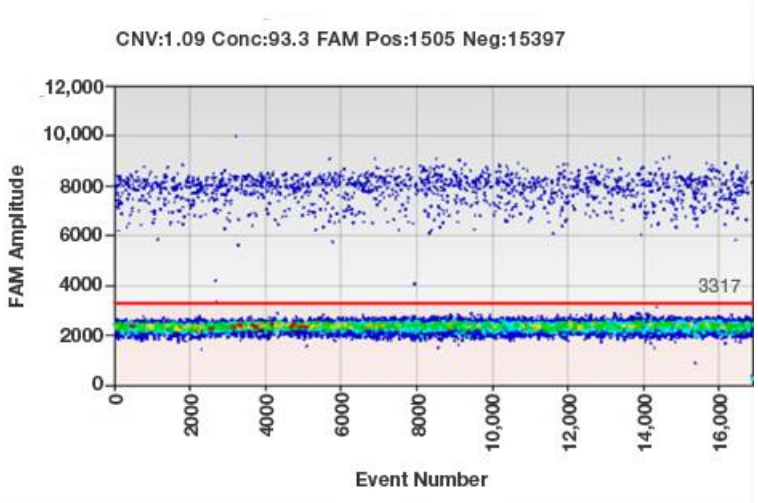

Figure 42: Sample results from a ddPCR experiment. Each droplet in a sample is plotted on a graph of fluorescence intensity versus droplet number. All positive droplets (those above the threshold intensity indicated by the red line) are scored as positive, and each is assigned a value of 1. All negative droplets (those below the threshold) are scored as negative, and each is assigned a value of 0 (zero). This counting technique provides a digital signal from which to calculate the starting target DNA concentration by a statistical analysis of the numbers of positive and negative droplets in a given sample. Source: http://www.bio-rad.com/en-ca/applications-technologies/droplet-digital-pcr-ddpcr-technology.

Furthermore, once all the data from each partition is collected computer software performs the necessary statistical calculations, using Poisson's law of small numbers, to determine the number of copies per microlitre of the target sample in the original DNA solution. This is based on the fact that the proportion of positive droplets detected following PCR is proportional to the concentration of target DNA in the sample. As a result, absolute quantification of the DNA sample can be carried out. This is done without the need for standard curves, or the amplification of an endogenous reference gene, as is the case with relative quantification when preforming traditional real-time Taqman® PCR.

Isothermal DNA amplification

A number of different methods have been developed for using an isothermal DNA amplification. These include helicase dependent amplification, loop-mediated isothermal amplification (LAMP), nucleic acid sequence-based amplification, nicking enzyme amplification reaction (NEAR), rolling circle

amplification, recombinase polymerase amplification (RPA), and strand displacement amplification. Of these LAMP, RPA and NEAR have been developed for LMO detection and identification. LAMP is described here as an example.

Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) is a DNA amplification procedure that is carried out at a single, isothermal, temperature, unlike traditional PCR where temperature cycling is required. It is preformed using a DNA polymerase that has high strand displacement capabilities and is functional at optimal temperatures around 60-65^{o}C. The method requires the use of 4-6 primers that target 6-8 distinct regions, as indicated in figure 43, which work in concert to amplify the DNA in two distinct phases.

1. *Non-cyclical phase*: this step results in the generation of the stem loop or "dumbbell" DNA structure that serves as the key intermediate from which amplification can continue, as shown in figure 43b.

2. *Cycling phase:* using the dumbbell structure as a starting point, the DNA polymerase continues the amplification process to generate concatemers of inverted repeats of the target amplicon, as outlined in figure 43c.

In addition to being performed at a constant temperature, the LAMP reaction can be completed within 30 minutes, yielding between 10-20µg of amplified DNA. The results can be visualized with the naked eye using simple colorimetric techniques, such as the formation of a precipitate or a colour change resulting from a change in pH as amplified DNA accumulates. These qualities make the LAMP technique suitable for use in the field with results being provided with minimal delay. However, the development and validation of an optimized LAMP assay can be laborious and the nature of the assay does not allow for the possibility of multiplexing.

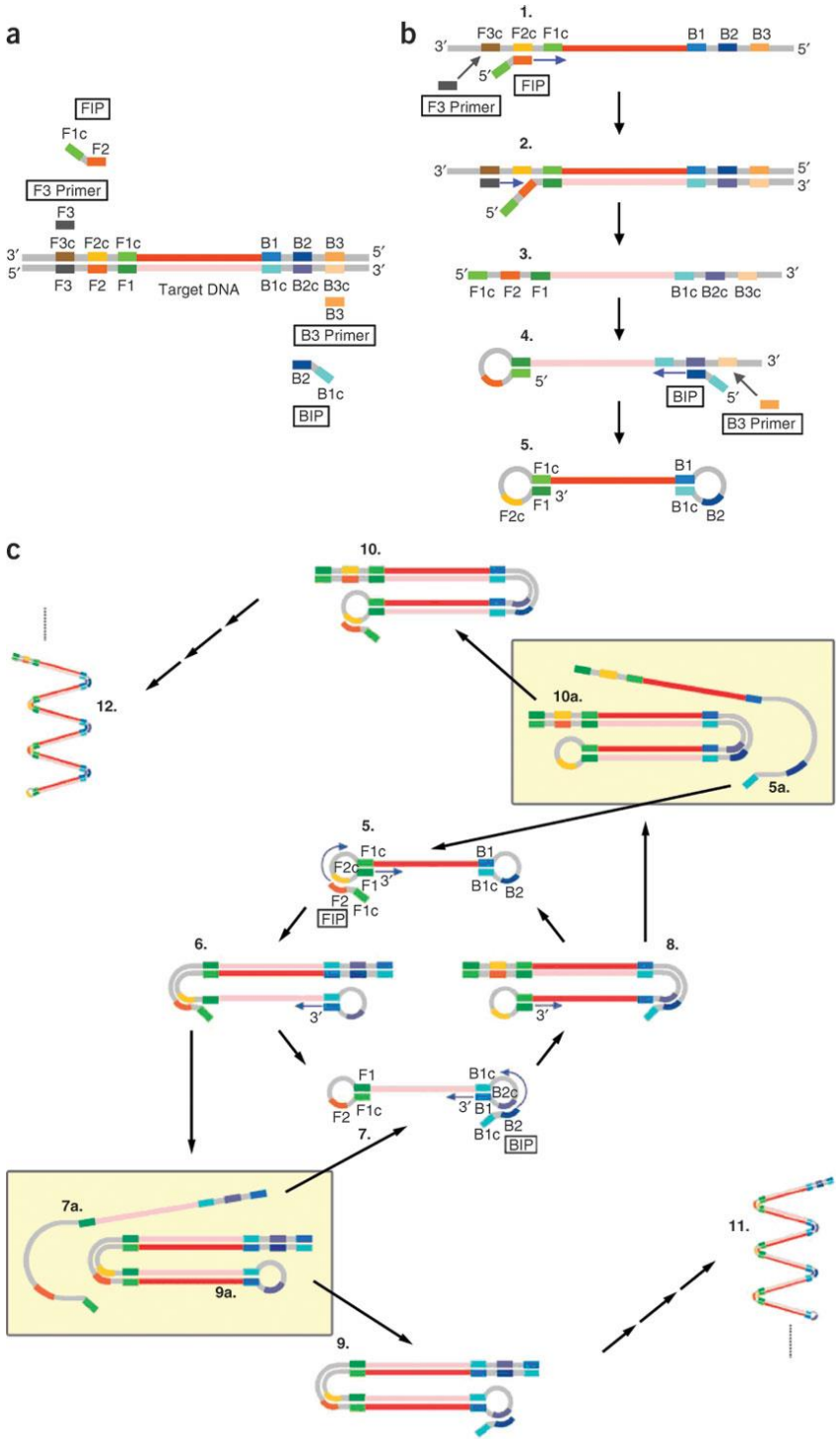

Figure 43: (a) Primer design of the LAMP reaction. For ease of explanation, six distinct regions are designated on the target DNA, labelled F3, F2, F1, B1c, B2c and B3 from the 5′ end. As c represents a complementary sequence, the F1c sequence is complementary to the F1 sequence. Two inner primers (FIP and BIP) and outer primers (F3 and B3) are used in the LAMP method. FIP (BIP) is a hybrid primer consisting of the F1c (B1c) sequence and the F2 (B2) sequence. **(b) Starting structure producing step.** DNA synthesis initiated from FIP proceeds as follows. The F2 region anneals to the F2c region on the target DNA and initiates the elongation. DNA amplification proceeds with BIP in a similar manner. The

F3 primer anneals to the F3c region on the target DNA, and strand displacement DNA synthesis takes place. The DNA strand elongated from FIP is replaced and released. The released single strand forms a loop structure at its 5′ end (structure 4). DNA synthesis proceeds with the single-strand DNA as the template, and BIP and B3 primer, in the same manner as described earlier, to generate structure 5, which possesses the loop structure at both ends (dumbbell-like structure). **(c) Cycling amplification step.** Using structure 5 as the template, self-primed DNA synthesis is initiated from the 3′ end F1 region, and the elongation starts from FIP annealing to the single strand of the F2c region in the loop structure. Passing through several steps, structure 7 is generated, which is complementary to structure 5, and structure 5 is produced from structure 8 in a reaction similar to that which led from structures 5–7. Specifically, intermediate structures 7a and 9a and structures 5a and 10a (in the yellow boxes) are produced from structures 6 and 8, respectively. Structures 9a and 10a then form structures 9 and 10, respectively, whereas the displaced strands 7a and 5a form the dumbbell-like structures 7 and 5, respectively. More elongated structures (11, 12) are also produced. Source: http://www.nature.com/nprot/journal/v3/n5/fig_tab/nprot.2008.57_F1.html

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Module 5:

Introduction to Quality Assurance/Quality Control Standards

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Introduction

An important aspect when establishing capacity for the detection, identification and quantification of LMOs is the implementation of a laboratory management system that ensures the provision of minimum performance criteria with respect to quality control and quality assurance (QA/QC) to confirm the adequate handling and processing of samples, as well as to ensure the quality of and confidence in the results obtained. While there is a wide variety of methodologies and instruments that can be used in a molecular biology laboratory for the detection and identification of LMOs, it is important to select and implement methods that will produce reliable and consistent results, while, at the same time, meeting minimum performance criteria.

The implementation of minimal performance criteria ensure that differences in methodology do not bias the results of laboratory analysis and form part of the QA/QC system that is required for laboratory accreditation. It is important that the analyte being measured is defined and that the unit of measurement is standard and understood in simple and internationally accepted language. Furthermore, it is important to use certified reference materials (CRMs) to confirm harmonization between laboratories and methods as well as for method validation and verification.

This section of the training manual provides an introduction to the minimum performance criteria that may be considered by laboratories when implementing their selected methods for the detection and identification of LMOs as well as information on the establishment of a QA/QC system and how to validate or verify such methods within a laboratory.

Laboratory documentation system

An overarching aspect of QA/QC is the need to substantiate critical aspects of all laboratory activities with documentation. Establishing sound documentation practices contributes to ensuring that minimum performance criteria are implemented and maintained in the laboratory.

Documentation of laboratory activities including the minimum performance criteria include but are not limited to the following:

- Standard operating procedures for the different methods used to process samples;
- Standard operation procedures for equipment used to process samples;
- Standard operating procedures for the preparation of reagents used to process samples;
- Safety manual for the laboratory.

In addition, information relating to the sample as it is processed should also be documented in order to allow for complete traceability of the procedure. This should include but is not limited to documentation of the following:

- Reagent preparation;
- Reagent lot numbers used during sample processing including controls;
- Temperature controlled equipment including fridges/freezers and incubators;
- Maintenance and verification/calibration of equipment.
- Raw data for samples and controls throughout the analysis process;
- The final report for the result of the analysis.

Additional information on specific documentation requirements are provided under relevant sections in this chapter.

Laboratory set-up requirements and environment

A well-organized laboratory is one of the most important considerations in an LMO testing facility in order to produce reliable and consistent results as well as minimize the possibility of sample contamination. Separate rooms or areas for each processing step should be used wherever possible while maintaining a unidirectional forward workflow. This may include, as appropriate, dedicated areas for sample reception and/or sample homogenization, analyte extraction, reagent preparation, PCR set-up, PCR, agarose electrophoresis and/or data analysis. Such considerations are particularly important in multipurpose laboratories where shared spaces are used for diagnostic as well as research purposes.

Each separate laboratory area must have dedicated equipment that cannot be moved from one laboratory or area to another without carrying out the necessary cleaning and decontamination steps to prevent contamination. Furthermore, each laboratory area must be furnished with dedicated laboratory wear, stationary and personal protective equipment, such as laboratory coats and gloves to facilitate the implementation of good laboratory practice by personnel.

Good laboratory practice includes implementing a cleaning routine of laboratory areas before and after use to minimize the risk of contamination. This includes:

1. Cleaning and decontamination of the working area using detergent and decontamination reagent. It is important that when decontamination reagent is used that the area also be rinsed with water to remove any residual decontamination reagent from working surfaces.

2. Cleaning and decontamination of the sampling and homogenization equipment using detergent and decontamination reagent. It is important that when decontamination reagent is used that the equipment also be rinsed with water to remove any residual decontamination reagent.

3. The sample preparation work area can also be vacuumed after processing a sample to remove large particulate matter.

1. Sample reception and homogenization

Samples that are submitted for analysis need to be received, catalogued and homogenized. Samples that have been prepared this way are referred to as retention or retained samples. Once prepared they are stored in a dedicated space that provides an appropriate storage environment and is secure. Retention samples can be stored at room temperature provided that the sample integrity is maintained for the period of storage. For example, high temperatures and/or high humidity may lead to the destruction of the sample as a result of the growth of microbes and result in subsequent DNA degradation. Under the latter conditions, it would be necessary to store retention samples in a dedicated fridge. Some retention samples may not store well at room temperature or in a fridge, for example seed potatoes, such samples may therefore be stored in a freezer. It is important that precaution is taken not to dispose of any remaining non-homogenized sample in a way that can result in its accidental introduction into the environment.

Sample homogenization should be performed in a dedicated room where no other laboratory procedures are performed and does not share ventilation with other rooms to avoid the spread of possible dust resulting from sample homogenization. Good laboratory practice must be implemented to ensure that dust particles produced by the sample do not contaminate other laboratory samples considering the high

sensitivity of subsequent analytical methods. Ideally, the procedure should be carried out in a dedicated fume hood or dust extraction cabinet.

It is important that standard operating procedures include the cleaning and decontamination of the sample reception and homogenization area as well as any equipment. To validate whether a cleaning and decontamination method is effective, either of the following tests can be performed:

3. After processing an LMO sample, a non-LMO sample is processed and then tested for the presence of LMO material; or a sample of one species (for example corn) is processed followed by a second sample from another species (for example soybean). The second sample is then tested for the presence of the first species.

4. Working surfaces and equipment can be swabbed with sterile PCR grade water and the swab placed in a tube with a minimal amount of PCR grade water. Following a brief incubation period, including vigorous shaking using a vortex and centrifugation, the same volume of liquid of water from the swab is added to a PCR reaction to test for the presence and contamination that my result in DNA amplification.

2. DNA extraction

DNA is extracted from samples using a combination of approaches as listed in module 3. The DNA extraction area is considered a "dirty" environment, meaning that there is potential to for contamination to take place. Care must therefore be taken to ensure that good laboratory practice is followed to minimize potential cross contamination between samples as they are processed in this area. In this area dilutions of extracted DNA can also be done.

3. PCR master mix set-up

In this area, also known as the "clean room", reagents, such as primers, probes and buffers, are prepared, stored, and aliquoted in a clean environment that is free of any nucleic acids. The process of aliquotting stock solutions into smaller working volumes is an important step in managing the spread of contamination and prevents excessive waste of unused reagents if contamination occurs. Furthermore, making aliquots minimizes the frequency of freeze/ thaw cycles that stock solutions may be exposed to therefore helping to maintain the quality of the reagents that may be sensitive to changes in temperature. Other procedures that take place in this area include the preparation of PCR master mixes prior to the addition of the sample DNA.

4. PCR sample preparation

This area is dedicated to the addition of the extracted and positive control DNA to the PCR master mixes. The addition of sample and positive control DNA to the PCR reaction can also take place in the PCR amplification area.

5. PCR amplification

This area is dedicated to the amplification of the PCR target and contains the PCR machines. The PCR area can also be combined with post-PCR if the laboratory area allows a suitable separation of work activities. The extracted DNA as well as PCR controls can be added to the PCR master mix in a dedicated area in PCR if a separate PCR sample addition area is not available.

6. Post PCR

This area is dedicated to post-PCR activities but can also be combined with the PCR amplification area depending on the available laboratory space. Post-PCR primarily deals with gel electrophoresis as well as other possible activities such as DNA sequencing. Ideally, PCR machines should be in separate areas to that of other post-PCR activities including gel electrophoresis.

Sample tracking

All samples that are received by the laboratory must be assigned a unique sample tracking number. This unique tracking number is used to identify the sample as it is processed. The use of a tracking number helps maintain traceability and confidentiality of the sample information. Furthermore, the unique sample number can be used to facilitate the systemic storage of samples and/or extracted DNA.

Minimal performance criteria and requirements for quality assurance

Quality assurance is defined by ISO9000:2015 as "part of quality management focused on providing confidence that quality requirements will be fulfilled".[18] It is a multifaceted procedure that is put in place by the laboratory to ensure that the results of testing are precise, accurate and reproducible. The laboratory quality assurance system also outlines the necessary corrective action to be taken if required.

The following section presents an overview of specific areas where quality assurance measures are applied and the impact they have on maintaining quality. Understanding these parameters and being able to detect any deviations from set criteria during routine testing ensures that results are consistent and remain within acceptable ranges of variability.

1. Personnel

Similar to other fields of molecular diagnostics, LMO detection requires that it is performed by competent and trained personnel. New personnel should undergo formal training and demonstrate competency that is documented before being allowed to work unsupervised. Trained laboratory personnel should periodically undergo retraining such that they remain up to date on testing methodologies. Furthermore, the roles of personnel should be clearly defined with a clear reporting structure.

2. Analytical controls

There are several potential sources of contamination or DNA degradation as a sample is processed, such as:

1. Contamination of extracted DNA or controls by PCR amplified target DNA;
2. Cross-contamination between samples during DNA extraction and addition of DNA to PCR reactions;
3. Degradation of DNA due to the use of decontamination reagents to clean equipment and bench surfaces.

The first two can lead to false positive results whereas the third will produce a possible false negative result. In order to obtain reliable results the use of analytical controls, which are tested in parallel with test

[18] https://www.iso.org/obp/ui/#iso:std:iso:9000:ed-4:v1:en:term:3.6.5.

samples, must always be used to monitor the performance of the DNA extraction and PCR for the presence of contaminants (ISO 24276)[19] (table 23).

Table 23. Common controls that can be used to monitor reagent and environmental conditions during DNA extraction and PCR

Name	Description	Purpose	Expected result
Environmental / PCR blank control	Nucleic acid free water	Monitor contamination during PCR set-up and in the PCR reagent	Negative
Extraction blank control	Control in which the sample is substituted by nucleic acid-free water and that follows all the extraction steps	Monitor contamination during the extraction procedure and in the DNA extraction reagent	Negative
Positive extraction control	Control sample positive for the target analyte (it can be certified reference material)	Monitor the extraction and PCR amplification of a sample containing the target analyte	Positive
Positive PCR control	DNA positive for the target analyte that can be used at an amount of DNA or copy number that reflects the limit of detection or limit of quantification	Monitor that the PCR assay can amplify the target analyte at the limit of detection or limit of quantification	Positive
Negative LMO control/positive taxon control	Positive for the taxon-specific target DNA but negative for the LMO target	Monitor the absence of false LMO positives during the extraction method and PCR and confirm that the taxon-specific target can be PCR amplified	Positive for the taxon-specific target but negative for the LMO target
PCR inhibition control	Dilution of the sample DNA, detection of a taxon-specific target or the addition of positive target DNA to the sample DNA	Monitor PCR inhibition in the sample	Positive

[19] ISO 24276:2006 Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - General requirements and definitions.

Positive controls

The purpose of the PCR positive control is to confirm that the testing procedure was performed correctly and can also be used to monitor that the target analyte is detected at the necessary limit of detection (LOD) or limit of quantification (LOQ). A sample containing the target DNA can be included as extraction control and analysed with other samples to ensure that the extraction method and PCR assay is acceptable.

Negative controls

Contamination may occur during the extraction and purification of the target DNA, as well as during the preparation of the amplification reaction mixture. The negative control, therefore, confirms that no contaminants were introduced into the testing reagents during the testing process and thus assures that the results obtained from the test samples actually reflect the content of the test sample. An extraction control blank containing sterile PCR grade water can be included in each batch of extractions to monitor if contamination of the extraction and PCR reagent occurs. Furthermore, a negative LMO target/positive taxon-specific DNA control can be used to: 1) confirm the absence of the LMO target and 2) confirm that the taxon target is amplifiable.

Inhibition controls

An inhibition control can be used to ensure that the PCR test result is not a false negative due to sample inhibition. The degree to which a PCR is inhibited is proportional to the concentration of inhibitors that may have been co-extracted with the DNA from the sample. Sample inhibition can be determined by either a dilution of the sample, the detection of an endogenous taxon-specific target in the sample or by spiking the sample by the addition of a known amount of positive control DNA.

3. Equipment

The maintenance and/or verification/calibration of laboratory equipment is an essential component in ensuring the precision, accuracy and reproducibility of laboratory results. The routine maintenance and calibration of equipment is vital to maintaining the QA/QC programme within a laboratory. The interval for maintenance and/or verification/calibration of equipment as well as the results of such verification/calibration should be well documented.

The equipment verification/calibration procedure must be appropriate for the intended use of the equipment and performance criteria and can often be obtained from the manufacturer's operating manuals. Equipment that is not performing as required should either be removed from the laboratory area where it is used or labelled as "out of order" and not used in any further sample processing. Equipment can be calibrated using external service providers; however, this may be cost prohibitive for some laboratories. Where the appropriate expertise is available in the laboratory, an internal verification/calibration system can be adopted.

Equipment must be regularly cleaned and checked to ensure that it is performing as expected. Where the measurement of units is critical for the performance of equipment, such as the accuracy of an analytical balance or the temperature of a heating block, verification can be carried out using a calibrated measure of the unit. For example, a calibrated thermometer can be used to check the accuracy of temperature sensitive equipment such as heating blocks, or calibrated weights can be used to check the accuracy of an analytical balance. In turn, the verified analytical balance can be used to check the accuracy of a pipette by weighing the required volume of pipetted water by weight. In addition, equipment used to measure the intensity of color development (e.g for ELISA) or fluorescence should also be regularly calibrated.

Table 24. Sample checklist of information that can be kept in an instrument maintenance log-book. Source: Adapted from UNOCD 2009.

Checklist of information to be kept in the instrument maintenance log-book
• **Name of the equipment and associated software**
• **Name of the manufacturer, model and/or type Serial number**
• **Date of receipt of equipment in laboratory/Date equipment was placed in service by the laboratory**
• **Details and date of checks made for compliance with relevant calibration or test standard specification**
• **Current location in the laboratory, if appropriate**
• **Copy of the manufacturer's operating instruction(s)**
• **Details of maintenance carried out and records of the subsequent performance check**
• **Maintenance/verification/calibration intervals**
• **Maintenance/verification/calibration record**
• **Identity of the individual performing maintenance check and/or verification/calibration**

4. Reagent quality control

Reagents can either be prepared in the laboratory and/or commercially acquired as appropriate. Reagents that are prepared in the laboratory should be labelled as applicable with the following information as needed: identity of the reagent, molarity or concentration, date of preparation, the lot number of the reagent, whom it was prepared by and the expiration date. Commercially acquired reagents should also be labelled as applicable with the date opened and by whom and the expiration date. Quality control documentation, especially of laboratory prepared reagents, should be used to maintain a written record of the information listed above. It is also important to document the safety information regarding reagents as applicable regarding managing reagent spills and disposal.

New batches of laboratory prepared and commercially acquired reagent must be tested to ensure correct functionality. For example, new batches of reagent can be tested in parallel with an existing batch of the same product. The functionality of reagent used for DNA extraction or PCR, can be tested for functionality using appropriate controls.

In the case of lateral flow strips, they have a built-in reagent quality control in the form of a control line, which should always show up for the test to be considered acceptable.

5. Reference materials

The European Commission's Joint Research Centre (JRC) defines a reference material as "reliable quality assurance tools that improve confidence in test results obtained by laboratories. They play a key role in the calibration of laboratory instruments by providing precise reference values and data".[20] Reference material is an important tool in the verification and/or validation of methods.

There are different forms of reference material that can be used. These include: reference material containing a known analyte including CRM, DNA that has been extracted from a sample containing a known analyte, plasmid copies of the target analyte or a PCR amplicon of a target analyte. It is also possible to use synthetic oligomers as a target analyte if its sequence is known. It is important that reference materials are stored under the correct conditions as specified by the manufacturer in order to

[20] https://ec.europa.eu/jrc/en/reference-materials.

minimize target analyte degradation. For example, reference material in matrix format or extracted DNA can ideally be stored at -20°C. Reference materials, like any other reagent, must be tested for reliability every time a new lot is used in the laboratory.

Method validation/verification

As previously mentioned, any method that is being used in an LMO detection laboratory must meet minimum performance criteria.

New methods that have been developed by the laboratory need to undergo a validation process, which involves a comprehensive analysis of the methods, to ensure they meet these performance criteria.

On the other hand, methods that have already been validated by other laboratories and are being implemented in another laboratory need to undergo a method verification process. Verification of an already validated method is carried out to ensure that the method functions according to validated performance criteria in the hands of the implementing laboratory.

The performance criteria for validated/verified methods should include an analysis of the following criteria, as applicable: accuracy, precision, specificity, LOD, LOQ, reproducibility and linearity/range of detection. Below is a brief definition of each of the criteria and an overview of how they can be tested for.

1. Dynamic range, R^2 coefficient and PCR efficiency:

Amplification efficiency for real-time PCR: The rate of amplification that leads to a theoretical slope of –3.32 with an efficiency of 100% in each cycle. The PCR efficiency is calculated by determining the slope of a standard curve for the target analyte. The standard curve can be obtained by making a serial dilution of a known amount of target analyte. The efficiency of the reaction can be calculated by the following equation.

$$Efficiency = 10^{\left(\frac{-1}{slope}\right)} - 1$$

Acceptance criterion: The slope of the standard curve should be in the range of $-3.1 \geq \text{slope} \geq -3.6$.

R^2 coefficient for standard curves: The R^2 coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis. Acceptance criterion: The R^2 should be ≥ 0.98.

These parameters are verified by testing a sample over 5-8 concentration points with 2-5 replicates per concentration. Acceptance criteria for these tests include assessing the quality specific data as shown in figure 44:

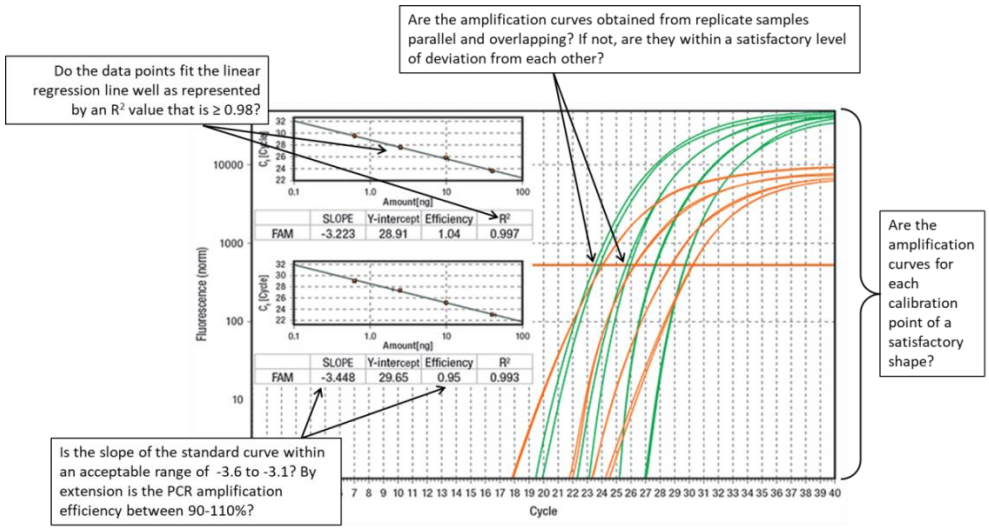

Figure 44: Considerations for acceptance criteria when verifying data for dynamic range, R^2 coefficient and PCR efficiency. Source: Adapted from http://www.sigmaaldrich.com/technical-documents/articles/biology/roche/kapa-multiplex-rtpcr.html.

2. Trueness/precision and relative repeatability standard deviation:

Precision - relative reproducibility standard deviation (RSD_R): The relative standard deviation of test results obtained under repeatability conditions. Repeatability conditions are conditions where test results are obtained with the same method, on identical test items, in the same laboratory, by the same operator, using the same equipment within short intervals of time. Acceptance criterion: The relative repeatability standard deviation should be ≤25% over the whole dynamic range of the PRC method, and ≤15% for an ELISA method.

Trueness: The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias. Acceptance criterion: The trueness for a PRC result should be within ±25% of the accepted reference value over the whole dynamic range.

These parameters can be tested using CRMs at 2-3 known LMO concentrations. For PRC they are analysed by comparing the data obtained from for example 2 sets of extracted material each of which is analysed using PCR that is tested in duplicates on each of 4 different PCR plates; therefore at least 16 data points are collected per concentration of CRM, as shown in figure 45.

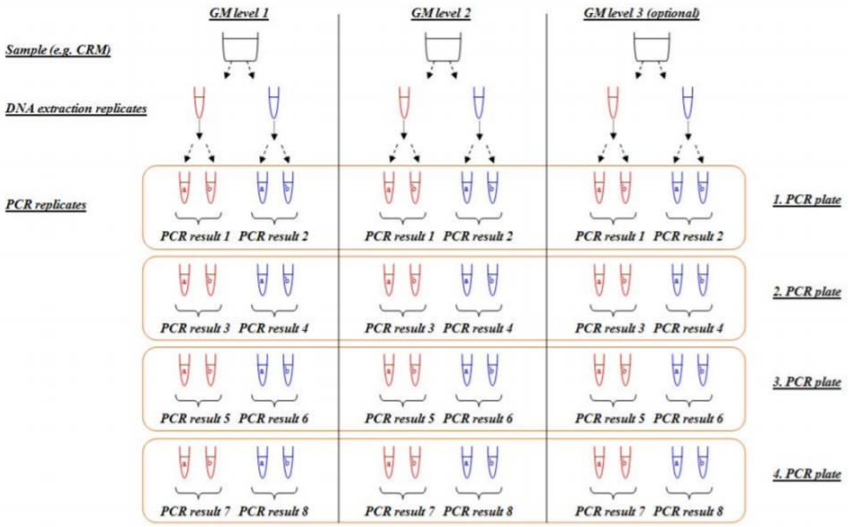

Figure 45: Schematic representation of the experimental design for the analysis of trueness/precision and relative repeatability standard deviation indicating the number of replicates for each sample. Source: http://gmo-crl.jrc.ec.europa.eu/doc/ENGL%20MV%20WG%20Report%20July%202011.pdf.

The acceptance criteria for verifying trueness/precision is that the calculated concentration based on the tests conducted should be within 25% of the CRM concentration. Furthermore, the acceptance criteria for RSD_r is less than 25%.

3. Limit of detection (LOD) and limit of quantification (LOQ):

LOQ: The limit of quantification is the lowest amount of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy. Acceptance criterion: the LOQ should be less than 1/10th of the value of the target concentration with an RSDr ≤25%. The target amount should be intended as the threshold relevant for legislative requirements.

LOD: The limit of detection is the lowest amount of analyte in a sample, that can be reliably detected, but not necessarily quantified. Acceptance criterion: The LOD should be less than 1/20th of the target amount. Experimentally, quantitative methods should detect the presence of the analyte at least 95% of the time at the LOD, ensuring ≤5% false negative results. The target amount should be intended as the threshold relevant for legislative requirements.

Similarly to measuring trueness/precision, LOD and LOQ are verified by testing multiple samples over a range of low copy number concentrations of CRM. They can be tested using CRMs that contain very low copy numbers of an LMO's DNA. They are analysed by e.g. comparing the data obtained from 10 replicates at each concentration that have been amplified using PCR, as shown in figure 46.

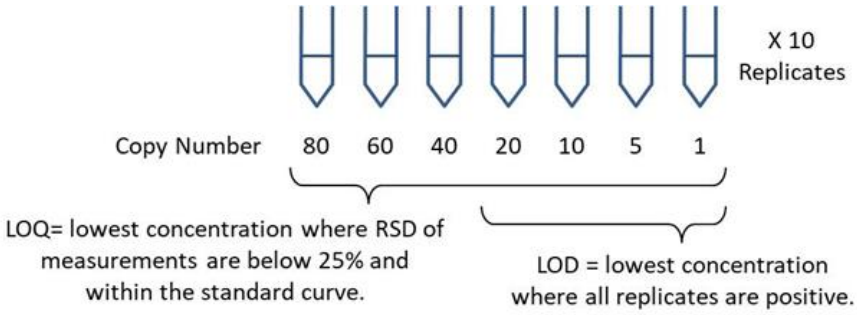

Figure 46: Outline of an approach for verifying LOD and LOQ, including guidance on the acceptance criteria.

The acceptance criteria for verifying LOD is the copy number at which all replicates are positive. With regards to LOQ the acceptance criteria for a PCR method are the lowest concentration at which the RSDr is less than 25% and the values are within the standard curve. For an ELISA method the criteria for the LOQ is a RSDr less than 15%.

4. Robustness:

Robustness is the capacity for a method to withstand small changes or deviations from experimental conditions. Verifying for robustness does not need to be carried out for methods that have already been formally validated. However, if deviations from the validated method have been introduced then verifying robustness may be necessary. Verifying robustness can be carried out by performing the method altered concentrations of primers and probes or by testing the method against DNA originating from different matrices. The acceptance criteria for this verification experiment are that the method performs equally well as the method as originally described in spite of the changes in the parameters.

5. Specificity:

Specificity is when the method being verified is capable of producing data that accurately and specifically determines the presence of the LMO that is being tested for in spite of the presence of other components within the sample. As with robustness, this parameter does not necessarily need to be verified if the method has already been formally validated. However, specificity may need to be verified for possible cross-reactivity against newly developed LMOs that have been introduced into the market and may be found in samples.

Proficiency testing

Participation in proficiency testing schemes facilitates the independent assessment of laboratory performance in comparison with performance data from other laboratories when using approved technical procedures. Ideally, a laboratory should participate in at least one proficiency test per year. The choice of proficiency test depends on the methods that are in use in the laboratory and the type of samples that are commonly analysed. While laboratories may use many methods for testing it may not be practical or feasible to assess all of them at the same time as each proficiency test covers only a limited number of LMOs and matrices. Therefore, it is recommended that the laboratory prepares a long-term plan for its participation in proficiency tests to ensure that it assesses a wide range of methods and matrices on a regular basis based on the laboratory's needs and compliance with the relevant requirements of any accreditation bodies, as appropriate. There are various institutions that offer proficiency tests including:

- BIPEA (https://www.bipea.org/content/pt-programs)

- FAPAS (https://fapas.com)
- ISTA Proficiency Test (PT) Programme (https://www.seedtest.org/en/home.html)
- EURL GMFF (http://gmo-crl.jrc.ec.europa.eu/Comparative-Testing.html)
- USDA (https://www.gipsa.usda.gov/fgis/proficiencyprogram.aspx)

Non-conformances, root cause analysis and corrective actions

Any activity in the laboratory that does not comply with standard operating procedures is referred to as a "non-conformance". Non-conformances can be administrative/clerical or technical in nature.

As part of a laboratory's QA/QC plan, a strategy has to be in place to direct staff towards taking appropriate action in the event a non-conformance is observed, for example contamination, and following root cause analysis, an outline of the steps for corrective action. The goals of this process is to document a non-conformance, identify the possible causes of the problem and the corrective action and implement a solution to avoid recurrence. The process of root cause analysis and corrective action should be consultative with the necessary staff to determine the most appropriate solution. Any non-conformance should be reported to the necessary authority in the laboratory, such as the laboratory manager, and documented, for example in a logbook for non-conformances. A root cause analysis should be performed to determine why the non-conformance occurred and the appropriate corrective action should be implemented as necessary and documented.

An administrative/clerical non-conformance can, for example, be an error in data transcription. The root cause analysis may identify that there has been an error due to inaccurate in data transcription. The corrective action may be to require that transcribed data be checked and signed off as being correct or to introduce a secondary check by another member of the laboratory. A technical non-conformance may occur when a method is not followed as described in the standard operating procedures or if a test result, for example of controls, do not meet minimum performance criteria. Repetitive failure of controls may indicate a systemic problem in sample processing.

Databases of methods for LMO detection

Databases containing accurate and reliable information on methods, reference materials and DNA sequences, if available, are an important tool to enable countries to effectively detect and identify LMOs. To be useful to the public at large, such databases must, at a minimum, be available online and open to the public, contain accurate and up-to-date information, and have user-friendly mechanisms for searching and retrieving information. The scope of the database may vary in the type of methods (e.g. DNA and/or protein; validated or not), type of LMOs (e.g. crops, vaccines, etc) or geographic area (e.g. local or global).

Below is information on databases that meet these criteria. They include databases of detection methods and reference materials.

Databases of methods for the detection of living modified organisms

European Union Database of Reference Methods for GMO Analysis[21]

This database was developed by the Joint Research Centre's European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF). It is maintained by the EURL-GMFF in collaboration with the European Network of GMO Laboratories (ENGL). This database contains fully validated detection methods for LMOs using, mostly, real-time PCR methods as well as some PCR methods. The collection includes methods that are event-specific, construct-specific and element-specific methods for screening as well as taxon-specific methods for species identification. For each method a complete protocol with the information needed to conduct the test and all the validation data are provided.

Event-specific detection methods validated by the EURL-GMFF can also be found by searching the "Status of Dossiers" list on the EURL-GMFF website.[22]

European GMO Initiative for a Unified Database System (EUginius)[23]

EUginius (EUropean GMO INItiative for a Unified database System) is an initiative of the German Federal Office of Consumer Protection and Food Safety (Berlin, DE) and Dutch RIKILT Wageningen UR (Wageningen, NL). The initiative is enlarging through participation of partner organizations from Austria, Italy, Poland and Belgium.

In order to support competent authorities, and private users, they compile accurate information on GMOs. EUginius provides accurate information of major and relevant issues regarding the presence, detection and identification of GMOs, with a focus on the situation in the European Union, but as well as world-wide coverage.

Element-specific, construct-specific and event-specific detection methods can be found by searching and filtering.

GMO Detection Method Database (GMDD)[24]

GMO Detection Method Database (GMDD) was developed and is maintained by the GMO Detection Laboratory at Shanghai Jiao Tong University in China. It contains information on event-specific detection methods for many LMOs, as well as real-time PCR and PCR methods that are element-specific, gene-specific or taxon-specific. Some protein-based methods are also included. Not all the methods in the database are validated through collaborative trial studies; however, if available, information on the validation status of a method is provided. The primer and probe sequences are provided with citations to the relevant publications. The database also includes a large collection of publicly available LMO sequence information. In addition, links to GenBank and other publications containing information on the sequence of the inserts is provided. Finally, there is also information on relevant CRMs, if available.

[21] The event-specific detection methods are available at http://gmo-crl.jrc.ec.europa.eu/gmomethods/.

[22] The compendium of detection methods is available at http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx.

[23] EUgenius detection information is available at http://www.euginius.eu/euginius/pages/detection_index.jsf.

[24] The GMO Detection Method Database is available at http://gmdd.shgmo.org/.

CropLife International Detection Methods Database[25]

This database is maintained by CropLife International, a global federation of the plant biotechnology industry, and contains both DNA- and protein-based methods. It currently contains approximately 40 methods related to about 27 LMOs. The detection methods available have been developed and validated by the technology providers for their own proprietary technologies and products. Not-for-profit laboratories have free access to the methods, whereas laboratories that undertake "fee-for-service" testing must request a license to access the methods.

Screening matrices for the detection of living modified organisms

German laboratory network screening table[26]*/EUginius GMO matrix*

The German laboratory network has developed a "GMO method matrix" (also known as the Waiblinger screening table), which is based on eight methods targeting specific genetic elements and constructs that are most frequently present in commercialized LM crops. It comprises a set of real-time PCR methods to detect:

1. The cauliflower mosaic virus 35S promoter (P-35S);
2. The nopaline synthase terminator derived from *Agrobacterium tumefaciens* (T-nos);
3. The ctp2-cp4epsps junction of the chloroplast-transitpeptide (CTP2) from *Arabidopsis thaliana* and the epsps gene from *A. tumefaciens* strain CP4 (epsps);
4. The bar gene from *Streptomyces hygroscopicus*;
5. A sequence from the P35S-pat junction of the CaMV P-35S promoter and the synthetic pat gene;
6. The synthetic fusion gene cry1 Ab/Ac derived from *Bacillus thuringiensis*;
7. The figwort mosaic virus 34S promoter (P-FMV);
8. The nopaline synthase promoter derived from *Agrobacterium tumefaciens* (P-nos).

All eight methods have been fully validated and are included in the EURL-GMFF method database. Furthermore, a table listing the accessibility of publicly available reference materials and their sources is provided. The screening table is maintained by the German NRL and the Excel spreadsheet with implemented filter-functionalities and a list of available LMO reference materials can be downloaded from the Internet.[27] The matrix is maintained in parallel on the EUginius platform (www.euginius.net).

GMOseek matrix[28]

The GMOseek matrix is a "GMO target matrix" and provides a comprehensive and user-friendly overview of 273 genetic elements and their occurrence in 328 LMOs5. The GMOseek matrix is freely available online as an Excel spreadsheet. Filtering functions allow users to search for events that fit into a

[25] CropLife International Detection Methods Database is available at www.detection-methods.com.

[26] The Waiblinger screening table is available at http://www.bvl.bund.de/SharedDocs/Downloads/09_Untersuchungen/screening_tabelle_gvoNachweis.xls?__blob=publicationFile&v=2.

[27] The compilation of reference materials is available at http://www.bvl.bund.de/SharedDocs/Downloads/06_Gentechnik/nachweis_kontrollen/referenzmaterialien.pdf?__blob=publicationFile&v=6.

[28] The GMOseek matrix is available at http://www.gmoseek.com/gmoseek.

defined pattern of genetic elements based on their absence or presence in an LMO. It also helps users in identifying genetic elements that could be targeted during the screening phase of LMO analysis. The GMOseek matrix is not maintained and new LMOs are not included.

JRC GMO-Matrix[29]

The JRC GMO-Matrix is a "GMO method matrix". It takes advantage of the DNA sequence information compiled in the JRC's Central Core DNA Sequence Information System (CCSIS). The JRC receives DNA sequence information on the insertions in LMOs from plant biotechnology companies, as part of their legal obligations in the EU. In addition they extract sequence information from nucleotide or patent sequence databases, as well as the primer and probe sequences of the detection methods compiled in the EURL-GMFF reference method database.

The user selects a set of plant species and/or LMOs and a set of detection methods and after in silico simulations of PCR amplification using bioinformatics tools, the results are displayed in a table with predictions of possible amplification(s).

GMOfinder[30]

The GMOfinder is a compilation of data to construct a combination of both, a "GMO target matrix" and a "GMO method matrix". It is based on an MS Access database and has integrated algorithms that facilitate the interpretation of the results of screening analyses. The tabular matrix provides information on selected genetic elements originating from the literature, LMO notifications and other sources. This information is integrated in a tabular format for 15 real-time PCR methods partly targeting the same genetic element but having different ranges of specificity. The recording of the sources of information facilitates a careful evaluation of the screening results and the tracing back of possible errors in the conclusions of the screening analysis. The GMOfinder is available free of charge upon requests addressed to the authors.

Combinatory qPCR SYBR®Green screening[31]

Combinatory qPCR SYBR® Green screening (CoSYPS) is a "GMO method matrix" based on the SYBR® Green qPCR analysis method for detecting the presence of the following genetic elements in LMOs: the cauliflower mosaic virus 35S promoter and terminator, the nos promoter and terminator derived from *Agrobacterium tumefaciens*, the figwort mosaic virus promoter, the rice actin promoter, the nptII gene from *Escherichia coli*, the epsps gene from *Agrobacterium tumefaciens* CP4, the epsps gene from *Zea mays*, the pat gene from *Streptomyces viridochromogenes*, the bar gene from *Streptomyces hygroscopicus*, the barnase gene from *Bacillus amyloliquefaciens* and several cry genes (cry1Ab, cryAc, cryF, cry3Bb) from *Bacillus thuringiensis*. In addition, a set of plant taxon-specific method is included in the CoSYPS testing platform.

The analytical results obtained with the CoSYPS matrix are interpreted and evaluated in combination with a "prime number"-based algorithm, by which the nature of the subsets of corresponding LMOs in a sample can be determined.

[29] The JRC GMO-Matrix is available at http://gmo-crl.jrc.ec.europa.eu/jrcgmomatrix/.

[30] GMOfinder matrix is available at http://link.springer.com/article/10.1007%2Fs12161-012-9378-6.

[31] The CoSYPS matrix is available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2836468/pdf/216_2009_Article_3286.pdf.

GMO Checker[32]

This screening application was developed as a real-time PCR array and is a "GMO method matrix". The platform can be used for the comprehensive and semi-quantitative detection of LM crops. It is a combination of 14 event-specific and 10 element-specific methods. The specificity and sensitivity of the PCR assays were evaluated experimentally and are tabulated in the publication. An Excel spreadsheet application for the evaluation of analytical results concerning the presence of LM crops has been developed and can be downloaded from the Internet.

Other relevant databases

Biosafety Clearing-House[33]

The Biosafety Clearing-House (BCH) central portal contains an LMO Registry with detailed descriptions for each LMO, including a unique identifier, if available, and detailed information on the transformation method, modified genetic elements, and vector as well as links to other registries in the BCH such as for risk assessments and countries' decisions. Each entry in the LMO registry also contains links to relevant detection methods for many of the commercialized LMOs.

The BCH also contains two other registries which are closely related to the LMO registry: the Organism Registry and the Genetic Element Registry. The Organism Registry includes information on the donor organisms and the recipient or parental organisms for the registered LMOs. The Genetic Element Registry contains records of the genes and other genetic elements that were modified in the LMOs. For each entry there is a brief description of the element and links to associated LMOs. Due to the confidential nature of the information, actual sequence information is available for only a few of the genetic elements.

BioTrack Product Database[34]

The BioTrack Product database is maintained by the Organisation for Economic Co-operation and Development (OECD). It compiles a list of Unique Identifiers (UIs) for LM plants that have been approved for commercial application in at least one country, in terms of food, feed or environmental safety. UIs are codes of a fixed length of 9 alphanumeric digits specific for a single transformation event and are intended to be used as "keys" to access and share information on a particular LMO.

BIOTradeStatus[35]

The BIOTradeStatus database is maintained by the Biotechnology Industry Organization and contains information on approvals, commercialization and seed sale of common commercially available LMOs.

[32] The GMO Checker is available at http://cse.naro.affrc.go.jp/jmano/UnapprovedGMOChecker_v2_01.zip.

[33] The LMO, Organism and Genetic Element Registries of the BCH can be found at http://bch.cbd.int/database/organisms/.

[34] The BioTrack Product Database is available at http://www2.oecd.org/biotech/default.aspx.

[35] The BIOTradeStatus is available at http://www.biotradestatus.com/.

International Service for the Acquisition of Agri-biotech Applications Database[36]

The International Service for the Acquisition of Agri-biotech Applications (ISAAA) is a not-for-profit international organization that, among other things, maintains a GM approval database that draws on information from biotechnology clearing-houses of approving countries and from country regulatory websites.

CERA LM Crop Database[37]

The Center for Environmental Risk Assessment (CERA), established by the food-industry funded, non-profit International Life Science Institute Research Foundation (ILSI), also maintains an LM crop database which includes not only plants produced using recombinant DNA technologies (e.g., genetically engineered or transgenic plants), but also plants with novel traits that may have been produced using more traditional methods, such as accelerated mutagenesis or plant breeding. The database provides information on the genetic elements construct, vector as well as the LMO characteristics (traits, common use etc.), risk assessments and regulatory decisions.

GMOtrack [38]

This programme generates cost-effective testing strategies for traceability of LMOs and computes the optimal set of screening assays for a two-phase testing strategy.

Overview of relevant accreditations and international standards

International guidance and useful standards about method performance criteria are well documented and available from several sources. These documents include ISO guidelines, Codex Alimentarius standards, guidelines from international associations (ISTA, AOAC), metrology institutions, as well as official and peer reviewed references regarding proficiency trials for method validation procedures.

- o ISO Standards (e.g. ISO17025)
- o ISO standards specific to GMO detection (ISO 21569, ISO 21570, ISO 21571, ISO 21572, ISO 24276)
- o Codex Standards and guidelines (Methods and LLP)
- o Role of Standards Developing Agencies
- o ISTA guidelines

[36] ISAAA GM Approval Database is available at http://www.isaaa.org/gmapprovaldatabase.

[37] The CERA LM Crop Database is available at http://cera-gmc.org/index.php?action=gm_crop_database.

[38] GMOtrack is available at http://kt.ijs.si/software/GMOtrack/.

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Module 6:

Reporting

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Introduction

The outcome of a sample analysis is presented in the form of a written report prepared by laboratory officials. The report is intended to inform the requesting authority about the laboratory's findings regarding whether or not LMOs are present in the sample and, when needed, the corresponding estimated quantities. The report is written according to the laboratory policies[39] in compliance with national and international regulations and practices.

A well drafted report is a vital component of the laboratory's work. It is the primary channel of communication between the laboratory and the requesting authority. A well written report aims to transmit an interpretation of the complex scientific data generated by the laboratory in a clear and concise manner without the use of potentially confusing scientific jargon.

While the format of the report may be presented in numerous ways, there are, nonetheless, several key elements that should be included to ensure that the report is thorough and comprehensive. Additional information can be included to supplement the information contained in the report, based on the national regulatory requirements and the laboratory's policy.

Laboratory documentation requirements

A well compiled laboratory case file is a key component in facilitating the drafting of an informative and thorough report. It allows for efficient traceability from the raw data to the final report and it is therefore important to ensure that the case file is complete and contains all the relevant information needed. All the information in a case file should be compiled in such a way that any other trained laboratory member can follow and understand all the steps and decisions taken during the analysis.

While variations do exist between laboratories with respect to the level of details included in a case file, there are a few basic pieces of information that comprise a thorough case file. This may include:

- ***Case submission information:*** This is a standard form that is to be submitted along with the samples by the authority requesting the testing. The form should include date of sample receipt, name and contact information of the requesting authority, a general description of the items received such as sample type, sample matrix and sample weight, the name and contact information of the laboratory receiving officer, as well as information on the sample's chain of custody and packaging. Upon the submission of a case to the laboratory a file number should be assigned in order to facilitate administrative follow-up and as part of laboratory good management practices.

- ***Test record information:*** raw data from laboratory testing is normally recorded on standard work forms that are developed by the laboratory. Such forms encourage consistent and standard recording of the required information and facilitate the traceability of the raw data. The forms are compiled in the relevant laboratory case file along with relevant references to the location of electronic data sets. These data can be referred to as needed while drafting a report.

- ***Case report:*** once the report is written, the analytical outcome also becomes a component of the case file and a copy is retained within the file.

[39] See module 5: Introduction to Quality Assurance/Quality Control Standards.

Report writing sections and contents

The contents and scope of the report may vary based on the specific requests made by the requesting authority. This will influence both the types of tests carried out and the specificity of the details included in the report in order to adequately answer the questions posed by the requesting authority. The following points are guidelines for the minimum content that may be considered for inclusion in the report. Several of these pieces of information also make up a major component of the case file, which highlights the importance of ensuring completeness of the case file.

1. Report introduction

The introductory section of the report provides the reader with a description of the requests and/or instructions given to the laboratory by the requesting authority. For example, the requesting authority may ask for testing for the presence of LMOs in general (screening), or for performing the analysis for the identification and quantification of the specific LMOs that may be present in a sample.

This section should also include logistic information pertaining to the case such as the laboratory's unique identification numbers for the case and its associated specimens; the name of the requesting authority; the date the specimens were received by the laboratory; the condition in which the specimens arrived, and finally the date when the report was issued.

2. Summary of sample handling procedures

The description of the sample submitted for testing and unambiguous identification is an important component of the report. This is relevant to provide the reader with the context in which the tests are being carried out *vis-a-vis* the quality of the sample, as it was submitted to the laboratory. The summary may include information on whether or not the size of the sample was adequate to carry out the analyses requested, as well as information on the sample matrix from which it was taken, and the visual presence of contaminants. Any possible limitations arising from a poorly submitted sample may affect the results obtained by the laboratory and, therefore, will have to be indicated in the report. These elements will serve to identify sources of uncertainty and aid data interpretation.

Further to this description, additional information shall be included regarding the procedures followed by the laboratory for subsampling and the sample processing steps. These additional manipulations of the sample influence the overall quality of data and conclusion of the report and, as such, should be described within the report. An example of appropriate phrasing for this includes "A 2Kg sample of maize grains was submitted to the laboratory for analysis. The whole sample was homogenized following the procedure outlined in the laboratory SOPs for processing maize grains and mixed. From the homogenized sample a 10g subsample was used to…"

3. Summary of preparation method

The report should clearly specify what tests were conducted on the sample in order to provide the reader with the context within which the data was interpreted. This includes, for example, a description of the methods and analyses that were used to investigate for the presence of LMOs. This can be done by listing the procedure's laboratory reference number in the report, for example "…a 10g subsample was used to extract DNA using method number NO-1234…" or "…genetic sequences for NOS terminator were detected using method number NO-5678…" Similarly, reference to the specific screening matrix that was utilized to analyse the results would be useful information to add to the report. Furthermore, the inclusion

of information on reference parameters such as the specificity, and sensitivity of the methods performed (e.g. detection and/or quantification limits) is also useful.

In some laboratories, an annex is included with the report to provide the reader with a detailed technical summary of the methods used including an explanation of the experimental design and the rationale behind the choice of the analytical tests that were used to analyse the sample.

4. Results of the analysis

The results and conclusions of the report provide a description and interpretation of the analytical findings produced by the laboratory. Laboratory personnel that are responsible for drafting the report should take into consideration the intended recipients, which may include individuals who may not have a scientific background, such as customs officials. It is therefore important that the report is presented in a well-structured and understandable manner that allows for an easy exchange of information. This can be achieved through the use of standardized terminology to explain the analytical data to the reader. A brief glossary or an appendix might serve this purpose. Therefore, accurate terminology is important as it allows for clearer communication of results without generating confusion or giving misleading information.

An example of appropriate terminology regarding the detection of an LMO using an event-specific method could be *"the sample tested positive with the […] method that is specific to […] LMO"* rather than *"the sample is…"*.

Furthermore, caution must be observed when test results may be subject to inferred interpretation. For example, if laboratory protocols test only for the presence of an LMO using element-specific methodology or some construct-specific methodologies, then it would not be appropriate to speculate as to the presence of a particular LMO not being tested. It can only be reported that "*the sample tested positive with the XXX method that is specific to […] genetic element"*.

If however an appropriately validated screening matrix is used to infer the presence of a specific LMO when using a set of element-specific methodologies, then, terminology such as "the sample was found to contain…" can be used. It must be noted that in such cases, the report should also specify if it is possible that the result is ambiguous due to potential mixing or due to the presence of genetically modified material from another commodity, for example LM maize that has been intentionally or unintentionally mixed with LM soy.

In cases where the analysis of a sample indicates that the sample does not contain an LMO, care has to be taken when reporting the data so that that the reader understands that a negative result has to be interpreted in the context of the technical limitations of the test preformed. Therefore, terminology such as "not detected" or "none detected" may be used. This type of terminology is preferable to statements like LMO is "not present", "does not contain" or "negative" because it provides the reader with a framework that recognizes the limitations of the methods performed, and that the LMO may be present in quantities that are below the limits of detection. To provide additional clarity to the reader, it is useful to state within the report what the limit of detection of the test is, together with the conditions in which the analysis was performed. It is also particularly important in such cases, that control tests are introduced and well documented, as they provide relevant information on the quality of the data.

An example of a negative report for LMOs would therefore indicate: A 2Kg sample of maize grains was submitted to the laboratory for analysis. The whole sample was ground up and mixed. A 10g subsample was used to extract DNA using method number NO-1234. No genetic sequences from MON-ØØ6Ø3-6 were detected using method number NO-5678 with a detection limit of 0.01%cp/cp in this matrix.

A positive report would similarly indicate: A 2Kg sample of maize grains was submitted to the laboratory for analysis. The whole sample was ground up and mixed. A 10g subsample was used to extract DNA using method number NO-1234. Genetic sequences for the presence of MON-ØØ6Ø3-6 were detected using method number NO-5678 with the Waiblinger (ref.) screening matrix.

If quantitative analyses were performed, amounts may be provided as relative or absolute values, and calibration curves or standard reference materials used need to be mentioned.

Technical and administrative review

A *technical review* is an in-depth review of the analysis records that is carried out prior to the issuance of a report to confirm the validity of results and conclusions.

The technical review is carried out with the view to:

- Ensure that the appropriate analyses have been conducted.
- Ensure that the conclusions of the reporting analyst are reasonable, consistent with the documented data, and within the constraints of validated scientific knowledge.
- Confirm that verifications have been documented.
- Ensure that technical language in the report is clear, accurate, and complete.
- Ensure that there is sufficient supporting documentation.

In addition, the technical reviewer and the analyst who drafted the report are equally responsible for ensuring the accuracy of the technical aspects of the case record.

An *administrative review* is a procedure that checks the case file documentation and reports for consistency with laboratory policy and for editorial correctness prior to issuing a report to the requesting authority.

An administrative review ensures that the content of the report follows laboratory policy and procedure by ensuring that the language in the report is clear, accurate, and complete, in addition to proofreading the report for clerical errors. Furthermore, the administrative review checks that the technical review has been properly conducted and documented.

Technical and administrative reviews may be combined as one process and carried out by the same person. However, neither the technical nor the administrative reviews are to be conducted by the analyst who completed the work.

Report issuance

Once the technical and administrative reviews have been completed and documented in the case file, the report is signed by an authorized signatory as the person(s) accepting responsibility for the content of the report, as per laboratory policy. The authorized signatory is to include their signature, date of signature, title, or equivalent identification, printed name and laboratory of employment and date of the reviews.

Laboratories must retain an exact copy of all reports issued within the relevant case files. These copies must be retained securely and be readily available for the time period specified in the laboratory's documented policies, since they are subject of audit and review.

In the event that an amendment has to be made to a report after it has been issued, it has to be communicated in the form of a supplementary report that quotes the original report as a reference. If it is necessary to issue a replacement report, this should be uniquely identified and should contain a reference to the original document that it replaces.

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