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CONFERENCE OF THE PARTIES TO THE
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THE CARTAGENA PROTOCOL ON BIOSAFETY
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Item 12 of the provisional agenda[*]

DRAFT TRAINING MANUAL ON THE DETECTION AND IDENTIFICATION OF LIVING MODIFIED ORGANISMS

Note by the Executive Secretary

1. In its decision BS-V/9, the Conference of the Parties serving as the meeting of the Parties to the Cartagena Protocol on Biosafety (COP-MOP) established an electronic network of laboratories with a view to bringing together representatives of laboratories involved in the detection of living modified organisms (LMOs). Furthermore, COP-MOP requested the network to carry out online discussion forums and workshops to exchange information and experience on the implementation of relevant standards and methods involved in the detection and identification of LMOs.

2. At its seventh meeting, COP-MOP reiterated the importance of the detection and identification of LMOs in the implementation of the Protocol by, among other things, requesting the Network of Laboratories to continue its work with a view to assisting Parties in fulfilling the requirements under Article 17 and making progress towards the relevant outcomes of the Strategic Plan.

3. In response to the above decisions, the Network of Laboratories for the Detection and Identification of Living Modified Organisms, at its last face-to-face workshop, included a plan of action for the development of training material for capacity-building activities on the detection and identification on LMOs to assist countries in fulfilling their relevant obligations under the Protocol and achieving the outcomes of the Strategic Plan that are relevant to the detection and identification of LMOs.

4. Following the workshop and in accordance with the agreed plan of action for the development of the training material, the Secretariat initiated the development of the training material, in consultation with relevant participants in the workshop and the Network, as appropriate.

5. The annex to this document contains the draft training manual on the detection and identification of LMOs as work in progress. Further feedback and inputs from participants of the Network of Laboratories is envisaged for further improving the manual.

[*] UNEP/CBD/BS/COP-MOP/8/1.

Draft Training Manual on the Detection and Identification of Living Modified Organisms

Module 1:

Overview of Biosafety and the Cartagena Protocol on Biosafety

Contents of this module

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 marks a significant achievement in the overall policy of the United Nations on the environment. Several documents resulting from that meeting constitute the basis of the international law on biosafety, such as 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 the natural resources. Its chapter 16 addresses the "Environmentally sound management of biotechnology" (see box below) by recognising that modern biotechnology can make a significant contribution to enhancing food security, health and environmental protection, and outlining the need for international agreement on principles to be applied to risk assessment and management and set out the implementation of safety mechanisms on regional, national, and international levels.

Agenda 21, chapter 16, paragraph 29

"There is a need for further development of internationally agreed principles on risk assessment and management of all aspects of biotechnology, which should build upon those developed at the national level. Only when adequate and transparent safety and border-control procedures are in place will the community at large be able to derive maximum benefit from, and be in a much better position to accept the potential benefits and risks of, biotechnology."

Source: UNCED (1992a).

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 dramatic 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) of the CBD.

More specifically, in Article 8(g), Parties to the CBD are called upon 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. In Article 19(3) the Parties are called upon 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 8(g). In-situ Conservation of the Convention on Biological Diversity

"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).

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

"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 into account the provisions above, 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 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 September 2011, 161 Parties had acceded/ratified the Protocol.

What is Biosafety?

In its broad sense, the term biosafety refers to the protection of human health and the environment from potential harm due to biological agents.

Under the Convention on Biological Diversity (CBD), and more specifically under the Cartagena Protocol on Biosafety (hereinafter "the Protocol")[1], the term biosafety essentially 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 comprises multidisciplinary scientific fields including, but not limited to biology, ecology, microbiology, molecular biology, animal and plant pathology, entomology, agriculture and medicine as well as legal and socio-economic considerations, and public awareness.

What are living modified organisms?

According to the Cartagena Protocol on Biosafety:[2]

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".

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

2 Article 3, paragraphs (g) and (i).

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).

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 - Advanced Informed Agreement (AIA)

The Advanced Informed Agreement (AIA) 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 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).3 In its decision, the Party of import may approve[4] 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 Advanced 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)).
- 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 (LMO-FFPs) (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 (LMO-FFPs)

According to Article 11 of the Protocol, 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(1)).

Competent National Authorities

Each Party should designate one or more competent national authorities (CNAs) who will perform the administrative functions required by the Protocol and are authorized to take decisions on the LMOs for which they are designated (see Module 2).

3 Unless article 10, paragraph 2(b) applies.

4 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.

Detection and identification in the context of the Cartagena Protocol

The sampling, detection and identification of living modified organisms (LMOs) is a cross-cutting and broad reaching activity, whose successful implementation can facilitate the application of several articles of the Cartagena Protocol on Biosafety.

With the adoption of the Strategic Plan for implementation of the Protocol for the period 2011-2020 by the COP-MOP 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); and
- 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 have a direct impact on the application of several articles of the Cartagena Protocol on Biosafety, as represented in Figure 2 below due to the cross-cutting nature of detection and identification.

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.

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.

Detection and identification therefore serves as a corner stone in facilitating Parties' capacity to make informed decisions on the handling, transport and use of LMOs with the view to effectively implementing the Protocol as it provides a scientifically based tool to facilitate decision making in the context of the Protocol.

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 languages of the UN.

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 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.

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 socio-economic 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.

International Plant Protection Convention

The International Plant Protection Convention (IPPC; www.ippc.int) is a multilateral treaty for international cooperation in plant protection. It aims to protect plant health while facilitating international trade. The IPPC applies to cultivated plants, natural flora and plant products and includes both direct and indirect damage by pests (including weeds). The IPPC was adopted by the Conference of the FAO in 1951. There are currently 173 contracting Parties to the IPPC.

The governing body of the IPPC is the Commission on Phytosanitary Measures (CPM). The CPM has adopted a number of International Standards for Phytosanitary Measures (ISPMs) that provide guidance to countries and assist contracting Parties in meeting the aims of the convention. The IPPC is recognized by the World Trade Organization as the relevant international standard setting body for plant health. Application of ISPMs is not mandatory; however under the WTO-SPS Agreement (see below) phytosanitary measures based on international standards do not need additional scientific or technical justification.

ISPM No. 11 (IPPC, 2004) describes the factors to consider when conducting a pest risk analysis (PRA) to determine if a pest is a quarantine pest. The main text of the standard (indicated with "S2" throughout the text) and particularly Annex 3 of this ISPM includes guidance on conducting PRA on LMOs.

In order to increase member countries' capacity to conduct pest risk analysis, the IPPC has developed a training course and training materials.[5]

Codex Alimentarius Commission

The Codex Alimentarius Commission (CAC; www.codexalimentarius.net) is a subsidiary body of the 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 166 members.

Codex Alimentarius, which means "food code", is a compilation of standards, codes of practice, guidelines and recommendations on food safety prepared by the Commission. In the area of foods derived from biotechnology, the Codex provides guidance on human health risk analysis in its

5 The IPPC training materials are available at https://www.ippc.int/index.php?id=186208 .

"Principles for the Risk Analysis of Foods Derived from Modern Biotechnology" (CODEX, 2003) and in its "Working Principles for Risk Analysis for Food Safety for Application by Governments" (CODEX, 2007).

Food and Agriculture Organization

The Food and Agriculture Organization (FAO; www.fao.org) of the United Nations 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".

World Organisation for Animal Health

The World Organisation for Animal Health (OIE; www.oie.int) is an international intergovernmental organization founded in 1924 for improving animal health worldwide. As of June 2010, the OIE had 176 member countries.

The objectives of the OIE are to: (a) guarantee the transparency of animal disease status world-wide; (b) collect, analyze and disseminate veterinary scientific information, (c) provide expertise and promote international solidarity for the control of animal diseases; and (d) guarantee the sanitary safety of world trade by developing sanitary rules for international trade in animals and animal products.

Within the mandates of the OIE, the principal aim of import risk analysis is to provide importing countries with an objective and defensible method of assessing the disease risks associated with the importation of animals, animal products, animal genetic material, feedstuffs, biological products and pathological material.

Organisation for Economic Cooperation and Development

The Organisation for Economic Cooperation and Development (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.

With regard to risk assessment, the OECD has published the "Recombinant DNA Safety Considerations" (OECD, 1986) and consensus documents, which focus on the biology of the recipient organisms or introduced traits and are useful in background preparation for an LMO risk assessment.[6]

World Trade Organization

The World Trade Organization (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

6 Available at http://www.oecd.org/science/biotrack/consensusdocumentsfortheworkonthesafetyofnovelfoodsandfeeds.htm.

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 IPPC, 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.

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.[7]

7 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

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 for the development of more productive, pest resistant crops that produce better or different quality of products than previous ancestral lines. Such changes involve 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.

There are a number of disadvantages to traditional hybridization and selection methods. One major disadvantage is that breeders often wish to introduce single selected traits rather than transferring and recombining entire genomes. Also, the selection and sorting of genetically stable varieties is a slow process.

These drawbacks may be alleviated through the use of modern biotechnology techniques. Under the 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 such techniques result in the production of living modified organisms (LMOs) and/or genetically modified organisms (GMOs). The protocol specifically focuses on LMOs which are any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology. Other bodies and legislations use the term GMO to describe organisms whose genetic material has been modified in a way, which does not occur in nature under natural conditions of cross-breeding or natural recombination. In either case the resulting organism must be a biological unit that is able to multiply or to transmit genetic material.

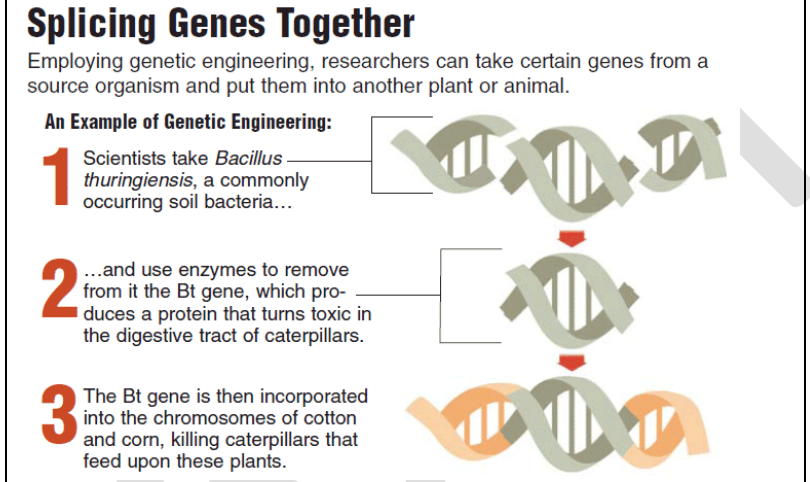
In the context of agricultural crops, the terms refer to plants in which genetic material from different species have been stably introduced into a host genome using modern biotechnology techniques resulting in the production of a gene product, i.e. a protein, or in some cases, the silencing the expression of an endogenously produced protein, i.e. gene silencing. 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 alteration of nutrient biosynthesis.

Overview of techniques used in modern biotechnology

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. The terms genetically modified organism (GMO) as well as genetically engineered or transgenic organism are often used interchangeably with LMO. The Cartagena Protocol emphasizes the "living" nature of the organism, and some of its provisions also apply to processed materials that originate from LMOs and contain detectable novel combinations of replicable genetic material obtained through the use of modern biotechnology.

Figure 1* – In vitro *nucleic acid techniques



Source: North Carolina State University (website).

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 containing 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.

Commonly used methods for genetic modification of plants

Production of LMOs through genetic modification is a multistage process that can be achieved through a variety of methodologies. Methods that are commonly used in the development of LM plants can be summarized as follows:[8]

- Once a gene of interest has been identified and isolated from a donor organism, it is manipulated in the laboratory such that it can be inserted effectively into the intended recipient organism. The manipulation may, for example, include changes to the sequence of

8 Adapted from IUCN (2003).

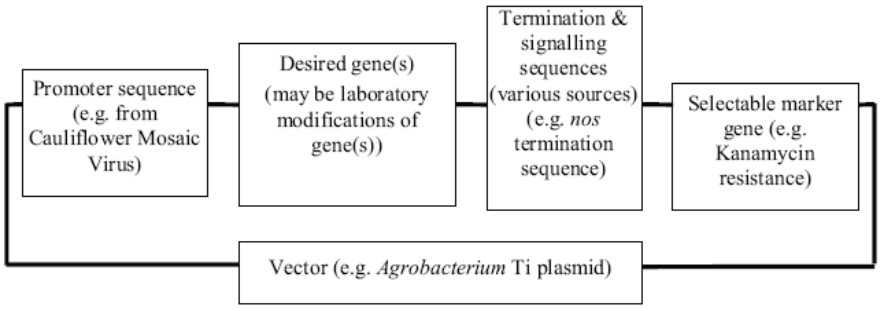
nucleotides so as to enhance or modulate the expression of the gene once it is introduced into the intended recipient organism.

- One or more genes of interest, as well as other nucleotide sequences needed for the proper functioning of the gene(s) of interest, may then be built in an orderly sequence into a "transformation cassette",[9] as shown in figure 2. The transformation cassette typically includes a "promoter sequence" and "termination sequence" which are necessary to ensure that the gene is expressed correctly in the recipient organism. Different promoter sequences control gene expression in different ways; some allow the continuous expression of the gene (these promoters are known as "constitutive"), while others switch the expression of the gene on or off in different tissues, organs and/or developmental stages of the organism or in reaction to other external influences. Some promoters may be highly specific to the point that they regulate gene expression only in a few cells of the organism and during short, specific developmental stages.

- A "marker gene" is often 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. identify or select cells or organisms.

- Finally, the transformation cassette may be incorporated into a larger DNA molecule to be used as vector.[10] The purpose of the vector is to assist the transfer of the transformation cassette into the recipient organism.

Figure 2 – Scheme of a transformation cassette and vector



Note: Transformation cassettes currently used may include multiple elements – for example, several promoter sequences and desired genes.

Source: IUCN (2003).

9 A transformation cassette comprises a group of DNA sequences (e.g., parts of a vector and one or more of the following: a promoter, the 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".

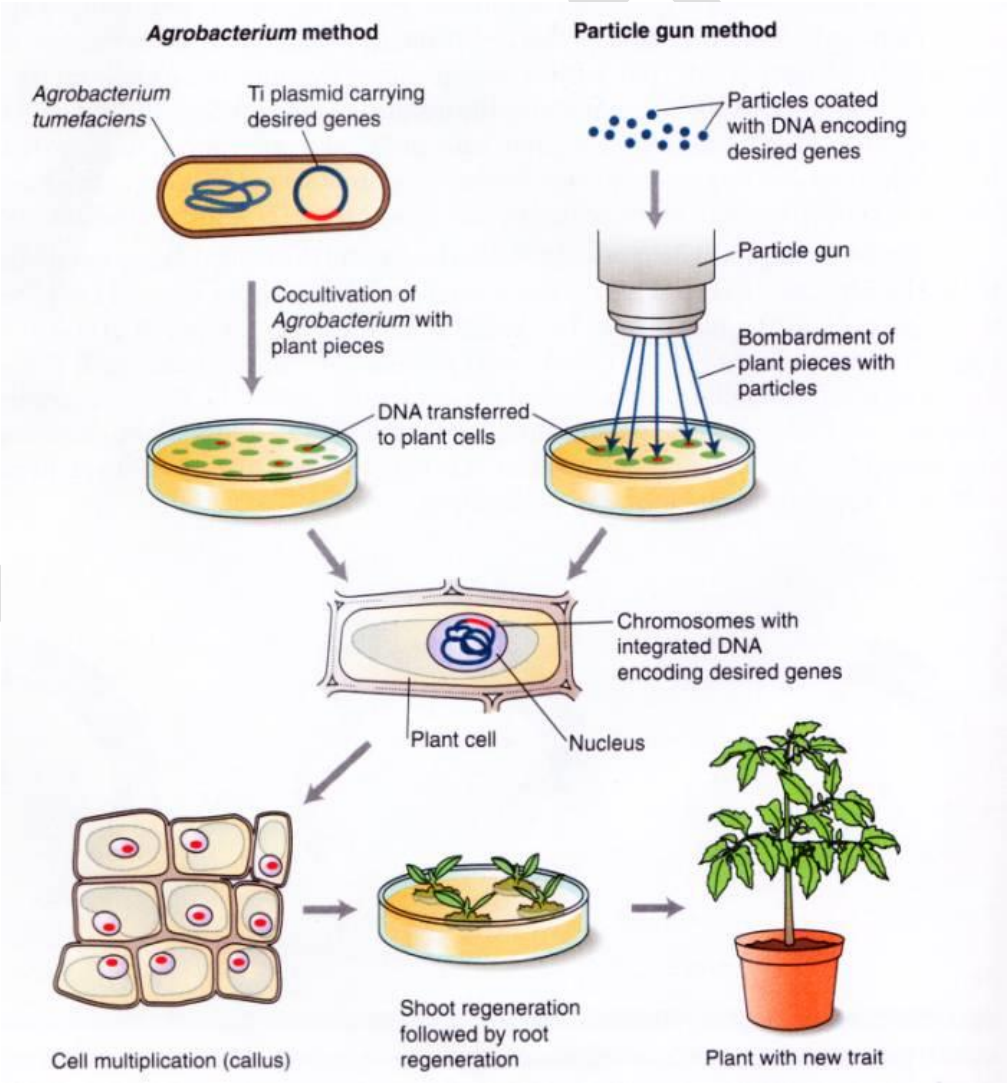
10 In the context of genetic modification, a vector is an organism (e.g., virus) or a DNA molecule (e.g., plasmid, nucleic acid cassettes) used to assist the transfer of genetic material from a donor organism to a recipient organism.

The transformation cassettes are integrated into the genome of the recipient organism through a process known as transformation, as outlined in figure 3. This can be carried out through different methods such as infection using *Agrobacterium*, particle bombardment or microinjection.

Transformed cells are then selected, e.g. with the help of a marker gene, and regenerated into complete LMOs. The subsequent step is the further selection of the modified organisms that contain the desired transgene(s)[11] or modification, and express the desired characteristics. Through selection, many experimental LMOs are discarded and only a few events may reach the stage of commercialization.

In the case of LM plants, cross-breeding to introduce the transgene(s) into other recipient varieties is also common.

Figure 3 – Genetic modification of plants



Source: **Mirkov (2003).**

11 A nucleic acid sequence in an LMO that results from the application of modern biotechnology as described in Article 3 (i) (a) of the Protocol.

Overview of available detection and identification methods[12]

A number of methodologies and techniques are available to detect, identify and quantify living modified organisms (LMOs). These methodologies 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. 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. For service-oriented laboratories, particularly those servicing regulatory authorities, the selection of methods is guided by, amongst other things, the country's specific regulatory requirements in accordance with national biosafety laws. The methodologies may therefore 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. Below is a brief overview of some of the more commonly used methodologies for the detection, identification and quantification of LMOs, their strengths and limitations.

LMOs are often developed by inserting one or more "genes of interest" which are DNA molecules encoding proteins that confer particular traits of interest, such as insect resistance or herbicide tolerance. Either the DNA or the protein can be targeted for the detection or identification of such LMOs. Both approaches, i.e. protein- or DNA-based methods, have advantages and disadvantages and the adoption of one over the other, or both, will depend largely on the available expertise, infrastructure to handle samples, laboratory equipment and regulatory requirements.

Protein-based methods for LMO detection

LMO specific proteins (i.e. those produced by the inserted genes) can be detected by 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-well format as an enzyme-linked immunosorbent analysis (ELISA) or a gel electrophoresis protein immunoblot (also known as western blot)

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. Protein-based detection methods require the use of antibodies to detect 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. For lateral flow strip testing, the 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 that it is simple to perform, requires little technical expertise or equipment and can be performed at the point of sampling. Electronic devices have also been developed that allow a semi-quantitative interpretation of the result. A disadvantage of this method is that its sensitivity is dependent on the binding affinity between the antibody and the protein.

The ELISA based approach to LMO detection follows the same crude protein extraction step as the lateral flow strip. However, the antibody is used to pre-coat the inside of a micro-well plate. Following a series of steps which allow the target protein to bind to the antibody, the cell debris, including other proteins, are removed from the plate through a series of wash steps. The bound protein

12 This section has been adapted from the SCBD Technical Tools and Guidance for the Detection and Identification of LMOs 2014. Available at http://bch.cbd.int/protocol/cpb_detection/toolsandguidance.shtml

is detected through a colour reaction that can be read through visual inspection or by 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 are included on the plate and an optical plate reader is used to evaluate the intensity of the colour reaction resulting from antibody recognition of the target protein in the LMO. The advantages of ELISA LMO testing are that it can produce a qualitative or quantitative result and is more sensitive than the lateral flow strip method.

For western blotting, the extracted crude proteins are separated according to their size by gel electrophoresis. Following this, the proteins are transferred from the gel to a membrane for detection of the target protein. Usually this step of the process involves two antibodies: firstly 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 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 disadvantage of this method is that the primary antibody may cross react with native forms of the same protein that may be present in the organism.

DNA-based methods for LMO detection

DNA-based methods for LMO detection and identification are based mainly on the use of the polymerase chain reaction (PCR). PCR is a method that employs 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 detected to determine whether or not DNA originating from an LMO is present in a sample.

DNA-based methods require the extraction of DNA prior to PCR detection. It is important to determine which DNA extraction method is appropriate for a particular LMO. While most extraction methods employ a cetyltrimehylammonium bromide (CTAB) protocol following mechanical homogenization of the LMO, different crop types may require additional steps for optimal DNA extraction. It is also important to note that the pre-analytical sampling regime employed and the amount of laboratory sample used will also impact on the accuracy and sensitivity of LMO detection.

Following the extraction of DNA from a sample, target sequences only found in the LMO are amplified using primers that have been designed to specifically bind the target sequence during the PCR reaction. The resulting PCR product can either be detected in real-time during the amplification process or after the PCR is completed.

Detection for the presence of the target sequence post PCR, also known as end-point PCR, is done through the process of gel electrophoresis where the amplified target sequence is separated based on its size through a gel matrix under the influence of an electric current. The fragment of amplified DNA corresponding to the inserted DNA in the LMO can be visualised through colour detection using a dye that binds to double stranded DNA and fluoresces under ultraviolet light.

Real-time PCR technology allows the detection of the amplified target sequence during the PCR amplification process using either a fluorescent DNA binding dye or fluorescence tagged probe. The

DNA binding dye simply detects the level of PCR amplification but does not discriminate between specific and non-specific amplification. In contrast, the use of a fluorescent probe can be used to verify that the specific target sequence was amplified during the PCR process. If real-time PCR technology is used in conjunction with the necessary standards, the percentage content of an LMO event in a sample can be determined. The overall applicability of this method must be evaluated in terms of the availability of equipment, its practicability and cost efficiency.

PCR has the advantage that it can be used to screen a sample for the presence LMOs by using primers that target sequences that are commonly found in a variety of different LMOs. Depending on the combination of primers used, the PCR detection can be gene-specific, construct-specific or event-specific. The differences between each type of PCR target region is represented in Figure 1. The advantage of PCR technology is that it is versatile and can be used to simultaneously screen a sample for LMO content, identify the specific LMO gene or event present and, when used in a real-time PCR platform, quantify the amount of LMO present in the sample. The disadvantage of a PCR based approach is that it requires specialized expertise and equipment.

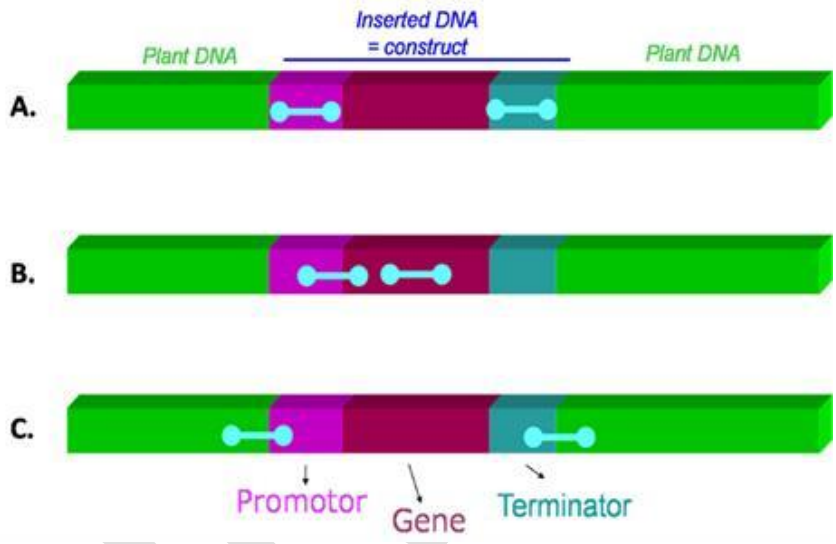


Figure 1 : Different types of PCR target regions A) common regulatory elements (such as promoters, terminators) B) gene-specific or construct-specific (junction between two genetic elements within the construct) C) event-specific (junction between the inserted construct and the plant genome) Source: National Institute of Biology

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

Both protein- and DNA-based approaches to LMO detection are useful and each serves different purposes. Protein-based methods, such as strip testing and ELISA, are simple, time efficient (several minutes to a few hours). Strip testing is useful for LMO testing at the point of sampling. However, protein detection of LMOs requires a different test for the detection of individual LMO traits and cannot be used to distinguish between different LMO events that may be present in a single sample. In contrast, western blots and PCR approaches 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 developed by inserting DNA fragments that produce a protein, i.e. transgenes, but not for detecting inserted genetic elements that do not

produce a protein, such as regulatory sequences. Protein-based methods rely on the specific recognition of an antigen in the transgenic protein by an antibody and therefore, any changes in the tertiary structure of the protein renders the method ineffective. Such conformational changes are sometimes induced during sample processing where the samples are subjected to heat and/or chemical treatment. The detection capability in protein-based methods is also affected by the expression level of the transgenic protein that 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 the transgenic protein in a specific tissue and may not be necessarily present in the part of the organism being tested.

In contrast, DNA has a greater chemical stability as compared to proteins which allows it to withstand chemical and heat 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. In addition, PCR can be applied qualitatively, to detect specific genes or events, and quantitatively, to determine the percentage level of a particular LMO event in a sample.

Challenges in LMO detection and new technology developments

There are several considerations and challenges for the application of LMO detection. These include the use of validated methods, availability of reference material for specific LMO events and access to information on the LMO event including sequence data for detection method development. With the development of each new LMO, their sampling, detection and identification becomes more challenging and complex. However, progress in the field of LMO detection technologies will lead towards making LMO detection more accessible.

Method validation and quality control

An important consideration in the application of LMO detection is that standardized operating procedures (SOPs) for validated methods and equipment should be used to ensure that results are reproducible and accurate. It is therefore important that criteria to test the performance of the methods be harmonized internationally to make results comparable throughout the world taking into account the availability of resources in different countries. The development of harmonized performance criteria should aim at simplifying and increasing the accessibility to LMO detection technologies by countries with less capacity and fewer resources. Thus a challenge for LMO detection is to ensure that methods are validated and meet the necessary minimum performance criteria for quality control purposes. This means that the LMO detection laboratory is required to develop and maintain the necessary quality control measures to ensure the reliability, sensitivity and reproducibility of its methods.

Access to information and reference material for method design and validation

Another challenge in LMO detection is the difficulty for LMO detection laboratories to gain access to sequence data for genetic elements inserted into LMOs in order to design detection systems that are specific to a transformation event. While many countries require notifiers to provide a method to detect the LMO, in many cases validation of the method is not required. In addition, most regulatory LMO detection laboratories do not have access to reference material for the specific LMO event in order to validate the detection method. 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. Furthermore, since it is costly to develop and validate a new method to detect an LMO, developers could be required to provide a method for the detection of an LMO event, the necessary sequence information so that the method can be verified and to cover, as appropriate, the costs of method validation by the laboratory performing LMO detection as part of the regulatory requirements of a country.

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 LMO events that detection laboratories must detect. As a result, the potential presence of unapproved LMO events is also increasing and detection laboratories are faced with having to potentially detect multiple LMOs, some of which may be approved while others may be unauthorised or illegal within the same sample. One approach to this challenge is to follow a matrix approach in detecting multiple genetic elements commonly used in the transgene construct in an attempt to widen the screening capabilities. When used in conjunction with bioinformatics software, the matrix results can be used to identify which potential LMOs are present in a sample. This approach can be customized to include as many LMOs as the laboratory is required to detect but is only applicable to PCR-based methods.

Emerging technologies for LMO development and detection methods

New and emerging technologies for developing LMOs are proving to be a challenge to the detection and identification of the LMOs. For example, new gene silencing technologies increasingly include using double stranded RNA (dsRNA) molecules to confer desirable traits in LMOs. In such cases, a transgenic protein is not produced therefore only DNA-based detection methods can be used.

On the other hand, the continued development of new technologies is making LMO detection more readily accessible, especially in countries with fewer resources. For example, digital PCR and other chip-based technologies will soon enable the routine detection of LMOs in the field with hand held 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, amongst others.

Pipeline for the development of LMO Crops

(To be completed)

Examples of commercialized LMOs

In 1978, the first commercialized LMO was produced with the creation of an *Escherichia coli* strain (a bacteria) that produces the human protein, insulin. In 1996, the first genetically modified seeds were planted in the United States for commercial use.[13]

To date, the most broadly commercialized LMOs introduced into the environment are agricultural crops. 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 2009, the cultivation of LM crops accounted for 170 million hectares (James, 2012). Soy, maize,

13 FLAVR SAVR™ Tomato by Calgene Inc.

cotton, and rapeseed that are resistant to herbicides and/or able to produce pesticidal proteins account for the majority of LM crops being currently commercialized (see LMO Registry in the Biosafety-Clearing House at http://bch.cbd.int/database/lmo-registry).

In 2009, a goat that produces an anticoagulant drug for humans was the first LM animal to be approved for commercial production.[14] Zebra fish containing fluorescent protein genes are another example of LM animals on the market. Moreover, a number of LM vaccines for humans and animals have been commercialized.

To date, there are no examples of the commercialization of LMOs resulting from cell fusion.

Considerations for National Strategies towards the detection and identification of LMOs

There are several layers of considerations that need to be examined by a country that is working towards establishing a national detection and identification laboratory. These include determining their existing capacities in terms of technical expertise and the availability of the necessary equipment to perform the testing procedures as well as establishing channels of communication amongst the relevant authorities that will be sampling shipments at the border and laboratory personnel in order to ensure a viable workflow within the country.

As outlined in the Technical Tools and Guidance for the Detection and Identification of LMOs a strategy for the efficient sampling, detection and identification of LMOs may start by the country defining each of the following parameters in order to establish the scope and procedure of LMO testing within the country:

1. The national monitoring and control objectives/targets;
   Possible targets include the monitoring of (transboundary) movements of authorised LMOs and/or the detection and control of unauthorised LMOs.

2. The perceived, potential, or known parameter that shall be monitored and controlled;
   Possible parameters include maintaining a paper trail of any (trans-boundary) movement of material that could be LMO or contain (traces of) LMOs. Details of this paper trial can be agreed upon at the international level. In addition, or alternatively, the parameter of interest could also be the presence or absence of LMO material in sampled consignments. This would require first an appropriate capability of statistically representative sampling and second an appropriate capability to analyse the samples. Depending on the intended control level, the required analytical capability may be qualitative (presence/absence) or quantitative (e.g. if certain threshold require different measures.) The main text of this report relates to the issue of adequate analytical capability – assuming that representative samples of good quality reach the laboratories.

3. The resources (financial, human) that can be made available in the foreseeable future.
   Besides the resources required for laboratory testing capacities, countries also need to develop adequate capacities to sample LMOs, including training of their border control officers and field inspectors. The role of sampling is critical in that it determines the quality of the results and the overall outcomes of a system to monitor detect and identify LMOs. Taking the importance of sampling into account, where resources are limited, and efforts may be more

14 http://www.gtc-bio.com/atryn-antithrombin-recombinant.

efficiently allocated if focused on sound field sampling combined with basic, qualitative laboratory capacities.

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Module 3:

Sample Preparation and Extraction

Contents of this module

Introduction

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 sample preparation and extraction of the analyte from the sample submitted by the competent national authorities.

The procedures for sample preparation and extraction are vital in obtaining reliable results therefore proper procedures have to be followed in order to ensure that the sample obtained is homogeneous and representative of the original sample.

Sample Management

When samples are received by the laboratory there needs to be procedures in place for the submission, receipt, labelling, storage, sample preparation and destruction of samples received from clients.

The procedures set out for receiving, handling, storage, preparation and destruction of samples, including procedures for documenting each step provide for traceable records to ensure the preservation of the samples' integrity and minimising contamination. Below is a description of several steps to consider during sample receipt.

Sample Submission

All samples accepted by the Laboratory should be accompanied by the 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 client submitting the information. This information is used by the laboratory direct them to the correct contact person in the event 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 client's signature.
2. A description of each sample submitted to the laboratory is provided by the client, including information on the type of sample, for example if it is food, feed or seed.
3. An indication of what the target organism may be and what type of analysis is requested.
4. Information on whom or 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.

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 client 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 client 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 the following section) has to be made. The sample is weighted and a note is made if the weight of the sample size 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 client 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 sidebar) 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

MASS REDUCTION

Mass reduction is the part of the sample preparation procedure to reduce the mass of a laboratory sample through subsampling (ISO/FDIS 6498:2011). 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 its particle sizes are above 6 mm 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 have been carried out the samples have to be registered within the laboratory's information management system.

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

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 lab 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-five digit number, beginning with the number one (001) assigned to the first case submitted in the calendar year

Once review is completed the laboratory personnel that received the samples and carries 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 sample size of any given sample depends on the sample type and matrix which is usually defined in mass. Below is a table recommending appropriate laboratory sample sizes based on the type of matrix.

Plant species	**Mean mass of 1000 kernels (in g)**
Barley	37
Linseed	6
Maize	285
Millet	23
Oat	32
Rapeseed	4
Rice	27
Rye	30
Soybean	200
Sugar beet	11
Sunflower	100
Tomato	4
Wheat	37

Table 1: Recommended laboratory sample sizes according to the type of matrix (Adapted from the JRC's "Guidelines for sample preparation procedures in GMO analysis")

For seeds and commodity grains where the recommended laboratory sample size corresponds to the mass equivalent of a certain number of kernels or grains. Table 2 that provides information on the mean mass of 1000 kernels of various plant species from which the appropriate laboratory sample size can be extrapolated.

Products	**Recommended laboratory sample size**
Seeds	Mass equivalent of 3000 kernels
Commodity grains	Mass equivalent of 10000 grains
First transformation products (semolina, flour, grits, oilcake etc.)	From 100 g to 1 kg
Liquids	500 ml

Doughy and viscous products	500 g
End products (e.g. packed rice noodles)	From 100 g to 1 kg

Table 2: Data concerning the mean mass of 1000 kernels for different plant species (Adapted from the JRC's "Guidelines for sample preparation procedures in GMO analysis")

It should be noted that for each plant species there may be a large number of varieties that are encountered in the laboratory. Each of these varieties may have different sized kernels therefore a variety with bigger kernels (compared to the average values of maize kernels), may contain less than 1000 kernels in in a mean sample mass.

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 sample with bigger kernels it would be good practice to, on a case-by-case basis as appropriate, 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 preform a mass reduction step on a 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.

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 most samples may be stored at room temperature without any negative effect on the sample's quality, it is important to ensure that the environmental conditions 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, therefore leading to a deleterious change in the quality of the sample. 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, care must be taken that there is no accidental release of the seeds into the environment. In this case is advisable to homogenise the sample as soon as possible to eliminate the risk of an accidental release.

Sample Homogenisation

In general the whole laboratory sample is used to obtain a 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 homogenisation is particle size reduction. The smaller the particle size of a sample the more DNA or protein can be extracted from the test portion. Homogenization is the step with the highest risk of error and contamination.

The risk of error arises due to the fact that, as indicated in Box XX, a laboratory sample can range from 100g – 3kg in mass, depending on the plant species. Whereas the resulting test potion that will be used to carry out the analysis can range from 20mg – 1g in mass. This represents a 200- 10,000 fold difference in mass and, as such represents the biggest possible source of error. Therefore a specific procedure should be followed during the sample homogenisation to manage and reduce the likelihood of error.

The contamination risk that occurs during the homogenisation 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 minimising the degradation of the target analyte, be it DNA or proteins, in the test sample.

The homogenisation 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 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.

Species	**Matrix**	**Possible Homogenisation method**	**Test portion**
Soybean	Grain / seed	Retsch ZM200 rotor mill	2 x 200 mg
	Leaves	Small pieces from different leaves, combined and homogenized as one sample with FASTprep instrument or manually crushed with LN_2	100 pieces
Maize	Grain / seed	Retsch ZM200 rotor mill	2 x 200 mg
	Leaves	Small pieces from different leaves, combined and homogenized as one sample with FASTprep instrument or manually crushed with LN_2	100 pieces
Rapeseed	Grain / seed	Retsch GM200 knife mill + LN_2 or coffee grinder + LN_2	2 x 200 mg
	Leaves	Small pieces from different leaves, combined and homogenized as one sample with FASTprep instrument or manually crushed with LN_2	100 pieces

Flax	Grain / seed	Retsch GM200 knife mill + LN_2 or coffee grinder + LN_2	2x 200 mg
	Leaves	Retsch GM200 knife mill + LN_2 ,	100 pieces
Rice	Grain	Retsch ZM200 rotor mill	2 x 200 mg
Potato	Tuber	Bioreba	2 x 500mg
Tomato	Fruit	Bioreba	2 x 500mg
	Leaves	Small pieces from different leaves, combined and homogenized as one sample with FASTprep instrument or manually crushed with LN_2	100 pieces
Wheat	Grain	Retsch ZM200 rotor mill	2x 200 mg
Sunflower	Grain	Retsch ZM200 rotor mill	2x 1 g

Table 3: Various commonly encountered sample matrices and possible equipment / method used for their homogenisation (Adapted from ŽEL et. al. Extraction of DNA from different sample types - a practical approach for GMO testing)

However, grinding using a mill with an integrated sieve gives you control and uniformity over particle size. In the case of difficult samples that may be prone to degradation during the homogenisation step, the use of successive grinding steps with sieves of decreasing mesh sizes or repeated grinding with the same mesh size sieve for fatty samples 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.

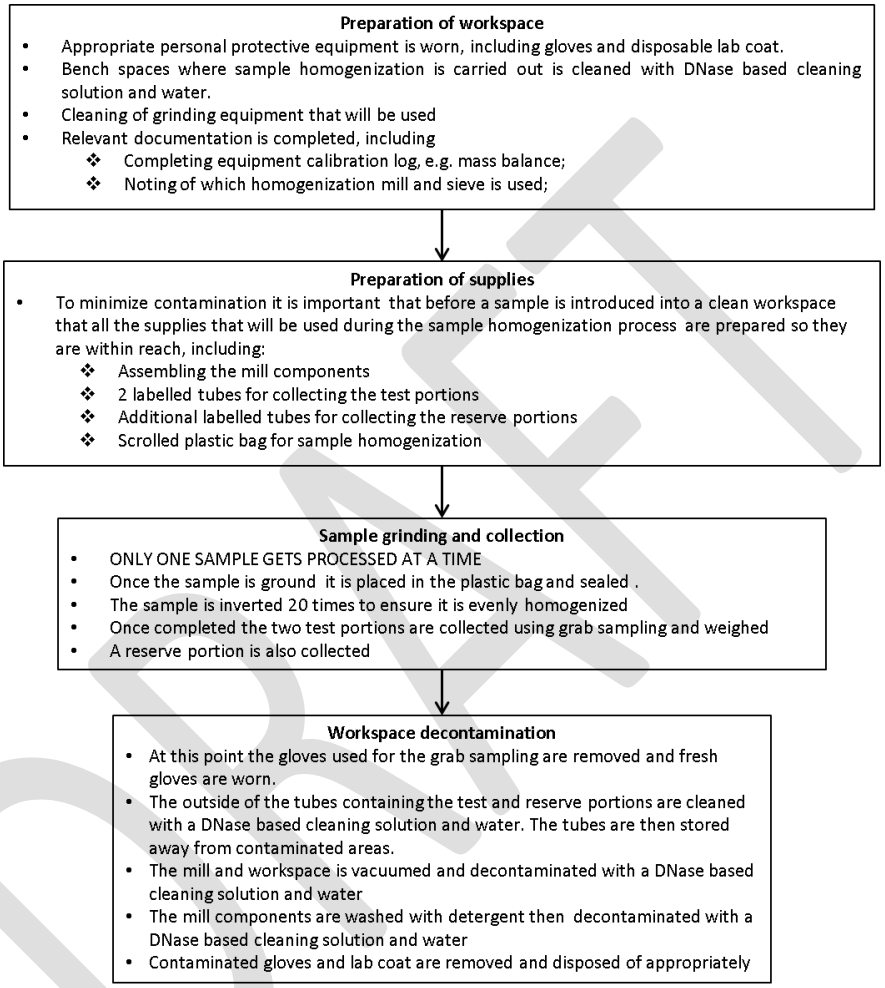
After grinding, the laboratory sample should be thoroughly mixed, or blended, to obtain a very homogeneous analytical sample. In cases where the laboratory sample is flour or liquid, homogenization is not needed but thoroughly mixing or shaking the sample is necessary. This is carried out by placing the homogenised sample in a disposable plastic bag or other suitable container, that is, at a maximum, half full and inverting the contents of the container 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 minimise gravitational segregation of the homogenised particles, which may affect the homogeneity of the sample. For each sample two 200mg test portions are taken from the prepared sample. 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 degrees 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. 3 months), would need to be stored at -20 degrees for the period time specified before it is either used or destroyed.

Once the test portions are successfully processed and analysed the resulting DNA 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.

Below is a flow chart outlining a step by step guide on practical, hands on procedures that can be followed in order to successfully homogenise a sample.



Considerations for QA/ QC during Sample Homogenisation

The application of stringent QA/QC procedures during the sample homogenisation step is vital since, as previously indicated, it is the step with the highest contamination risk and the quality of the homogenisation will affect the efficiency of downstream processes. 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 homogenised particle size: Ensuring that the particles produced during the homogenisation 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 homogenisation 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/ GMO sample followed by a non-LMO/ non-GMO sample then analytically testing for the presence of any traces of LMO/ GMO 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 lab sample and the test portion, validation test have to be carried to ensure the representativeness of lab sample. Similarly to the approach provided above, this involves either:

1. Processing a sample that has been spiked with 1 LMO/ GMO grain in 10,000 non-LMO/ non-GMO 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 homogenised samples.

Sample DNA Extraction

Extraction and purification of nucleic acids is the first step in most molecular biology studies and in all recombinant DNA techniques. The objective of nucleic acid extraction methods is to obtain purified nucleic acids from various sources with the aim of conducting specific analyses using 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 that may inhibit the performance of a PCR analysis. Examples of such inhibiting contaminants are listed in Table 1.

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%

Table 1: Examples of PCR inhibitors.

In order to avoid false negative results arising from the presence of PCR inhibitors in the sample, it is highly 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.

Extraction methods

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

The ideal cell 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 solubilise cellular membranes and strong chaotropic salts to inactivate intracellular enzymes. One such solution is cetyltrimethylammonium bromide (CTAB), an iconic 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 already been tested in validation trials for GMO 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.

Cell 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 1.

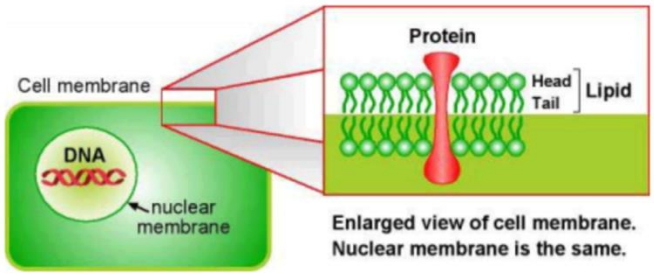


Figure 1: Simplified representation of cellular membranes.

In the CTAB method, disruption of the cellular membrane is achieved by treating the homogenised 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 solubilisation using a detergent is shown in Figure 2.

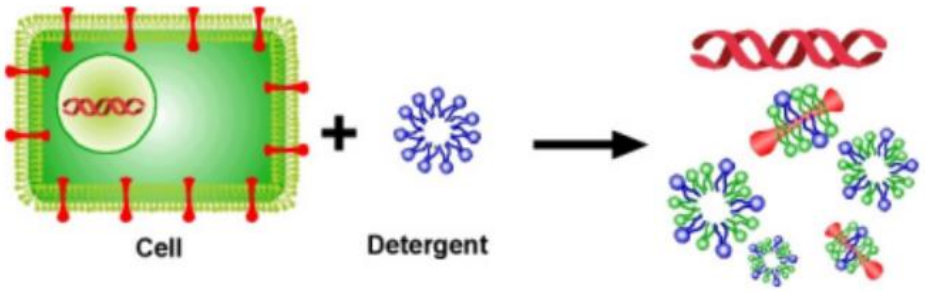


Figure 2: Schematic representation of the disruption of the cellular membrane and extraction of genomic DNA.

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, amongst others. Magnesium ion is a DNase cofactor therefore the presence of EDTA reduces the amount of bioavailable magnesium ions and, therefore, minimises 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 homogenisation and addition of the CTAB buffer should be minimised.

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 1). 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. 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. 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. 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 utilises 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*: utilised 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:* utilises 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 forced 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 2-3 times in order to ensure that the maximum amount of contaminants have 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 of 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 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.

DNA Quantification

Following the extraction and purification of DNA from a sample it is useful to get an assessment of the nucleic acid concentration. 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.

1. *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:

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

where:

A: Absorbance
OD: Optical Density
ε: Molar extinction coefficient
c: Concentration
l: Cuvette pathlength

The maximum absorbance of DNA and RNA solutions is at a wavelength of 260nm. 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 recognised 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 280nm, and protein solutions

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

partially absorb light at 260 nm, the ratio between the readings at 260nm and 280nm (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 230nm 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 325nm 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.

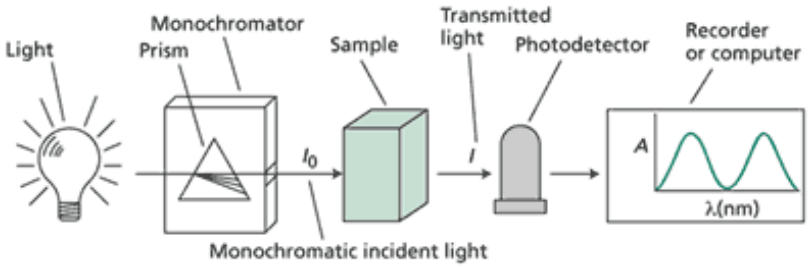


Figure 4: Schematic representation of the components of a spectrophotometer http://6e.plantphys.net/topic07.01.html

As shown in figure 4, 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 1ml to microvolume cuvettes with a capacity of 5 to 70µl. 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.

1. Select the correct cell pathlength based on the choice of cuvette being used.
2. Set the target molecule; dsDNA, ssDNA or RNA

3. 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.
4. 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 0.2ml then 5µl of DNA extract is diluted in 195µl 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 5µl 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: $c(pmol/\mu l) = A_{260}/0.027$

Double-stranded DNA: $c(pmol/\mu l) = A_{260}/0.02$

Single-stranded RNA: $c(pmol/\mu l) = A_{260}/0.025$

Oligonucleotide: $c(pmol/\mu l) = A_{260}*100/1.5NA+0.71NC+1.2NG+0.84NT$

An example of absorbance readings of highly purified calf thymus DNA suspended in 1x TNE buffer assuming that the reference DNA is dsDNA with concentration of DNA was nominally 25µg/ml.

For a 10 mm pathway 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.

2. *Fluorometric quantification*[16]

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 fluormeter, as shown in figure 5.

16https://www.promega.com/~/media/files/products%20and%20services/instruments/detection/tbs%20technical%20support%20docs/s-0041.pdf
http://www.biotechniques.com/BiotechniquesJournal/2007/August/Protocol-for-high-sensitivitylong-linear-range-spectrofluorimetric-DNA-quantification-using-ethidium-bromide/biotechniques-42715.html
http://nar.oxfordjournals.org/content/24/13/2623.full
http://www.phenixresearch.com/Images/TN_FluorecentProbeQuantitation.pdf

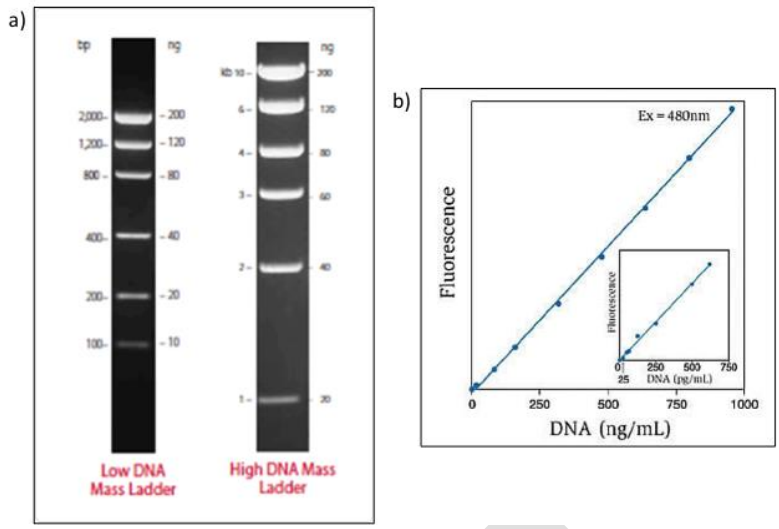


Figure 5: a) DNA mass ladders consist of equimolar mixtures of DNA fragments for the estimation of the mass of unknown DNA samples on gels. https://www.thermofisher.com/ca/en/home/life-science/dna-rna-purification-analysis/nucleic-acid-gel-electrophoresis/dna-ladders/dna-size-markers-mass-ladders.html 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. 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.

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Module 4:

Techniques for detection and identification[17]

Contents of this module

17 Sections of this module were adapted, with permission, from The Training Course on the Analysis of Food Samples for the Presence of Genetically Modified Organisms User Manual of the European Commission Joint Research Centre Institute for Health and Consumer Protection.

Introduction

A number of methodologies and techniques are available to detect, identify and quantify living modified organisms. These methodologies generally target once of two components, specifically, the proteins that are expressed by the living modified organism or the DNA insert that was transformed 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. Each of these methodologies have their advantages and drawbacks which 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, amongst 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.

Analysis	Test	Measures	Advantages	Disadvantages
Immunologic	Strip test	Protein	Because the test is rapid and can be performed onsite, this method is very useful as an initial screen for seed and grain	Often low sensitivity (Limit of detection 0.1-1%)
				Because the test is not performed with laboratory controls, operator error resulting in inaccurate test results can sometimes be an issue.
				Not appropriate for processed products
				GM protein levels may vary between different commercial GM cultivars and different parts of the same GM plant
	ELISA	Protein	High sensitivity (Limit of detection 0.01-0.1%)	Not appropriate for processed products
				GM protein levels may vary between different commercial GM cultivars and different parts of the same GM plant
				Must be performed in a laboratory
Genetic	PCR	DNA	High sensitivity (limit of detection 0.01%) and specificity	Must be performed in a laboratory
			Capable of detecting all GMOs	
			Allows definitive quantification	
			Effective with broad range of sample types	
			Industry standard used worldwide in surveillance and testing labs	

Table 1: Summary of available testing options for the detection and identification of LMOs, including the advantages and drawbacks of each method. http://www.gmotesting.com/Testing-Options

Protein-based methods

Introduction

One approach for the 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 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.

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. In addition, certain proteins may be under the

control of selective promoters and may, therefore, only be expressed in specific parts of the plant or during specific stages of physiological development.

- The expression levels of transgenic proteins in plants can be up to 2% of the total soluble protein, even when strong constitutive promoters are used to drive expression therefore the expression levels may be too low for immunological testing to be successful.
- All immunoassays are based on the specific binding of antibody to antigen therefore the sample and/or proteins of interest should not be significantly processed or degraded in order for the integrity of the antigen to be maintained.

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[18]

A lateral flow assay is a simple and easy to use detection method that is based on the use of immunochromatography to confirm for the presence or absence of a specific analyte. The assay is available in the form of a test strip that is impregnated with antibodies that bind a specific antigen that is the target of the testing. The test strips are composed of the following components, as shown in figure 1:

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 that 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. The as the sample passes through this section of the strip the antibodies recognise 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 immobilised 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 immobilised antibodies that bind the secondary antibody regardless of 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.

18 http://www.sciencedirect.com/science/article/pii/S131961031400129X

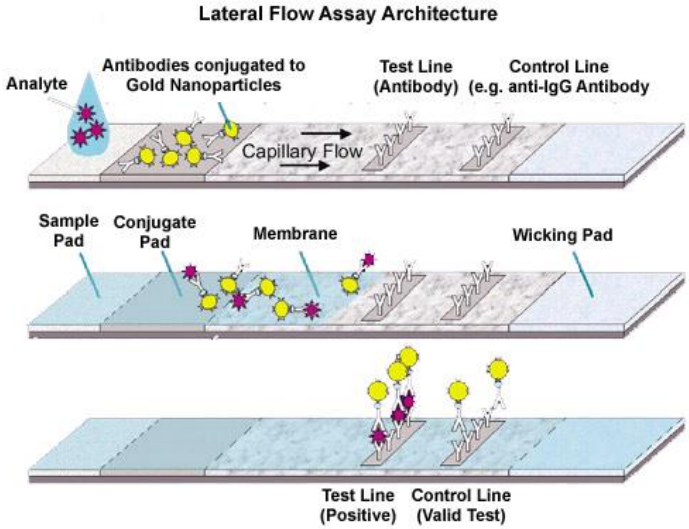

Figure 1: Schematic diagram of the structure and components of a lateral flow assay test strip. http://www.cytodiagnostics.com/store/pc/Lateral-Flow-Immunoassays-d6.htm

In the context of LMO testing using the lateral flow assay, the test strip is placed in a 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 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 2.

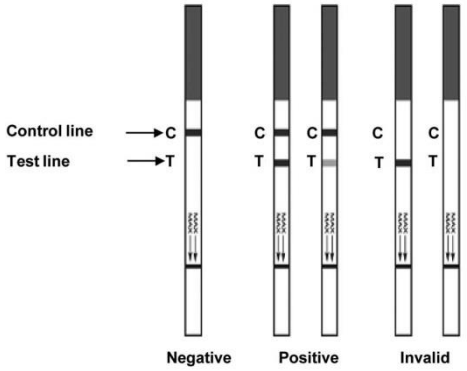

Figure 2: Representative results of a lateral flow assay. Adapted from https://www.spandidos-publications.com/ijmm/30/5/1041

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 point of sampling. Electronic devices have also been developed that allow a semi-quantitative interpretation of the result. A disadvantage of this method is that its sensitivity is dependent on the binding affinity between the antibody and the protein which can be compromised by a number of factors such as degradation due to processing.

ELISA Technique[19]

The enzyme-linked immunosorbent assay, ELISA, is another type of biochemical testing procedure that is based on the use of the specific interaction that takes place between antibodies and their

19 http://www.elisa-antibody.com/ELISA-Introduction 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

antigens, which in the case of the detection of GMOs, are the proteins synthesised by the newly introduced genes.

There are three different forms of the ELISA assay, as shown in figure 3, each of which is based on the same basic principal of immobilising 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.

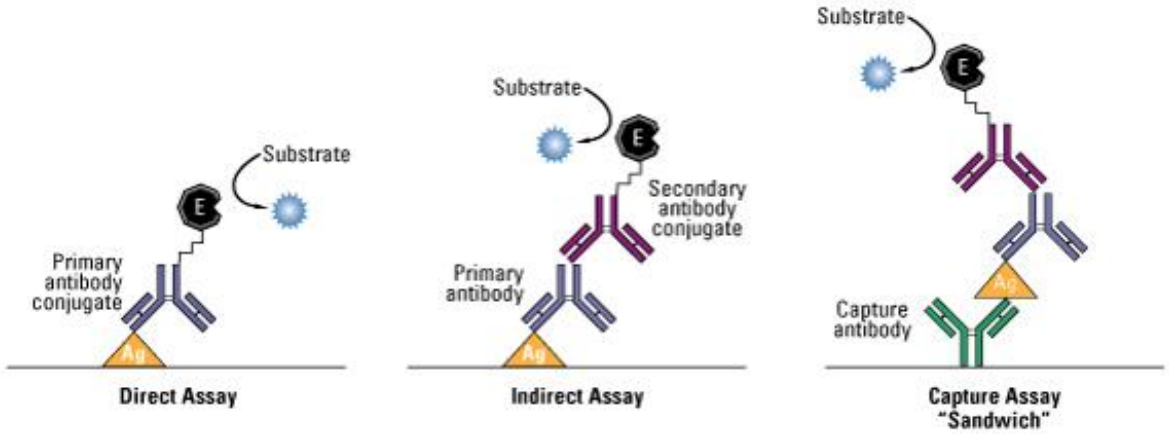

Figure 3: Schematic diagram of the three types of commonly used ELISA assays. https://www.thermofisher.com/ca/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-elisa.html

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

Similarly to 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 immobilised. 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 unlabeled primary and conjugated secondary antibodies (indirect detection) may be used to carry out the detection step, as shown in figure 3.

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 4.

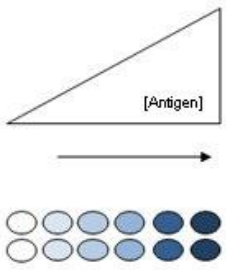

Figure 4: Schematic representation of the correlation between signal intensity and antigen concentration. https://www.bio-rad-antibodies.com/elisa-types-direct-indirect-sandwich-competition-elisa-formats.html

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.

In the context of LMO detection, ELISA has been widely applied to evaluate the expression level of the protein(s) synthesised by the newly introduced gene at the experimental stage. Information regarding production and use of specific antibodies can be therefore found in many articles describing the developments of transgenic plants. However, only a few specific antibodies directed against proteins that are the products of transgenes used in approved genetically engineered crops are commercially available[20]. These include antibodies against various forms of the Cry protein, 5-enolpyruvylshikimate-3-phosphate synthase from the Agrobacterium sp. strain CP4 and the phosphinothricin acetyltransferase, amongst others. 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. ELISA tests can therefore detect the presence of GMOs at concentrations ranging from 0.1% to 5% of the crude extract.

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 revolutionised 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, 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 GMO detection.

Nature of DNA

Components of DNA

20 http://www.envirologix.com/solutions/catalog/?filtering=1&filter_target=gmo&filter_technology=protein

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

1. A triphosphate group;
2. A pentose sugar molecule, knows as deoxyribose; and
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.

A nucleotide is formed when a purine or pyrimidine base is bound to the pentose ring by an N-glycosydic bond and the phosphate group is bound to the 5' carbon atom of the sugar by a diesteric bond, as shown in figure 1.

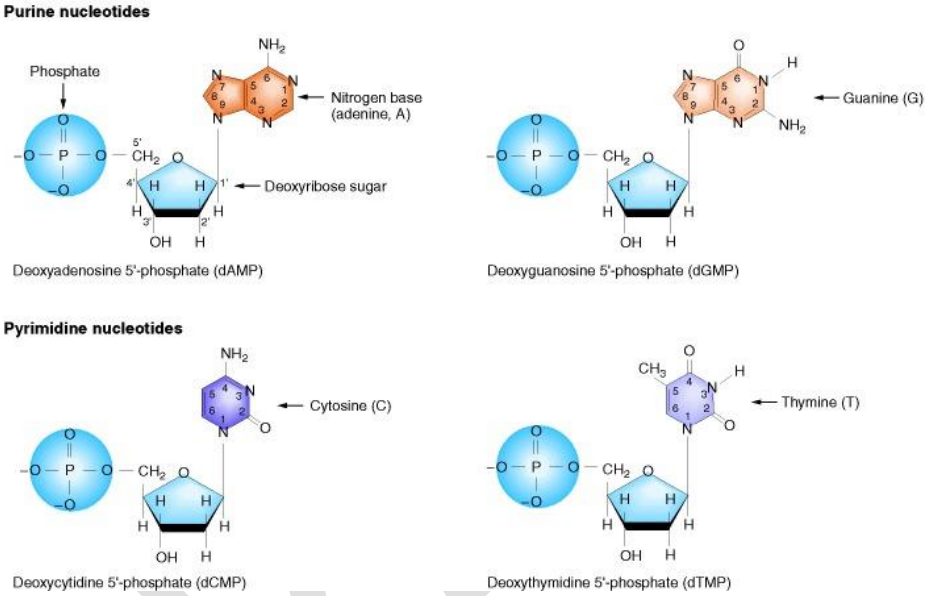

Figure 1: Chemical structure of the four nucleotides that form the building blocks of DNA. Griffiths AJF, Gelbart WM, Miller JH, et al. Modern Genetic Analysis. New York: W. H. Freeman; 1999. The Nature of DNA. Available from: https://www.ncbi.nlm.nih.gov/books/NBK21261/

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. 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 sugars-phosphate backbone forms an external hydrophilic layer.

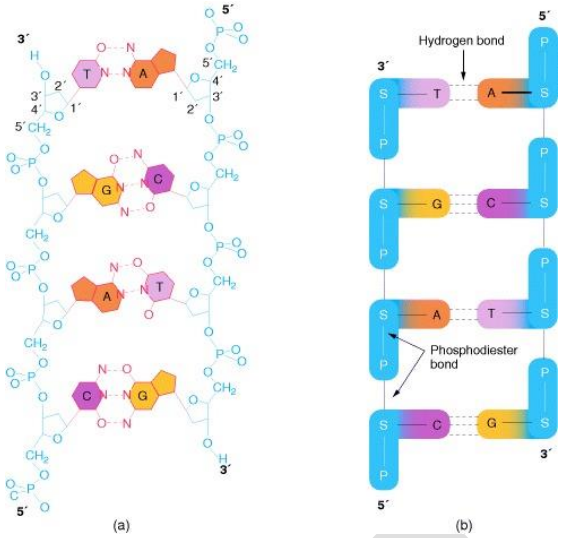

Figure 2: A schematic representation of a)the base pairing between the bases of complementary DNA stands and b) the antiparallel orientation of the DNA strands within a double helix. Griffiths AJF, Gelbart WM, Miller JH, et al. Modern Genetic Analysis. New York: W. H. Freeman; 1999. The Nature of DNA. Available from: https://www.ncbi.nlm.nih.gov/books/NBK21261/

DNA Replication

The aim of DNA replication is to synthesise 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 synthesised DNA helices consists of one parent strand and daughter strand.

Several enzymes are required to in the DNA replication process, as indicated in figure 3.

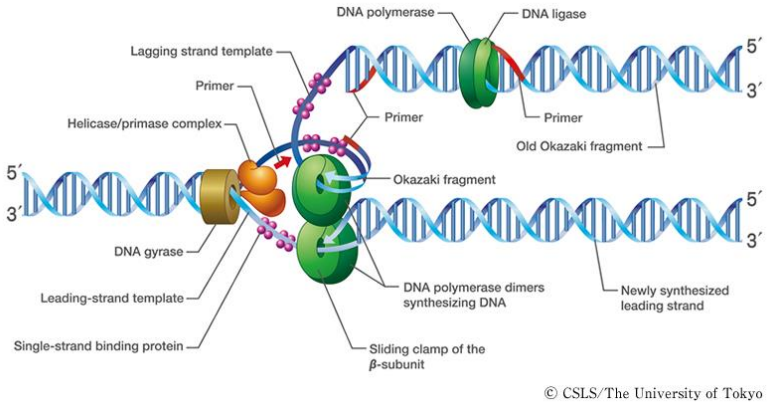

Figure 3: Schematic representation of the enzymes involved in DNA replication at the replication fork. http://csls-text3.c.u-tokyo.ac.jp/active/07_04.html

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 minimise 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 the that of the 5'→3' template, otherwise known as the lagging strand due to the fact that DNA con only be synthesised from 5'→3'.

Synthesis of the leading strand proceeds directly, from 5'→3', as the helix unwinds. However, DNA polymerase cannot start synthesising *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 synthesises 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 synthesising 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 RNase H and the gap are filled in by a DNA polymerase.

Synthesis of the lagging strand occurs in a more fragmented manner in order to accommodate for the fact that the direction of synthesis is opposite to the direction of the growing replication fork. Synthesis takes place in spurts of short 100 base pair fragments of DNA as described above. These short fragments are known as Okazaki fragments, as shown in figure 4. The Okazaki fragments are then joined together with the enzyme DNA ligase.

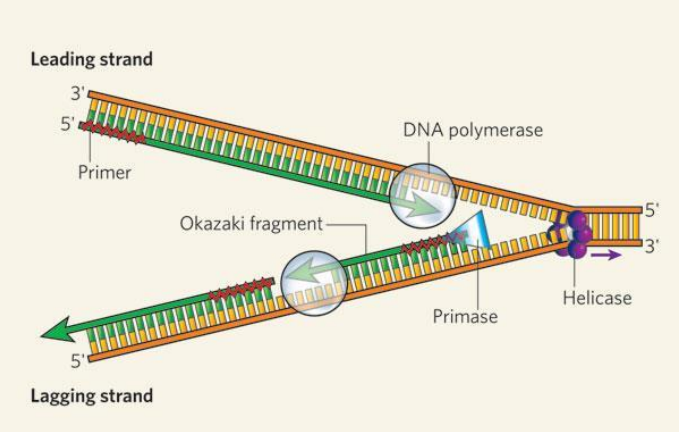

Figure 4: Schematic diagram of DNA replication of the leading strand and the lagging strand, including Okazaki fragments. http://www.nature.com/scitable/content/dna-replication-of-the-leading-and-lagging-14668888

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 templates which are then duplicated and rewound. To successfully preform 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 reaction 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 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, PCR can be performed if at least one intact copy of the target sequence is present, however, this amount of DNA allows for the availability of a greater number of target copies which increases the probability of a successful amplification. Furthermore, this amount of DNA compensates 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 complementary to the regions flanking the segment of DNA that is targeted for amplification. Generally, primers are between 16-30 nucleotides in length with a melting temperature between 52-60^{o}C. Primers are usually added to a PCR at a concentration of 1µM. The presence of primers at higher concentrations can result in mispriming and amplification non-target sequences. Conversely, PCR efficiency is reduced in 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, in 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.

- ***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 *E. 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 an enzyme incorporates into DNA before detaching itself from the template. Taq, for example, synthesises 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™, DeepVent™, Pfu and UITma™, should only be added after the reaction has started, or alternatively, chemically modified primers should be used.
- **Hotstart Activation:** Polymerases with Hotstart properties, such as AmpliTaqGold™, are inactive at room temperature, and can only be activated following an incubation period at 94°C. This provides for increases specificity, sensitivity and yield.

- ***Deoxyribonucleoside triphosphates***

Free deoxyribonucleoside triphosphates (dNTPs) are required for DNA synthesis, as they are the building block 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; and
- Stabilise 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.

- ***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 Mastermix

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 mastermix 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 mastermix is then aliquotted into individual tubes and the template DNA is added. The advantages of using a mastermix 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; and
- Fewer pipetting transfers reduced 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 5, are: a) DNA denaturation, b) Primer annealing / hybridisation; and c) Primer extension.

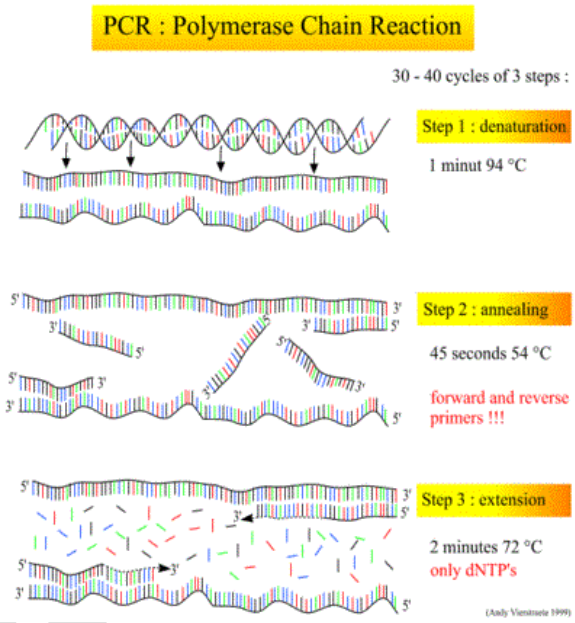

Figure 5: The steps of a PCR amplification (Picture: Andy Vierstraete, 1999) http://users.ugent.be/~avierstr/principles/pcr.html

- ***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 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 all the double stranded DNA becomes single stranded. Furthermore, during this step all enzymatic reactions stop due to the elevated temperature conditions.

- ***Primer annealing / hybridisation***

The primer annealing / hybridisation 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 hybridised 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 hybridisation 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 synthesises 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 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 particularly long, however, for the majority of PCR experiments an extension time of 1 min 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.

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 could 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 7.

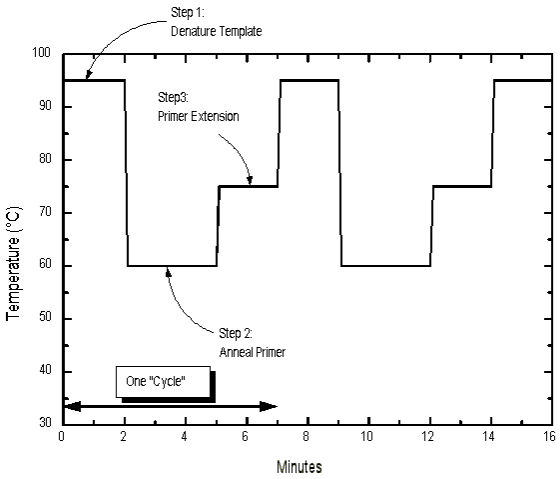

Figure 7: PCR temperature cycling profile

Exponential Amplification

After each PCR cycle, the newly synthesised 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 6.

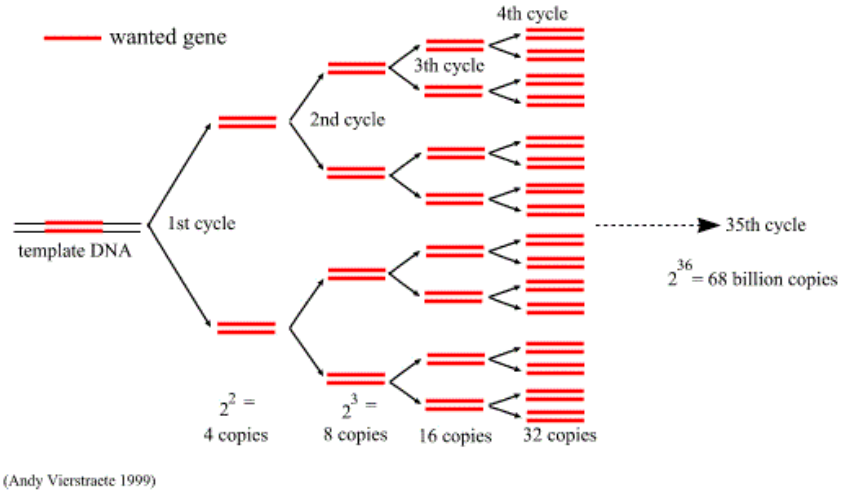

Figure 6: The exponential amplification of DNA in PCR (Picture: Andy Vierstraete, 1999) http://users.ugent.be/~avierstr/principles/pcr.html

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 optimised.

Cycle number and plateau effect

The number of amplification cycles necessary to produce enough target DNA so that it can be visualised 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 stages of PCR. This may be caused by reagent degradation, reagent depletion, by-product 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 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 3.

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 on 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 optimised results, more than 104 copies of the target sequence should be used as a starting template in order to obtain a signal within 25-30 cycles.

While it may be possible to successfully amplify less than 10 copies of a target sequence, such cases would require the use of more than 30 PCR cycles in order to detect a signal by gel electrophoresis. As previously discussed this should be avoided and alternate methods should be employed.

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

Table 3: Comparison of genome size of some plant species and corresponding genome copies in defined amount of DNA

Specialised PCR

In addition to the amplification of a target DNA sequence by the typical PCR procedures already described, several specialised types of PCR have been developed for specific applications.

Nested PCR

Nested sets of primers can be used to improve PCR yield of the target DNA sequence (Newton and Graham, 1994). PCR with nested primers is performed for 15 to 30 cycles with one primer set and then for an additional 15 to 30 cycles, with a second primer set, for an internal region of the first amplified DNA product. Thus, the larger fragment produced by the first round of PCR is used as the template for the second PCR. Using the nested PCR method can dramatically increase the sensitivity and specificity of DNA amplification. The specificity is particularly enhanced because this technique almost always eliminates any spurious non-specific amplification products. This is because after the first round of PCR any non-specific products are unlikely to be sufficiently complementary to the nested primers to be able to serve as a template for further amplification, thus the desired target sequence is preferentially amplified. However, the increased risk of contamination is a drawback of this extreme sensitivity, and great care must be taken when performing such PCRs, particularly in a diagnostic laboratory.

Multiplex PCR

Whereas standard PCR usually uses one pair of primers to amplify a specific sequence, Multiplex PCR uses multiple pairs of primers to amplify many sequences simultaneously. The presence of many PCR primers in a single tube could cause many problems, such as the increased formation of misprimed PCR products, "primer dimers", and the amplification discrimination of longer DNA fragments (Atlas and Bey, 1994).

For this type of PCR amplification, primers are chosen with similar annealing temperatures. The lengths of amplified products should be similar; 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 contain Taq polymerase additive, which decreases the competition among amplicons and the discrimination of longer DNA fragments during Multiplex PCR.

Multiplex PCR products can be further hybridised with a gene-specific probe for verification.

PCR in practice

As already illustrated in the previous sections, PCR is widely used and is a powerful analytical and preparative technique. However, because of the nature of this procedure, trace amounts of DNA contaminants could serve as templates, resulting in amplification of the wrong target nucleic acid (false positives). Thus, it is critical to perform PCR amplification in a DNA-free environment. Providing physically separate working areas with dedicated equipment reduces the risk of contamination. Strict compliance with decontamination requirements (decontamination of nucleic acids, prevention of aerosols etc.) is the most important prerequisite to reduce the rate of false-positive results to a minimum. PCR contamination can be caused by 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.

This section provides some recommendations, with the aim of defining the routine requirements for the establishment and maintenance of a clean environment for any PCR-based assay system, regardless of the number of samples being processed (Roth et al., 1997).

Physical prevention methods

Laboratory facilities. In order to avoid contamination, physically separate working areas should be set up as follows:

1. Sample preparation area: This room consists of an area where all the steps prior to amplification of the template DNA are performed (e.g. isolation and purification of DNA).
2. PCR set-up room: This "clean" room is devoted to the procedures related to the preparation of the PCR reaction (e.g. mastermix, primers dilutions etc.).
3. Post-PCR area: The area is dedicated to the amplification of the target DNA sequence, and the detection and analysis of the PCR products.

In addition, the following general rules should be observed:

- All the rooms should contain dedicated equipment (coats, gloves, reagents and supplies).
- Reagents and other devices must be labelled with content and date of preparation.
- Use a one-way flow system, i.e. never move material, samples or equipment from post-PCR areas into pre-PCR locations.
- Use disposable PCR reaction tubes, which are DNase and RNase free.
- Use special aerosol-resistant pipette tips and a dedicated (used only for PCR) set of pipettes, preferably positive displacement pipettes.
- If possible, set up PCR reactions under a fume hood that is equipped with UV light. Under the fume hood, store a microcentrifuge and disposable gloves that are used only for PCR.
- Periodically wash benches and shelves with 10% bleach followed by 70% ethanol.

Sample handling

- Use sterile techniques and always wear fresh gloves when working in the areas previously described. Change gloves frequently, especially if you suspect they have become contaminated with solutions containing template DNA.
- Always use new and/or sterilised glassware, plasticware and pipettes to prepare PCR reagents and template DNA.
- Autoclave all reagents and solutions that can be autoclaved without affecting their performance. Of course, primers, dNTPs and Taq DNA Polymerase should not be autoclaved.
- Have your own set of PCR reagents and solutions that are only used for PCR, and store these reagents in small aliquots.
- When pipetting DNA, avoid creating aerosols that can carry contaminants.
- Always include control reactions, for example a negative ("no DNA") control, which contains all reaction components except the template DNA, and a positive

- control that has been successfully used in previous PCRs.

Biochemical prevention methods

Uracil-DNA Glycosylase. The polymerase chain reaction (PCR) can amplify a single molecule over a billionfold. Thus, even minuscule amounts of a contaminant can be amplified and lead to a false positive result. Such contaminants are often products from previous PCR amplifications (carry-over contamination). Therefore, methods to avoid such contamination have been developed.

One common strategy is substituting dUTP for dTTP during PCR amplification, to produce uracil-containing DNA (U-DNA) (Longo et al., 1990). Treating subsequent PCR reaction mixtures with Uracil-DNA Glycosylase (UNG) prior to PCR amplification and subsequent cleavage of pyrimidinic polynucleotides at elevated temperature (95°C) under alkaline conditions (during the initial denaturation step) will remove contaminating U-DNA from the sample (see Figure 8).

This method, of course, requires that all PCR-reactions in the lab have to be carried out with dUTP instead of dTTP. Note the following when using dU-containing PCR products in downstream applications:

- PCR products containing dU perform as well as those containing dT when used as hybridisation targets or as templates for dideoxy sequencing.
- PCR products containing dU can be cloned directly, if they are transformed into UNG–bacterial hosts.
- A dU-containing substrate is readily digested by some common restriction enzymes (e.g. EcoR I and BamH I), while others show reduced activity (e.g. Hpa I, Hind II, Hind III) on these substrates.
- The use of dU-containing DNA is not recommended for protein-binding or DNA- protein interaction studies.

DNase I, exonuclease III. Other biochemical methods are based on the treatment of the contaminated DNA with DNase I, exonuclease III or with a restriction enzyme, containing a recognition sequence within the target DNA. However, because of the harsh reaction condition required, these enzymes present the disadvantage of reducing the efficiency of the PCR amplification.

Other novel technologies/strategies for LMO Detection

To be completed

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Module 5:

Introduction to Quality Assurance/Quality Control Standards

Contents of this module

Introduction

An important aspect when building capacity for the detection, identification and quantification of living modified organisms (LMOs) is to apply minimal quality control and quality assurance standards to ensure the adequate handling and processing of samples, as well as to ensure the quality and confidence in the results obtained. A wide variety of methodologies and instruments can be put in place by a molecular biology laboratory that wishes to undertake activities pertaining to the detection and identification of LMOs. Deciding which of the available methods is sufficiently reliable, accurate and more suitable to each laboratory's needs, including considerations such as costs of implementation, can sometimes be challenging. In addition, ensuring that the selected methods produce reliable and consistent results, while, at the same time, meeting minimum performance criteria, is essential.

Minimal performance criteria represent an advantageous set of parameters for selecting and implementing routine methodologies within each laboratory. Inconsistencies in results can have a major impact on the reliability of data produced. For example, the use of different measurement platforms, whether for the analysis of protein or nucleic acids involving the use of different instruments, or different algorithms to interpret the data, can cause serious discrepancy if no basic quality and performance criteria are established upfront. Understanding the relevance of minimal performance criteria, and monitoring some of the method's parameters on a routine basis, allows the lab-facility to establish a quality assurance and quality control system (QA/QC) and standard operating procedures (SOP) that can be used later on for corroborating laboratory proficiency. Additionally, when installing performance quality control measures, the procedure facilitates compliance with the requests from accreditation bodies. To ensure consistency, it is important that the analyte intended to be measured is first defined and that the units in which the measurement is expressed are common standard as well as ensuring the use of common vocabulary for better understanding. Use of certified reference materials is fundamental for protocol harmonization between laboratories and method validation. This is of particular interest when it becomes necessary to allow for result comparison and harmonized interpretation.

This section of the training manual aims to provide an introduction to the criteria that may be considered by individual laboratories when selecting and implementing methods for the detection and identification of LMOs within their national frameworks, for setting up an adequate quality assurance and quality control system as well as minimal performance criteria that are needed in a laboratory performing detection and identification of LMOs, how to validate such methods such that they are shown to be fit for purpose and the establishment of internal QC/QC programmes within their laboratory.

Definition

"Fit for purpose" is the extent to which the performance of a method matches the criteria agreed between the analyst and the end-user of the data

Source: Zel et al 2008

Laboratory set-up requirements and environment

A well-organized laboratory is one of the most important precautions in an LMO testing laboratory in order to prevent cross-contamination between samples. Ideally laboratories should be organised such that there is a unidirectional flow of the sample as it is processed. Separate rooms or chambers for each testing step should be used wherever possible. This may include, as appropriate, separate areas for the receiving of samples, sample homogenization, analyte extraction, reagent preparation, PCR set up, and analysis. Such considerations are particularly important in multipurpose laboratories where shared spaces are used for diagnostic testing as well as research purposes. A well thought out plan will maximise the use of limited space while reducing the risk of cross-contamination.

In this section we cover some specific precautions that need to be considered when planning the setting up an LMO detection laboratory.

1. Sample Storage and Homogenisation

Samples received from competent authorities need to be received and stored in a dedicated space that provides an appropriate storage environment and is secure. The availability of environmentally controlled storage space is required to preserve the integrity of the sample. Samples can be stored at room temperature provided that the environment is cool and dry. This may not be appropriate in areas where unfavourable environmental conditions exist, for example, fluctuations in temperature or high humidity that may lead to the growth of mould and DNA degradation. Such laboratories may require the need for dedicated fridges and freezers to provide a controlled environment for their samples. Furthermore, in the case of seeds, precautions have to be put in place to prevent their accidental introduction into the environment.

The vast majority of laboratory samples must be ground up prior to extraction. The grinding of samples results in the formation of wide spreading dust particles. If good laboratory practices and cleaning practices are not employed following the grinding of each sample, the residual dust could contaminate subsequent laboratory samples due to the high sensitivity of analytical methods. As such, stringent QA/ QC practices have to be put in place at the sample preparation stage to avoid cross-contamination while minimising degradation of the target analyte, be it DNA or proteins, in the test sample.

Ideally, sample homogenisation is carried out in a dedicated space where no other testing steps take place and does not share ventilation with such areas since dust can travel through ventilation systems. If laboratory space is limited and a separate room is not available then the grinding procedure can be carried out in a dedicated laminar flow hood, which would contain the spread of dust into the laboratory space.

In addition to having in place a physical separation of sample homogenisation, good housekeeping practices need to be outlined and strictly followed. This involves outlining cleaning procedures of the space where homogenisation takes place and the sampling device (grinding and mixing equipment). Such procedures may include:

1. Cleaning the working area and sampling device prior to each use with water and DNA decontamination reagent that removes DNA and DNases from lab equipment and surfaces.
2. Vacuuming the working area and sampling device after grinding each sample to remove large particulate matter.

3. Decontamination of the components of the sampling device by through washing and scrubbing with an appropriate detergent followed by a DNA decontamination reagent and water.
4. Decontamination of the working surfaces using water and a DNA decontamination reagent followed by water again to remove any residual reagent.

To test 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 before collecting the next sample, a test can be preformed to validate the procedures. This involves either:

3. Processing a LMO/ GMO sample followed by a non-LMO/GMO sample then analytically testing for the presence of any traces of LMO/GMO material in the sample; or
4. Processing a sample of species A (for example corn) followed by a sample of species B (for example flax) then analytically testing for the presence of traces of species A, in this case corn, in the test portion of species B, the flax.

Once the sample is homogenised and test portions and back up aliquots have been obtained they have to be appropriately stored. Samples that have been ground up are ideally stored at -20^{o}C. Furthermore, the location of the samples must be documented in the case notes such that they can be easily found when needed. The issue of sample storage is discussed in more detail under section XXX.

2. PCR Laboratory facilities

The purpose of PCR is to synthesise millions of copies of specific DNA fragments, which may lead to PCR product being a source of contamination if steps are not taken to mitigate this risk. Therefore, in a PCR based testing laboratory the establishment of a unidirectional workflow is essential to minimise such a risk. In such a set up laboratories and equipment are organised in a manner that allows the workflow to move from clean, pre-PCR areas towards, post-PCR areas.

The designation of laboratory space for each of the following modules of the analytical process involved in GMO/LMO detection will help ensure the unidirectional transfer of samples. Each of these areas 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 amongst personnel.

a) PCR set-up Room

In this area, also known as the “clean room”, reagents, such as primers and buffers, are stored, prepared, and aliquotted in an environment that is free of nucleic acids that originate from GMOs/LMOs, including DNA extracts, samples, cloned materials and PCR products. Other procedures that take place in this area include the preparation of PCR master mixes prior to the addition of the sample DNA. If a dedicated room is not available then a chamber devoted to sample preparation can be an adequate alternative.

In the Laboratory: Aliquotting

Aliquotting stock solutions into smaller working volumes is an important step in the PCR preparatory process as it is a means of managing the spread of contamination when it is detected and prevents an excessive waste of unused reagents in the event that contamination occurs. Furthermore, aliquotting minimises the frequency of freeze/ thaw cycles that stock solutions may be exposed to therefore helping maintain the quality of the reagents that may be sensitive to changes in temperature

b) The Sample preparation area

This area is dedicated to preforming all the steps that take place prior to the amplification of template DNA. This includes the extraction of DNA from the homogenised sample and the addition of the extracted DNA and positive control to the previously prepared PCR master mixes. This area must also remain free of PCR products, plasmids and stocks of cloned materials.

c) Post PCR area

This area is dedicated to the amplification of the target DNA sequence and the detection and analysis of the resulting PCR products. PCR machines are located in this area as well as equipment to analyse the PCR products such as sequencing machines and gel electrophoresis systems.

To complement the physical separation of working areas good laboratory practice must also be exercised to further minimise the risk of contamination. This includes regular hand washing, changing gloves and lab coats between the separate areas and cleaning work surfaces before and after every use.

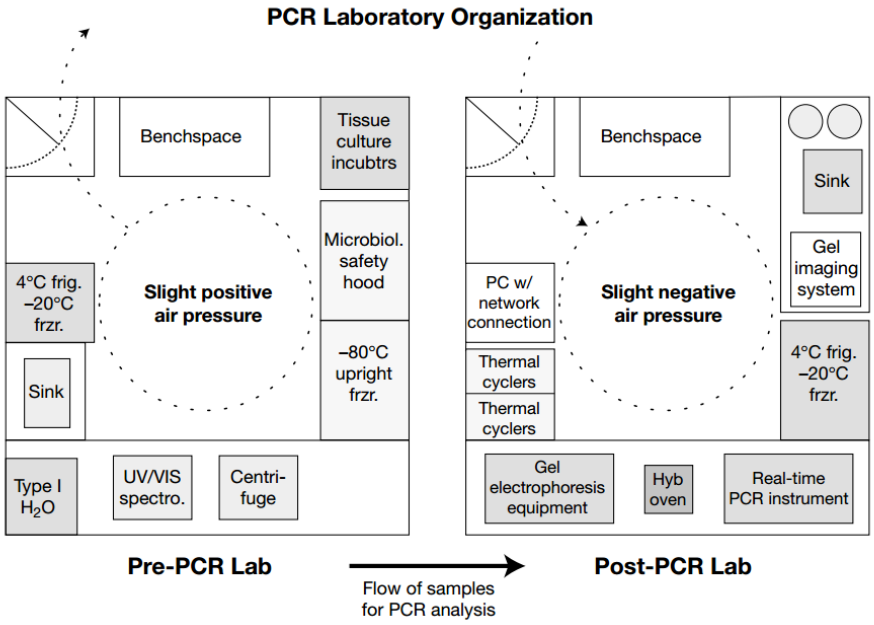

Figure 1: . Organization of a PCR laboratory with separate pre- and post-PCR rooms. Source: Mifflin, 2003

In the event that provisioned laboratory facilities do not have enough space to accommodate setting up the laboratory in this manner alternate measures can be put in place in order to maximise use of existing laboratory space while mitigating and preventing contamination. At a minimum, an LMO detection laboratory will need to be physically separated into two areas: a pre-PCR area and a post-PCR area, each with its own dedicated equipment.

Additional specific arrangements would be needed to be put in place to create dedicated spaces, in the pre-PCR room for specific tasks to mirror the separation requirements seen when multiple rooms are available. Such arrangements include:

1. **PCR set-up space:** as previously described, this space will be dedicated to preparing reagents, such as primers and buffers as well as PCR master mixes. These reagents will require dedicated freezers, pipettes, lab ware and workspace. The workspace can be separated from other laboratory activities by containing it within a laminar flow hood or, if that is cost prohibitive, a dedicated bench space separated by a glass barrier or within a chamber. This space is to be placed as far away as possible from work areas where DNA or sample handling takes place.
2. **Sample preparation space:** this space is dedicated to the extraction of DNA from the homogenised sample and the addition of the extracted DNA and positive control to the previously prepared PCR master mixes. This space should also contain dedicated freezers for extracted DNA storage, pipettes, lab ware and workspace. The workspace can be separated from other laboratory activities by containing it within a laminar flow hood or, if that is cost prohibitive, a dedicated bench space separated by a glass barrier or within a chamber. This space is to be placed as far away as possible from work areas where reagent preparation takes place.

In laboratories where space is shared within the pre-PCR area it would also be useful to plan the laboratory's activities in such a way that, in addition to the provision of spatial separation of activities there is also a temporal separation of the work carried out during the day. For example, a workday would be arranged such that any pre-PCR testing modules are performed before any post-PCR manipulation. This temporal separation of work carried out in the lab adds an additional layer of prevention against the risk of contamination.

In such shared spaces, good housekeeping and cleaning procedures have to be stringently followed, this includes:

1. Cleaning the working area and equipment, such as pipettes, prior to each use with water and DNA decontamination reagent.
2. Decontamination of the working surfaces using water and a DNA decontamination reagent followed by water again to remove any residual reagent.

Other environmental considerations that would further facilitate the mitigation of the risk of contamination include:

1. Personal Protective Equipment: an adequate availability of laboratory coats, gloves, booties and any other personal protective equipment should available for working in each unique work space since aerosolized contaminants can cling to such surfaces and be transferred between workspaces. Furthermore, working materials, such as notebooks, pens or memory sticks also cannot be transferred between workspaces.
2. UV Radiation: DNA is sensitive to UV radiation, which causes chemical modification in DNA and changes its molecular structure by the formation of dimers. This sensitivity to UV light can be used to decontaminate PCR laboratories by exposing surfaces to UV light overnight.
3. Air Flow: Ideally, the air pressure should be controlled to avoid contamination resulting from airflow through ventilations systems between pre-PCR and post-PCR working areas. In the pre-PCR laboratory, there should be a slight positive pressure, and in the post-PCR laboratory, a slightly reduced pressure to prevent dispersal of amplified DNA from samples.

4. Laboratory Temperatures: While this does not affect the risk of contamination, laboratory temperatures need to be controlled and maintained to within ±3^{o}C to ensure the accuracy of pipetting volumes.

Sample Tracking

As previously outlined under the sample preparation module, all samples that are received in by the laboratory are assigned a unique sample tracking number that is logged in the laboratory information management system and in the case file. This unique tracking number is used to follow each sample as it is processed through the laboratory. The use of a tracking number helps maintain traceability and confidentiality of the sample.

Furthermore, the unique tracking number can be used to facilitate the systemic storage of samples. This includes the storage of an aliquot of homogenate and the extracted DNA from the two test portions. These measures facilitate maintaining traceability of the sample in the event that retesting is needed or if the sample needs to be used for research or proficiency testing purposes.

Minimal Standard Criteria and Requirements for Experimental Quality Assurance

Quality assurance is defined by ISO9000:2015 as "part of quality management focused on providing confidence that quality requirements will be fulfilled"21. It is a multifaceted procedure that is put in place by the laboratory to ensure that the results of testing are accurately reflecting the sample composition.

The procedures involve applying various measures to monitor the quality of data produced during testing which allows laboratory personnel to confirm the precision and accuracy of their measurements and, if needed, apply corrective measures if any drifts are observed. These measures include the use of (certified) reference materials, control samples and duplicates to monitor the performance of a method and ensure the validity of analytical results.

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

The meticulous and detail oriented nature of the work that is carried out in an LMO detection laboratory calls for the need to have competent and trained personnel that have the knowledge, skills, abilities, experience and training to perform assigned duties, such as equipment operation performing the technical steps of the testing procedure and subsequent analysis of results. Personnel have to be trained to understand the importance of the QA/QC measures that are enforced by the laboratory and to be reliable, precise and motivated in their enforcement.

21 https://www.iso.org/obp/ui/#iso:std:iso:9000:ed-4:v1:en:term:3.6.5

New personnel should be formally trained and familiarised with laboratory procedures and demonstrate competency in their execution before beginning independent casework. Experienced Laboratory personnel should be continuously educated and trained. The evolving nature of the field of LMO development and, by extension, the field of LMO detection and identification requires that staff are encouraged and supported to stay up to date with the most recent advances in their field. Therefore it is recommended that the laboratory establishes goals for the continuing education and training of all personnel to meet the present and anticipated needs of the laboratory

Furthermore, managerial and technical tasks and their associated responsible personnel should be clearly defined; including substitutes in the case the primary contact is absent.

2. Analytical Controls

As previously indicated, there are several potential sources of contamination throughout an laboratory, including samples, laboratory staff, ventilation, equipment and reagents. Contaminants can affect analytical results in various ways, such as:

1. Carry-over contamination of amplified target DNA from previous PCRs;
2. Cross-contamination between samples, resulting in transfer of target DNA from one sample to another;
3. Cross contamination from past sample preparations; or
4. Degradation of DNA template or products from decontamination reagents.

The first three forms of contamination would produce false positives whereas the latter type would result in false negative results. In order to obtain reliable results, the use of analytical controls, which are tested in parallel with test samples, must always be used during a PCR as means of validating the test run and monitoring for the presence of contaminants, as listed in table 4,

Control	Method
No template control Tests for the presence contaminants in the PCR reagents	PCR of the mastermix only without the addition of any DNA template
Positive control Confirmation of the integrity of the PCR mastermix	PCR of the mastermix with the addition of a DNA template that is known to amplify
Reaction Sensitivity	PCR of the mastermix with the addition of a dilution series of DNA template to verify that the desired conditions and yields are fulfilled

Table 4: Some common controls that can be used during PCR monitor performance. Source: Adapted from JRC-IHCP 2006

In general analytical controls comprise positive and negative controls.

Positive controls

The positive control is present to confirm that the testing procedure was performed correctly and that the performance level was within acceptable ranges of variability. Ideally, limits of detection should be given as genomic equivalents, which would allow the production of defined sensitivity controls, with small copy numbers. As a rule, a reference preparation, containing a known concentration of the target DNA under investigation, should be available.

Negative controls

Contamination may occur during the isolation 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.

The use of analytical controls should be specified for each method that is used in the testing process. This includes defining the purpose of each control and instructions on the parameters and criteria to follow in order to ensure that the data obtained from the analytical controls are within acceptable limits. The instructions should also specify procedures that need to be followed in the event an analytical control is non-conformant. Additional information on the actions to follow in the event a non-conformance is discovered is covered under section XX.

3. Equipment

Calibration and maintenance of laboratory equipment is an essential component in ensuring the validity of laboratory results. Not only does it prolong the life span of equipment but it is also a way to monitor changes in the performance levels that may lead to a drift in the accuracy of its parameters and, by extension, affecting test results. These changes may occur due to environmental variations, moving or servicing equipment, or the aging of critical components within the equipment.

The establishment of a calibration and maintenance plan is therefore vital to maintaining the QA/QC programme within a laboratory. A calibration and maintenance plan is a procedure that is documented within the laboratory's quality manual that outlines the frequency and protocol to follow for monitoring the performance and calibration status of each equipment item. In verifying the performance and calibration status traceable physical parameters should be measured, as appropriate depending on the item being calibrated, for example mass, volume, temperature wavelength etc. Appropriate calibrators and standards should be used in each case to obtain an estimation of uncertainty/ standard deviation for each piece of equipment, where possible.

Equipment Calibration and Verification

For any piece of laboratory equipment, procedures have to be put in place for its calibration and/or performance verification in the laboratory's QC/QC manuals. The calibration procedures have to be appropriate for the intended use of the equipment and outline criteria for determining if calibration is satisfactory. Information on appropriate calibration procedures can be obtained by consulting the manufacturer's operating manuals to determine the correct calibration method and interval.

These procedures have to be implemented on new equipment, or any piece of equipment which leaves the control of the Laboratory, prior to being used in testing. Moreover, equipment which should not be used if satisfactory calibration cannot be achieved or the calibration date has passed. Infrequently used equipment, would also have to be calibrated and verified prior to use. Laboratory equipment requiring calibration should be labelled to indicate the calibration status, including the date when last calibrated and the due date for recalibration.

Equipment can be calibrated using external service providers. Calibration certificates from these providers should contain the measurement results, including the measurement uncertainty and/or a statement of compliance with an identified metrological specification. This method of equipment calibration and verification may be cost prohibitive for some laboratories. As such, where appropriate expertise is available in the laboratory, an internal calibration and verification system may be adopted.

In addition to calibration and verification procedures, preventative maintenance procedures for each equipment item should also be enforced and performed according to a regular schedule. Preventive maintenance should also be documented in the maintenance records.

In ensuring that each piece of equipment is calibrated and maintained on a regular basis, the laboratory must keep through written records to reflect this for each piece of equipment, as out lined in the table below. In addition to this, some laboratories also choose to maintain control charts to record quantitative data of some equipment such as scales and pipettes. This form of record keeping will not only indicate whether or not the routine maintenance was actually carried out it will also serve as a historical record of the equipment's performance levels to verify that equipment is functioning as expected and if there is a drift in the accuracy of its parameters then it can be easily traced.

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
• Condition when received (new, used)
• Details and date of checks made for compliance with relevant calibration or test standard specification
• Date equipment was placed in service by the laboratory
• Current location in the laboratory, if appropriate
• Copy of the manufacturer's operating instruction(s)
• Performance criteria defined according to the requirements of the type of analyses to be carried out with this instrument
• Details of maintenance carried out and records of the subsequent performance check
• History of any damage, malfunction, modification or repair and records of the subsequent performance check
• Maintenance plan and frequency of checking the performance criteria
• Description of Adjustments or repairs made
• Identity of the individual performing calibration.

Table 1: Checklist of information to be kept in the instrument maintenance log-book. Source: Adapted from UNOCD 2009

4. Reagent Quality Control

The quality of reagents have a direct impact on the on the quality of the testing procedures they are used in. Therefore the reliability of reagents must be verified prior to their use in routine casework to ensure it complies with the specifications or requirements defined in the testing procedures. Furthermore, procedures are needed to ensure the reliability of reagents is routinely monitored. This may be achieved by, amongst other things, the use of analytical controls to check the performance of reagents as they are used in routine casework.

Reagents may be prepared in the laboratory or commercially purchased. Reagents that are prepared in the laboratory should be labelled with, at a minimum, their identity, date of preparation, and date of expiration. Quality control records should also include information on lot numbers, the person who prepared the reagent, and the results of reliability testing. New batches of commercially produced laboratory reagents have to be tested for reliability in parallel with an existing batch of the same

product that is known to perform well. The new batch should also be marked with an expiration date that has either been established by the manufacturer or, if none is provided, an expiration date that has been established through validation studies of the reagent's performance over time.

In the Laboratory

An example of ensuring continuity in the quality of reagents as different lots are acquired is with new orders of primers and probes.

When new orders are received to replenish existing ones they must be tested in parallel with lots that are already in use in the laboratory and are known to work up to the required levels of quality. Some parameters that are specifically compared between lots, in the case or qPCR, include Ct values, amplification efficiency and the shape of the amplification curve. Similarities and consistencies of these values between different lots help determine and ensure the quality of the reagents.

5. Reference materials

The European Commission's Joint Research Centre (JRC) defines a reference material as a "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"[22]. There are vital tools in the validation of methods and procedures as well as their use as analytical positive controls to facilitate the confirmation of results obtained during the testing process.

Reference materials are available in various formats and can be selected based on the laboratory's needs. They can be purchased in a matrix format, for example as ground seed, for qualitative analysis, which can be useful for validating extraction procedures. They can also be purchased in varying percentage of LMO content and, once the DNA is extracted, can be used for quantitative PCR based analysis. Ideally, reference materials that are purchased in matrix format should be stored in their original container under the conditions specified by the manufacturer. They can also be extracted and the DNA can be stored at -20^{o}C.

Reference materials, like any other reagents, have to be tested for reliability every time a new lot is ordered in the laboratory. This is done by extracting the new lot of reference material and testing it in parallel with a lot of reference material that is currently in use. The new lot should preform in a manner that is comparable to the reference material that is currently in use. If there are differences, which may arise due to variations in the extraction efficiency between the different lots, then adjustments would have to be made to the stock solutions of the extracted DNA to ensure that the calibration curves obtained when using them are accurate. It should also be noted that purchased reference materials are certified for the presence of a specific LMO at a specific concentration however they may also contain low level contaminants of other LMOs so it is possible that these will be detected.

Certified reference materials for commonly encountered, commercialized LMOs can be purchased from various suppliers, as indicated below:

1. The American Oil Chemists' Society (AOCS)[23]

22 https://ec.europa.eu/jrc/en/reference-materials
23 http://www.aocs.org/attain-lab-services/certified-reference-materials-(crms)

2. The European Commission's Joint Research Centre (JRC)[24]
3. Sigma-Aldrich[25]

Reference materials can also be purchased as plasmid DNA fragments. It should be noted that purified plasmid DNA fragments can overestimate the LMO concentration present in a case sample since the PCR efficiency of such samples can be higher that that of extracted sample DNA. It is therefore important to carry out a reliability test on such reference materials and make the necessary adjustments. In some cases, for example for locally produced LMOs or those that that are not commonly commercialised, alternate sources of reference materials would have to be sought. This may include samples that have previously tested positive.

Method Validation

As mentioned in previous sections of this manual, there are several types of analytical methods that target different analytes and use a wide variety of chemistries and strategies. For instance, for DNA based molecular methods, detection may involve the amplification of a GM event or its genetic markers by simple PCR reaction, hydrolytic probes, isothermal amplification or high resolution melting strategies amongst others either in single reaction, arrays or in a multiplex.

The laboratory may select from this wide array of test methods based on, among other things, each country's specific regulatory requirements, operational costs, infrastructure and technical capability as well as ensuring that the needs of the customer are met. Regardless of which method is selected they all will need to have been validated. The goal of the validation is to evaluate the performance characteristics and limitations of an analytical method. The validation process for new methods takes place either within each laboratory or, ideally, through inter-laboratory comparisons to ensure that they meet minimum performance criteria and that they provide accurate results that are, preferably, published as international, regional, or national standards. Validation studies should be performed prior to the implementation or use of:

- Technical procedures or methods that are developed by the laboratory
- Non-standard methods
- Standard methods used outside their intended scope
- Modifications of methods

Some of the parameters reported during the validation process are used to determine the acceptability of the method and include parameters such as applicability, specificity, dynamic range, robustness, limits of quantification, limits of detection and efficiency, amongst others. These elements also allow for measuring the method's level of measurement uncertainties, determining its overall performance, for which, precision and trueness complement the set of measured parameters. It is important to mention that the method used is validated with appropriate reference samples, which have been critically evaluated and a proper value assigned by metrologically sound procedures, which is normally available through National Metrology Institutes or Designated Institutes.

There are several steps involved in the validation of a method that has been developed by a laboratory before it can be first used in laboratory casework:

24 https://ec.europa.eu/jrc/en/reference-materials/catalogue
25 http://www.sigmaaldrich.com/analytical-chromatography/analytical-products.html?TablePage=9641474

1. Method development: a method may have been developed in-house by the laboratory in response to a particular need based on appropriate research and development procedures by qualified personnel. Once a method is developed it would still have to go through the validation process to ensure that it is appropriate for use in casework.
2. In house validation: Once a method is fully developed the laboratory that developed it evaluates its performance and parameters within the laboratory to ensure that it complies with performance parameters that are appropriate for casework.
3. Prevalidation: To ensure that the method is robust and that it can preform under a wider range of laboratory conditions, a prevalidation of the method can be preformed. This includes the transfer of the method to a limited number of laboratories who will test the performance of the method's parameters.
4. Full validation: To fully validate a method an inter-laboratory study, or ring trial, should be preformed. Under this step a larger number of laboratories evaluate the method according to internationally acceptable standards to ensure its parameters preform consistently under varying laboratory conditions.
5. Method Verification: A novel method that was developed by an external laboratory may be adopted as part of standard laboratory procedures. Before it is used as part of standard casework its performance characteristics have to be verified in-house to ensure that they preform to acceptable standards under in house laboratory conditions. During the method verification process the laboratory may wish to modify the method parameters to suit the laboratory's needs, for example, reducing the reaction volume or changing the choice of PCR master mix. Once the method is fully verified the appropriate accreditation body should be informed as appropriate.

Method Verification and Verification Records

Verification studies of in-house procedures and methods should be planned and performed by qualified personnel following an experimental plan that was previously prepared and documented. This documentation should include information on:

1. The draft standard operating procedure of the method that is to be introduced. This includes a summary of the experimental plan followed to carry out the method verification and a clear definition of the parameters being tested and the corresponding method acceptance criteria.
2. If modifications of the method are made that deviate from the procedure described in the method validation report they have to be documented in the verification procedure to determine the effect(s) of the modification on the method performance. This documentation includes data obtained from an analysis of identical samples tested in parallel using the method as defined as published and the method as modified by the laboratory.
3. The documentation of the experimental plan should also include details of the laboratory personnel that preformed the verification experiments and details of the instruments used for each experimental procedure.
4. A detailed documentation of the data obtained from the verification procedure as evidence that the method is reliable as compared to the characteristics reported during the method validation report.

The experimental plan of the verification study should address and document the following criteria, as appropriate:

1. Accuracy and precision of the procedure over the range of parameters expected in casework as determined by examining accuracy, detection limits, selectivity of the method, linearity,

limit of repeatability and/or reproducibility, robustness against external influences, and/or cross sensitivity against interference from the matrix of the sample.

2. Apparatus and equipment.
3. Reference standards and reference materials.
4. Environmental conditions.
5. Uncertainty or the procedure for estimating uncertainty.

A detailed definition of each of the performance parameters, and an outline of how to preform their practical assessment, is outlined in table 1.

Designing a method verification experimental plan

A well-designed experimental plan for method verification allows the analyst to use time and resources efficiently in order to gather the relevant information needed to verify a method.
Such plans may include one of several strategies:

(To be completed)

Parameter	Definition	Expression	Requirements	Practical Assessment
Accuracy	The closest agreement between a test result and the accepted reference value			
1. Precision	The closeness of agreement between independent test results obtained under stipulated conditions	• Standard deviation s or **SD** • relative standard deviation s_{rel} or **RSD** • % coefficient of variation **%CV** or **%RSD** = 100* **RSD**=100*SD/X • Repeatability limit **r** = 2.83xSDr and reproducibility limit **R** = 2.83 x SDR • Confidence interval **CI**= X±C (*)	• **RSD_r** = 0.5-0.6 times theoretical values determined by the **Horwitz function** = 2 exp (1 – 0.5 log C) with C= analyte concentration in decimal fraction • **RSD_R** = 0.5-2 times theoretical values determined by the Horwitz function • **Horrat** Value = RSD_R(trial)/RSD_R(Horwitz)? 2 • **RSD_r** and **RSD_R** values according to AOAC Peer verified Programme	• Minimum of 3 concentration levels over full range of analytical method (~at LOD, in middle, high) • Minimum of 3 repeats per concentration level • Calculate repeatability precision SR_r, RSD_r, r=2.8xSD_r, C, CI • Calculate intermediate precision SR_{int}, RSD_{int}, r=2.8xSD_{int}, C, CI • Calculate reproducibility precision SR_R, RSD_R, r=2.8xSD_R, C, CI • Document in **bar chart** or in **control chart**
1.1 Repeatability Precision (intra-run precision)	Precision under conditions where independent 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 time intervals			
1.2 Intermediate Precision (inter-run precision)	Precision under conditions where independent test results are obtained with the same method on identical test items in the same laboratory but by different analysts using different equipment over an extended period of time			
1.3 Reproducibility Precision (inter-laboratory precision)	Precision under conditions where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment			
2. Trueness	The closeness of agreement between the expectation of the test result(expected mean value) and an accepted reference value (true value)	<u>If CRMs are used:</u> **Bias**= % error: difference between measured value and true value **Z-score:** difference between measured value and certified reference value% (**) <u>If no CRMs are available:</u> % recovery if known, spiked amount of analyte	<u>General:</u> compare results with second, validated reference method <u>if CRMs are used:</u> **Z-score** ≤ |2| <u>if spiking method is used:</u> **% recovery** according to according to USP/ICH guidelines (depending on analyte concentration level)	• Minimum of 3 concentration levels over full range of analytical method (~at LOD, in middle, high) • Minimum of 3-10 repeats per concentration level • <u>Use of CRMs:</u> calculate bias (% error) and/or Z score • <u>No CRMs:</u> spike a typical matrix and a blank sample with known amount of analyte; calculate % recovery = 100x [spike $_{matix\ sample}$/ spike $_{blank}$]

Recovery	The fraction of the analyte added to the test sample (fortified or spiked) prior to analysis which is measured by the method	**% recovery** of known, spiked amount of analyte	**% recovery** according to USP/ICH guidelines (depending on analyte concentration level)	• Minimum of 6 repeats of matrix planks or samples unfortified and fortified with the analyte at different concentrations • Calculate % recovery = 100x [spike $_{matrix\ sample}$/spike $_{blank}$]
Specificity	The ability of the method to determine accurately and specifically the analyte of interest in the presence of other components in a sample matrix (that may be expected to be present in the sample matrix) under the stated conditions of the test (specificity = 100% selectivity)	NOTE: Cannot be expressed, must be demonstrated; depends on type and purpose of methods For identification tests: **% correct classification** of ● non-analyte containing samples as negative ● analyte containing samples as positives the **ability to discriminate** with compounds of closely related structures (negative results) For quantitative tests: **% recovery** of samples, spiked with possible interferents	for identification tests: **% correct classification** ~100 for quantitative tests : **% recovery** according to USP/ICH guidelines (depending on analyte concentration level)	Identification tests: • Determine % false positives for a minimum number of blank samples • Determine % false negatives for a minimum number of positive samples (RMs) • Test a minimum number of structurally similar or closely relates samples which must be negative Impurity and assay tests: • Spike different samples with possible interferents and calculate % recovery • If no interfering compounds are available, compare with second method
Limit of detection (LOD)	The lowest concentration or amount of analyte - that can be reliably distinguished from zero - that can be detected/ measured with reasonable statistical certainty	NOTE: LOD is expressed as a concentration or amount of analyte which is derived from the measured signal (***) **Lowest Signal:** $X_L = X_{bl}+3S_{bl}$ **Lowest concentration/ amount:** $LOD = q_L(C_L) = X_L/S = 3S_{bl}/S$ NOTE: a signal of 3 times the SD blank corresponds to a % RSD of 33%		• Measure, each once, a minimum of either 10 independent sample blanks ($X_L = X_{bl}+3S_{bl}$) or 10 independent sample blanks fortified at lowest acceptable concentration ($X_L = 3s_x$) • choose a number of low concentration levels near the expected LOD • set up the calibration curve: signal x = A.concentration+ B and thus X_L=A.LOD • Determine the slope A and calculate the $LOQ = X_L/A = [X_{bl} + 3S_{bl}]/A$

Limit of Quantification (LOQ)	The lowest concentration or amount of analyte that can be determined quantitatively with an acceptable level of repeatability precision and trueness	NOTE: LOQ is expressed as a concentration or amount of analyte which is derived from the measured signal Lowest Signal: Xl= Xbl+10Sbl Lowest concentration/ amount: LOQ= qL(CL)=XL/S=10Sbl/S NOTE: a signal of 10 times the SD blank corresponds to a % RSD of 10%		• Measure a minimum of 10 independent sample blanks each once ($X_L= X_{bl}+10S_{bl}$) • choose a number of low concentration levels near the calculated LOD • set up the calibration curve: signal x = A.concentration+ B and thus X_L=A.LOQ • Determine the slope A and calculate the LOQ = $X_L/A= [X_{bl} + 10S_{bl}]/A$
Linearity **Linear range= Working range= Linearity limits**	The ability of the method to obtain test results proportional to the concentration of analyte (within a given range) The range of concentrations/ amounts of analyte over which • the method gives test results proportional to the concentration of analyte OR • a linear calibration model can be applied with a known confidence level	NOTE: <u>Linearity</u> cannot be expressed, must be demonstrated <u>Range:</u> A **range** con concentrations between lower and upper **linearity limits**	<u>For assay tests:</u> **Range**= 80-120% of analyte level <u>For impurity tests:</u> **Range=** LOQ(or 50%) -150%, 50=150% or 0-1050% according to USP/ICH and IUPAC Guidelines **Lower linearity limit =** LOQ	• First rough estimation of linear range: measure blank + min. of 6 independent concentration levels, set up calibration curve and estimate linear range visually • choose CRMs or spiked samples of minimum 6 different concentrations within estimated linear range • calculate **residual Y values** from calibration curve and plot them as function of concentration • if *randomly* distributed → *linearity*; if *systemic* trends → *non-linearity* • calculate **relative signals** = signal/ concentration and plot them as function of concentration • if *horizontal line* → linearity; *linearity limits* correspond to 95% and 105% relative signal values → *range*

Ruggedness **Robustness**	The (intra laboratory tested) behaviour of an analytical process when small changes in the environmental and/or operating conditions are made (generally used term) A measure of the capacity of the analytical procedure to remain unaffected by small but deliberate variations in method-performance parameters, which provides in indication of its reliability during normal usage (a term used by USP/ ICH only)	Ruggedness: measure of variability (reproducibility of results obtained under a variety of conditions); expressed as %RSD (inter-laboratory)		• Evaluate the effect of different small changes in parameters (in days, instruments, analysts, reagents, material, amount of sample material used etc.) individually • Calculate precision data
Sensitivity	The change in the response of a measuring instrument divided by the corresponding change in stimulus	The **slope** of the calibration curve (arbitrary)		

Table 1: Summary of method-performance parameters: definitions, ways of expression, requirements or acceptance criteria and guidelines for practical assessment. Source Taverniers et al 2004

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, all staff involved in casework should participate in at least one proficiency test per year. Proficiency tests can be used to quantitatively and/ or qualitatively test for the presence of various LMOs from different species (corn, soy etc.) in various types of commodities, such as food, feed or seed and in several types of matrices such as baked products, animal feed or mixed flours.

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.

There are various organisations that offer proficiency tests. Depending on your regulatory requirements some may be mandatory, for example the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) organises mandatory comparative testing for National Reference Laboratories, designated by each EU Member State[26]. Tests from other providers are also available and can be performed to target specific areas where the laboratory would like to test its proficiency. Such providers include:

- GeMMA proficiency testing scheme; Administered by FAPAS[27]
- USDA/GIPSA Proficiency Program[28]
- ISTA Proficiency Test (PT) Programme[29]

It should be noted that while participating in proficiency testing schemes represents a cornerstone of a laboratory's QA/QC programme, they can however, be costly and time consuming. 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. Such a plan would outline which methods and/ or matrices the laboratory needs to have tested for proficiency every year since it would not be cost or time effective to test all methods and/ or matrices with every proficiency test. For example, methods that are used most frequently in the laboratory may not need to be subjected to proficiency testing as often due to the availability of control charts that give an indication of the performance of the method within that laboratory.

Non-Conformances and Corrective Actions

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 an outline of the steps to follow towards corrective actions. The goals of this corrective action are to identify the root cause of a problem; correct non-conformities; implement a solution to avoid

26 http://gmo-crl.jrc.ec.europa.eu/Comparative-Testing.html
27 http://fapas.com/
28 https://www.gipsa.usda.gov/fgis/proficiencyprogram.aspx
29 https://www.seedtest.org/en/proficiency-test-_content---1--1078.html

recurrence; and maintain the highest level of quality. Non-conformances can be administrative/ clerical or technical in nature.

An administrative/ clerical non-conformance, for example a transcription error, can be corrected on the spot by the individual who discovers the error or by the original examiner when administrative or technical review is returned.

A technical non-conformance, can be identified through examining information from analytical controls, for example if amplification is observed in the negative control, or, conversely if there is no amplification in a positive control which may indicate a failure in the reaction and its source needs to be identified. Other forms of non-conformance may include failure to identify the presence or absence of a particular analyte or inconsistencies in proficiency test results. If a technical non-conformance is observed during a particular procedure then the appropriate responsible staff should be informed and consulted to evaluate the non-conformity and decide on the most appropriate way forward. This may include, depending on the severity of the non-conformance, identifying the problem and fixing it on the spot or suspending the use of the procedure until the problem has been resolved, and the procedure can be shown to work as expected once more.

Throughout the process of following up on a non-conformance and the applicable corrective actions, through documentation must be maintained. Depending of the laboratory's choice of procedures and the nature of the non-conformance this may consist of documenting the relevant remedial actions in the relevant case file and/or in a separate laboratory log of non-conformity and corrective action records, as applicable. The documentation of non-conformity may include information on the following:

1. The non-conformity.
2. Event(s) which identified the non-conformity.
3. Extent of the non-conformity.
4. Effect(s) of the non-conformity on the quality of work.
5. Short term response.
6. Root cause(s) of the non-conformity. Examples of findings or root causes may include equipment failure; incomplete or non-existent procedures; non-compliance with procedures and regulations; improper collection, storage, handling, or preparation; calculation errors or transcription errors; and lack of training.

Databases of methods and screening matrices[30]

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 Living Modified Organisms (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).

30 This section has been adapted from the SCBD Technical Tools and Guidance for the Detection and Identification of LMOs 2014. Available at http://bch.cbd.int/protocol/cpb_detection/toolsandguidance.shtml

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[31]

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 gel-based PCR methods. The collection includes methods that are event-specific, construct-specific and element-specific methods for screening as well as plant 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. [32]

GMO Detection Method Database (GMDD)[33]

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 gel-based PCR methods that are element-specific, gene-specific or reference gene 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 certified reference materials (CRM), if available.

CropLife International Detection Methods Database[34]

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

The number of commercially available LMOs is huge and is growing at the global scale. It is therefore not efficient to only employ event-specific methods when testing for LMO presence. Fortunately, there is a considerable body of knowledge in the scientific literature that contains

31 The event-specific detection methods are available at: http://gmo-crl.jrc.ec.europa.eu/gmomethods/

32 The compendium of detection methods is available at: http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx

33 The GMO Detection Method Database is available at: http://gmdd.shgmo.org/

34 CropLife International Detection Methods Database is available at: www.detection-methods.com

valuable information on strategies that have been developed for the identification of LMOs. The most cost efficient alternative strategy to detect the potential presence of an LMO in a sample is to use a screening approach which employs the combination of element-specific detection methods selected on the basis of information on presence or absence of the target in a list of LMOs.

There are two different types of commonly used matrices as described in the recently drafted European Technical Specification for the application of PCR-based screening strategies using the so called "matrix approach". According to this document, the first type of screening matrix is the "GMO method matrix", 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.

The second type of screening matrix, the "GMO 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 "GMO method matrices", "GMO target matrices" are independent from a particular detection method.

German laboratory network Screening Table[35]

The German laboratory network has developed a "GMO method matrix" (also known as the Waiblinger Table), which is based on five 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 *nos* 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;* and
5. a sequence from the P35S-pat junction of the CaMV P-35S promoter and the synthetic pat gene.

All five 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.[36].

35 The Waiblinger Screening Table is available at: http://www.bvl.bund.de/SharedDocs/Downloads/09_Untersuchungen/screening_tabelle_gvoNachweis.xls?__blob=publicationFile&v=2

36 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

GMOseek matrix[37]

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 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.

JRC GMO-Matrix[38]

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[39]

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[40]

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.

37 GMOseek matrix is available at: http://www.gmoseek.com/gmoseek

38 The JRC GMO-Matrix is available at http://gmo-crl.jrc.ec.europa.eu/jrcgmomatrix/

39 GMOfinder matrix is available at: http://link.springer.com/article/10.1007%2Fs12161-012-9378-6

40 The CoSYPS matrix is available at:
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2836468/pdf/216_2009_Article_3286.pdf

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.

GMO Checker[41]

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[42]

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[43]

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[44]

The BIOTradeStatus database is maintained by the Biotechnology Industry Organization and contains information on approvals, commercialization and seed sale of common commercially available LMOs.

41 The GMO Checker is available at: http://cse.naro.affrc.go.jp/jmano/UnapprovedGMOChecker_v2_01.zip

42 The LMO, Organism and Genetic Element Registries of the BCH can be found at: http://bch.cbd.int/database/organisms/

43 The BioTrack Product Database is available at: http://www2.oecd.org/biotech/default.aspx

44 The BIOTradeStatus is available at: http://www.biotradestatus.com/

International Service for the Acquisition of Agri-biotech Applications Database[45]

The International Service for the Acquisition of Agri-biotech Applications (ISAAA) is a not-for-profit international organization that, amongst 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[46]

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 [47]

This program 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

(To be completed)

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Module 6:

REPORTING

Contents of this module

Introduction

The outcome of a sample analysis is presented in the form of a written report prepared by the laboratory. The report is intended to inform the requesting authority of the laboratory's findings regarding whether or not LMOs are present in the sample. The report is written according to laboratory policy in compliance with national and international regulations and practices.

A well drafted report is a vital component of the laboratory's work. It 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, none the less, several key elements that should be included to ensure that the report is through and comprehensive. Additional information can be included to supplement the report based on the national regulatory requirements and laboratory policy.

Laboratory Documentation Requirements

A well compiled laboratory case file is a key component in facilitating the drafting of an informative and through report. It allows for efficient traceability of raw data to the final report and is therefore important to ensure that the case file is complete and contains all the relevant information needed before writing a report. All the information in a case file should be compiled in such a way that another trained laboratory member can follow and understand all the steps and decisions taken during the analysis.

While variations do exist between laboratories in what they choose to include 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, and information on the sample's chain of custody and packaging.
- *Test record information:* raw data from laboratory testing is normally recorded on standard work forms that are developed by the laboratory[48]. Such forms encourage consistent and standard recording of required information and facilitate the traceability of raw data. The forms are compiled in the relevant laboratory case file along with relevant references to the location of electronic data which can be referred to as needed while drafting a report.
- *Case report:* once the report is written, it too becomes a component of the case file and a copy is retained within.

48 Write something about this in QA section and reference

Report Writing Sections and Contents

The contents and scope of the report may vary based on the specific requests for testing 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 are guidelines for the minimum content that can be considered for inclusion in the report. As you may notice several of these pieces of information also make up a major component of the case file which highlights the importance of ensuring that the case file is complete.

1. Report introduction

The introductory section of the report provides the reader with a description of the background of the case may be useful to give the reader an overview of the purpose of the testing and the requests and/or instructions given to the lab by the requesting authority. For example the requesting authority may request for testing for the presence of LMOs or testing for the identification and quantification of the LMOs that may be present in a sample.

This section should also include logistical information pertaining to the case such as the laboratory's unique identification numbers for the case and each sample or specimen; the name of the requesting authority; the date the specimen was received by the laboratory and finally the date the report was prepared.

2. Summary of Sampling Procedures

A description and unambiguous identification of the sample submitted for testing is an important component of the report 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 lab. This 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 and the visual presence of contaminants. Any possible limitations arising from a poorly submitted sample may affect the result obtained by the laboratory and have to, therefore, be disclosed within the report.

Further to this description, additional information is also included regarding the procedures followed for laboratory sub-sampling techniques and sample processing. These additional manipulations of the sample are also a contributing factor to the overall quality of data and conclusion of the report and, as such, should be disclosed within the report to provide clarity to the reader. 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 ground up and mixed and a 10g subsample was used to…"

3. Summary of Method of Preparation

The report should clearly specify what tests were conducted in order to investigate for the presence of LMOs in order to provide the reader with a context within which the data was interpreted. 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. In some laboratories, an annex is included with the report to provide a technical summary to the reader including an

explanation the experimental design and choice of analytical tests that were used to analyse the sample.

4. Results of the Analysis[49]

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 be mindful of the intended recipients, which may include individuals who do 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 easier exchange of information. This can be achieved through the use of standardised terminology to explain the analytical data to the reader is therefore important as it allows a clear communication of the results obtained without giving misleading information.

An example of appropriate terminology regarding the detection, in accordance with the laboratory protocols, of an LMO using an event specific methodology would be reported as "the sample was found to contain…" rather than "the sample is…" the LMO that was tested for. Furthermore, if laboratory protocols only test for the presence of LMOs using element specific methodology or some construct specific methodologies then it would not be appropriate to speculate as to the presence of a specific LMO. It can only be reported that "the sample was found to contain the […] genetic element" that was tested for. 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 it should also be specified within the report if it is possible that the result is ambiguous due to potential mixing or presence genetically modified material from another commodity, for example maize that has been contaminated 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 in such a way that the reader understands that a negative result has to be interpreted in the context of the limitations of the test preformed. Therefore, terminology such as "not detected" or "none detected" may be used. This type of terminology is preferable to stating that an LMO is "not present", "does not contain" or "negative" because it provides the reader with a framework of the limitations of the test performed, and that the LMO may be present in quantities that are below the limit of detection. To provide additional clarity to the reader is may be useful to state within the report what the limit of detection of the test is.

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 Roundup Ready Maize were detected using method number NO-5678 with a detection limit of 0.01% in this matrix.

And 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

49 Insert a table of terminologies

of Roundup Ready maize were detected using method number NO-5678 with the Waiblinger Screening matrix.

Technical and Administrative Review

A *technical review* is an in-depth review of 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 the appropriate analyses have been conducted.
- Ensure 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 there is sufficient supporting documentation.

In addition the technical reviewer and the author are equally responsible for ensuring the accuracy of the technical aspects of the case record.

An *administrative review* is a procedure that checks case file documentation and reports for consistency with laboratory policy and for editorial correctness prior to the issuance of a report to the requesting authority.

An administrative review ensures that the contents of the report follows laboratory policy and procedure by ensuring that language in the report is clear, accurate, and complete in addition to proofreading the report for clerical errors. In addition, the administrative review checks that the technical review has been conducted and documented.

Technical and administrative reviews may be combined as one process and carried out by the same person. However, neither the technical or 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 authorised signatory as the person(s) accepting responsibility for the content of the report, as per laboratory policy. The authorised signatory is to include their signature and title, or equivalent identification, printed name and employing laboratory.

Laboratories must retain an exact copy of all reports issued within the relevant case file. These copies must be retained securely and be readily available for the time period specified in the laboratory's documented policies.

In the event that a material amendment has to be made to a report after it has been issued, it is 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 that it replaces.

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