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The analysis of food samples for the presence of Genetically Modified Organisms

User Manual

JRC F7 – Knowledge for Health and Consumer Safety

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Foreword

The Joint Research Centre of the European Commission is the Knowledge and Science service, which supports EU policies providing independent scientific evidence along the whole policy cycle. One of the main tasks of the JRC is to create, manage and make sense of knowledge as well as develop innovative tools and make them available to policy makers.

Over the years, the Joint Research Centre has acquired significant level of knowledge in relation to Genetic Modified Organism (GMO) detection and quantification, and has designed adapted or validated methods for their detection and quantification[1]. Its capacity building in the field also led to the creation and support of different Networks for GMO testing across the globe facilitating trade.

To achieve this, since 2000[2], several training courses and workshops have been organised by the JRC and in some cases jointly with the World Health Organisation (WHO). The purpose was to assist staff of control laboratories to become accustomed with molecular biology techniques, and to help them adapt their facilities and work programmes to include analyses that comply with worldwide regulatory acts in the field of biotechnology.

The Manual was first prepared in 2000, to support the courses and to provide background, theoretical and practical information to the participants. The former Molecular Biology & Genomics Unit staff developed it, describing some of the techniques used in their premises:

The following areas were covered throughout the courses:

- DNA extraction from raw and processed materials
- Screening of foodstuffs for the presence of GMOs by conventional Polymerase Chain Reaction (PCR)
- Quantification of GMOs by real-time PCR
- Quantification of GMOs by the Enzyme-Linked ImmunoSorbent Assay

This current version of the Manual prepared in 2020, tries to integrate and update information presented in previous published versions (2004, 2010). As already mentioned in the forewords of the previous versions, the Manual does not aim at substituting scientific literature. Instead, it provides background information, and technical details, as well as the updated regulatory framework of the European Union. Whenever retained necessary additional reading is suggested.

To facilitate diffusion and consultation, this publication is also available online at:

https://gmo-crl.jrc.ec.europa.eu/capacitybuilding/documentation.htm

[1] http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx
[2] http://gmo-crl.jrc.ec.europa.eu/capacitybuilding/training.htm

JRC staff members who participated in the preparation of this manual were supervised by Maddalena Querci and are indicated as co-authors. A special recognition and acknowledgment are also given to all present and past colleagues who, even not individually mentioned, contributed to the preparation and updating of the manual.

Maddalena Querci, PhD

March 2020

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All JRC colleagues that over the years have contributed to the optimization of the protocols included in this User Manual.

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Abstract

The User Manual - background information and didactical guide for the participants attending the training courses on 'The analysis of Food Samples for the Presence of Genetically Modified Organisms' organised by the Joint Research Centre - provides the theoretical and detailed practical information on the methodologies and protocols for GMO detection used during the training. Structured in 12 Sessions, it covers a wide variety of techniques for the detection, identification, characterisation, and quantification of GMOs.

Session 1

Overview, General Introduction on Genetically Modified Organisms (GMOs), EU Legislation

Introduction

Apart from the range of genetically modified crop lines deployed across parts of the globe's agricultural regions, all currently grown crop cultivars are the products of intensive domestication from their original wild state through continuous selection and controlled breeding to be more productive, pest resistant, or to produce a better or different quality of product than previous ancestral lines. Such changes, which have been going on since the first domestication of plants for human exploitation, involve the exchange or recombination of desired traits, or genes, through continuous crossing over time within species or between closely related, sexually compatible species groups. In recent decades the production of crosses both between plants that are cross compatible in nature, and between plants that are considered as naturally cross sterile became possible. Examples of techniques used in those cases are embryo-rescue techniques, *in vitro/in vivo* embryo cultivation, ovary and ovule cultures, *in vitro* pollination and *in vitro* fertilisation. In addition, mutational changes could be obtained, for instance, by irradiation of seeds.

There is a number of disadvantages to traditional hybridisation and selection procedures. 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 seem to be alleviated by the application of recombinant DNA and transformation technologies. The term genetically modified organisms (GMOs) has been introduced to describe organisms whose genetic material has been modified in a way, which does not occur in nature under natural conditions of crossbreeding or natural recombination. The GMO itself must be a biological unit that is able to multiply or transmit genetic material. Applied to crops, the term refers to plants in which a gene or genes *from different species* have been stably introduced into a host genome using techniques of genetic transfer and where in most cases such introduced genes produce a gene product (a protein). The process of introducing genes into unrelated species and getting them to function is known as "*genetic transformation*".

The analysis of notifications for experimental releases in the EU shows the following most frequently tested traits: herbicide tolerance, male sterility/fertility restoration, Bt-derived insect resistance, virus resistance, fungal resistance and alteration of starch biosynthesis (for further details see https://gmoinfo.jrc.ec.europa.eu/).

Genetic modification in plants

Although there are many variations on the plant transformation theme, there are few main methods for genetic modification in plants. The earliest of the technologies, developed in the 1980s, uses a bacterial species (*Agrobacterium tumefaciens*) to deliver the gene of interest into the host plant.

Agrobacterium, a microorganism that causes plant disease, has been known since the turn of the 20th century. In nature, *A. tumefaciens* has the exceptional ability to transfer a particular DNA segment (T-DNA) of its tumour-inducing (Ti) plasmid into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed, causing the crown gall disease. The T-DNA fragment is flanked by 25-bp direct repeats, which act as a *cis* element signal for the transfer apparatus.

Scientists take advantage of the fact that any foreign DNA placed between these T-DNA borders can be transferred to plant cells to develop *Agrobacterium* strains in which disease-causing genes have been replaced with specifically chosen DNA.

Since this discovery, considerable progress in understanding the *Agrobacterium*-mediated gene transfer process to plant cells has been achieved. However, *A. tumefaciens* naturally infects only dicotyledonous plants and many economically important plants, including cereals (which are monocotyledonous), have remained largely inaccessible for genetic manipulation. For these cases, alternative direct transformation methods have been developed, such as polyethyleneglycol-mediated transfer, microinjection, protoplast, and intact cell electroporation and gene gun (biolistic) technology.

However, *Agrobacterium*-mediated transformation has various advantages over direct transformation methods, as it reduces the copy number of the transgene, potentially leading to fewer problems with transgene co-suppression and instability.

In both cases, the cells (both those infected by *Agrobacterium* and shot by the "biolistic" gun) are regenerated into whole plants, which then carry the new gene, or genes, of interest. These plants are tested, intensively reproduced, and ultimately provide the seed for a new generation of genetically modified plant lines.

In addition to the ones mentioned above, other methods are currently being applied to obtain new plant varieties containing the desired characteristics generally called new breeding techniques (Lusser et al., 2012). These new plant-breeding techniques have been reviewed and described in various documents, among which the Scientific Advice Mechanism of the European Commission (Scientific Advice Mechanism, 2017). Site-directed nuclease (SDN) techniques trigger different repair outcomes, and their use can lead to changes ranging from point mutations to large insertions and deletions. The

European Food Safety Authority in its scientific opinion[3] describes the mechanism of SDN as follows:

- In SDN-1 applications, only the SDNs are introduced generating site-specific mutations by non-homologous end-joining.
- In SDN-2 applications, homologous repair DNA (donor DNA) is introduced together with the SDN complex to create specific nucleotide sequence changes by homologous recombination. The SDN-2 technique can result in minor or more substantial changes to the nucleotide sequences of the target gene.
- In the SDN-3 technique a large stretch of donor DNA (up to several kilobases) is introduced together with the SDN complex to target DNA insertion into a predefined genomic locus. The predefined locus may or may not have extensive similarity to the DNA to be inserted.

In the case of point mutations, the detection methods widely used in the field of GMO detection, are not able to distinguish these products from the conventional counterparts (Lusser et al., 2011). The main challenge is to demonstrate that a point mutation has been a product of gene editing technology rather than a random mutation occurring in nature. Similar challenges and concerns have been addressed by the European Network of GMO Laboratories (ENGL) in a recent document[4]. Currently at EU level discussions are ongoing on whether the products of new breeding techniques should be included in the existing legislation[5]. As both the methodologies and the regulatory framework are not yet determined, the new breeding techniques will not be addressed in this manual.

[3] EFSA Panel on Genetically modified organisms (GMO). Scientific opinion addressing the safety assessment of plants developed using Zinc Finger Nuclease 3 and other Site-Directed Nucleases with similar function. EFSA Journal 2012;10(10):2943. [31 pp.] doi:10.2903/j.efsa.2012.2943. Available online: https://efsa.onlinelibrary.wiley.com/doi/abs/10.2903/j.efsa.2012.2943

[4] http://gmo-crl.jrc.ec.europa.eu/doc/JRC116289-GE-report-ENGL.pdf

[5] European Court of Justice, C-528/16 - Judgement of 25 July 2018. http://curia.europa.eu/juris/document/document.jsf?docid=204387&mode=req&pageIndex=1&dir=&occ=first&part=1&text=&doclang=EN&cid=515140

EU legislation[6,7]

The use of genetically modified organisms - their release into the environment, cultivation, importation and particularly, their utilisation as food or food ingredients - is regulated in the European Union by a set of strict procedures. The first community legal instruments (Council Directive 90/220/EEC and Council Directive 90/219/EEC) were produced in 1990 with the specific scope to protect human and animal health and the environment.

Regarding the release into the environment, the principal community legal instrument, considered as the horizontal legal frame governing biotechnology in the EU, is **Directive 2001/18/EC[8]** on the deliberate release into the environment of genetically modified organisms. Directive 2001/18/EC repeals Council Directive 90/220/EEC and strengthens previously existing rules on the release of GMOs into the environment, *inter alia* introducing principles for environmental risk assessment, mandatory post-market (environmental) monitoring, mandatory supply of information to the public, mandatory labelling and traceability at all stages of placing on the market, and establishes a molecular register[9].

The consent for the release into the environment of GMOs is granted for a maximum period of ten years, starting from the date on which the authorization is issued, and can be renewed following the procedure laid down in Article 17 of Directive 2001/18/EC. After the placing on the market of a GMO as or in a product, the notifier shall ensure that post-market monitoring and reporting is carried out according to the conditions specified in the consent[10].

Directive 2001/18/EC (amended by Directive 2008/27/EC[11]) is implemented in each Member State by national laws. The Directive deals with both small-scale field trials (voluntary releases carried out for experimental purposes, dealt with in part B of the Directive) and the marketing provisions of GMOs (dealt with in part C).

Directive 2001/18/EC has been also amended by Directive (EU) 2015/412[12] to grant the right to Member States to restrict or prohibit the cultivation of genetically modified organisms (GMOs) in their territory.

A series of vertical legal instruments deal more specifically with the approval and safe use of GMOs intended for human and animal consumption:

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[6] See a non-exhaustive list of the most relevant EU Regulations/Directives pertaining GMOs in Table 1.
[7] Status on 24.08.2017
[8] Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001
[9] A sister directive, Directive 2009/41/EC of the European Parliament and of the Council, repealing Council Directive 98/81/EC of 26 October 1998 deals with the contained use and risk assessment of genetically modified micro-organisms.
[10] The updated information and status of the application dossiers can be found at http://gmoinfo.jrc.ec.europa.eu/gmc_browse.aspx
[11] Directive 2008/27/EC of the European Parliament and of the Council of 11 March 2008
[12] Directive (EU) 2015/412 of the European Parliament and of the Council of 11 March 2015

Regulation (EC) No.1829/2003 introduces for the first time, specific rules on GM feed, strengthens the rules for safety assessment of GMOs and enshrines labelling requirements for GM food and feed. As a main feature, this Regulation introduces one single authorisation procedure covering both food and feed use, meaning that operators may file a single application for the GMO and all its uses if they wish, where a single risk assessment is performed and a single authorisation is granted for the GMO and all its uses.

Under Regulation (EC) No. 1829/2003 the applicant shall submit a full dossier, including a detection method for the genetically modified event in question. The dossier, and in particular, the environmental and food safety risk assessment parts, are evaluated by the European Food Safety Authority (EFSA), established by **Regulation (EC) No. 178/2002**[13]. The detection methods provided by the applicant are evaluated and validated by the European Union Reference Laboratory for GM food and feed (EURL[14] GMFF), as established by Regulation (EC) No. 1829/2003.

Similarly to the provisions of Directive 2001/18/EC for the release of GMOs into the environment, the authorisation for GM food and feed can last a maximum of 10 years and can, afterwards, be renewed following the procedure laid down in Article 11 and Article 23 of the Regulation. The full and updated list of authorised GM food and feed can be consulted through the EU register web page[15] .

A 'sister' Directive governs the contained use of genetically modified micro-organisms (**Council Directive 98/81/EC** of 26 October 1998 amending Council Directive 90/219/EEC on the contained use of genetically modified micro-organisms).

In addition to the Directives mentioned above, a series of vertical legal instruments have been elaborated and implemented over the years, dealing more specifically with the approval and safe use of GMOs intended for human consumption. The placing on the market within the Community of novel foods or novel food ingredients was, until recently, regulated by a vertical piece of legislation: **Regulation (EC) No 258/97.** The specific issue of labelling of GM food has been addressed by several legal instruments. Labelling requirements were first mentioned in **Regulation (EC) No 258/97** (Novel Foods Regulation), but specific GM maize and soybean lines were subsequently subjected to labelling by the introduction of **Council Regulation (EC) No. 1139/98**.

Council Regulation (EC) 1139/98 provided a model for labelling based on the principle that a GM food or ingredient is no longer considered to be equivalent to an existing, non-GM one, if DNA or protein resulting from the genetic modification is detectable. Additives were

[13] Regulation (EC) No. 178/2002 of the European Parliament and of the Council of 28 January 2002
[14] Originally defined Community Reference Laboratory (CRL)
[15] The complete list of authorised GM food and feed can be found in the EU register at the page https://webgate.ec.europa.eu/dyna/gm_register/index_en.cfm

excluded from the labelling requirements until **Commission Regulation (EC) 50/2000** was introduced.

Regulation 1139/98 was then amended by the so-called "threshold regulation" (**Commission Regulation (EC) 49/2000** of 10 January 2000 amending Council Regulation (EC) No 1139/98) that tried to cope with the problem of unintended contamination and introduced the concept of threshold.

This Regulation stipulated that foodstuffs shall not be subject to the additional specific labelling requirements where material, derived from the genetically modified organisms, was present in food ingredients in a proportion no higher than 1% of the food ingredients considered individually.

In addition, in order to establish that the presence of this material was adventitious, operators had to supply evidence that appropriate steps to avoid using genetically modified organisms were taken.

Several reasons, including the controversial opinion of different users associations in relation to GMOs, difficulty in interpretation and application of the legal instruments issued over time, the fact that no specific EU legislation on GM feed was in place, among others, highlighted the need for unified, updated and complete legal instruments on this issue.

Finally, in October 2003, two Regulations were published that, amending or repealing previous legal instruments, provided a more complete and informative guidance on these matters.

The EU recognizes the consumers' right for information and labelling as a tool to make an informed choice. In **Regulation (EC) 1829/2003**, rules for safety assessment have been strengthened and expanded. This Regulation introduces, for the first time, specific rules on GM feed and enshrines labelling requirements for GM food and feed, so far only partially covered by Council Regulation (EC) 1139/98, and Commission Regulation (EC) 49/2000. As a main feature, this Regulation implements the "one key-one door" approach: one single authorisation covers both food and feed use, therefore filling the legal gap for feed products approval, whilst abandoning the simplified procedure based on the concept of "substantial equivalence".

Furthermore, Regulation (EC) No. 1829/2003 introduces a minimum labelling threshold for authorised GM food and feed, set at 0.9% of the total ingredients. Meaning that below this threshold, the GM does not have to be labelled, if its presence is adventitious or technically unavoidable.

Regulation (EC) No. 1830/2003 reinforces the labelling rules on GMOs. Mandatory labelling in accordance with the 0.9% threshold is extended to all food and feed irrespective

of detectability. It also provides the definition of traceability as the ability to trace GMOs and products produced from GMOs at all stages of their placing on the market throughout their production and distribution chains. Under Regulation (EC) 1829/2003 (in force since 18 April 2004) the applicant shall submit a full dossier, including a detection method of the particular genetically modified event in question. The dossier, and in particular, the environmental and food safety risk assessment parts, are evaluated by the European Food Safety Authority (Established by Regulation (EC) 178/2002 of the European Parliament and of the Council of 28 January 2002). The detection methods provided by the applicant are evaluated and validated by the Community Reference Laboratory (Established by Regulation (EC) 1829/2003). Methods are thus necessary, not only to detect the presence of a GMO in a food/feed matrix but also to identify the specific GMO and to quantify the amount of GMOs in different food and feed ingredients.

Qualitative detection methods can be used as an initial screening of food/feed products, to investigate whether GMO specific compounds (DNA and/or proteins) are present. Qualitative analysis could, thus, be performed on products, sampled from retailers, from supplies stored in stockpiles, or from points further up the supply chain.

If the qualitative analysis provides an indication of the presence of GMOs, the subsequent quantitative test must give a decisive answer concerning the labelling requirements.

Regulation (EU) No. 619/2011[16], the so called "Low Level Presence" (LLP) Regulation, has been adopted in the EU for GM feed material. This Regulation refers to the GM material for which the authorisation procedure is pending for more than 3 months in the EU but has been authorized elsewhere in the world (asynchronous authorization), or of which the authorisation has expired. The Regulation foresees that samples containing less than 0.1% mass fraction of GM material are to be considered compliant with the EU legal framework, taking into account the margin of error (measurement uncertainty), 'where *the event-specific quantitative methods of analysis submitted by the applicant have been validated by the EURL and provided that the certified reference material is available*'.

The growing use of GMOs worldwide led to the establishment of the Cartagena Protocol on Biosafety[17] in 2000. Within this international agreement, the impact of GMOs on biosafety is taken into account. The Biosafety Clearing House[18] (BCH) is the main available instrument to exchange information on GMOs import/export, national legislation and regulatory decisions (approval/prohibition). The European Union, being Party to this international agreement, has implemented the rules on transboundary movement of GMOs

[16] Commission Regulation (EU) No. 619/2011 of 24 June 2011
[17] https://bch.cbd.int/protocol
[18] https://bch.cbd.int/

by means of Regulation (EC) No. 1946/2003[19], which deals with the requirements needed for export of GMOs to third countries and Directive 2001/18/EC as regards imports of GMOs into the EU.

A comprehensive list of the most relevant pieces of legislation in the GMO detection field is presented in Table 1. More in-depth information on legislation on GMOs can be found in the publication "The EU Legislation on GMOs - An overview"[20].

The EURL GMFF

An essential integral component of the EU legislative procedure is the EURL GMFF. In the context of Regulation (EC) No. 1829/2003, the Directorate General Joint Research Centre (JRC) of the European Commission has been appointed as the EURL GMFF and is assisted by the European Network of GMO Laboratories (ENGL)[21].

The EURL GMFF has the mandate to provide and distribute control samples to the National Reference Laboratories (NRLs), to evaluate the data provided by the applicant, validate the analytical methods for the detection and identification of the transformation event (ensuring that they are “fit for the purpose of regulatory compliance”) and to submit evaluation reports to EFSA. It should also provide scientific and technical advice in case of disputes. Under Regulation (EC) No. 882/2004[22], amended by Regulation (EU) 2017/625[23], the duties of the EURLs include provision of analytical methods, organisation of comparative testing, training courses and capacity building activities.

The EURL GMFF has the responsibility of the chairmanship and secretariat of the ENGL[24]; which enforces the network among GMO related laboratories and is composed of around 100 control laboratories from all EU Member States and associated countries (i.e. Switzerland, Norway and Turkey). The main purpose of the ENGL is to act as a unique platform for experts that are involved in the sampling, detection, identification and quantification of GMOs - in seeds, grains, food, feed and environmental samples - and where technical items can be put forward and discussed. In the framework of the network activities, training courses are one of the main tools to achieve the objectives.

[19] Regulation (EC) No 1946/2003 of the European Parliament and of the Council of 15 July 2003
[20] D. Plan, G. Van den Eede, The EU Legislation on GMOs - An overview. EUR 24279 EN – 2010. ISBN 978-92-79-15224-5. ISSN 1018-5593. doi:10.2788/71623, https://ec.europa.eu/jrc/en/publication/eur-scientific-and-technical-research-reports/eu-legislation-gmos-overview
[21] http://gmo-crl.jrc.ec.europa.eu/
[22] Regulation (EC) No. 882/2004 of the European Parliament and of the Council of 29 April 2004
[23] Regulation (EU) 2017/625 of the European Parliament and of the Council of 15 March 2017
[24] http://gmo-crl.jrc.ec.europa.eu/ENGL/ENGL.html

The European Food Safety Authority (EFSA).

The European Food Safety Authority is an agency of the European Commission legally established by the General Food Law (Regulation 178/2002). Its task in the field of GMOs is to evaluate their safety before a market authorization decision is made. EFSA performs the risk assessment of the potential impact of GMOs on human health, animal health and the environment. The risk assessment is integral part of the decision making process for the authorization of the GMOs[25].

EFSA applies the principles of GMO risk assessment considering the following points (as described in its website[26]):

- Molecular characterisation: assessment of the molecular structure of the newly created proteins, their functioning and their potential interactions.
- Comparative analysis: comparison of the GM plant with its conventional counterpart. The aim is to detect differences in the plant's observable appearance such as height and colour – phenotypic characteristics – and its agronomic characteristics such as yield. The analysis also compares the nutritional values of the GM plant and its conventional counterpart.
- Evaluation of potential toxicity and allergenicity.
- Evaluation of potential environmental impact.

GMO authorisations for the EU market are valid for 10 years, after which EFSA re-assesses it.

[25] https://ec.europa.eu/food/sites/food/files/plant/docs/gmo_auth_decision-making-process.pdf
[26] http://www.efsa.europa.eu/en/topics/topic/gmo

Table 1. Key EU legislation relevant to GMO detection[27,28]

Number	Topic	Publication	Key Provisions
Directive 2001/18/EC	Deliberate release into the environment of GMOs	OJ L 106 17.04.2001	Community procedure for authorisation of deliberate release (experimental or commercial) of GMOs into the environment Definition of a GMO Mandatory labelling of GMOs Registers for recording public information on GMOs
Regulation (EC) No 178/2002	Food safety	OJ L 31 01.02.2002	General principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety
Regulation (EC) No 1829/2003	Genetically Modified Food and Feed	OJ L 268 18.10.2003	Community procedure for authorisation of both GM food and GM feed (including one door-one key authorisation process, allowing approval of a GMO under Regulation (EC) No 1829/2003 both for food/feed uses and for cultivation) Mandatory labelling of GM food and feed, irrespective of detectability of DNA or protein resulting from the genetic modification 0,9% labelling threshold for the adventitious or technically unavoidable presence of GM material in food or feed Mandatory submission of detection methods and samples of GM food/feed, including validation by the Community Reference Laboratory (CRL)
Regulation (EC) No 1830/2003	Traceability and Labelling of GMOs and food feed produced from GMOs	OJ L 268 18.10.2003	Operators must transmit the following information to the operator receiving the product: - an indication that the product contains GMOs - the unique identifier(s) assigned to those GMOs

[27]As from The EU Legislation on GMOs - An overview. https://ec.europa.eu/jrc/en/publication/eur-scientific-and-technical-research-reports/eu-legislation-gmos-overview;
See also https://ec.europa.eu/food/plant/gmo/legislation_en
[28] The full text of all legislations can be found in Eur-Lex, the Official Journal of the European Union, EU case law and other resources for EU law, at http://eur-lex.europa.eu/homepage.html?locale=en

Regulation (EC) No 1946/2003	Transboundary Movement of GMOs	OJ L 287 05.11.2003	Specific requirements for exports of GMOs from the EU to third countries in order to ensure compliance with the obligations in the Cartagena Protocol on Biosafety (including information to be provided to third countries and to the Biosafety Clearing House BCH)
Regulation (EC) No 65/2004	System for assignment of Unique Identifiers for GMOs	OJ L 10 16.01.2004	Unique Identifiers should be assigned to GMOs according to the format defined in the Annex and should appear in the GMO authorisation
Decision 2004/204/EC	Detailed arrangements for the registers recording information on GMOs	OJ L 65 03.03.2004	Details about the information to be recorded in the GMO registers provided for in article 31 of Directive 2001/18/EC
Regulation (EC) No 641/2004	Detailed rules for Implementation of Regulation (EC) No 1829/2003 on GM food feed	OJ L 102 07.04.2004	Details regarding the contents of an application for GM food feed authorisation, in particular regarding method validation and reference material
Recommendation 2004/787/EC	Technical guidance for sampling and detection of GMOs	OJ L 348 24.11.2004	Technical guidance in particular about sampling protocols and analytical test protocols (incl. unit of measurement for percentage of DNA)
Regulation (EC) No 882/2004	Official controls performed to ensure compliance with feed and food law	OJ L165 30.04.2004 (*corrigendum* in OJ L 191 28.05.2004)	Community harmonised framework on official controls performed to ensure compliance with feed and food law Designation and activities of Community Reference Laboratories and National Reference Laboratories (incl. on GMOs)
Regulation (EC) No 1981/2006	Detailed rules for Implementation of article 32 of Regulation (EC) No 1829/2003 on the CRL for GMOs	OJ L 368 23.12.2006	Detailed rules concerning: - the contribution to the costs of the tasks of the Community Reference Laboratory and of the National Reference Laboratories - the establishment of National Reference Laboratories assisting the CRL for GMOs
Directive 2008/27/EC	Release into the environment of GMOs	OJ L 81 20.3.2008	Deliberate release into the environment of genetically

			modified organisms, as regards the implementing powers conferred on the Commission
Regulation (EU) No 619/2011	Analytical methods for GMOs	OJ L 166 25.6.2011	Methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired
Regulation (EU) No 503/2013	Applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003	OJ L157 08.06.2013	Replaces 641/2004 and tackles the same issues of 641/2004 with some additional information requested for the authorisation procedure
Directive (EU) 2015/412	Restriction or prohibition of GMO cultivation in Member States	OJ L 68 13.3.2015	Possibility for the Member States to restrict or prohibit the cultivation of genetically modified organisms (GMOs) in their territory
Regulation (EU) No 2017/625	Official controls for food and feed GMOs	OJ L 9507 04.2017	Official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products
Directive (EU) 2018/350	Environmental Risk assessment	OJ L 67 9.3.2018	Amendment to Directive 2001/18/EC as regards environmental risk assessment (ERA) of GMOs

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Session 2

Manual Presentation, Working Methods and Course Introduction

How to detect GMOs

Transgenic plants are characterised by the insertion of a new gene (or a new set of genes) into their genomes. The new gene(s) are translated and the new protein(s) expressed. This gives the plant a new characteristic, such as resistance to certain insects or tolerance to herbicides. The basis of every type of GMO detection technology is to exploit the difference between the unmodified variety and the transgenic plant. This can be done by detecting the new transgenic DNA that has been inserted, or the new protein expressed, or (if the protein acts as an enzyme), by using chemical analysis to detect the product of the enzymatic reaction.

Traditionally, the two approaches that have been mostly used for detecting genetic modification in crops such as soybeans, corn, cotton and others are **ELISA** (Enzyme-Linked ImmunoSorbent Assay), and **PCR** (Polymerase Chain Reaction). ELISA involves testing for the presence of specific proteins by exploiting the specificity of binding between expressed antigen and target antibody, while PCR is based on the detection of novel DNA sequences inserted into the plant genome. These methods detect the absence or presence of a GMO in the sample and can also give an indication of the quantity (percentage) in a tested sample.

The first method validated in the EU was a PCR-based screening method able to detect most of the GMOs approved for commercialization at the time (Lipp et al., 1999). This method, developed by Pietsch et al. (1997), is based on the detection of the control sequences flanking the newly introduced gene, namely the 35S promoter and the *nos* terminator. The validation was coordinated by the Joint Research Centre (JRC) Food Products and Consumer Goods Unit of the former Institute for Health and Consumer Protection (IHCP), in collaboration with the former Institute for Reference Materials and Measurements (IRMM), which was responsible for the production of appropriate Certified Reference Materials (CRMs). Since then many other PCR-based methods have been developed and validated (http://gmo-crl.jrc.ec.europa.eu/gmomethods/). Research efforts were also directed at the development of protein-based methods and one of the first immunological approaches validated was a highly specific method for the detection of Roundup Ready® soybean using ELISA (Lipp et al., 2000).

Inherent advantages and limitations of DNA-based and protein-based approaches

The DNA-based approach

Analytical methods based on PCR technology are increasingly used for the detection of DNA sequences associated with GMOs. PCR allows the selective amplification of specific

segments of DNA occurring at low copy number in a complex mixture of other DNA sequences. In PCR, small complementary DNA pieces referred to as primers are used in pairs. The primer pair is designed to hybridise to complementary sequence recognition sites on opposite strands of the target DNA of interest. Through a series of repetitive differential thermal cycles, a DNA polymerase aids the replication and the exponential amplification of the sequence between the primer pair. Finally, these amplified pieces are subjected to standard gel electrophoresis so that their presence can be visualized based on their size determination.

Numerous PCR-based methods, which can detect and quantify GMOs in agricultural food and feed crops, have been developed. Moreover, the determination of genetic identity allows for segregation and traceability (identity preservation) throughout the supply chain of genetically modified (GM) crops. A prerequisite for GMO detection comprises knowledge of the type of genetic modification, including the molecular make-up of the introduced gene and the regulatory elements (promoters and terminators) flanking it. For analysis purposes, a minimum amount of sample material containing intact DNA comprising the target gene is required. PCR is a laboratory-based technique, requiring trained staff and specialised equipment.

Some of the key characteristics of PCR diagnostics are as follows:

- Compared to protein approaches, it is more sensitive, capable of detecting one or a few copies of a gene or target sequence of interest within an entire organism's genetic material, or genome. As a result of this high sensitivity, very low levels of contamination can result in false positives. Therefore, attention must be paid to prevent cross contamination.
- It requires little reagent development time compared to immunological assays (primer synthesis versus antibody production).
- All reagents needed are commercially available and can be easily obtained from a number of sources. However, some of these require a license for use in commercial diagnostic applications.
- Sample analysis time requires approximately one day.
- PCR is capable of discriminating between different types of genetic modification (also referred to as transgenic events) if properly developed. Diagnostic methods for identifying specific transgenic events require additional development time and validation efforts.

The protein-based approach

The protein-based test method uses antibodies specific for the protein of interest. ELISA detects or measures the amount of protein of interest in a sample that may contain other numerous dissimilar proteins. ELISA uses one antibody to bind the specific protein, a second antibody to amplify the detection (optional), and an antibody-conjugated to an enzyme whose product generates a colour reaction that can be easily visualised and quantified based on comparison of a standard curve of the protein of interest. Trained personnel and specialised equipment are required for proper execution of the test.

The Lateral Flow Tests (LFT) are variation of the ELISA format. Lateral flow strips have the advantage that the reaction takes place on one solid support, exploiting the protein solution flux through the absorbent strip. Results are obtained in a few minutes and the method, although not quantitative, is very economical.

ELISA, LFT and of immunological methods:

- Are Less sensitive than PCR methods;
- Do not require the amplification of the target and, therefore are also less susceptible to 'false positives' caused by possible minor levels of contaminations;
- Rely on the commercial development and production of antibodies and protein standards;
- Even though protein-based methods are initially time-consuming and expensive during method development and the generation of antibodies and protein standards, they are characterised by a very low per sample cost once optimised for routine use;
- Compared to PCR, the ELISA method is considered a rapid test;
- Are trait-specific and cannot discriminate among different transgenic events containing the same transgene or expressing proteins with similar characteristics;
- Cannot be used to detect GMOs in samples that have undergone processing, e.g. industrial treatment.

Taken into account these considerations, immunological methods (both ELISAand LFT) and PCR should be regarded as complementary rather than exclusive to each other. See Table 1 for a comparison of DNA-based and protein-based approaches.

Table 1. Summary and comparison of DNA-based and protein-based approaches. For further details, refer to Session 12.

Analysis	Test	Target	Advantages	Disadvantages
Protein-based	LFT*	Protein	The test is rapid and can be performed on-site, very useful as an initial screen for seed and grain	Low sensitivity (LOD* 0.25% to 1%) Does not allow quantification Not suitable for processed samples GM* protein levels susceptible to variability in life cycle moments and parts of the plant
	ELISA	Protein	Less prone to false positives (LOD 0.01%-0.1%)	Not suitable for processed samples GM protein levels susceptible to variability in life cycle and parts of the plant Must be performed in a laboratory
DNA-based	PCR	DNA	High sensitivity (LOD 0.01) and specificity Capable of detecting all GMOs Allows quantification (real-time PCR) Effective with a broad range of sample types Used worldwide in GMO testing laboratories	Must be performed in a laboratory and requires trained personnel and equipment. Time-consuming

*LFT= Lateral Flow Test; LOD= Limit of Detection; GM= Genetic modification

General considerations and presentation of the Manual

Method validation is critical to both laboratories and control authorities. Ideally, each method should be confirmed for performance verification by a limited number of skilled laboratories to provide reproducible, accurate, and specific results. The JRC of the European Commission was the first to validate the ELISA and PCR methods for raw materials consisting of Roundup Ready® soybean and a PCR method for Maximizer maize (Bt-176) and to validate a PCR method for both Roundup Ready® soybean and Maximizer maize (Bt-176) in processed food fractions (Lipp et al., 1999, 2000 and 2001). Since 2004, various other methods for both qualitative and quantitative analysis have been developed and validated. For updated information on validated methods for GMO detection and quantification, please see http://gmo-crl.jrc.ec.europa.eu/gmomethods/.

Sample preparation for both DNA-based and protein-based methods is critical for detection and/or quantification. It is important to know the limitations of each procedure depending on the qualitative or quantitative information required. Both the sample size and sampling procedures dramatically impact on the conclusions that may be drawn from any of these testing methods.

The availability of suitable **Certified Reference Materials** is a fundamental requirement for each detection method. The samples used during the course are Certified Reference Materials produced at the former JRC IRMM[29] (Trapmann et al., 2008 and Broothaerts et al., 2009). The characteristics and corresponding certificates are presented in Session 3. Another critical step is **sample homogenisation**.

Figure 1 summarises the different steps performed during the course. Optimised **DNA extraction** is fundamental to ensure the presence and quality of extracted and PCR amplifiable DNA. This aspect is particularly important as most food commodities on the market made from soybeans or maize are highly processed. It is well known that DNA may degrade considerably during food processing, particularly by thermal treatment and in the presence of water. Thus, the amount of DNA fragments that are still sufficiently long enough, and still containing the intact target of interest to allow the detection of the presence of GMOs in processed food, might decrease the more the food is processed. In addition, a proper, suitable DNA extraction method should ensure the removal of inhibitory substances present in the sample. This topic will be covered in Session 4. Several methods for DNA extraction have been developed and many commercial companies have produced specialised – ready to use – kits. Performance and validity of the different available protocols will be discussed during the course. However, in order to avoid direct implications with commercial companies, it was decided to perform DNA extraction using the so-called

[29] Current name: JRC, Directorate for Health, Consumers & Reference Materials

Cetyltrimethyl ammonium bromide (CTAB) method, a validated and versatile protocol that has demonstrated its suitability for a variety of different matrices.

After DNA extraction, the samples (as well as PCR products) are analysed by **agarose gel electrophoresis** (Session 5).

The principles, advantages, and drawbacks of the **PCR** will be presented in Session 6.

As mentioned above, the efficient utilisation of modern techniques for GMO detection depends on the availability of accurate information. GMO detection requires at least a partial knowledge of the expected target gene sequence and type of genetic modification. The specific characteristics of transgenic lines MON810 maize, Bt-11 maize and Roundup Ready® soybean, are presented in Session 7.

Different PCR approaches have been developed for the detection of approved GMOs. PCR specificity depends upon accurate choice of primers. PCR primers can be directed to different elements used in the transformation process. "Broad range" PCR detection systems, generally called "screening methods", can be obtained by designing primers specific to the most common sequences of genetic elements used in transformation. These are generally the regulatory sequences (promoter and terminator). Genetically modified plants can also be divided into "categories" according to the structural gene introduced. An additional way to direct specificity of the reaction is to choose primers specific to DNA sequences located in different genetic elements (e.g. promoter-structural gene, structural gene-terminator). Finally, provided that the specific and complete sequence information is available, in order to produce really specific methods for a given genetically modified plant, "line specific" (transformation event specific) systems can be developed by selecting a "unique" sequence combination, present only in that specific transformed line. This is generallyobtained by designing primers hybridising in the DNA region spanning the integration site junction. The junction between inserted DNA (T-DNA) and host-DNA offers a unique nucleotide sequence providing an ideal target for a highly specific PCR test. The methods performed during the course are summarised in Figure 1 and described in detail in Session 8. The experimental part of the methods and protocols can be found in Session 9.

As indicated above, the need of quantifying the events of GMO present in a sample led to the development of many PCR-based protocols, which allow not only a qualitative answer (presence/absence), but also a more or less precise (depending on the method chosen) indication of the relative quantity of GMO present in a given sample. The most common DNA-based approach is **real-time PCR** (Session 10). Real-time PCR is performed using specific and sophisticated instrumentation, currently available from different commercial companies. The protocols that will be followed during the course can be found in Session 11.

Finally, Session 12 gives a general introduction to the protein-based approach for the detection of genetically modified organisms. In particular, the **ELISA** and the **LFT** techniques will be explained.

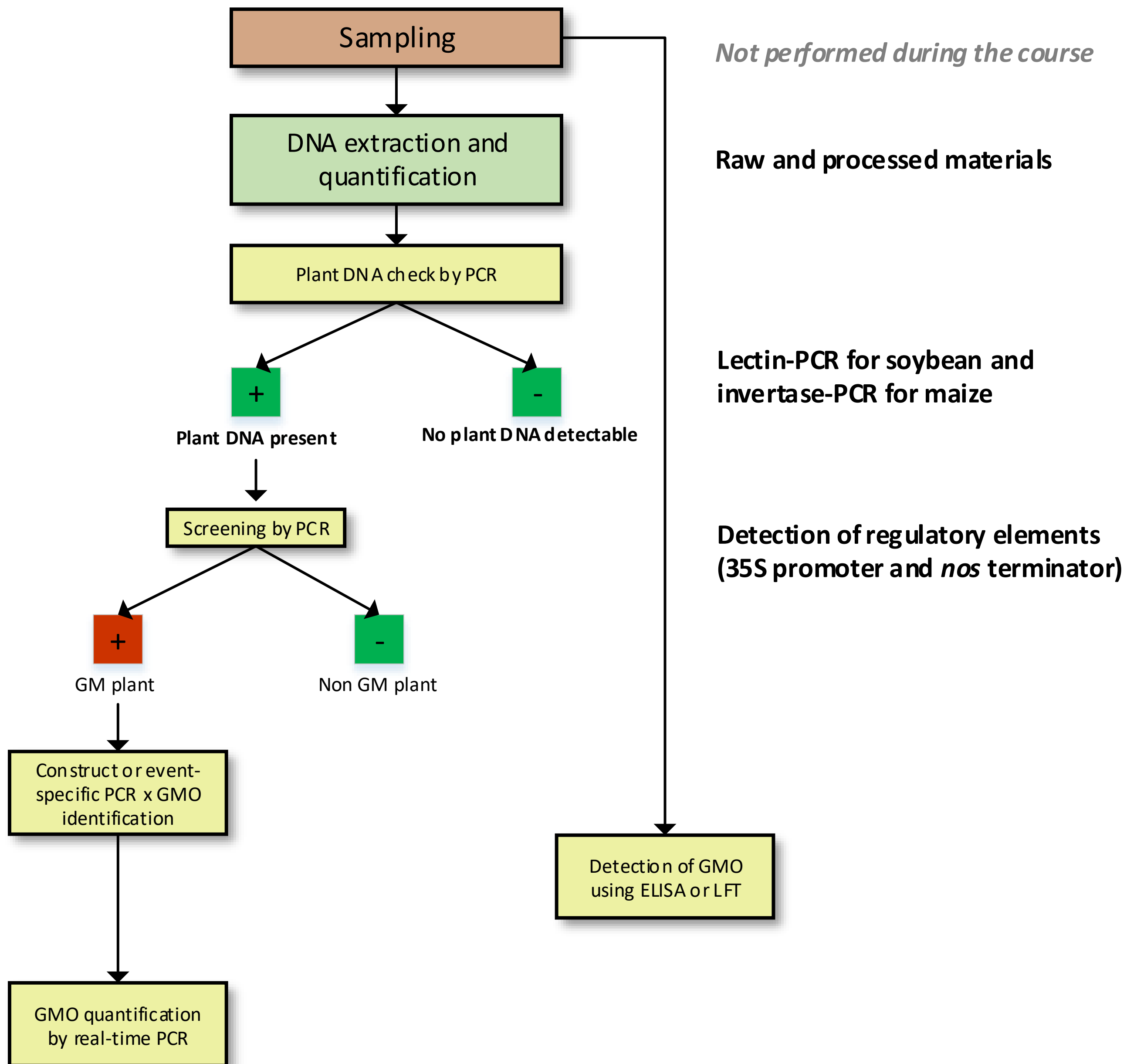

Figure 1. Flowchart of methods.

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Session 3

Samples Used during the Course

Introduction

Sample preparation and homogenization are crucial steps for Genetically Modified Organisms (GMO) detection. More exhaustive information can be found in the European Network of GMO Laboratories (ENGL) technical report on "Guidelines for sample preparation procedures in GMO analysis" (https://ec.europa.eu/jrc/en/publication/eur-scientific-and-technical-research-reports/guidelines-sample-preparation-procedures-gmo-analysis). The aim of this technical report is to provide guidelines for a correct sample preparation in GMO analysis of food, feed, seed, plants and propagating material samples. It sets *ad-hoc* standard operating procedures for sample preparation including, for instance, the evaluation of possible cross-contamination and the safety of the operator. The document also details the correct procedure for handling a sample upon its arrival to the laboratory to obtain homogenous and representative test portions and how to avoid cross-contamination.

Sampling is an important step in GMO analysis and it is covered by the EU legislation. Particularly **Regulation (EC) No. 152/2009** and its amendment, **Regulation (EU) No. 691/2013,** deals with sampling and analysis of feed. **Regulation (EU) No. 619/2011** deals with sampling and analysis of GMOs for which the authorization has expired or is ongoing; **Recommendation 2004/787/EC** endorses the use of DNA copy number (c-value) for GMO quantitation and highlights the importance of using Certified Reference Materials (CRMs) for GMO testing laboratories. Harmonized rules for the sampling of food for GMO detection have not yet been established due to the variety of available products containing GM material on the market.

Certified Reference Materials

One of the main challenges in GMO detection and quantification is the availability of CRMs. The majority of CRMs intended for the detection and quantification of GMOs are powders produced from seeds or vegetables. These matrix materials are mixtures of non-GMO material with GMO material that have been gravimetrically certified for their mass fraction (expressed in g/kg) for a specific GMO event. The available concentrations differ for individual GM events (and the set of CRMs) and range from nominal 0 g/kg up to 1000 g/kg. The two major developers and producers of reference materials in the world are the American Oil Chemists' Society (AOCS, http://www.aocs.org/index.cfm) and the Joint Research Centre of the European Commission (JRC) (https://ec.europa.eu/jrc/en/reference-materials/catalogue).

How to choose samples and CRMs

During GMO detection and quantification, it is important to use samples that are representative of the market and the different situations that may be encountered in a GMO testing laboratory. The more the product is processed the lower the quality and quantity of DNA can be (Gryson, 2009). In GMO testing, CRMs are used either for qualitative purposes (e.g. positive controls) or for the calibration and quality control of GMO quantification measurements based on quantitative real-time Polymerase Chain Reaction (qPCR). More information on the correct use of CRMs can be found at https://ec.europa.eu/jrc/en/publication/eur-scientific-and-technical-research-reports/training-manual-gmo-quantification-proper-calibration-and-estimation-measurement-uncertainty.

Samples used during the course

All samples mentioned in this manual are only given as examples for didactical purposes.

During the course, we will use different methods to detect the presence of MON810 maize, and Roundup Ready® soybean in different matrices. For this purpose, we will use mixtures of non-GM and GM maize (MON810) and non-GM and GM soybean (Roundup Ready® soybean), at different concentrations (Table 1, 2). Two types of materials will be used:

- Raw materials
- Processed materials

Raw materials

For the scope of this training, series of CRMs and a mixture of CRMs at different concentrations (mixed flour) were will be used as testing samples as described below.

CRMs series. Raw plant materials used during the course are CRMs ERM-BF410K (Roundup Ready® soybean) and ERM-BF413K[30] (MON810 maize). ERM-BF410K and ERM-BF413K consist of two sets of CRMs of dried soybean and maize powder, respectively, with different GM mass fractions (Roundup Ready® soybean: 0 g/Kg, 1 g/Kg, 10 g/Kg and 100 g/Kg corresponding respectively to 0, 0.1, 1 and 10% GM nominal value; MON810 maize: 0 g/Kg, 4.9 g/Kg, 19.8 g/Kg and 99 g/Kg corresponding respectively to 0, 0.5 and 2% GM nominal value).

[30] It should be noted that CRMs might be produced in different batches therefore the reference code will change accordingly. An example is CRM series IRMM-413 (reference material for MON810) reported in the former version of this manual, which, in the current version, has been substituted by CRM series ERM-BF413K. For updated information on available CRMs, please see: https://ec.europa.eu/jrc/en/reference-materials.

The dried soybean powder containing GM Roundup Ready® soybean has been produced from whole seeds of a non-modified soy line (Asgrow A1900) and the genetically modified event 40-3-2 Roundup Ready® soybean (Asgrow line AG5602 RR). The dried maize powder containing GM MON810 maize has been produced from whole kernels of the non-modified cultivar EXP258B and MON810 cultivar DKC57-84.

Mixed Flour. In order to obtain 1% of each GM event in a total of 100 mg mixed flour, the following recipe was used:

- 50 mg (total dry weight) of 1% of RR soya (ERM-BF410DK)
- 25 mg (total dry weight) of 2% MON810 (ERM-BF413EK)
- 25 mg (total dry weight) of 0% MON810 (ERM-BF413AK).

CRM powders were weighed and added directly to reaction tubes ready for DNA extraction.

The total amount of mixed flour prepared (100 mg) may vary depending on the DNA extraction method to be used. Please note that even though samples like flour and milk are considered homogenous, this does not mean that they should not be mixed or shaken.

Processed materials

Snack food crumb. This sample is derived from a GMO proficiency testing scheme to which the European Reference Laboratory for GM Food and Feed (EURL GMFF) participated (FAPAS, Genetically Modified Material Analysis Scheme -GeMMA, Round 06, test material GMO-06B). The material was prepared from commercially available non-GM soya-based dried snack food (containing no detectable GM DNA) and GM-containing soya snack food.

Snack food crumb	1372 g GM-free soya snack food, 28 g GM soya snack food

Before mixing, both materials were ground and sieved to give a homogenous crumb mix and then tumble-blended overnight. Finally, materials were mixed for approximately one hour using a rotary blender. Storage of the materials was at –20°C.

Biscuit. The original material was produced at the JRC and was used to validate a PCR method for both Roundup Ready® soybean and Maximizer maize (Bt-176), in processed food fractions (Lipp et al., 2001).

Any other material, similar to the one mentioned above, would be suitable for the purposes of this manual.

Dry soybean and maize derived flours were weighed and mixed with the other ingredients in the proportion indicated below.

Biscuits # 1	250 g maize (0% GMO), 250 g soybean (0% GMO), 300 g wheat, 200 g sugar, 100 g butter, 10 g salt, 16 g vanilla baking powder, 2 eggs

The ingredients were carefully mixed with 600 ml water and homogenised, spread out evenly on a baking plate and baked in a pre-heated oven at 180°C with recirculating air for 10 min. The material was removed from the oven, covered to avoid contamination and cooled down at room temperature. Storage was at -20°C.

Soy milk powder. This sample is derived from Round 05 FAPAS-GeMMA proficiency testing scheme.

A total of 1700 g of US soybean milk powder were tumble blended overnight with 300 g of Roundup Ready® soybean protein isolate. Individual sub-samples (10 g) were dispensed into screw-topped plastic containers and stored at ambient temperature prior to distribution.

Biscuits MON810. This material was produced at the JRC (EURL-CT-01/13, Detection and Quantification of GM Events in Biscuit Powder (98140 maize, MON 810 maize, MON 863 maize).

Dry maize derived flour was weighed and mixed with the other ingredients in the proportions indicated below.

Biscuits MON810	200 g wheat flour, 100 g maize flour* (2% GMO), 150 g sugar, 100 g butter, 1 egg

*2% MON810 maize flour was obtained by adding wild-type maize flour to 100% MON810 flour and mixing for 30 minutes.

The ingredients were carefully mixed, spread out evenly on a baking tray and baked in a pre-heated oven at 180°C with recirculating air for 10 min. The material was removed from the oven, covered to avoid contamination and allowed to cool at room temperature. Storage was at 4°C until required.

List of samples distributed during the course and expected results

Table 1. Expected quantitative results. GMO content is expressed in mass fraction (%) for each ingredient (soybean or maize).

Sample	% GMO* (for each specific ingredient)	
	RR soybean	**MON810 maize**
Biscuits #1	0%	0%
Mixed flour	1%	1%
Flour MON810	-	1%
Snack food crumb	2.2%	-
Soya milk powder	8.9%	-
Biscuits MON810	-	2%

*For a more detailed explanation on quantitative detection of GMOs, please see Session 10 and 11.

Table 2. Expected qualitative results. The plus (+) symbol means that the correspondent result was positive, while the minus (-) means that the result was negative.

SAMPLE	***Zein****	***Lectin****	**35S***	***nos****	***E35S/hsp70(b)****	**CTP/EPSPS***
ERM-BF410AK (0%)	-	+	-	-	-	-
ERM-BF410BK (0.1%)	-	+	+	+	-	+
ERM-BF410DK (1%)	-	+	+	+	-	+
ERM-BF410GK 10%)	-	+	+	+	-	+
ERM-BF413AK (0%)	+	-	-	-	-	-
ERM-BF413CK (0.5%)	+	-	+	-	+	-
ERM-BF413EK (2%)	+	-	+	-	+	-
ERM-BF413GK (10%)	+	-	+	-	+	-
Biscuits #1	+	+	-	-	-	-
Mixed flour	+	+	+	+	+	+
Flour MON810	+	-	+	-	+	-
Snack food crumb	-	+	+	+	-	+
Soya milk powder	-	+	+	+	-	+
Biscuits MON810	+	-	+	-	+	-

*A more detailed explanation on qualitative detection of GMOs can be found from Session 7 to 9.

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Session 4

Extraction and Purification of DNA

Introduction

The extraction and purification of nucleic acids is the first step in the detection and quantification of GMOs. The objective of nucleic acid extraction methods is to obtain purified nucleic acids from various sources with the aim of conducting GM specific analysis using the Polymerase Chain Reaction (PCR). The quality and purity of the extracted nucleic acids is one of the most critical factors for PCR analysis.

As a wide variety of methods exists for extraction and purification of nucleic acids, the choice of the most suitable technique is generally based on the following criteria:

- Target nucleic acids
- Organism
- Starting material (e.g. tissue, leaf, seed, processed material)
- Desired results (e.g. yield, purity, purification time required)
- Downstream application (e.g. PCR, cloning, labelling, blotting, real-time PCR, cDNA synthesis)

In recent years, DNA extraction methods have been developed for different matrices shifting from a "same strategy for all matrices" approach to a "matrix centred" approach. In fact, it is very important to know the nature of the sample for successful DNA extraction. For example, it is important to determine whether the sample's origin is from raw or processed material, if it is rich in fat/oil or salt, etc. As an example, extraction from highly processed food is more challenging due to the reduced number of intact DNA copies available for amplification. At the same time if two methods have a similar extraction efficiency for different matrices, then choosing only one helps optimize resources.

The following paragraph describes the principles of the main steps of DNA extraction and purification as well as considerations on inhibition during PCR as a consequence of different extraction procedures.

Inhibition

Inhibitors are a series of substances of different nature that can be present in the sample and that may interfere with the different steps of a PCR analysis. Their presence can be related to the extraction buffers or the intrinsic composition of the samples themselves (Table 1). The presence of inhibitors can lead to false negative results in the PCR. To minimise this effect the most appropriate extraction methods, depending on the matrix to be analysed, shall be chosen. In order to detect the presence of PCR inhibitors in the sample, it is highly recommended to perform a control experiment to test PCR inhibition. For this purpose, a plant-specific (eukaryote or chloroplast) or species-specific PCR analysis is commonly used to perform what is referred to as an "inhibition test" or "inhibition run".

This is described in more detail in session 10, since it is important in real-time PCR when event specific targets are being quantified.

Table 1. Examples of PCR inhibitors reported in the literature and methods to minimize inhibition.

Inhibitor	Description and inhibitory concentration for PCR	Methods to minimize inhibition
TAB	≥ 0.005%; ≥ 0.01%	70% ethanol wash
EDTA	≥ 0.5mM; ≥ 1 mM	Reduce the concentration of EDTA to 0.1 mM in the TE buffer or simply use Tris-HCl (10 mM) to bring DNA in solution. DNA can also be brought in pure water (but the DNA cannot be stored for long term use)
Ethanol	> 1% (v/v)	Dry pellet and resuspend
Fat		Lipase or hexane treatment and chloroform extraction.
Isopropanol	> 1% (v/v)	Dry pellet and resuspend
Phenol	> 2% (v/v); ≥ 0.2%	Incorporation of 1.2% citric acid at the DNA extraction step neutralized the inhibitory effect of chlorogenic acids
Polysaccharides	Acidic polysaccharides such as dextran sulphate are inhibitory. Dextran sulphate: > 0.1%; ≥ 0.001% Pectin: > 0.5% Xylan: > 0.0025%	Use CTAB buffer and chloroform extraction. Treatment with enzymes such as pectinase, cellulase, hemicellulase and α-amylase can be used to remove polysaccharides. High salt precipitation
Protein	1% casein hydrolisate in PCR mixture caused inhibition.	Use SDS, CTAB or guanidinium buffers, proteinase K
SDS	≥ 0.005%	Wash with 70% ethanol

Sodium Acetate	≥ 5 mM	Wash with 70% ethanol
Sodium Chloride	≥ 25 mM	Wash with 70% ethanol or use silica-based purification

CTAB= Cetyltrimethyl ammonium bromide; EDTA= Ethylenediamine tetra acetic acid; SDS= Sodium dodecyl sulfate; Tris-HCl= Tris[hydroxymethyl] aminomethane hydrochloride

Extraction methods

The extraction of nucleic acids from biological material requires the cell lysis, the inactivation of cellular nucleases and denaturation of nucleoproteins, the removal of contaminants, and the DNA precipitation. Often, the ideal lysis procedure is a compromise of techniques and must be rigorous enough to disrupt the complex starting material (e.g. tissue), yet gentle enough to preserve the target nucleic acid. Common lysis procedures include:

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

Cell membrane disruption and inactivation of intracellular nucleases can be combined. For instance, a single solution may contain detergents to solubilise cell membranes and strong chaotropic salts to inactivate intracellular enzymes. After cell lysis and nuclease inactivation, cellular debris can be removed either by filtration and/or precipitation.

Purification methods

Methods for purifying nucleic acids from cell extracts are usually combinations of two or more of the following techniques:

- Extraction/precipitation
- Chromatography
- Centrifugation
- Affinity separation

A brief description of these techniques is provided in the following paragraphs (Zimmermann et al., 1998).

Extraction/Precipitation

Solvent extraction is used to eliminate contaminants from nucleic acids. For example, a combination of phenol and chloroform is frequently used to remove polar molecules such

as proteins. Precipitation with isopropanol or ethanol is used to concentrate nucleic acids. If the amount of target nucleic acid is low, an inert carrier (such as glycogen) can be added to the mixture to increase precipitation efficiency. Other precipitation methods of nucleic acids include selective precipitation using high concentrations of salt ("salting out") or precipitation of proteins using changes in pH.

Chromatography

Chromatography methods may utilise different separation techniques such as gel filtration, ion exchange, selective adsorption, or affinity binding. Gel filtration exploits the molecular sieving properties of porous gel particles. A matrix with 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 chromatography is another technique that utilises an electrostatic interaction between a target molecule and a functional group on the column matrix. Nucleic acids (highly negatively charged, linear polyanions) can be eluted from ion exchange columns with simple salt buffers. In adsorption chromatography, nucleic acids adsorb selectively onto silica or glass in the presence of certain salts (e.g. chaotropic salts), while other biological molecules do not. A low salt buffer or water can then elute the nucleic acids, producing a sample that can be used directly in downstream applications.

Centrifugation

Selective centrifugation is a powerful purification method. Frequently, centrifugation is combined with DNA precipitation or the use of DNA extraction or purification columns which rely on gel filtration 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 purify selectively one type of nucleic acid.

Affinity separation

In recent years, more and more purification methods have combined affinity immobilisation of nucleic acids with magnetic separation using magnetic beads linked to particles that "trap" DNA or RNA based on their chemical properties or charge. For instance, poly(A) + mRNA may be bound to streptavidin-coated magnetic particles by biotin-labelled oligo(dT) and the particle complex removed from the solution (and unbound contaminants) with a magnet. 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 separation step.

CTAB extraction and purification method

The cetyltrimethylammonium bromide (CTAB) protocol, which was first developed by Murray and Thompson in 1980 (Murray and Thompson, 1980), was successively published by Wagner and co-workers in 1987 (Wagner et al., 1987). The method is appropriate for the extraction and purification of DNA from plants and plant derived foodstuffs and is particularly suitable for the elimination of polysaccharides and polyphenolic compounds that otherwise affect DNA purity and therefore quality. This procedure has been widely applied in molecular genetics of plants and already been tested in validation trials in order to detect GMOs (Lipp et al., 1999; 2001). Several additional variants have been developed for a wide range of raw and processed food matrices (Hupfer et al., 1998; Hotzel et al., 1999; Meyer et al., 1997*;* Poms et al., 2001).

Principles of CTAB method: lysis, extraction and precipitation

Plant cells can be lysed with the ionic detergent CTAB, which forms an insoluble complex with nucleic acids in a low-salt environment. Under these conditions, polysaccharides, phenolic compounds and other contaminants remain in the supernatant and can be washed away. The DNA complex is solubilised by raising the salt concentration and precipitated with ethanol or isopropanol. In this section, the principles of these three main steps, lysis of the cell membrane, extraction of the genomic DNA and its precipitation will be described.

Lysis of the cell membrane. As previously mentioned, the first step of the DNA extraction is the rupture of the cell and nucleus wall. For this purpose, the homogenised sample is first treated with the extraction buffer containing EDTA Tris/HCl and CTAB. All biological membranes have a common overall structure comprising lipid and protein molecules held together by non-covalent interactions.

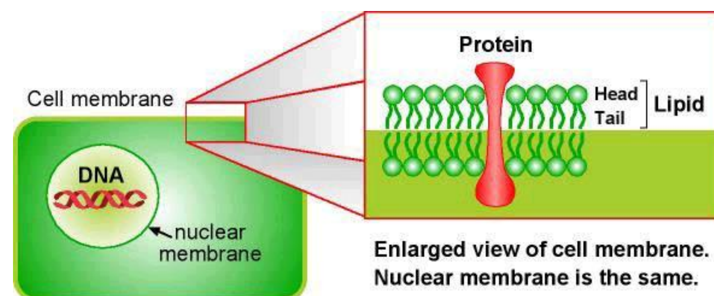

Figure 1. Simplified representation of the cell membranes[31]

As shown in Figure 1, the lipid molecules are arranged as a continuous double layer in which the protein molecules are “dissolved”. The lipid molecules are constituted by

[31] Pictures originally taken from the Genetic Science Learning Center, University of Utah

hydrophilic ends called "heads" and hydrophobic ends called "tails". In the CTAB method the lysis of the membrane is accomplished by the detergent (CTAB) contained in the extraction buffer. Because of the similar composition of both the lipids and the detergent, the CTAB component of the extraction buffer has the function of capturing the lipids constituting the cell and nucleus membrane. The mechanism of solubilisation of the lipids using a detergent is shown in Figure 2.

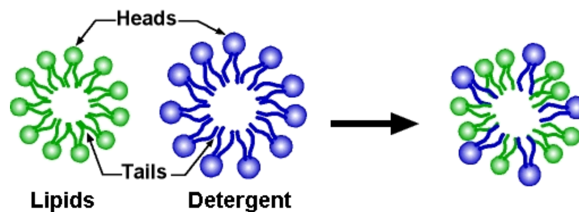

Figure 2. Lipid solubilisation[32]

Figure 3 illustrates how, when the cell membrane is exposed to the CTAB extraction buffer, the detergent captures the lipids and the proteins allowing the release of the genomic DNA. In a specific salt (NaCl) concentration, the detergent forms an insoluble complex with the nucleic acids. EDTA is a chelating component that among other metals binds magnesium (Mg). Magnesium is a cofactor for DNase. By binding Mg with EDTA, the activity of present DNase is decreased. Tris/HCl gives the solution a pH buffering capacity (a low or high pH damages DNA). It is important to notice that, since nucleic acids can easily degrade at this stage of the purification, the time between the homogenisation of the sample and the addition of the CTAB buffer solution should be minimised. After the cell and the organelle membranes (such as those around the mitochondria and chloroplasts) have been broken apart, the purification of DNA can be performed.

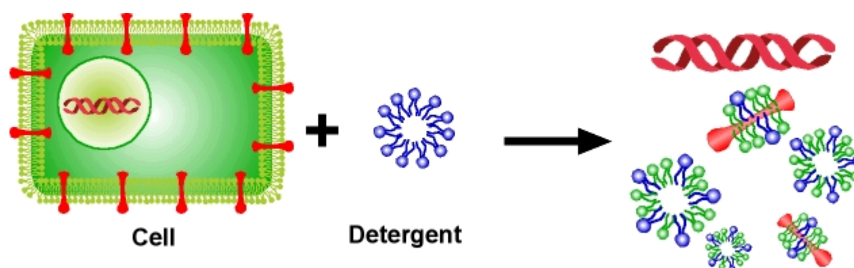

Figure 3. Disruption of the cellular membrane and extraction of genomic DNA[2]

Extraction. In this step, polysaccharides, phenolic compounds, proteins and other cell lysates dissolved in the aqueous solution are separated from the CTAB nucleic acid complex. The elimination of the polysaccharides, as well as phenolic compounds, is

[32] Pictures originally taken from the Genetic Science Learning Center, University of Utah

particularly important because of their ability to inhibit a great number of enzymatic reactions. Under low salt concentration (< 0.5 M NaCl), the contaminants of the nucleic acid complex do not precipitate and can be removed by extraction of the aqueous solution with chloroform. The chloroform denatures the proteins and facilitates the separation of the aqueous and organic phases. Normally, the aqueous phase forms the upper phase. However, if the aqueous phase is dense because of salt concentration (> 0.5 M), it will form the lower phase. In addition, the nucleic acid will tend to 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 extraction with chloroform is performed two or three times in order to remove completely the impurities from the aqueous layer. To achieve the best recovery of nucleic acid, the organic phase may be back-extracted with an aqueous solution that is further added to the prior extract. Once the nucleic acid complex has been purified, the last step of the procedure, i.e. precipitation, can be accomplished.

Precipitation. In this final stage, the nucleic acid is co-precipitated with salt. DNA precipitation is performed by using high salt concentrations of sodium chloride or sodium acetate, and alcohol, either ethanol or isopropanol. The purpose of DNA precipitation is to remove the detergent and other chemicals used during the extraction as well as to concentrate the DNA. Following this, the precipitate is treated or washed with 70% ethanol to remove excess salt from the DNA. Following DNA precipitation and washing, the precipitated DNA is dissolved in a solvent such as a diluted concentration of tris-EDTA (TE) buffer or sterile molecular biology grade water.

Commercially available kits

Nowadays, extraction and purification kits are commercially available in many different variants. As previously stated, a kit does not necessarily apply to every kind of matrix but more than one kit can be suitable for the same sample. Kit extraction methods can also be combined with different extraction protocols to aid in column purification of the extracted DNA.

References about the suitability of different extraction methods for different matrixes are available at the end of this chapter.

Quality of extracted DNA

Once extraction and purification procedures have been completed, the quality of DNA needs to be assessed. Some examples of DNA quantification methods are:

- UV Spectrophotometry
- Fluorometry
- Agarose Gel Electrophoresis (which will be discussed in session 5)

Each of these methods has advantages and disadvantages, so it is important to evaluate which one is fitter for the purpose, in terms of the resources available in the laboratory.

It is important to assess the quality and purity of the DNA, both for detection and quantification because, if the DNA is highly damaged or inhibited, the analysis could result in a false negative or in an underestimation of GMO content, respectively.

Quantification of DNA by spectrophotometry

DNA, RNA, oligonucleotides and even mononucleotides can be measured directly in aqueous solutions in a diluted or undiluted form by measuring the absorption *A* (also defined as optical density, *OD*) in ultraviolet light (but also in the visible range). If the sample is pure (i.e. without significant amounts of contaminants such as proteins, phenol or agarose), the spectrophotometric measurement of the amount of ultraviolet irradiation absorbed by the bases is simple and reasonably accurate. For this method, aqueous buffers with low ion concentrations (e.g. TE buffer) are ideal. The concentration of nucleic acids is usually determined by measuring at 260 nm against a blank. Interference due to contaminants can be recognised by the calculation of a "ratio". Since proteins absorb at 280 nm, the ratio A_{260}/A_{280} is used to estimate the purity of nucleic acid. Pure DNA should have a ratio of approximately 1.8, whereas pure RNA should give a value of approximately 2.0. Absorption at 230 nm reflects contamination of the sample by substances such as carbohydrates, peptides, phenols or aromatic compounds. In the case of pure samples, the ratio A_{260}/A_{230} should be approximately 2.2. It is important to consider that when using the spectrophotometric approach the chemicals used in the extraction of the DNA can influence the concentration determination as well as the A_{260}/A_{280} ratio.

Principles of spectrophotometric determination of DNA

A spectrophotometer makes use of the transmission of light through a solution to determine the concentration of a solute within the solution. The apparatus operates on the basis of a simple principle in which light of a known wavelength passes through a sample and the amount of light energy transmitted is measured with a photocell on the other side of the sample.

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 the illumination intensity, the 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 later investigation.

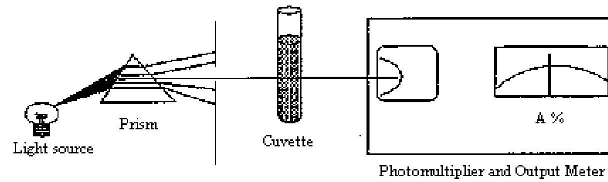

Figure 4. Schematic light transmission

All molecules absorb radiant energy at a specific wavelength, from which it is possible to extrapolate the concentration of a solute within a solution. According to the Beer-Lambert law there is a linear relationship between the absorbance *A* and the concentration of the macromolecule given by the following equation:

$$A = OD = \varepsilon l c \qquad (1)$$

Where ε is the molar extinction coefficient, *c* is the concentration; and *l* is the path length of the cuvette.

Proteins and nucleic acids absorb light in the ultraviolet range within wavelengths of between 210 and 300 nm. As previously explained, the maximum absorbance of DNA and RNA solutions is at 260 nm whereas the maximum absorbance of protein solutions is at 280 nm. Since, both DNA and RNA solutions do partially absorb light at 280 nm, and protein solutions partially absorb light at 260 nm, the ratio between the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of the nucleic acids. Pure preparations of DNA and RNA have A_{260}/A_{280} values of 1.8 and 2.0 respectively. 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. If there is contamination with protein, the A_{260}/A_{280} will be significantly less than the values given above and accurate quantification of the amount of nucleic acid will not be possible. It is important to mention that impurities in DNA solutions caused by RNA cannot be confidently identified by spectrophotometry. An absorbance of 325 nm can be used to indicate the presence of debris in the solution or that the cuvette itself is dirty.

Determination of the concentration of nucleic acids

Choice of the cuvette. The amount of nucleic acid solution used for the measurement of the absorbance *A*, depends on the capacity of the cuvette. A suitable cuvette should be chosen depending on sample concentration range, dilution factor and available sample volume. In most of the procedures used for the detection of GMO the volume of genomic DNA collected is between 50 and 100 µl. Several types of microvolume cuvettes with a capacity of 5 to 70 µl are utilised for the spectroscopic quantification of small volumes of nucleic acids.

Set up. In order to calibrate the spectrophotometer, it is important:

- to set the correct cell path length
- to set the correct factor (select between dsDNA, ssDNA, RNA)
- to measure a blank solution (set reference) (A_{260} = 0) containing the same solvent used to dissolve the DNA in after precipitation
- to measure the OD of the sample

When determining the concentration of multiple samples, it is advisable to set the blank periodically in between batches of sample. It is also useful to use a known amount of pure nucleic acid in order to check the reliability of the spectrophotometer.

Measurement of an unknown sample. Depending on the capacity of the cuvette used, specific amounts of DNA solution are used for the concentration evaluation (e.g. for cuvette of capacity lower than 0.2 ml, 5 µl of DNA is diluted in 195 µl of water or ideally, the same solvent used to dissolve the DNA). After calibrating the spectrophotometer and the addition of the nucleic acid solution, the cuvette is capped, the solution mixed, and the absorbance measured. In order to reduce pipetting errors, the measurement should be repeated at least twice and at least 5 µl of the DNA solution should always be used. A_{260} readings lower than 0.02 or between 1 and 1.5 (depending on the instrument used) are not recommended to be considered reliable because there might be 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:* $c(pmol/\mu l) = A_{260}/0.020$
- *Single-stranded RNA:* $c(pmol/\mu l) = A_{260}/0.025$
- *Oligonucleotide:* $c(pmol/\mu l) = A_{260}100/1.5N_A+0.71N_C+1.20N_G + 0.84N_T$

where A_{260} is the absorbance measured at 260 nm.

An example of absorbance readings of highly purified *calf thymus* DNA suspended in 1x TNE buffer assuming that the reference DNA is dsDNA with A_{260} = 1 for 50 μg/ml in a 10 mm path length cuvette is shown in Table 2. The concentration of DNA was nominally 25 μg/ml.

Table 2. Absorbance reading of highly purified *calf thymus* DNA in 1x TNE buffer

Wavelength	Absorbance	A_{260}/A_{280}	Conc. (μg/ml)
325	0.01	-	-
280	0.28	-	-
260	0.56	2.0	28
230	0.30	-	-

This method is still in use and, in the latest years, it has been improved and refined. In fact, the use of traditional spectrophotometers requires a bigger amount of DNA compared to the most recent developments of this method requiring only 1 µl of undiluted DNA solution for quantification, this allows a quicker analyses and minor "waste" of useful DNA.

A disadvantage of this technique is the fact that the signal is more susceptible to contaminants. In other words, the signal of the contaminant can interfere with the quantification. Therefore, fluorometry has been developed to overcome this problem.

Quantification of DNA by fluorometry

Fluorometry applies the same technology as spectrophotometry but with an extra feature: the addition of a fluorescent dye that binds to double stranded DNA. The fluorometric method is considered more accurate than the spectrophotometric method. A standard concentration of DNA (usually supplied with the reagent kit) is used to determine the concentration of double stranded DNA in the sample (more information on this procedure at http://www.nanodrop.com/Library/PicoGreen%20-%20dsDNA%20protocol.pdf).

Experimental

Equipment

REMARK
All of the equipment must be sterilised prior to use and any residue of DNA must be removed. In order to avoid contamination, barrier pipette tips that are protected against aerosol should be used.

- Instruments for size reduction like a sterile surgical blade or a mortar
- Water bath or heating block
- Microcentrifuge
- Micropipettes
- Vortex mixer
- 1.5 ml microcentrifuge tubes
- Weight boats or equivalents
- Spatulas
- Balance capable of 0.01 g measurement
- Loops
- Rack for microcentrifuge tubes
- Optional: vacuum desiccator to dry DNA pellets
- Powder free nitrile or latex gloves

Plastic ware has to be sterile and free of DNases, RNases and nucleic acids. Filter pipette tips protected against aerosol should be used.

Reagents

REMARK
All chemicals should be of molecular biology grade. Deionised water and buffers should be autoclaved prior to use. In addition, all chemicals should be DNA and DNase free.

- Cetyltrimethylammonium bromide (CTAB) CAS 56-09-0
- Chloroform CAS 67-66-3
- Isopropanol CAS 67-63-0
- Na_2EDTA CAS 139-33-3
- Ethanol CAS 64-17-5
- NaCl CAS 7647-14-5
- Proteinase K CAS 39450-01-6

- RNase A CAS 9001-99-4
- Tris-HCl CAS 1185-53-1
- Sterile deionised water
- Liquid nitrogen

2 % CTAB-buffer 200 mL

20 g/l CTAB	4 g
1.4 M NaCl	16.4 g
0.1 M Tris-HCl	3.15 g
20 mM Na_2EDTA	1.5 g

Alternatively 20 ml of a 1 M ready-to-use solution of Tris-HCl pH 8.0 can be used:

- add 100 ml of deionised water
- adjust pH to a value of 8.0 with 1M NaOH
- fill up to 200 ml and sterilize
- store buffer at room temperature for maximum 12 months

0.5 % CTAB-precipitation solution

5 g/l CTAB	1 g
0.04 M NaCl	0.5 g

- Add 100 ml of deionised water
- Adjust pH to a value of 8.0 with 1 M NaOH
- Fill up to 200 ml and filter sterilize
- Store solution at 4°C for maximum 6 months

NaCl 1.2 M

- Dissolve 7.01 g of NaCl in 100 ml deionised water
- Autoclave and store at room temperature

Ethanol-solution 70 % (v/v)

70 ml of pure ethanol are mixed with 30 ml of sterile deionised water.

NaOH 1M

- Dissolve 2 g of NaOH in 50 ml of sterile water.
- NaOH 0.1M
- Dilute 10 ml of NaOH 1M in 90 ml of sterile water

***RNase A* 10 mg/ml (store at –20°C)**

- Dissolve the RNase A at a final concentration of 10 mg/ml in sterile water.
- If required from RNase A preparation supplier: boil the RNase A solution at 95°C for 15 min in order to remove any residual nuclease activity.

***Proteinase K* 20 mg/ml (store at –20°C)**

Dissolve the Proteinase K at a final concentration of 20 mg/ml in sterile distilled water according to the supplier's specifications.

Ready-to-use 1 x Tris-EDTA buffer solution pH 8.0

A ready-to-use 1 x TE solution BioUltra, for molecular biology, pH 8.0

0.2 x TE buffer

TE 0.2 x buffer is obtained through five-fold dilution of the 1 x TE buffer.

Procedure

The procedure should be run under sterile conditions. Contamination can be avoided during sample preparation by using single-use equipment and decontamination solutions.

1. Transfer 100 mg of a homogeneous sample into a sterile 1.5 ml microcentrifuge tube
2. Add 300 µl of sterile deionised water, mix with a loop or vortex
3. Add 500 µl of CTAB-buffer, mix with a loop or vortex

4. Add 20 μl Proteinase K (20 mg/ml), mix and incubate at 65°C for 30-60 min [*]
5. Add 20 μl RNase A (10 mg/ml), mix and incubate at 65°C for 5-10 min [*]
6. Centrifuge for 10 min at about 13,000 *rpm*
7. Transfer the supernatant to a microcentrifuge tube containing 500 μl chloroform, mix completely by inverting the tubes several times for 30 sec
8. Centrifuge for 10 min at 13,000 *rpm* until phase separation occurs
9. Transfer 500 μl of upper layer into a new microcentrifuge tube containing 500 μl chloroform, mix completely by inverting the tubes several times for 30 sec
10. Centrifuge for 5 min at 13,000 *rpm*
11. Transfer upper layer into a new tube
12. Add 2 volumes of CTAB precipitation solution, mix completely by pipetting
13. Incubate for 60 min at room temperature
14. Centrifuge for 15 min at 13,000 *rpm*
15. Discard supernatant
16. Dissolve precipitate in 350 μl NaCl (1.2 M)
17. Add 350 μl chloroform and mix completely by inverting the tubes several times for 30 sec
18. Centrifuge for 10 min at 13,000 *rpm* until phase separation occurs
19. Transfer the upper layer to a new microcentrifuge tube
20. Add 0.6 volumes of isopropanol and mix gently and completely by inverting the tube
21. Centrifuge for 10 min at 13,000 *rpm*
22. Discard the supernatant
23. Add 500 μl of 70% ethanol solution, mix gently and completely by inverting the tube
24. Centrifuge for 10 min at 13,000 *rpm*
25. Discard the supernatant
26. Dry pellets and re-dissolve DNA in 100 μL 0.2xTE buffer.

The DNA solutions may be stored in a refrigerator for a maximum of two weeks, or in the freezer at - 20°C for longer periods.

It is good practice to store a certain amount of backup material in case something goes wrong during the subsequent steps of the analysis and the extraction needs to be repeated.

[*] These additional optional steps are commonly introduced to the CTAB extraction method to enhance the yield of genomic DNA from highly complex matrixes.

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Session 5

Agarose Gel Electrophoresis

Introduction

Gel electrophoresis is a method that allow separation of macromolecules on the basis of their size, electric charge and other physical properties. The term electrophoresis describes the migration of charged particles under the influence of an electric field. "Electro" refers to electricity and "Phoresis", from the Greek word *phoros*, meaning, "to carry across." Thus, gel electrophoresis refers to a technique in which molecules are forced across a span of a gel matrix, under an electrical current. The driving force for electrophoresis is the voltage applied to electrodes at either end of the gel. The ionic properties and size of a molecule determine how rapidly it will move through a gelatinous medium under an electric field. Many important biological macromolecules (e.g. amino acids, peptides, proteins, nucleotides and nucleic acids) possess ionisable groups and, at any given pH, exist in solution as electrically charged species either as cations (+) or anions (-). Depending on the nature of the net charge, the charged particles will migrate either to the cathode or to the anode. For example, when an electric field is applied across a gel at neutral pH, the negatively charged phosphate groups of the DNA cause it to migrate toward the anode (Westermeier, 1997).

Agarose gel electrophoresis is a standard method used to separate, identify and purify DNA fragments. The technique is simple, rapid to perform, and capable of resolving fragments of DNA that cannot be separated adequately by other procedures. Furthermore, the location of DNA within the gel can be determined by staining with a low concentration of fluorescent DNA intercalating dye such as ethidium bromide or other less harmful commercial DNA stain.

The following sections will outline the physical principles, components (gel matrix, buffer, loading buffer and marker) and procedures for the preparation of agarose gel electrophoresis (Sambrook et al., 1989).

Physical principles of agarose gel electrophoresis

Gel electrophoresis is a technique used for the separation of nucleic acids and proteins. Separation of macromolecules depends upon two variables: charge and mass. When a biological sample, such as DNA, is mixed in a buffer solution and applied to a gel matrix, these two variables act together. The electrical current from one electrode repels the molecules while the other electrode simultaneously attracts the molecules. The frictional force of the gel material acts as a "molecular sieve", separating the molecules by size. During electrophoresis, macromolecules are forced to move through the pores and their rate of migration through the electric field depends on the following:

- The strength of the field
- The size and shape of the molecules
- The relative hydrophobicity of the samples
- The ionic strength and temperature of the buffer in which the molecules are moving
- The concentration of the gel matrix that determines the pore size

To understand completely the separation of charged particles in gel electrophoresis, it is important to look at the simple equations relating to electrophoresis. When a voltage is applied across the electrodes, a potential gradient, E, is generated and can be expressed by the equation:

$$E = V/d \quad (1)$$

where V, measured in volts, is the applied voltage and d the distance in cm between the electrodes.

When the potential gradient, E, is applied, a force, F, on a charged molecule is generated and is expressed by the equation:

$$F = Eq \quad (2)$$

where q is the charge in coulombs bearing on the molecule. This force, measured in Newtons drives a charged molecule towards an electrode.

There is also an additional force, the frictional resistance, that slows down the movement of charged molecules. This frictional force is a function of:

- the hydrodynamic size of the molecule
- the shape of the molecule
- the pore size of the medium in which electrophoresis is taking place
- the viscosity of the buffer

The velocity v of a charged molecule in an electric field is a function of the potential gradient, charge and frictional force of the molecule and can be expressed by the equation:

$$v = Eq / f \quad (3)$$

where f is the frictional coefficient.

The electrophoretic mobility, M, of an ion can then be defined by the ion's velocity divided by the potential gradient:

$$M = v / E \quad (4)$$

In addition, from equation (3) one can see that electrophoretic mobility M can be equivalently expressed as the charge of the molecule, q, divided by the frictional coefficient, f:

$$M = q / f \quad (5)$$

When a potential difference is applied, molecules with different overall charges will begin to separate due to their different electrophoretic mobilities. The electrophoretic mobility is a significant and characteristic parameter of a charged molecule or particle and depends on the pK value of the charged group and the size of the molecule or particle. Even molecules with similar charges will begin to separate if they have different molecular sizes, since they will experience different frictional forces. Linear double stranded DNA migrates through gel matrices at rates that are inversely proportional to the $\log_{10}$ of the number of base pairs. Larger molecules migrate more slowly because of the greater frictional drag and because of the less efficient movement through the pores of the gel.

The current in the solution between the electrodes is conducted mainly by the buffer ions with a small proportion being conducted by the sample ions. The relationship between current I, voltage V, and resistance R is expressed as in Ohm's law:

$$R = V / I \quad (6)$$

This equation demonstrates that for a given resistance R, it is possible to accelerate an electrophoretic separation by increasing the applied voltage V, which would result in a corresponding increase in the current flow I. The distance migrated will be proportional to both current and time. However, the increase in voltage, V, and the corresponding increase in current, I, would cause one of the major problems for most forms of electrophoresis, namely the generation of heat. This can be illustrated by the following equation in which the power, W, (measured in Watts) generated during the electrophoresis is equal to the product of the resistance times the square of the current:

$$W = I^2R \quad (7)$$

Since most of the power produced in the electrophoretic process is dissipated as heat the following detrimental effects can result:

- An increased rate of diffusion of sample and buffer ions leading to broadening of the separated samples
- The formation of convection currents, which leads to mixing of separated samples;
- Thermal instability of samples that are rather sensitive to heat (e.g. denaturation of DNA)
- A decrease of buffer viscosity hence a reduction in the resistance of the medium

Components of agarose gel electrophoresis

Agarose

Agarose, a natural colloid extracted from seaweed, is a linear polysaccharide (average molecular mass ~12,000 Da) made up of the basic repeated unit agarobiose, which comprises alternating units of galactose and 3, 6-anhydrogalactose. Agarose is very fragile and easily destroyed by improper handling. Agarose gels have large "pore" sizes and they are used primarily to separate large molecules with a molecular mass greater than 200 kDa.

Agarose gels can be processed reasonably quickly, but they have limited fragment resolution and DNA/fragment bands formed tend to be fuzzy/diffuse. This is a result of pore size and it cannot be controlled. Agarose gels are obtained by suspending the dry powdered agarose in an aqueous buffer, and by boiling the mixture until the agarose melts into a clear solution. Furthemore, the solution is poured into a gel-tray containing a comb that is used to mould wells in the gel into which the DNA sample will the loaded. The gel is cooled down at room temperature to become rigid. Upon hardening, the agarose forms a matrix whose density is determined by its concentration, the comb can be removed and the form wells are used to load the DNA samples.

Electrophoresis buffer

The electrophoretic mobility of DNA is affected by the composition and ionic strength of the electrophoresis buffer. In the absence of ions, electrical conductance is minimal and DNA migrates slowly, if at all. In a buffer of high ionic strength, electrical conductance is very efficient; however, conductance might result in the generation of heat. In extreme cases of heat generation, if the voltage applied is too high for too long, the gel will melt and the heat may damage the electrophoretic apparatus.

Several buffers are available for electrophoresis of native double-stranded DNA. These contain EDTA (pH 8.0) and Tris-acetate (TAE), Tris-borate (TBE), or Tris-phosphate (TPE) at a concentration of approximately 50 mM (pH 7.5 - 7.8). Electrophoresis buffers are usually prepared as concentrated solutions and stored at room temperature. TBE was originally used at a working strength of 1 x for agarose gel electrophoresis. However, a working solution of 0.5 x provides more than enough buffering power and almost all agarose gel electrophoresis is now carried out using this buffer concentration.

Agarose concentration

A DNA fragment of a given size migrates at different rates through a gel depending on the concentration of agarose. For a specific concentration of agarose and/or buffer, it is possible to separate DNA segments containing between 20 and 50,000 bp. In horizontal gels, agarose is usually used at concentrations between 0.7% and 3% (see Table 1).

Table 1. Recommended agarose gel concentration for resolving linear DNA molecules

% agarose	DNA size range (bp)
0.75	10.000 - 15.000
1.0	500 - 10.000
1.25	300 - 5000
1.5	200 - 4000
2.0	100 - 2500
2.5	50 - 1000

Marker DNA

For a given voltage, agarose gel and buffer concentrations, the migration distance depends on the molecular weight of the starting material. A marker generally contains a defined number of DNA fragments of known size that makes it easier to determine the size of the unknown DNA. Marker DNA should be loaded into wells on both the right and left sides of the gel to be able to determine if any systematic distortion of DNA migration through the gel occurred during the electrophoresis.

Loading buffer

The DNA samples to be loaded onto the agarose gel are first mixed with a loading buffer usually comprising water, sucrose or glycerol, and a dye (e.g. xylene cyanole, bromophenol blue, bromocresol green,). The minimum amount of DNA that can be detected by photography of ethidium bromide stained gels is approximately 2 ng in a 0.5 cm wide well band. If there is more than 500 ng of DNA in a well band of this width, the well will be overloaded, resulting in smearing. The loading buffer serves three purposes:

- Increases the density of the sample ensuring that the DNA drops evenly into the well
- Adds colour to the sample, thereby simplifying the loading process
- Imparts a dye to the sample that, in an electric field, moves toward the anode at a predictable rate

Experimental

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

Equipment

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

Reagents

- TBE Tris/Boric acid/EDTA buffer (10x)
- Deionised water
- Agarose, suitable for DNA electrophoresis
- Tris[hydroxymethyl] aminomethane (Tris) CAS 77-86-1
- Boric acid CAS 10043-35-3
- Na_2EDTA CAS 139-33-3
- Ethidium bromide CAS 1239-45-8
- Sucrose CAS 57-50-1
- Xylene cyanole FF CAS 2650-17-1
- DNA markers:
  - Lambda DNA EcoRI/HindIII digested (or other similar suitable marker)
  - 100 bp DNA ladder

10 x TBE buffer (1 litre)

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

Tris	54.0 g
Boric acid	27.5 g
Na_2EDTA	7.44 g

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

6 x loading buffer (10 ml)

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

Xylene cyanole FF	0.025 g
Sucrose	4 g

- Add sucrose and xylene cyanole FF to deionised water to obtain 10 ml of solution (the sucrose can be substituted with 20% (v/v) glycerol).
- Mix the solution, autoclave, and store at 4°C.

Procedure

- Seal the edges of a clean, dry plastic gel-tray either with tape or by other means. Position the appropriate comb so that complete wells are formed when the agarose solution is added.
- Dilute 10x TBE buffer to prepare the appropriate amount of 0.5x TBE buffer to fill the electrophoresis tank and to prepare the gel.
- Weigh powdered agarose depending on the dimensions of the amplicon (according to Table 1) and add it to an appropriate amount of 0.5x TBE buffer in an Erlenmeyer flask with a loose-fitting cap (usually 150 ml gel solution for a 15 x 15 cm gel-tray and 100 ml gel for a 15 x 10 cm gel-tray).
- Heat the slurry in a microwave oven or in a boiling water bath until the agarose dissolves (check the volume of the solution after heating and adjust with distilled water to compensate for evaporation).
- Cool the mixture down to 50 - 60°C and add ethidium bromide (from a stock solution of 10 mg/ml) to a final concentration of 0.2 µg/ml and mix thoroughly. If waste disposal is a challenge, the gel can be stained post electrophoresis in an ethidium bromide solution.

- Pour the solution into the gel-tray and allow the gel to set. The amount of gel used should correspond to a depth of approximately 3 - 5 mm.
- After the gel is completely set, carefully remove the comb and the tape and place the gel in the electrophoresis tank.
- Add enough 0.5 x TBE buffer to the electrophoresis unit to cover the gel to a depth of about 2 - 5 mm.
-

Prepare samples and marker for genomic DNA as follows:

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

Prepare samples and marker for PCR products as follows:

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

- Load 10 µl of each sample (including positive and negative controls) into consecutive wells and the appropriate DNA marker into the first and last lane.
- Close the lid of the gel tank and attach the electrical leads so that the DNA will migrate toward the anode and apply a voltage of 90-100 V/15 cm.
- Run the gel until the xylene cyanole has migrated the appropriate distance through the gel (~ 40 - 60 minutes).
- Turn off the current; remove the leads and the lid from the gel tank. Place the gel on a UV lightbox and photograph the gel. Gloves must be worn when handling the gel containing ethidium bromide and tools/surfaces that came in contact.
- Discard the gel into the solid waste bin and the electrophoresis buffer containing ethidium bromide, into the liquid waste container.

References

Sambrook J, Fritsch EF and Maniatis T, (1989). Gel electrophoresis of DNA. In: Sambrook J, Fritsch EF and Maniatis T (Eds.), Molecular Cloning: a Laboratory Manual. New York: Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, Chapter 6.

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Session 6

The Polymerase Chain Reaction (PCR)

Introduction

The invention of Polymerase Chain Reaction (PCR) by Mullis and co-workers in 1985 has revolutionised the molecular biology and molecular diagnostics (Saiki et al., 1985). The PCR is an *in vitro* technique used to enzymatically amplify a specific DNA region of a known DNA sequence. Whereas previously only minute amounts of a specific target DNA could be obtained, with the introduction of the PCR a single DNA target can be amplified to a million copies within a few hours.

PCR techniques have become essential for many common procedures such as cloning specific DNA fragments, detecting and identifying genes in diagnostics and forensics, and in the investigation of gene expression patterns. PCR has allowed the investigation of new fields such as the control of the authenticity of foodstuff, the presence of genetically modified DNA and microbiological contamination. In understanding the principles of PCR and its applications, the nature of the DNA molecule must first be considered, therefore the structure and the replication of DNA will be described in the following section.

Components, structure and replication of DNA

Components. A molecule of DNA is constituted of two parallel complementary twisted chains of alternating units of phosphoric acid and deoxyribose, linked by cross-pieces of purine and pyrimidine bases, resulting in a right-handed helical structure that carries genetic information encoded in the sequence of the bases. In eukaryotic cells, most of the DNA is contained within the nucleus and is referred to as chromosomal DNA. The nucleus is separated from the rest of the cell (cytoplasm) by a double layer membrane (nuclear envelope). In addition to chromosomal DNA, DNA can be found in the mitochondria and chloroplasts (extrachromosomal DNA).

The building blocks of DNA, called nucleotides, are:

- dATP, deoxyadenosine triphosphate
- dGTP, deoxyguanosine triphosphate
- dTTP, deoxythymidine triphosphate
- dCTP, deoxycytidine triphosphate

For convenience, these four nucleotides are called dNTPs (deoxynucleoside triphosphates). A nucleotide comprises three major parts: a purine base (adenine, A, and/or guanine, G), or a pyrimidine base (cytosine, C, and/or thymine, T), a pentose sugar molecule (deoxyribose) and a triphosphate group. As shown in Figure 1, a purine or pyrimidine base is bound to a pentose ring by a N-glycosidic bond and a phosphate group is bound to the 5’ carbon atom of the sugar by a diesteric bond. In the ribonucleic acid, RNA, thymine is substituted by uracil (U) and the deoxyribose molecule is replaced by ribose.

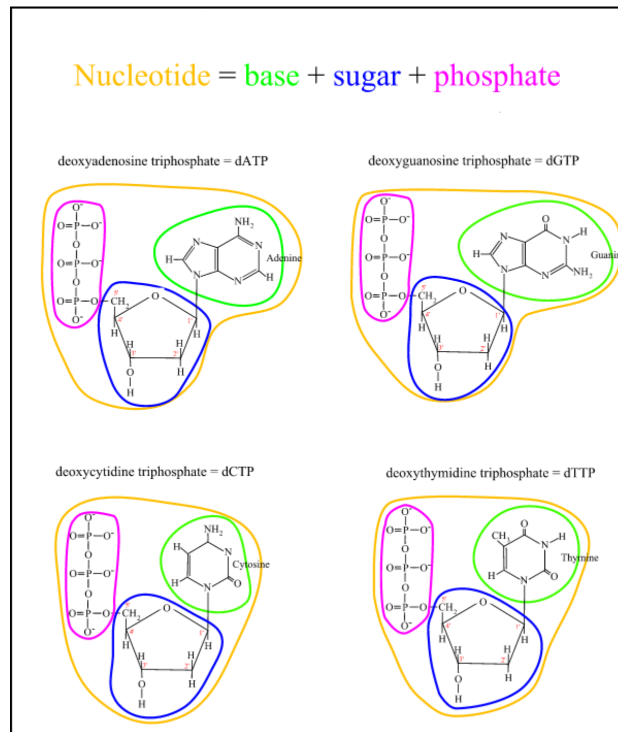

Figure 1. The components of nucleotides (source: Vierstraete, 1999)

Structure. Figure 2 shows how the nucleotides form a DNA chain. DNA is formed by coupling the nucleotides between the phosphate group from a nucleotide (which is positioned on the fifth C-atom of the sugar molecule) with the hydroxyl on the third C-atom on the sugar molecule of the previous nucleotide. To accomplish this, a diphosphate group is split off (with the release of energy). This means that new nucleotides are always added on the 3' side of the chain. As shown in Figure 3, DNA is double-stranded (except in some viruses), and the two strands pair each other in a very precise way. Each base in a strand will pair with only one kind of base from the opposing strand forming a base pair (bp): A is always paired to T by two hydrogen bonds; and C is always paired to G by three hydrogen bonds. In this way, the two chains are complementary to each other and one chain can serve as a template for the production of the other.

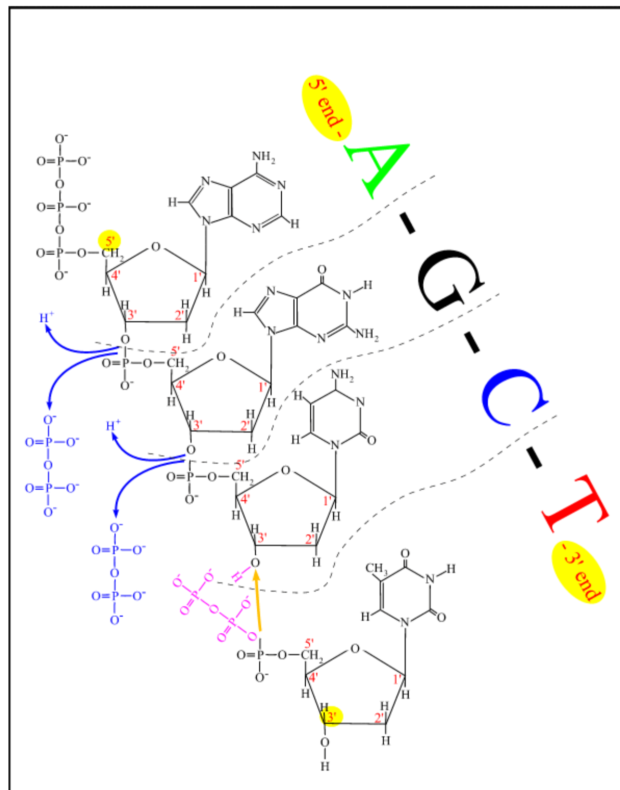

Figure 2. Formation of a DNA chain from individual nucleotides (source: Vierstraete, 1999)

The bases form a hydrophobic nucleus inside the double helix. The sugars and phosphate groups (in their anionic form) constitute the external hydrophilic layer of the molecule. Under *in vivo* and *in vitro* conditions, double-stranded DNA is more stable than a single-stranded DNA.

Replication. DNA contains the complete genetic information that defines the structure and function of an organism. Three different processes are responsible for the transmission of genetic information:

- Replication
- Transcription
- Translation

During replication, a double-stranded nucleic acid is duplicated to give an identical copy. This process propagates the genetic information during mitosis. During transcription, a DNA segment that constitutes a gene is read and transcribed into a single-stranded sequence of RNA. The RNA moves from the nucleus into the cytoplasm. Finally, during

translation, the RNA sequence is translated into a sequence of amino acids as the protein is formed (Alberts et al., 1983).

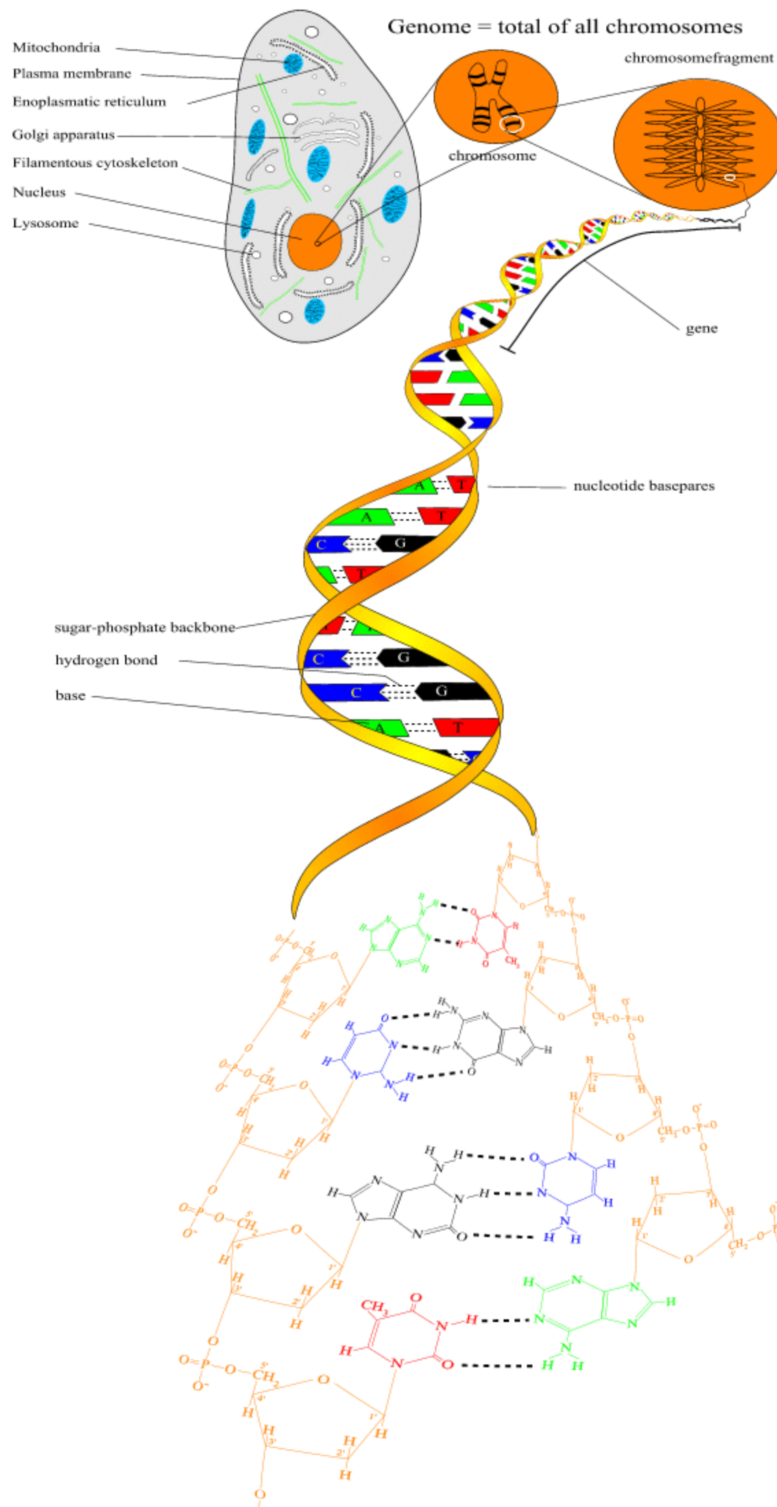

Figure 3. Structure of DNA in a cell (source: Vierstraete, 1999).

The replication of DNA is the process on which the PCR amplification is based, and will be described in detail.

During replication, the DNA molecule unwinds, with each single strand becoming a template for synthesis of a new, complementary strand. Each daughter molecule, consisting of one old and one new DNA strand, is the exact copy of the parent molecule.

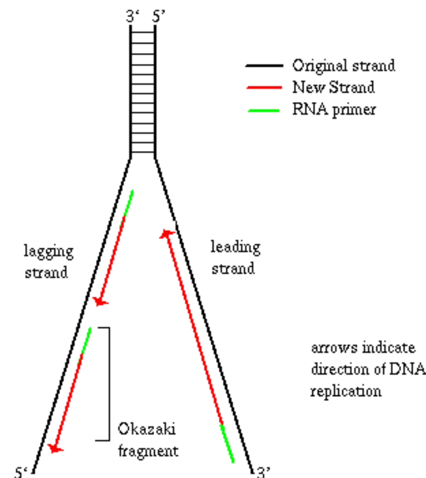

Figure 4. The replication fork (source: Vierstraete, 1999)

Several enzymes are required to unwind the double helix and to synthesise a new strand of DNA. Topoisomerase and helicase are responsible for the unwinding of the DNA by breaking the supercoiled structure and nicking a single strand of DNA. Then, primase (part of an aggregate of proteins called the primeosome) attaches a small RNA primer to the single-stranded DNA, to act as a 3-'OH end from which the DNA polymerase begins synthesis. This RNA primer is eventually removed by RNase H and the gap is filled in by DNA polymerase I. At this stage, DNA polymerase proceeds along a single-stranded molecule of DNA, recruiting free dNTPs to hydrogen bond with their appropriate complementary dNTP on the single strand (A with T and G with C), forming a covalent phosphodiester bond with the previous nucleotide of the same strand. The energy stored in the triphosphate is used to covalently bind each new nucleotide to the growing second strand. There are different forms of DNA polymerase and the DNA polymerase III is the one responsible for the progressive synthesis of new DNA strands. DNA polymerase only acts from 5' to 3'. Since one strand of the double helix is 5' to 3' and the other one is 3' to 5', DNA polymerase synthesises a second copy of the 5' to 3' strand (the lagging strand), in spurts (Okazaki fragments) (Ogawa and Okazaki, 1980). The synthesis of the new copies of the 5' to 3' strand is shown in Figure 4. The other strand, the leading strand, can proceed with synthesis directly, from 5' to 3', as the helix unwinds. DNA polymerase cannot start synthesising *ex novo* on a naked single strand but needs a primer with a free 3'-OH group onto which it can attach a dNTP.

Ligase catalyses the formation of a phosphodiester bond given an unattached but adjacent 3'-OH and 5'-phosphate. This can fill in the unattached gap left when the RNA primer is removed and filled in. It is worth noting that single-stranded binding proteins are important to maintain the stability of the replication fork. Single-stranded DNA is very labile, or unstable, so these proteins bind to it while it remains single-stranded, protecting it from degradation.

Principles of PCR

PCR is based on the mechanism of DNA replication *in vivo*: dsDNA is unwound to ssDNA, duplicated, and rewound. This technique consists of repetitive cycles of:

- Denaturation of the DNA through melting at elevated temperature to convert double-stranded DNA to single-stranded DNA
- Annealing (hybridisation) of two oligonucleotides used as primers to the target DNA
- Extension of the DNA chain by nucleotide addition from the primers using DNA polymerase as catalyst in the presence of magnesium (Mg^{2+}) ions.

The oligonucleotides typically consist of relatively short sequences that are complementary to recognition sites flanking the segment of target DNA to be amplified. The steps of template denaturation, primer annealing and primer extension corresponds to a single "cycle" in the PCR amplification methodology. The primer annealing and extension steps can be combined into one with only two temperature steps (annealing/extension and denaturation). Figure 5 illustrates the three major steps in a PCR amplification process.

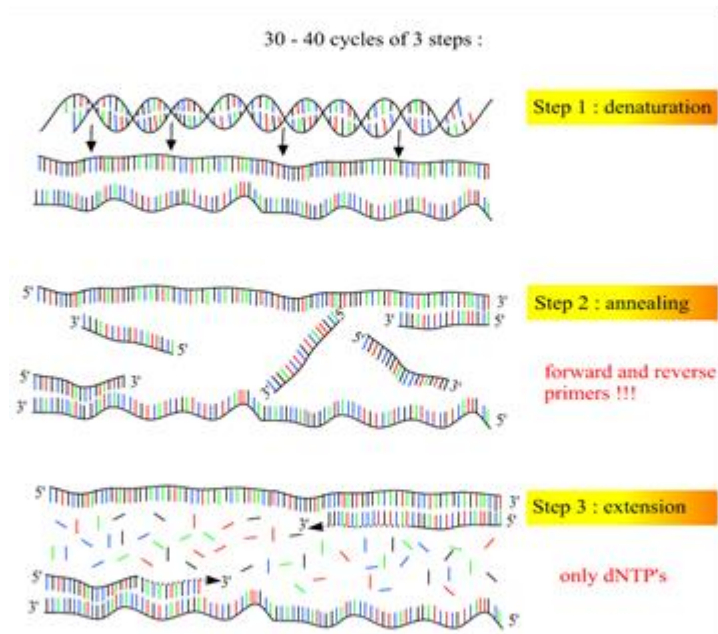

Figure 5. The steps of PCR amplification (source: Vierstraete, 1999)

After the completion of each cycle, the newly synthesised DNA strands can serve as new template for the next cycle. As shown in Figure 6, the major product of this exponential reaction is a segment of dsDNA whose termini are defined by the 5' termini of the oligonucleotide primers and whose length is defined by the distance between the primers. The products of the successful first round of amplification are heterogeneously sized DNA molecules, whose lengths may exceed the distance between the binding sites of the two primers. In the second round, these molecules generate DNA strands of defined length that accumulate in an exponential manner in subsequent rounds of amplification. Thus, amplification, as a final number of copies of the target sequence, is expressed by the following equation:

$$(2^n-2n)x \quad (1)$$

where n is the number of cycles, $2n$ is the first product obtained after the first cycle and second products obtained after the second cycle with undefined length, x is the number of copies of the original template. The value of $2n$ is negligible in comparison to 2^n.

Potentially, after 20 cycles of PCR, there will be a 2^{20}–fold amplification of the target sequence, assuming 100% efficiency during each cycle. The efficiency of a PCR varies from template to template and according to the degree of optimisation that has been carried out.

A detailed description of the three steps of PCR amplification (template denaturation, primer annealing and extension) is given in the following paragraphs (Sambrook et al., 1989).

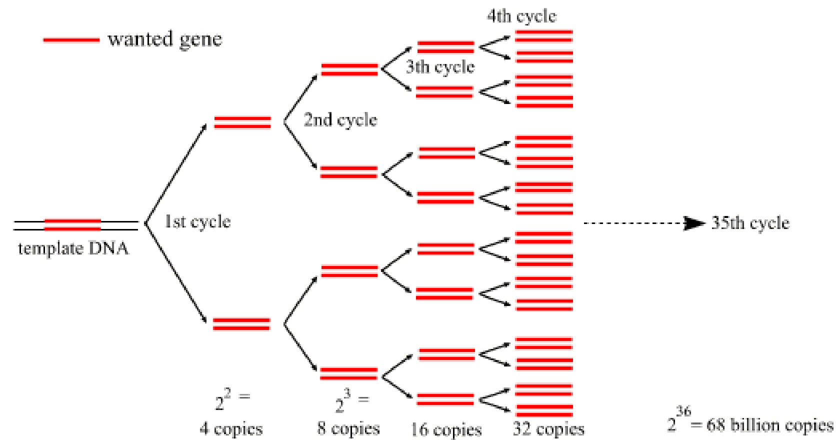

Figure 6. The exponential amplification of DNA in PCR (source: Vierstraete, 1999)

Template denaturation

During denaturation, the double strand DNA melts opening up to single-stranded DNA, and all enzymatic reactions stop (i.e. the extension from a previous cycle). The two complementary chains are separated by an increase in temperature. This is known as denaturation. To obtain the denaturation of DNA, the temperature is usually increased to ~ 93 - 96°C. In this way, the strong H-bonds are broken and the number of non-paired bases increases. The reaction is complete when all of the dsDNA becomes ssDNA. The temperature at which half of the dsDNA is single-stranded is known as the melting temperature (T_m).The type of solvent, the salt concentration and the pH used, influence the denaturation process. For example, in low salt concentrations, high pH and in the presence of organic solvents such as formaldehyde, the melting temperature, T_m, decreases. The concentration of G/C and T/A can also affect the value of T_m. The T_m of the DNA structure containing an elevated quantity of G/C is higher compared to that of DNA rich in T/A. For example, *Serratia marcescens* has approximately 60% G/C with a T_m of approximately 94°C, whereas *Pneumococcus* has approximately 40% G/C and a T_m of approximately 85°C.

Primer annealing

The annealing or re-hybridisation of the DNA strands takes place at lower temperature (usually 55 - 65°C). Once the temperature decreases, the two complementary ssDNA chains will reform into a dsDNA molecule. In this phase, the primers are flowing and hydrogen bonds are constantly formed and broken between the single-stranded primer and the single-stranded template. The more stable bonds last a bit longer (primers that exactly fit the template DNA) and on that small piece of double-stranded DNA (template and primer), the polymerase can attach and begins copying the template. Once there are

a few bases built in, the ionic bond is so strong between the template and the primer that it will not break.

Primer extension

In this step, the primers are extended across the target sequence by using a heat-stable DNA polymerase (frequently *Taq* DNA polymerase) in the presence of dNTPs resulting in a duplication of the starting target material. The ideal working temperature for the *Taq* DNA polymerase is 72°C. When the primers have been extended a few bases, they possess a stronger ionic attraction to the template, which reduces the probability of the reverse process. Primers that do not match exactly come loose again (because of the higher temperature) and do not give an extension of the fragment. The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTPs from 5' to 3', reading the template from 3' to 5'). The length of time of the primer extension steps can be increased if the region of DNA to be amplified is long, however, for the majority of PCR experiments, an extension time of 1 minute is sufficient to get a complete extension.

Instrumentation and components for the PCR

Instruments

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

a. The use of thermostable DNA polymerases, which resist inactivation at high temperatures. Thus, an initial aliquot of polymerase can last throughout numerous PCR cycles.
b. The development of thermal cyclers or PCR machines having blocks that can be heated and cooled in an automated and programmed manner.

Several designs of temperature cycling devices have been used. 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 temperature cycling profile for a three-step protocol is shown in Figure 7.

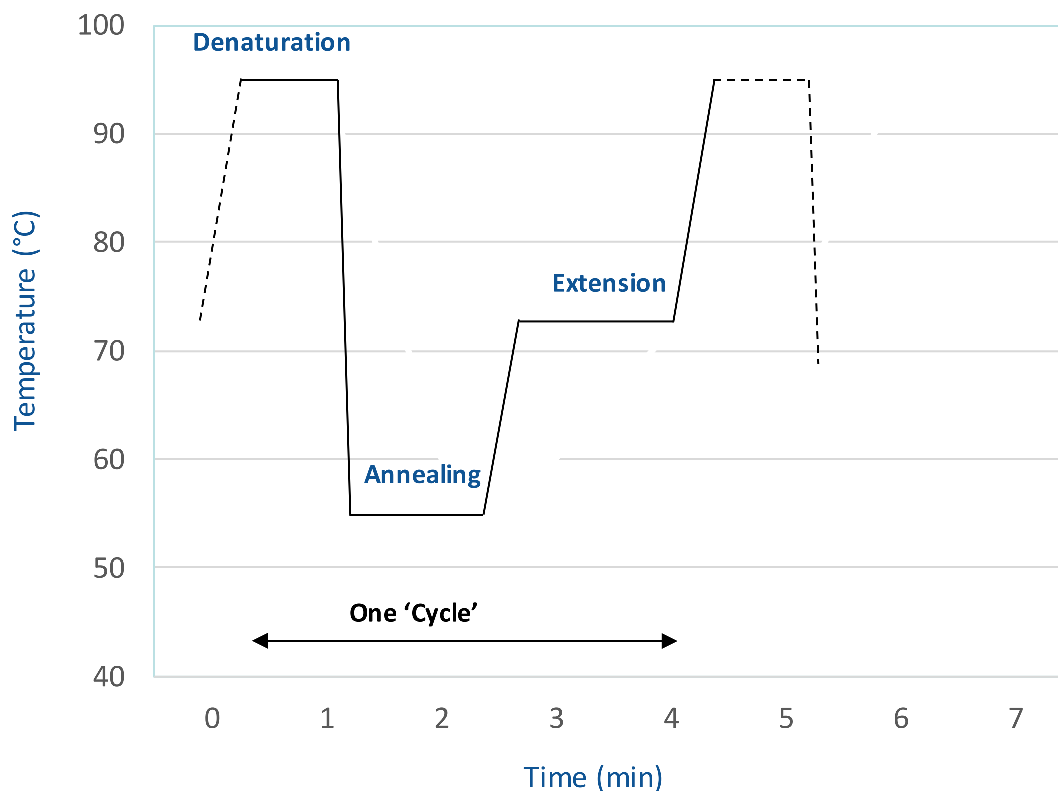

Figure 7. PCR temperature cycling profile (in-house graph).

Critical for a successful PCR are the thermal cycling parameters such as denaturation, primer annealing and primer extension already mentioned, as well as the components used and the cycle number described in the following paragraphs.

Target DNA

In principle, PCR amplification can be performed if at least one intact copy of the target DNA is present. A greater number of target copies enhance the probability of successful DNA amplification. Any damage, such as a nick in the target DNA, will prevent PCR amplification. The size of the target sequence can be anything from < 0.1 to a few kilobases. The total amount of DNA typically used for PCR is 0.05 to 1.0 µg; this allows detection of single copies of target sequence. Even if a sample does not need to be highly purified, some contaminants such as heparin, heme, formalin, Mg^{2+}-chelating agents, as well as detergents should be eliminated to avoid inhibition of the amplification process.

Primers

Generally, primers used are 16 - 30 nucleotides in length that allows the use of a reasonably high annealing temperature. Primers should avoid stretches of polybase sequences (e.g. poly dG) or repeat motifs – since these can hybridise inappropriately on the template. Inverted repeat sequences should be avoided so as to prevent formation of secondary structure in the primer, which would prevent hybridisation to template. Primers should not be complimentary to any other regions in the genome other than the target sequence. Furthermore, primers should not be complimentary to each other, particularly at their 3' ends, to avoid the formation of primer dimers. Ideally, the 3' end of the primer should be rich in G, C bases to enhance annealing of the end that will be extended. The distance between primers should be less than 10 Kb in length. Typically, substantial reduction in yield is observed when the primers extend from each other beyond ~3 Kb. Oligonucleotides are usually used at the concentration of 1µM in PCR. This is sufficient for at least 30 cycles of amplification. The presence of higher concentration of oligonucleotides can cause amplification of undesirable non-target sequences. Conversely, the PCR is inefficient with a limiting primer concentration.

History of DNA polymerase in PCR applications

The original method of PCR used the Klenow fragment of *E. coli* DNA polymerase I (Saiki et al., 1985). This enzyme, however, denatures at temperatures lower than that required to denature most template duplexes. Thus, in earlier experiments, fresh enzyme had to be added to the reaction after each cycle. In addition, samples have to be moved from one temperature bath to another to allow the individual steps of denaturation, annealing and polymerisation. The use of heat-resistant DNA polymerase has obviously facilitated the process because the addition of enzymes after every denaturation step is no longer necessary. Typically, DNA polymerases can only incorporate nucleotides from the 3’ end of a polynucleotide. The first thermostable DNA polymerase used was the ***Taq* DNA polymerase** isolated from the bacterium *Thermus aquaticus* (Saiki et al., 1988) living in a hot spring in Yellowstone National Park USA at temperatures close to 85°C. The optimal working temperature of this enzyme is 70 - 80°C. At this temperature, the bacterium

synthesises DNA at a rate of 35 - 100 nucleotides/sec. The average number of nucleotides, which an enzyme incorporates into DNA before detaching itself from the template, is known as the **processivity**. Even though this enzyme is probably the most widely used in PCR applications, several other DNA polymerases are commercially available.

Characteristics of polymerases suitable for PCR

Hot start polymerases. These enzymes are only activated at high temperature (generally at 95°C for 10 minutes) allowing the denaturation of all DNA molecules before starting the amplification process. This characteristic increases sensitivity and avoids the amplification of non-specific products especially when highly processed and complex samples are to be analysed (Gryson et al., 2004).

Polymerases with 5′ to 3′ exonuclease activity. Removing nucleotides ahead of the growing chain is particularly important in those real-time PCR experiments using hydrolysis probes (e.g. TaqMan probes, Session 10).

Polymerases with 3′ to 5′ exonuclease activity. Also called "**proofreading activity**", this property allows polymerases to check each nucleotide during DNA synthesis and excise mismatched nucleotides in the 3´ to 5´ direction being helpful in applications requiring small error rate. Nevertheless, exonuclease activity 3' to 5' can cause degradation of the primers. Therefore, the enzyme should only be added after the reaction has started, or alternatively, chemically modified primers should be used.

Low DNA (LD) polymerases. Consist in highly purified polymerases certified for the absence of contaminating bacterial DNA commonly present in recombinant protein preparations. They are particularly sensitive and used for applications requiring the absence of bacterial DNA contamination (e.g. detection of antibiotic resistance genes).

High fidelity. This kind of polymerases is particularly useful when doing sequencing because it has a reduced error rate compared to other conventional DNA polymerases.

Second-generation polymerases. Chimeric enzymes engineered substituting domains of the protein structure for the purpose of putting together the advantages of different polymerases, thus increasing accuracy (Yamagami et al., 2014), processivity, and resistance to environmental inhibitors (Baar et al., 2011).

Reaction buffers and $MgCl_2$ in PCR reactions

In addition to the reagents directly involved in the reaction, PCR requires a suitable buffer. The buffer composition depends on the type and characteristics of the enzyme being used and most suppliers usually provide a 10x buffer for use with the respective enzyme. The most common reaction buffer used with *Taq*/Ampli*Taq*® DNA polymerase contains:

- 10 mM Tris, pH 8.3
- 50 mM KCl

- 1.5-2.5 mM $MgCl_2$

The presence of divalent cations in PCR is critical. The $MgCl_2$ concentration in the final reaction mixture is usually between 0.5 to 5.0 mM, and the optimum concentration is determined empirically (Innis and Gelfand, 1990).

Mg^{2+} ions:

- Form a soluble complex with dNTPs which is essential for dNTP incorporation,
- Stimulate polymerase activity,
- Increase the T_m of primer/template interaction (and therefore they stabilise the duplex interaction).

Generally, a low Mg^{2+} concentration leads to low yields (or no yield) whereas a high Mg^{2+} concentration leads to accumulation of non-specific products (mispriming). It is important to avoid a high concentration of chelating agents such as EDTA or negatively charged ionic groups such as phosphate in the template DNA solution. Current literature includes discussions on various PCR buffers and additives, such as DMSO, PEG 6000, formamide, glycerol, spermidine and non-ionic detergents, used to increase the reaction specificity or efficiency (Roux, 1995). Certain DNA polymerases will indeed reach their optimum level of activity (Rolfs et al., 1992) only in the presence of such additives.

Deoxyribonucleoside triphosphates

Free deoxyribonucleoside triphosphates (dNTPs) are required for DNA synthesis. The dNTPs concentrations for PCR should be 20 to 200 µM for each dNTP and the four dNTPs should be used at equivalent concentrations to minimize mis-incorporation errors (Innis et al., 1988). High-purity dNTPs are supplied by several manufacturers either as four individual stocks or as a mixture of all four dNTPs. dNTPs stock solutions (usually 100 mM) should be adjusted to pH 7.0-7.5 with 1 M NaOH to ensure that the pH of the final reaction does not fall below 7.1 (Sambrook et al., 1989). However, many dNTPs stock solutions are now supplied with already adjusted pH.

Cycle number and plateau effect

The number of amplification cycles necessary to produce a band visible on an agarose gel depends largely on the starting concentration of the target DNA. In order to amplify 50 target molecules, 40 - 45 cycles are recommended, whereas 25 - 30 cycles are enough to amplify 3×10^5 molecules to the same concentration (Innis and Gelfand, 1990). This non-proportionality is due to the so-called *plateau* effect, which is the attenuation in the exponential rate of product accumulation in late stages of a PCR when the product reaches 0.3 - 1.0 nM. This may be caused by degradation of reagents (dNTPs, enzyme), reagent depletion (primers, dNTPs – the former a problem with short products, the latter with long products), end-product inhibition (pyrophosphate formation), competition for reagents by

non-specific products, or competition for primer binding by re-annealing of the concentrated (10 nM) product (Innis and Gelfand, 1990). If the desired product is not obtained in 30 cycles, a small sample (1 µl) of the amplified product can be taken, mixed and re-amplified 20 - 30 cycles in a new reaction mix, rather than extending the run to more cycles. In some cases where the template concentration is limiting, this re-amplification can produce a good product, whereas extension of cycling to 40 times or more does not. However, re-amplification is also prone to contamination especially in a routine diagnostic environment.

Design of primers for PCR

The most critical parameter for successful PCR is the design of primers. A poorly designed primer can result in a PCR reaction that will not work. The primer sequence determines several things such as the target position and length of the product, its melting temperature and ultimately the yield (Innis and Gelfand, 1994). A poorly designed primer can result in little or no product due to a lack of successful priming, non-specific amplification and/or primer-dimer formation, which can become competitive enough to suppress product formation. This application note is provided to give rules that should be taken into account when designing primers for PCR. More comprehensive coverage of this subject can be found elsewhere (Dieffenbach et al., 1995).

Primer selection

Several variables must be taken into account when designing PCR primers. Among the most critical are:

- Primer length
- Melting temperature (T_m)
- Specificity
- Complementary primer sequences
- G/C content and polypyrimidine (T, C) or polypurine (A, G) stretches
- 3'-end sequence

Each of these critical elements will be discussed in the following sections.

Primer length

Since specificity, temperature and time of annealing partly depend on primer length, this parameter is critical for successful PCR. In general, oligonucleotides between 18 and 24 bases are extremely sequence-specific, provided that the annealing temperature is optimal. Primer length is also proportional to annealing efficiency. In general, the longer the primer, the more inefficient the annealing. With fewer templates primed at each step, this can result in a significant decrease in amplified product. The primers should not be too

short unless the application specifically requires it. As discussed below, the goal should be to design a primer with an annealing temperature of at least 50°C.

The relationship between annealing temperature and melting temperature is one of the “Black Boxes” of PCR. A general rule-of-thumb is to use an annealing temperature that is 5°C lower than the melting temperature. Often, the annealing temperature determined this way will not be optimal and empirical experiments will have to be performed to determine the optimal one. This is most easily accomplished using a gradient thermal cycler.

Melting temperature (T_m)

It is important to keep in mind that there are two primers added to a site/target directed PCR reaction. The both oligonucleotide primers should be designed in a way to have similar melting temperatures. If primers are mismatched in terms of T_m, amplification will be less efficient or may not work at all since the primer with the higher T_m will misprime at lower temperatures and the primer with the lower T_m may not work at higher temperatures. The melting temperatures of oligos are most accurately calculated using nearest neighbour thermodynamic calculations with the formula:

$$T_m^{primer} = \Delta H\ [\Delta S + R\ \ln (c/4)]\ -273.15°C + 16.6\ \log_{10} [K^+] \quad (2)$$

where H is the enthalpy and S is the entropy for helix formation, R is the molar gas constant and c is the concentration of primers.

This is most easily accomplished by using primer design software packages already available on the market (Sharrocks, 1994). Fortunately, a good working approximation of this value (generally valid for oligos in the 18 - 24 base range) can be calculated using the formula:

$$T_m = 2(A+T) + 4(G+C) \quad (3)$$

where A, T, G, C are the purinic and pyrimidinic bases.

Table 1 shows the calculated values for primers of various lengths using this equation (known as the Wallace formula) and assuming 50% GC content (Suggs et al., 1981).

Table 1. Calculation of the Tm of the primers with Wallace's equation

Primer length	Tm = 2 (A+T) + 4(G+C)	Primer length	Tm = 2 (A+T) + 4(G+C)
4	12°C	22	66°C
6	18°C	24	72°C
8	24°C	26	78°C
10	30°C	28	84°C
12	36°C	30	90°C
14	42°C	32	96°C
16	48°C	34	102°C
18	54°C	36	108°C
20	66°C	38	114°C

The temperatures calculated using the Wallace's rule are inaccurate at the extremes of this chart. When calculating the melting temperatures of the primers, care must be taken to ensure that the melting temperature of the product is low enough to obtain 100% melting at 92°C. This parameter will help to assure a more efficient PCR but is not always necessary for successful PCR. In general, products between 100 - 600 base pairs are efficiently amplified in many PCR reactions. If there is a doubt, the product T_m can be calculated using the formula:

$$T_m = 81.5 + 16.6 (\log_{10}[K+] + 0.41 (\%G+C) - 675/length \quad (4)$$

Specificity

As mentioned above, primer specificity is partly dependent on primer length. That said, primers must be chosen so that they have a unique sequence within the template DNA that is to be amplified. A primer designed with a highly repetitive sequence will result in a smear when amplifying genomic DNA. However, the same primer may give a single band, if a

single clone from a genomic library is amplified. Because *Taq* DNA polymerase is active over a broad range of temperatures, primer extension will occur at the lower temperatures of annealing. If the temperature is too low, non-specific priming may occur, which can be extended by the polymerase if there is a short homology at the 3' end. In general, a melting temperature of 55° - 72°C gives the best results (note that this corresponds to a primer length of 18 - 24 bases using Wallace's rule).

Complementary primer sequences

Primers need to be designed with absolutely no intra-primer homology beyond 3 base pairs. If a primer has such a region of self-homology, "snap back", or "hair-pin", partially double-stranded structures can occur, which will interfere with annealing to the template. Another related danger is inter-primer homology. Partial homology in the middle regions of two primers can interfere with hybridisation. If the homology occurs at the 3' end of each primer, dimer formation will occur, preventing the formation of the desired product due to competition.

G/C content and polypyrimidine (T, C) or polypurine (A, G) stretches

The GC base content of primers should be between 45% and 55%. The primer sequence must be chosen in a way to avoid poly-G or poly-C stretches that can promote non-specific annealing. Poly-A and poly-T stretches are also to be avoided, as these will "breathe" and will open up the stretches of the primer-template complex. In this case, the efficiency of the amplification can be lowered. Polypyrimidine (T, C) and polypurine (A, G) stretches should also be avoided. Ideally, the primer will have a near random mix of nucleotides, a 50% GC content and be ~20 bases long. This will put the T_m in the range of 56° - 62°C (Dieffenbach et al., 1995).

3'-end sequence

The 3' terminal position in PCR primers is essential for the control of mispriming. The problem of primer homologies occurring in these regions has already been explored. Another variable to look at is the inclusion of a G or C residue at the 3' end of primers. This "GC Clamp" helps to ensure correct binding at the 3' end, due to the stronger hydrogen bonds of the G/C residues. This also helps to improve the efficiency of the reaction by minimising any "breathing" that might occur.

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 PCR is a modification of PCR that was designed to improve sensitivity and specificity. Nested PCR involves the use of two primer sets and two successive PCR reactions. The first set of primers are designed to anneal to sequences upstream from the second set of primers and are used in an initial PCR reaction. Amplicons resulting from the first PCR reaction are used as template for a second set of primers and a second amplification step[33].

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 can eliminate 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.

The products of the analysis can be further hybridised with a gene-specific probe for verification.

[33] https://www.sciencedirect.com/topics/neuroscience/nested-polymerase-chain-reaction

Multiplex PCR is very useful for "screening" purposes in case of GMO detection (see Session 9), because it reduces the amount of experiments. It is not possible to use the protocol of two single PCRs and put them together into a multiplex reaction because the amount of enzyme would be split among two or more targets as well as the quantity of reagents, lowering in this way the efficiency of the reaction. Nevertheless, there are many specific multiplex PCR protocols available.

PCR in practice

As already illustrated in the previous sections, PCR is widely used and it is a powerful analytical and preparative technique. However, because of the nature of this procedure, trace amounts of DNA contaminants can 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. Physically separate, distinct working areas with dedicated equipment reduces the risk of contamination. A forward workflow should be maintained throughout the process. Strict compliance with decontamination requirements (e.g. decontamination of nucleic acids and prevention of aerosols) 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 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 and primers dilutions).

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 forward work 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 filter and/or aerosol-resistant 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 or in a dead air box. Equipment and disposable gloves should be dedicated to such areas.
- 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. Reagents such as primers, dNTPs, and *Taq* DNA Polymerase cannot 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 ("not containing DNA") control, that 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 PCR can amplify a single molecule over a billion-fold. Thus, even tiny 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).

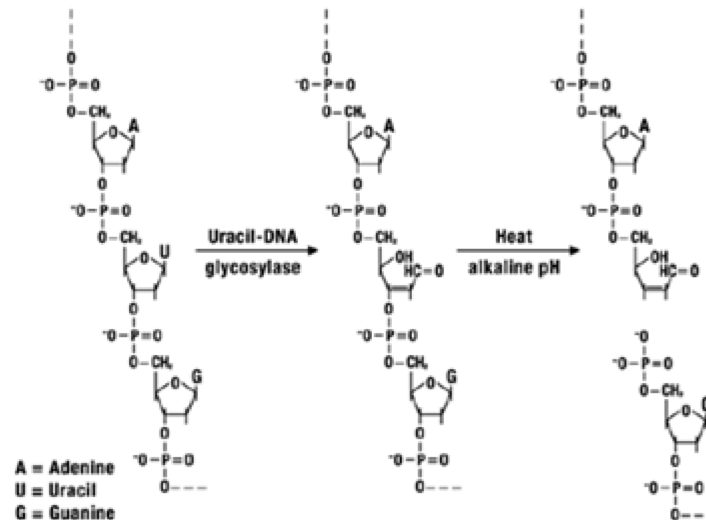

Figure 8. Uracil-DNA Glycosylase reaction

This method, of course, requires that all PCR-reactions in the lab are 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. *Eco*R I and *Bam*H I), while others show reduced activity (e.g. *Hpa* I, *Hind* II, *Hind* III) on these substrates.

  Note: 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.

Preparation of the mixture for the PCR reaction (Mastermix)

The essential reagent components for PCR are water, reaction buffer, a thermostable DNA polymerase, oligonucleotide primers, deoxynucleotides (dNTPs), template (target) DNA, and magnesium ions (Mg^{2+}). In general, all reagents (except the template DNA) are mixed in a single tube, in enough volume according to the number of reactions to be performed (mastermix). Commercial mastermix containing buffer, deoxynucleotides (dNTPs), and magnesium ions (Mg^{2+}) can also be used. The mastermix is then aliquoted into individual tubes and the template DNA is added. The use of a mastermix solution reduces the risk of contamination and improves the performance of the PCR reaction for the following reasons:

- A uniform quality of the solution is guaranteed for all the reagents for a series of analyses,
- The risk of contamination of the parent and resulting solutions is decreased,
- Larger volumes can be pipetted thus avoiding pipetting error,
- There are fewer pipetting stages and therefore time is saved.

Successful amplification of the region of interest depends on the amount and quality of the template DNA. The amount of template required is dependent upon the complexity of the DNA sample. Taking into account that the size of nuclear genome varies among organisms, the DNA concentration should be maintained constant (usually 10 ng/µl). A comparison of genome size of plant species frequently used in plant transformation and the corresponding number of genome copies (copy number) in a defined amount of DNA (ng), are given in Table 2 where 1C is the weight in picograms of the haploid genome.

Table 2. C values of the most frequent crops that have been genetically modified.

(*C values available at http://data.kew.org/cvalues/CvalServlet?querytype=1)

Taxon	1 C * (pg)	copy number in 100 ng
Soybean	1.13	88496
Maize	2.73	36630
Rice	0.5	200000
Rapeseed	1.15	86957
Cotton	2.4	41667
Sugar beet	1.25	80000
Tobacco	5.18	19305

For example, in a 4 kb plasmid containing a 1 kb insert, 25% of the input DNA is the target of interest. Conversely, a 1 kb gene in the maize genome (5 x 10^9 bp) represents approximately 0.00002% of the input DNA. Approximately 1,000,000-fold more maize genomic DNA is required to obtain the same number of target copies per reaction. For optimised results, > 104 copies of the target sequence should be used as a starting template to obtain a signal in 25 - 30 cycles. Even if in practice less than 10 copies of a target sequence can be amplified, in this case, more PCR cycles might be required to detect a signal by gel electrophoresis. General protocols routinely applied consider a number of cycles ranging between 30 and 40. Care should be taken in further increasing the number of cycles, since this may increase non-specific amplification.

The data contained in Table 2 was extrapolated using the following principle. If calculating the number of copies of DNA in a certain amount of extracted DNA, for example, from soybean (e.g. 200 ng of DNA), the following formula shall apply:

*(200 ng * 1000)/ 1.13 pg = 176991 copies*

where 1.13 is the average C value for soybean expressed in picograms.

Controls

As reported in the previous paragraphs, potential sources of contamination can be found throughout the laboratory. Samples, laboratory staff, air conditioning, equipment, and reagents can all be a source of contamination. Among contaminant agents, the following can occur:

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. Genomic DNA from previous sample DNA extraction
4. Degradation of DNA by decontamination chemicals

Whereas the first three forms of contamination produce false positives the last can produce a false negative result. This form of contamination, first observed by Niederhauser and co-workers in 1994, produces the inhibition of PCR reactions (Niederhauser et al., 1994). In fact, decontamination using the UNG method favours the formation of complexes with the primers.

In order to obtain reliable results, it is important to always perform the analysis with different controls monitoring malfunctioning of the amplification procedure, cross contamination, and inhibition (see Table 3).

Positive controls

The efficiency of the DNA extraction and its amplification has to tested against positive controls. 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 (carry-over of amplified products or nucleic acids) may occur during the isolation and purification of the target DNA, as well as during the preparation of the amplification reaction mixture. The use of a negative control with the amplification reaction mixture is therefore essential.

Inhibition controls

In contrast to positive and negative controls that have to go together with the sample analysis, it is appropriate to do the DNA inhibition test before the experiment, to avoid

wasting time and resources. Basically, inhibition is related to the concentration of inhibitors, and for this reason inhibition tests (runs) require the preparation of a set of serial dilutions of the extracted DNA. After their amplification, it should be possible to tell if the sample is highly inhibited or not based on the expected rate of PCR amplification in terms of the serial dilution. Determining whether PCR inhibition is present is important to avoid false negatives as well as during real-time PCR quantification. If a GMO is present in very low concentration and the extracted DNA is inhibited, the analysis could give a negative result even if the target DNA is present (false negative).

Table 3. Controls to be inserted within the PCR based tests according to ISO 24276

Name	Description	Purpose	Expected Result
Environmental control	Nucleic acid free water	Looking for cross contamination in laboratory environment	Negative
Extraction blank control	Control in which the sample is substituted by nucleic acid-free water and that follows all extraction steps	Looking for presence of contamination during extraction procedures	Negative
Positive extraction control	It contains the DNA under study (GMO), can be an appropriate Certified Reference Material	To assure that the procedure actually extracted DNA	Positive
Negative DNA target control	Contains taxon reference DNA but no GMO	To assure the absence of false positives	Negative
Amplification reagent control	Contains all reagents for amplification except the target DNA (sterile water instead)	To assure that there is no contamination	Negative
PCR inhibition control	Serial dilutions of the Certified Target DNA	To monitor inhibition	Within the range

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Session 7

Characteristics of Roundup Ready® Soybean, MON810 Maize, and Bt-11 Maize

Characteristics of Roundup Ready® soybean[34]

Brief identification

Designation (Unique identifier)	GTS 40-3-2 (MON-Ø4 Ø32-6)
Applicant	Monsanto Canada Inc.
Plant Species	*Glycine max* L. (soybean)
Novel Traits	Novel tolerance to glyphosate, the active ingredient of Roundup® herbicide
Trait Introduction Method	Particle acceleration (biolistics)
Proposed Use	Production of soybeans for animal feed (mostly defatted toasted meal and flakes) and human consumption (mostly oil, protein fractions and dietary fibre).

Background information

Soybean line GTS 40-3-2 was developed by Monsanto Canada Inc. to allow the use of glyphosate as an alternative weed control system in soybean production.

The development of GTS 40-3-2 was based on recombinant DNA technology, through the introduction of a glyphosate tolerant form of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene, isolated from *Agrobacterium tumefaciens* strain CP4, into the commercial soybean variety "A5403" (Asgrow Seed Company).

Description of the novel trait: Glyphosate tolerance

Glyphosate, the active ingredient of Roundup®, is a systemic, post emergent herbicide used worldwide as a non-selective weed control agent. Glyphosate acts as a competitive inhibitor of 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS), an essential enzyme of the shikimate biochemical pathway involved in the production of the aromatic

[34] Extracted from the Canadian Food Inspection Agency, Decision Document DD95-05.

aminoacids phenylalanine, tyrosine and tryptophan (Figure 1). The inhibition of EPSPS results in growth suppression and plant death.

The inserted glyphosate tolerance gene codes for a bacterial version (derived from the CP4 strain of *Agrobacterium tumefaciens*) of this essential enzyme, ubiquitous in plants, fungi and microorganisms and is highly insensitive to glyphosate. It can therefore fulfil the aromatic aminoacid metabolic needs of the plant.

The *EPSPS* gene is under the regulation of a strong constitutive promoter from Cauliflower Mosaic Virus (**P**-***CaMV*** **E35S**) and terminates with the nopaline synthase terminator (**T-*nos***) derived from *Agrobacterium tumefaciens* (Figure 2). A plant-derived DNA sequence coding for a chloroplast transit peptide (CTP4 from *Petunia hibrida*) was cloned at the 5' of the glyphosate tolerance gene. The signal peptide fused to the *EPSPS* gene facilitates the import of newly translated enzyme into the chloroplasts, where both the shikimate pathway and glyphosate sites of action are located. Once importation has occurred, the transit peptide is removed and rapidly degraded by a specific protease.

EPSP synthase is ubiquitous in nature and is not expected to be toxic or allergenic. When subjected to comparative analyses with sequence databases of toxic or allergenic polypeptides, the amino acid sequence of the enzyme showed no significant homology with any known toxin or allergen.

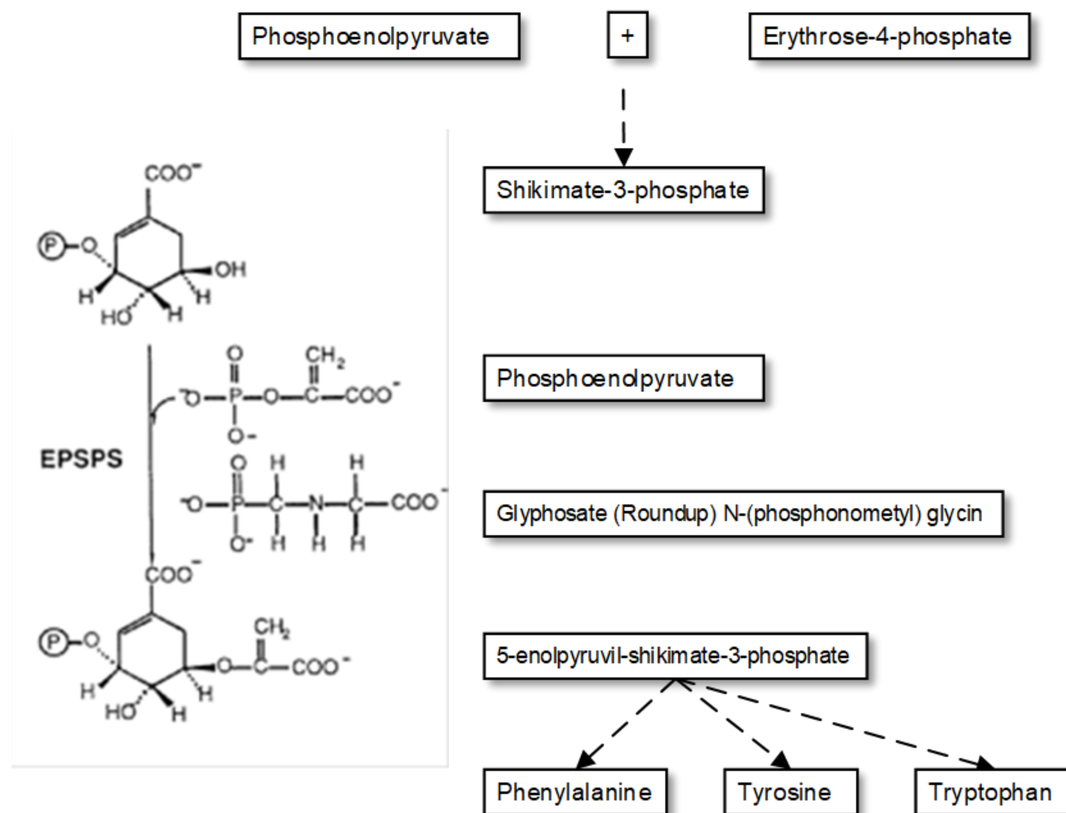

Figure 1. EPSPS catalyses the reaction of shikimate-3-phosphate and phosphoenolpyruvate (PEP) to form 5-enolpyruvylshikimate-3-phosphate (EPSP) and phosphate. EPSP is an intermediate for aromatic aminoacids synthesis. As a consequence of inhibition of this biochemical pathway, proteins' synthesis is disrupted, resulting in plant death. EPSPS is the only physiological target of glyphosate in plants, and no other PEP-utilising enzymes are inhibited by glyphosate.

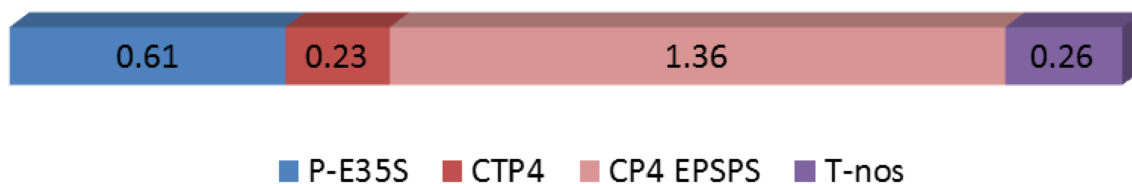

Figure 2. Schematic representation of the Roundup Ready® soybean gene cassette (Size of elements composing the construct in kilobases).

Development method

The commercial soybean variety A5403 (Asgrow Seed Co.) was transformed by means of gold particle bombardment with the PV-GMGT04 plasmid vector harvested from *Escherichia coli* (see Figure 3). The PV-GMGT04 plasmid contained the *CP4 EPSPS* gene coding for glyphosate tolerance, the *gus* gene for production of ß-glucuronidase as a selectable marker, and the *npt*II gene for antibiotic resistance (kanamycin). The original transformant selected showed two sites of integration, one with the *gus* selectable marker and the other with the glyphosate tolerance gene. These two sites subsequently segregated independently in the following sexual generation, and line GTS 40-3-2, upon analysis, was found to contain just one insertion site, in which only the glyphosate tolerance gene is integrated.

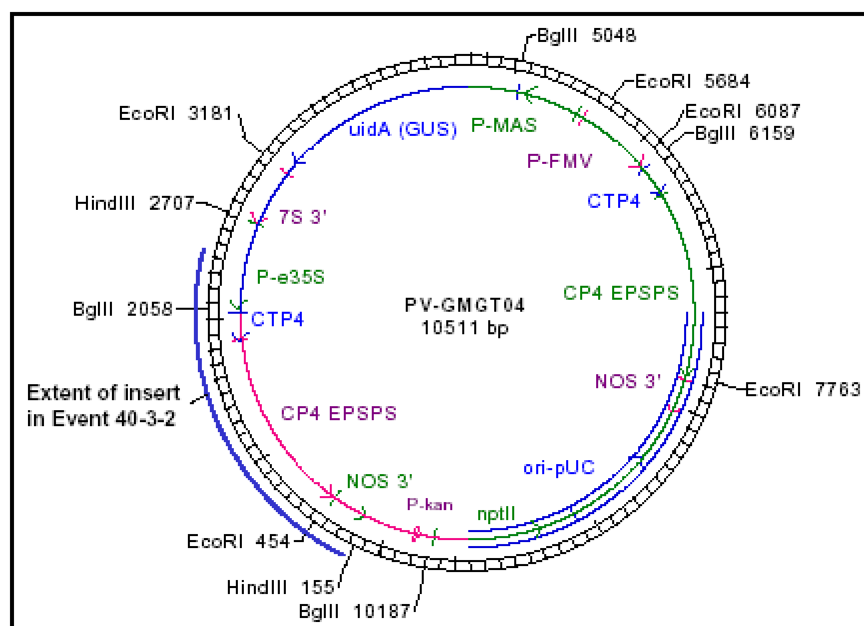

Figure 3. Plasmid map including genetic elements of vector PV-GMGT04 used in the transformation of RR soybean event 40-3-2 (taken from Monsanto, 2000)[35]

Stability of insertion of the introduced traits

The original data (Padgette et al., 1995, 1996) indicated that GTS 40-3-2 contained a single functional *CP4 EPSPS* gene cassette, consisting of the Cauliflower Mosaic Virus (CaMV) E35S promoter, a chloroplast transit peptide, the *CP4 EPSPS* coding sequence, and the *nos* polyadenylation signal.

No incorporation of any coding region from outside the fusion gene of the original plasmid vector was found. Subsequent generations demonstrated no further segregation of the

[35] Monsanto Company (2000). Updated Molecular Characterization and Safety Assessment of Roundup Ready Soybean Event 40-3-2. Monsanto Report, Product Safety Centre.

fusion gene described above, showing that line GTS 40-3-2 was homozygous for the fusion gene. DNA analyses over six generations showed that the insertion was stable.

More recent characterisation studies have shown that, during integration of the insert DNA several rearrangements occurred and that, in addition to the primary functional insert, Roundup Ready® soybean event 40-3-2 contains two small not functional segments of inserted DNA of 250 bp and 72 bp, respectively (Monsanto, 2000; Windels et al., 2001).

Regulatory decision

Roundup Ready® (RR) soybean was the first soybean line approved for marketing in the EU. After clearance in the US in 1994, consent for importation into the European Union was also given with Commission Decision 96/281/EC of 3 April 1996 (Commission Decision 96/281/EC) and from there renewed in 2006. The decision only allows for the importation of seed into the EU for industrial processing into non-viable products including animal feeds, food and any other products in which soybean fractions are used, while cultivation is not allowed.

Characteristics of maize MON810[36]

Brief identification

Designation (Unique identifier)	Event MON810 maize (MON-ØØ81Ø-6)
Applicant	Monsanto Canada Inc.
Plant Species	*Zea mays* L. (maize)
Novel Traits	Resistance to European Corn Borer (ECB) (*Ostrinia nubilalis*)
Trait Introduction Method	Particle acceleration (biolistics)
Proposed Use	Production of *Z. mays* for human consumption (wet or dry mill or seed oil), and meal and silage for livestock feed.

Background information

Maize event MON810 was developed by Monsanto Canada Inc. to be specifically resistant to European Corn Borer (ECB; *Ostrinia nubilalis*) and to provide a method to control yield losses due to damage through insect feeding caused by the ECB in its larval stages, without the use of conventional pesticides.

MON810 was developed using recombinant DNA technology and microprojectile bombardment of plant cells to introduce a gene encoding the production of a naturally occurring insecticidal protein (derived from *Bacillus thuringiensis* ssp. *kurstaki*). This protein is active against certain species of *Lepidoptera*, the insect order to which butterflies and moths belong, including ECB. More specifically, the protein expressed in MON810 is a truncated form of the insecticidal protein, CRYIA(b) δ-endotoxin, and protects the maize plants from leave and stalk damage caused by ECB larvae.

Description of the novel trait: Resistance to the European Corn Borer (ECB)

Bacillus thuringiensis ssp*. kurstaki* is an endospore-forming, gram-positive, soil-borne bacterium. In its sporogenic stage it produces several insecticidal proteins, including the δ-endotoxin CRYIA(b) protein active against certain lepidopteran insects such as the ECB, Spruce Budworm, Tent Caterpillar, Gypsy Moth, Diamondback Moth, Cabbage Looper,

[36] Extracted from the Canadian Food Inspection Agency, Decision Document 97-19.

Tobacco Budworm, and Cabbage Worm. The protein has been repeatedly shown to be non-toxic to humans, other vertebrates and beneficial insects (Lee et al., 1995).

MON810 was transformed with one copy of *cryIA(b)* gene (3.46 Kb) under the control of the strong constitutive enhanced CaMV 35S promoter, and the maize HSP70 intron leader sequence (Figure 4).

The *cryIA(b)* coding sequence from *Bacillus thuringiensis* ssp. *kurstaki HD-1* was modified to optimize and maximize the expression of the δ-endotoxin CRYIA(b) protein in plants. The protein becomes toxic for lepidopteran larvae following cleavage to a bio-active, trypsin-resistant core. The insecticidal activity is thought to depend on the binding of the active fragment to specific receptors present on midgut epithelial cells of susceptible insects and on the subsequent formation of pores, disrupting the osmotic balance and eventually resulting in cell lysis. Specific lepidopteran pests of maize sensitive to the protein are ECB and corn earworm.

The amino acid sequence of the toxin expressed in the modified maize was found to be identical to that occurring naturally, and equivalent to the protein produced as a biopesticide being widely used by the organic food industry.

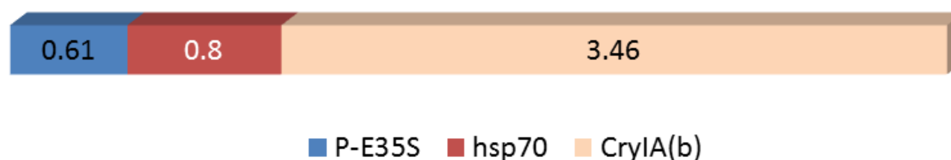

Figure 4. Schematic representation of the *cryIA(b)* construct from MON810, including the enhanced CaMV 35S-promoter, the maize hsp70 intron 1 and the synthetic *cryIA(b) gene*.

Development method

MON810 was obtained from maize genotype Hi-II by biolistic transformation with a mixture of plasmid DNAs, PV-ZMBK07 and PV-ZMGT10. The PV-ZMBK07 plasmid contained the *cryIA(b)* gene (Figure 5) and PV-ZMGT10 plasmid contained the *CP4 EPSPS* and *gox* genes. Both plasmids also contained the *npt*II gene (for bacterial selection) under the control of a bacterial promoter, and an origin of replication from a pUC plasmid (ori-pUC) required for replication of the plasmids in *E. coli*. The two vectors were introduced by microprojectile bombardment into cultured plant cells. Glyphosate tolerant transformed cells were selected and subsequently cultured in tissue culture medium for plant regeneration (Armstrong et al., 1991).

Molecular analyses provided by the authors indicated that only the elements from construct PV-ZMBK07 were integrated into the genome of line MON810 as a single insert, consisting of the enhanced CaMV 35S (E35S) promoter, the hsp70 leader sequence and the truncated

cryIA(b) gene. The *nos* 3' termination signal, present in plasmid PV-ZMBK07, was lost through a 3' truncation of the gene cassette and therefore was not integrated into the host genome (BATS, 2003).

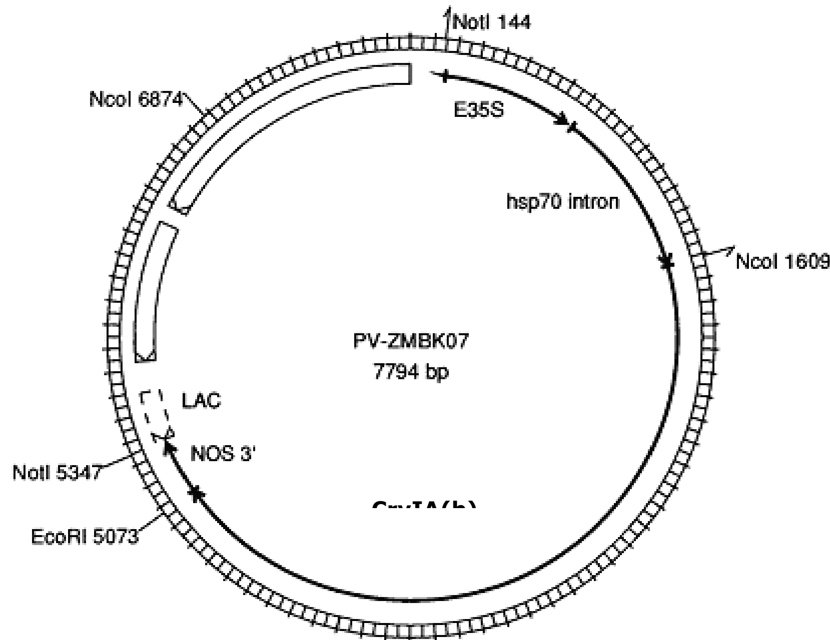

Figure 5. Schematic representation of the plasmid PV-ZMBK07 used in engineering MON810 (Sanders et al, 1998).

Stability of insertion of the introduced traits

Data provided by the authors show that segregation and stability were consistent with a single site of insertion of the *cryIA(b)* gene into the MON810 genome. The stability of the insertion was demonstrated through multiple generations of crossing. The maize line has been crossed with several different maize genotypes for 4 generations with protection against ECB maintained. MON810 was derived from the third generation of backcrossing. Stable integration of the single insert was demonstrated through all three generations by Southern Blot analysis.

Regulatory decision

Planting of maize line MON810 was approved in the United States in July 1996 by the Environmental Protection Agency. Commercialisation of this line of maize in the EU was authorised following Commission Decision 98/294/EC of 22 April 1998 (Commission Decision 98/294/EC).

The Canadian Food Inspection Agency issued the Decision Document 97-19 for its approval as food and feed. The MON810 line is also approved in Argentina, Australia, Japan, South Africa and Switzerland.

This line of maize is the only one cultivated in the EU and it is intended for human consumption (wet mill, dry mill or seed oil), meal and silage for livestock feed. The procedure for the renewal of the seeds for cultivation authorisation is currently ongoing (https://webgate.ec.europa.eu/dyna/gm_register/index_en.cfm, *accessed February 2020*).

Characteristics of maize Bt-11

Brief identification

Designation (unique identifier)	Event Bt-11 maize (SYN-BT Ø11-1)
Applicant	Syngenta Seeds SAS
Plant Species	*Zea mays* L. (maize)
Novel Traits	Resistance to the (ECB) (*Ostrinia nubilalis*), and tolerance to phosphinothricin (PPT) herbicide, specifically glufosinate ammonium.
Trait Introduction Method	Particle acceleration (biolistics)
Proposed Use	Production of *Z. mays* for human consumption (wet or dry mill or seed oil), and meal and silage for livestock feed.

Background information

Maize event Bt-11 was developed by Syngenta Seeds SAS to be specifically resistant to ECB (*Ostrinia nubilalis*) and to provide a method to control yield losses due to damage through insect feeding caused by the ECB in its larval stages, without the use of conventional pesticides.

Bt-11 was developed using recombinant DNA technology and microprojectile bombardment of plant cells, to introduce a gene encoding the production of a naturally occurring insecticidal protein (derived from *Bacillus thuringiensis* spp. *kurstaki*). This protein is active against certain species of *Lepidoptera*, the insect order to which butterflies and moths belong, including ECB.

It also contains phosphinothricin N-acetyltransferase (PAT) encoding gene from *Streptomyces viridochromogenes* to confer tolerance to phosphinothricin (PPT) herbicide, specifically glufosinate ammonium.

Description of the novel trait: Resistance to the ECB

Constitutive expression of the *cry1Ab* gene in Bt-11 is controlled by the 35S promoter derived from cauliflower mosaic virus (CaMV) modulated by the IVS6 intron (from maize alcohol dehydrogenase 1S gene), and the 3'-polyadenylation signal of the nopaline synthase (*nos*) gene from *Agrobacterium tumefaciens*. The phosphinothricin acetyltransferase (*pat*) gene is present as a selectable marker enabling identification of the transformed plant cells and to provide field tolerance to glufosinate ammonium. Constitutive expression of the *pat* gene is under the control of the CaMV 35S promoter, the IVS 2 intron, and NOS 3' terminator. For the properties of *cryIA(b)* gene see paragraph on MON810.

Tolerance to glufosinate ammonium

Bt-11 was also genetically modified to express the *pat* gene cloned from the common aerobic soil actinomycetes; *Streptomyces viridochromogenes* strain Tu494, which encodes a phosphinothricin-N-acetyltransferase (PAT) enzyme. The PAT enzyme was used as a selectable marker enabling identification of transformed plant cells as well as a source of resistance to the herbicide phosphinothricin (also known as glufosinate ammonium, the active ingredient in the herbicides Basta, Rely, Finale, and Liberty). Glufosinate ammonium acts by inhibiting the plant enzyme glutamine synthetase, the only enzyme in plants that detoxifies ammonia by incorporating it into glutamine. Inhibition of this enzyme leads to an accumulation of ammonia in the plant tissues, which kills the plant within hours of application. PAT catalyses the acetylation of the herbicide phosphinothricin and thus detoxifies glufosinate ammonium into an inactive compound. The modified maize line is protected from ECB and permits farmers to use phosphinothricin-containing herbicides for weed control in the cultivation of maize.

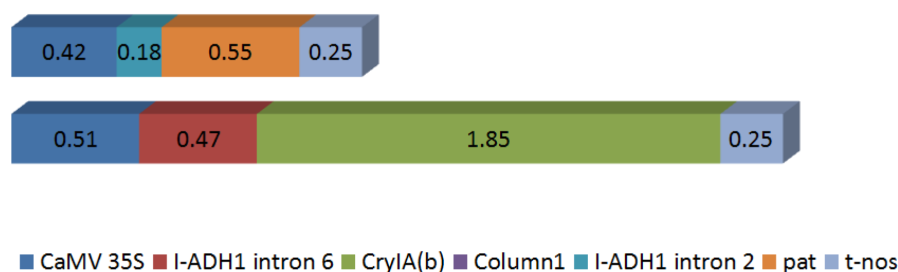

Figure 6. Schematic representation of the *cryIA(b)* and pat gene construct, including the enhanced CaMV 35S-promoters, the maize hsp70 intron 6 and 2 and the nos terminators.

Development method

The Bt-11 corn line was created through direct DNA transformation of plant protoplasts from the inbred maize line H8540 and regeneration on selective medium. A single plasmid, designated pZO1502, was used in the transformation event and contained a truncated

synthetic *cry1Ab* gene and a synthetic *pat* gene. Prior to transformation, the plasmid vector was treated with the restriction endonuclease *Not*I in order to remove the beta lactamase (*bla*) gene from the DNA fragment containing the *cry1Ab* and *pat* genes.

The plasmid pZO1502 also contained the beta lactamase (*bla*) gene included as selectable marker to screen transformed bacterial cells. The *bla* gene confers resistance to some beta-lactam antibiotics, including the moderate-spectrum penicillin and ampicillin. Bacterial cells that contained the pZ01502 plasmid were selected through their resistance to ampicillin. The *bla* gene was excised from the plasmid vector prior to transformation of the maize tissue.

Other genetic components incorporated included a non-functional *lac*Z gene, encoding a portion of the enzyme beta-galactosidase; and the pUC origin of replication derived from the plasmid pBR322. Following the transformation event, plants were regenerated and the pollen of maize plants (*Zea mays* L.) derived from transformation event Bt-11 was used to pollinate the female flowers of an inbred maize line. Descendants of the initial crossings were successively backcrossed to evaluate different maize lines carrying the Bt-11 event. Several hybrid maize varieties have been derived from the Bt-11 maize event.

Stability of insertion of the introduced traits

The stability of the inserted DNA in Bt-11 maize was demonstrated by a Mendelian inheritance pattern using Southern Blot analysis. Segregation analysis of the *cry1Ab* and *pat* genes over multiple generations demonstrated that they were closely linked, as they always segregated together. Restriction fragment length polymorphism (RFLP) mapping was used to determine the location of the novel genes in Bt-11. The single insertion site was mapped to the long arm of chromosome 8.

Regulatory decision

Bt-11 maize has been approved for import and feed and food use in the EU through Commission Decision 98/292/EC in 1998.

The placing on the market of Bt-11 sweet corn was approved in 2004 (2004/657/EC). The authorization has been renewed (2010/419/EU), for food and feed use, and for other uses other than food and feed with the exception of cultivation. Field maize and sweet corn are not currently approved for cultivation in the EU.

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COMMISSION DECISION (98/292/EC) of 22 April 1998 concerning the placing on the market of genetically modified maize (Zea mays L. line Bt-11), pursuant to Council Directive 90/220/EEC.

COMMISSION DECISION (98/294/EC) of 22 April 1998 concerning the placing on the market of genetically modified maize (Zea mays L. line MON 810), pursuant to Council Directive 90/220/EEC.

COMMISSION DECISION (2010/419/EU) of 28 July 2010 renewing the authorisation for continued marketing of products containing, consisting of, or produced from genetically modified maize Bt11 (SYN-BTØ11-1), authorising foods and food ingredients containing or consisting of field maize Bt11 (SYN-BTØ11-1) pursuant to Regulation (EC) No 1829/2003 of the European Parliament and of the Council and repealing Decision 2004/657/EC.

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Session 8

Characteristics of the Conventional and Real-time PCR Systems for Qualitative Analysis

Smart screening and matrix approach

The following section contains a general overview of the state of the art of screening for GM elements as well as the matrix approach.

It is appropriate, when testing for the presence of a GMO(s), to plan a stepwise approach. Screening aims at finding out if a sample contains GM material and if so what ingredient(s) it comes from. The screening regime can be set up in a manner that optimizes time, resources and reduces the workload. Testing a sample for every single GMO event authorized for marketing in the EU is time consuming and expensive. The "**matrix approach**" provides a cost and time effective approach to GM screening.

Tools like the **JRC GMO-Matrix** (available at http://gmo-crl.jrc.ec.europa.eu/jrcgmomatrix/) make possible to determine the effect of taxon, element, construct or event specific methods the sample tests positive or negative for. This can help minimize the analytical effort for further analyses. The matrix approach allows the identification of possible events based on the positive and negative methods used to test a sample. Alternatively, the GMOMETHODS database can be used to exclude events based on the taxon, element, construct or event specific methods the sample may test negative for, or include events based on positive test results. In this manner the events in the sample can be narrowed down and the most cost and time effective testing regime can be selected based on test results.

In Figure 1, the schematic representation of targets and corresponding methods.

When looking for GMOs in a sample, the best approach is to first test the sample with **element (2) or construct specific methods (3).** These methods can detect the elements of the transgene (for example a promoter or a terminator), or construct specific combinations of such elements (for example, a specific promoter in combination with a specific gene). In addition to this, **taxon specific methods (1) can be used to** identify the plant species present in the sample.

Once the number of events has been narrowed down to a manageable number by eliminating the possible events present, the sample can then be tested with different **event specific method(s) (4)** that detect the target to uniquely identify a specific GM event(s).

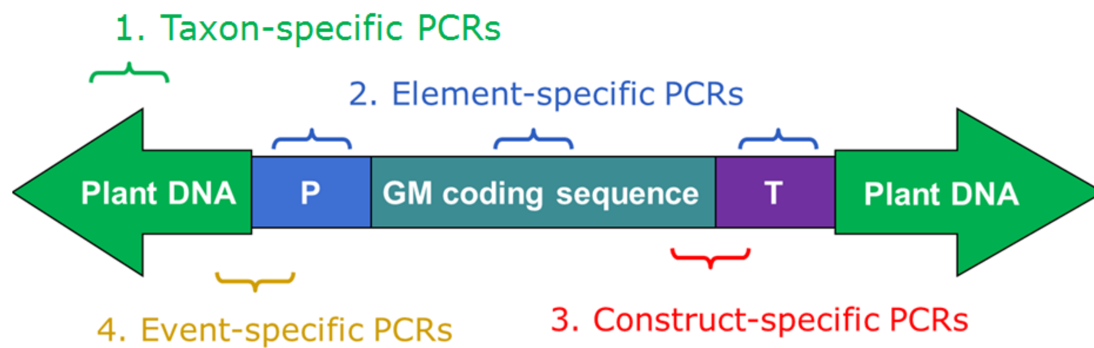

Figure 1. Schematic representation of methods and their targets. P: promoter; T: terminator

Characteristics of the qualitative PCR systems described in the manual

During this course, different detection systems will be used:

- Taxon-specific primers will be used to confirm the presence of soybean and maize and the quality (amplifiability) of the extracted DNA.
- Element specific "screening methods" will be used for the detection of the most common regulatory sequences, the 35S promoter and *nos* terminator.
- Construct specific and event-specific primers will be used to selectively detect and identify the events taken into consideration in the manual: Roundup Ready® soybean, MON810 maize and Bt11 maize.

Please note that the methods mentioned in this manual are only used for didactical scope. For more information on validated methods for detection and quantification of GMOs, please consult the GMOMETHODS database (http://gmo-crl.jrc.ec.europa.eu/gmomethods/).

Taxon specific PCR

Detection of the *lectin* gene

For the identification of soybean DNA a soybean specific method, which targets the species-specific *lectin* gene (*Le1*) will be used (Table 1) (Meyer et al., 1996).

As indicated above, the purpose is to confirm the presence of PCR amplifiable extracted DNA from soybean containing samples. The expected product is an amplicon of **118 bp**.

Table 1. Primers for *lectin* gene

Primer forward: **GM03**	GCCCTCTACTCCACCCCCATCC	target: *lectin* gene
Primer reverse: **GM04**	GCCCATCTGCAAGCCTTTTTGTG	target: *lectin* gene

Detection of the *invertase* gene

Invertase is an enzyme that catalyzes the hydrolysis of sucrose. The *invertase* 1 gene (*ivr1*) is used for the detection of *Zea mays*. A PCR assay (Table 2) will be used to confirm the presence and PCR amplifiable extracted DNA from maize-containing samples. If the target DNA is present, intact and amplifiable, the amplification band is expected to be **225 bp** (ISO/FDIS 21569:2005).

Other genes can be used for the detection of Zea mays, for example, *Alcohol dehydrogenase I (Adh I)* and the *high mobility group (hmg). Examples of taxon specific genes*, especially for use in quantitative analysis can be found at http://gmo-crl.jrc.ec.europa.eu/gmomethods/).

Table 2. Primers for *invertase* gene

Primer forward: **IVR1-F**	CCGCTGTATCACAAGGGCTGGTACC	Target: *ivr1* gene
Primer reverse: **IVR1-R**	GGAGCCCGTGTAGAGCATGACGATC	Target: *ivr1* gene

Screening method: Detection of the CaMV 35S promoter and nos terminator

The detection of the 35S promoter and *nos* terminator by PCR constitutes the so-called "screening methods" for the identification of genetically modified plant-derived foods. The use of the 35S promoter and *nos* terminator as target sequences allows the detection of the majority but not all GM events. In fact, from the GMO matrix it is evident that, for example, maize event MIR604 and soybean event CV127 do not contain these elements. The characteristics of some maize events approved for market introduction in the EU are listed in Table 3 as an example.

Table 3. Characteristics of transgenic maize events carrying the CaMV 35S promoter and/or the *nos* terminator.

Maize Event	35S promoter	*nos* terminator
1507	x	
3272		x
4114	x	
5307		x
59122	x	
Bt11	x	x
GA21		x
MIR604		x
MIR863	x	x
MON810	x	
MON87427	x	x
MON87460	x	x
MON88017	x	x
MON89034	x	x
NK603	x	x
T25	x	

Detection of the CaMV 35S promoter

The cauliflower mosaic virus promoter regulates the gene expression of many transgenic plants such as Roundup Ready® soybean and maize event MON810. For its specific detection, primers 35S-cf3 and 35S-cr4 (Table 4) will be used (Lipp et al., 2001). The expected amplicon size is **123 bp**.

Table 4. Primers for cauliflower mosaic virus promoter

Primer forward: **35S-cf3**	CCACGTCTTCAAAGCAAGTGG	Target: *CaMV* 35S promoter
Primer reverse: **35S-cr4**	TCCTCTCCAAATGAAATGAACTTCC	Target: *CaMV* 35S promoter

Detection of the *nos* terminator

Primers **HA-*nos*118-f** and **HA-*nos*118-r** (Lipp et al., 2001; Table 5) are used for the detection of the *nos* terminator. The *nos* terminator is present in the Roundup Ready® soybean and in several other transgenic lines (e.g. maize line Bt11). Amplification of the *nos* terminator results in the production of a DNA fragment of **118 bp**.

Table 5. Primers for nos terminator

Primer forward: **HA-nos118-f**	GCATGACGTTATTTATGAGATGGG	Target: T-*nos*
Primer reverse: **HA-nos118-r**	GACACCGCGCGATAATTTATCC	Target: T-*nos*

Multiplex detection of the 35S promoter and *nos* terminator by real-time PCR

Real-time PCR became popular for the possibility to quantify the target sequence in a reliable way. However, this technique is also more and more frequently used for qualitative detection purposes, thanks to its intrinsic specificity and due to the fact that it allows analysis and extrapolation of results directly from the instrument, omitting therefore the need for the identification and verification of the PCR product using gel electrophoresis.

For this reason, assays like the duplex real-time PCR for the detection of both 35S and T-nos have found application in routine GM analysis of food products (Table 6).

Even though real-time PCR approaches will be explained in Sessions 10 and 11, it is worth to mention their applicability to qualitative analysis in this section.

Table 6. Primers and probes for duplex real-time PCR for 35S promoter and *nos* terminator (Waiblinger et al., 2008).

Primer forward: **35S-FTM**	5'-GCCTCTGCCGACAGTGGT-3’	target: P-35S
Primer reverse: **35S-RTM**	5'-AAGACGTGGTTGGAACGTCTTC-3’	target: P-35S
Probe: **35S-TMP-FAM**	5'-FAM-CAAAGATGGACCCCCACCCACG-BHQ1-3’	target: P-35S
Primer forward: **180-F**	5’-CATGTAATGCATGACGTTATTTATG-3’	target:T-*nos*
Primer reverse: **180-R**	5’-TTGTTTTCTATCGCGTATTAAATGT-3’	target:T-*nos*
Probe: **TM-180YY**	5’-YY-ATGGGTTTTTATGATTAGAGTCCCGCAA-BHQ1-3’	target:T-*nos*

FAM: 6-Carboxyfluorescein; YY: Yakima Yellow; BHQ: Black Hole Quencher

Construct specific PCR

The amplification primers described in the following session have been designed to specifically detect the genetic structure inserted into the Roundup Ready® soybean, Bt11 and MON810 maize genomes. The methods are part of the ISO/FDIS 21569:2005. For detailed information, please also consult the GMO METHODS: EU Database of Reference Methods for GMO Analysis.

Specific detection of the junction region between the CaMV P-35S and the synthetic CTP sequence in Roundup Ready® soybean.

This method, being a construct specific method, can be considered specific for Roundup Ready® soybean because this is the only GM event carrying this construct. The method (Table 7) etects the junction between the Cauliflower Mosaic Virus 35S promoter (CaMV P-35S) and the chloroplast-transit-peptide (CTP) sequence from *Petunia hybrida epsps* gene. The expected amplicon size is **172 bp**.

Table 7. Primers for the specific detection of *CaMV* P-35S and CTP4

Primer forward: **p35S-af2**	TGATGTGATATCTCCACTGACG	target: *CaMV* P-35S
Primer reverse: **petu-ar1**	TGTATCCCTTGAGCCATGTTGT	target: CTP4

Specific detection of the junction region between IVS2 from the maize *adh1* gene and the synthetic *pat* gene in maize event Bt11.

Again, in Table 8, a construct specific method, detecting the junction between the Intron 2 (IVS2) from the maize alcohol dehydrogenase 1 (*adh1*) gene and the phosphinothricin N-acetyltransferase (*pat*) gene. The expected amplicon size is **189 bp**.

Table 8. Primers for the specific detection of IVS 2 *adh1* and *pat*

Primer forward: **IVS2-2**	CTGGGAGGCCAAGGTATCTAAT	target: IVS 2 *adh1*
Primer reverse: **PAT-B**	GCTGCTGTAGCTGGCCTAATCT	target: *pat*

Specific detection of 5' integration border region (IBR) between the synthetic gene and the host genome of maize event MON810

Finally, in Table 9, an event specific method that detects the 5' integration border region (IBR) between the insert of maize event MON810 and the maize host genome. The expected amplicon size is **170 bp**.

Table 9. Primers for the specific detection of 5' integration border region (IBR)

Primer forward: **VW01**	TCGAAGGACGAAGGACTCTAACG	Target: 5'-host genome
Primer reverse: **VW03**	TCCATCTTTGGGACCACTGTCG	Target: *CaMV* P-35S

References

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Session 9

Detection of MON810 Maize, Bt11 Maize and Roundup Ready® Soybean by Conventional PCR

Experimental

Introduction

The following protocols are PCR-based methods allowing the screening of GMOs (using the 35S promoter and the *nos* terminator) and the detection of specific GMOs (Roundup Ready® soybean, MON810 maize and Bt11 maize) in raw and processed food, by comparison with corresponding non-GM samples (soybean and maize).

The following methods allow only a qualitative result by indicating the presence/absence of the target sequence in the sample following gel electrophoresis to identify and verify the presence of the PCR product[37].

Equipment

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

REMARK

All plasticware should be DNA-free and where possible, sterilised prior to use.

In order to avoid contamination, filter pipette tips should be used to minimize aerosol formation.

Reagents

- dATP CAS 1927-31-7
- dCTP CAS 2056-98-6
- dGTP CAS 2564-35-4
- dTTP CAS 365-08-2
- 10 x PCR buffer (usually delivered from the same supplier as the *Taq* DNA polymerase)
- 25 mM $MgCl_2$
- *Taq* DNA polymerase 5 U/µL
- Upstream and downstream oligonucleotides (i.e. forward and reverse primers)

[37] Whenever methods of this version of the manual have substituted older ones, it is because validated and/or ISO standard methods have been preferred upon the older methods.

- Nuclease-free water

4 mM dNTP stock solution

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

20 µM primer solutions

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

- Prepare 20 µM primer solution according to the supplier's instructions.
  - 1 µM = 1 pmol/µL so 20 µM = 20 pmol/µL
  - X nmol primer + 10X µL sterile water = 100 pmol/µL = 100 µM
  - Incubate 5 min at 65°C, shake and incubate for another 3 min at 65°C
  - Dilution 1:5 ➔ Prepare 1 microcentrifuge tube with 400 µL sterile water and add 100 µL of the primer solution ➔ (100 µM) Final concentration: 20 µM
- Divide into small aliquots and store at -20°C. The aliquots stored at -20°C are stable for at least 6 months; the lyophilised primers are stable at -20°C for up to three years unless otherwise stated by the manufacturer.

10x PCR buffer

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

25 mM $MgCl_2$ solution

"PCR grade" $MgCl_2$ solution is generally supplied together with the *Taq* DNA polymerase and is ready to use. The solution should be mixed (vortex) before use and briefly centrifuged (to eliminate the formation of a concentration gradient that can form after prolonged storage). Store at -20°C.

Nuclease-free water aliquots

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

Taxon specific PCR: soybean-*lectin*

The identification of soybean DNA is performed using the *lectin* gene as PCR target.

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

Characteristics of primers GM03 and GM04

GM03	
Sequence	GCCCTCTACTCCACCCCCATCC
Length	22
Mol. Weight (g/mol)	6471.6
Melting point * (G/C)	65.1

GM04	
Sequence	GCCCATCTGCAAGCCTTTTTGTG
Length	23
Mol. Weight (g/mol)	6981.1
Melting point * (G/C)	59.6

*based on a [Na^+] of 50 mM

Controls

It is important to always include the necessary controls with every PCR reaction. A negative or blank control is used to check if the PCR reagents are contaminated with taxon specific DNA. A positive control for the specific DNA target is critical to confirm the the sensitivity and specificity of the PCR assay.

The following PCR controls must be included in each assay:

- Positive control: pure DNA, isolated from the conventional soybean

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

Mastermix preparation

The necessary reagents for a series of 10 samples (including positive/negative/no template controls) are mixed together according to the instruction given in Table 1. The following procedure applies to a sample containing 20 µL of GMO3/GMO4 Mastermix and 5 µL of DNA solution. All solutions are stored on ice during the preparation of the Mastermix.

Table 1. GM03/GM04 Mastermix

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

- Prepare a 1.5 ml microcentrifuge tube
- Add the reagents in the order given in Table 1
- Mix gently the GMO3/GMO4 Mastermix by pipetting and/or brief vortexing followed by centrifugation
- Divide the Mastermix into aliquots of 20 µL in 0.2 ml PCR reaction tubes
- Add 5 µL of the DNA solution to the aliquots of Mastermix (note: dilute the DNA at 10-20 ng/µL)
- Vortex and centrifuge briefly
- Place the PCR reaction tubes in the thermocycler

PCR program* (GM03/GM04)

Stage	Temperature	Time
Activation/Initial Denaturation	95°C	10 min
Denaturation	95°C	30 sec
Annealing	60°C	30sec
Extension	72°C	1 min
Number of cycles	35	
Final Extension	72°C	3 min
	4°C	∞

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

*<u>**Note**</u>: The use of a different thermocycler model or brand shall produce a similar result provided that the PCR programme is adapted and tested accordingly[38].

Analysis of PCR products

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

[38] The JRC does not endorse any equipment used during the training courses or mentioned in this manual. The analysis performed in our laboratories should be easily reproducible using alternative equipment, provided the differing characteristics of the system used are taken into account.

Interpretation of the results

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

The positive control will amplify a band at 118 bp. There should be no band(s) present in the negative and the no-template control after gel electrophoresis.

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

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

Taxon specific PCR: maize-*invertase*

The identification of maize DNA is performed targeting the *invertase* gene (ivr1).

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

Characteristics of primers IVR1-F and IVR1-R

IVR1-F	
Sequence	CCGCTGTATCACAAGGGCTGGTACC
Length	25
Mol. Weight (g/mol)	7643
Melting point * (G/C)	63.2

IVR1-R	
Sequence	GGAGCCCGTGTAGAGCATGACGATC
Length	25
Mol. Weight (g/mol)	7732
Melting point * (G/C)	62.8

*based on a [Na^+] of 50 mM

Controls

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

Mastermix preparation

The necessary reagents for a series of 10 samples (including positives/negative/no template controls) are mixed together as indicated in Table 2.

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

Table 2. IVR1-F/IVR1-R Mastermix

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

- Prepare a 1.5 ml microcentrifuge tube
- Add the reagents following the order given in Table 2
- Mix gently the IVR1-F/IVR1-R Mastermix by pipetting and centrifuge briefly
- Divide the Mastermix into aliquots of 20 µL in 0.2 ml PCR reaction tubes
- Add 5 µL of the DNA solution to the aliquots of Mastermix (note: dilute the DNA at 10-20 ng/µL)
- Vortex and centrifuge briefly
- Place the PCR reaction tubes in the thermocycler

PCR program (IVR1-F/IVR1-R)

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

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

Analysis of PCR products

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

Interpretation of the results

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

The positive control should amplify a DNA fragment of 225 bp.

The negative control and the no-template should not display any PCR amplification.

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

If the controls give the expected results and the sample shows absence of the 225 bp band, then, provided that the DNA is not inhibited, no amplifiable maize DNA is present in the sample. Please note that this method is being used only for didactical purposes and other options for the detection of taxon specific targets in maize are available.

Screening method for the detection of Genetically Modified Plants

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

In Roundup Ready® soybean as well as in Bt11, the identification of both the 35S promoter and the *nos* terminator is possible, whereas only the 35S promoter is present in the MON810 maize line.

Detection of the CaMV 35S promoter

Characteristics of primers p35S-cf3 and p35S-cr4

p35S-cf3	
Sequence	CCACGTCTTCAAAGCAAGTGG
Length	21
Mol. weight (g/mol)	6414.5
Melting point * (G/C)	57.4

p35S-cr4	
Sequence	TCCTCTCCAAATGAAATGAACTTCC
Length	25
Mol. weight (g/mol)	7544.2
Melting point * (G/C)	56.3

*based on a [Na^+] of 50 mM

Controls

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

Mastermix preparation

The necessary reagents for a series of 10 samples (including positive/negative/no template controls) are mixed together according to the instructions given in Table 3.

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

Table 3. p35S-cf3/p35S-cr4 Mastermix

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

- Prepare a 1.5 ml microcentrifuge tube
- Add the reagents following the order given in Table 3
- Mix gently the p35S-cf3/p35S-cr4 Mastermix by pipetting and centrifuge briefly
- Divide the Mastermix into aliquots of 20 µL in 0.2 ml PCR reaction tubes

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

PCR program (p35S-cf3/p35S-cr4)

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

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

Analysis of PCR products

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

Interpretation of the results

The primer pair p35S-cf3/p35S-cr4 is used for detection of the CaMV 35S promoter. This promoter regulates the gene expression of many transgenic plants such as Roundup Ready® soybean and maize line Bt11. If the extracted DNA is of sufficient amplification quality and contains the target, a specific band of **123 bp** will be observed on the gel.

The positive control should amplify a DNA fragment of 123 bp.

The negative control and the no-template should not display any PCR amplification.

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

If the controls give the expected results and the sample gives a band at 123 bp, it can be assumed that this sample contains GM DNA.

Detection of the *nos* terminator

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

HA-*nos* 118-f	
Sequence	GCATGACGTTATTTATGAGATGGG
Length	24
Mol. weight (g/mol)	7462.8
Melting point * (G/C)	56.2

HA-*nos* 118-r	
Sequence	GACACCGCGCGATAATTTATCC
Length	24
Mol. weight (g/mol)	7296.9
Melting point * (G/C)	61.2

*based on a [Na^+] of 50 mM

Controls

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

Mastermix preparation

The necessary reagents for a series of 10 samples (including positive/negative/no template controls) are mixed together according to the instructions given in Table 4.

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

Table 4. HA-*nos*118-f/HA-*nos*118-r Mastermix

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

- Prepare a 1.5 ml microcentrifuge tube
- Add the reagents following the order given in Table 4
- Mix gently the HA-*nos*118-f/HA-*nos*118-r Mastermix by pipetting and centrifuge briefly
- Divide the Mastermix into aliquots of 20 µL in 0.2 ml PCR reaction tubes
- Add 5 µL of the DNA solution to the aliquots of Mastermix (note: dilute the DNA at 10-20 ng/µL
- Vortex and centrifuge briefly
- Place the PCR reaction tubes in the thermocycler

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

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

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

Analysis of PCR products

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

Interpretation of the results

The primer pair HA-*nos*118-f/HA-*nos*118-r is used for detection of the *nos* terminator. This terminator is present in the Roundup Ready® soybean and other lines of transgenic plants (e.g. Maize line Bt11). If the extracted DNA is of sufficient amplification quality and contains the target, a specific band of **118 bp** will be observed on the gel.

The positive control should amplify a DNA fragment of 118 bp.

The negative control and the no-template should not display any PCR amplification.

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

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

Specific detection of Roundup Ready® soybean, MON810 maize and Bt11 by conventional PCR

Detection of Roundup Ready® soybean

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

Characteristics of primers

P35S-af2	
Sequence	TGATGTGATATCTCCACTGACG
Length	22
Mol. weight (g/mol)	6725,4
Melting point * (G/C)	53

petu-ar1	
Sequence	TGTATCCCTTGAGCCATGTTGT
Length	22
Mol. weight (g/mol)	6707,4
Melting point * (G/C)	53

*based on a [Na^+] of 50 mM

Controls

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

Mastermix preparation

The necessary reagents for one sample (including positive/negative/no template controls) are mixed together according to the instructions given in Table 5.

The following procedure applies to a sample containing 20 µL of P35S-af2/petu-ar1 Mastermix and 5 µL of DNA solution. All solutions are stored in ice during the preparation of the Mastermix.

Table 5. P35S-af2/petu-ar1 Mastermix

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

- Prepare a 1.5 ml microcentrifuge tube
- Add the reagents following the order given in Table 5
- Mix gently the P35S-af2/petu-ar1 Mastermix by pipetting and centrifuge briefly
- Divide the Mastermix into aliquots of 20 µL in 0.2 ml PCR reaction tubes
- Add 5 µL of the DNA solution to the aliquots of Mastermix (note: dilute the DNA at 10-20 ng/µL
- Vortex and centrifuge briefly
- Place the PCR reaction tubes in the thermocycler

PCR program (P35S-af2/petu-ar1)

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

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

Analysis of PCR products

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

Interpretation of the results

The primer pair P35S-af2/petu-ar1 is used for detection of the construct present in Roundup Ready® soybean and stacked events of this GM crop. If the extracted DNA is of sufficient amplification quality and contains the target, a specific band of **172 bp** will be observed on the gel.

The positive control should amplify a DNA fragment of 172 bp.

The negative control and the no-template should not display any PCR amplification.

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

If the controls give the expected results and the sample gives a band at 172 bp it can be assumed that in this sample Roundup Ready® soybean DNA is present.

Detection of maize Bt11

This is a construct specific method for the detection of genetically modified *Bacillus thuringiensis* toxin-producing Bt11 maize (Syngenta, former Novartis) in raw materials by PCR amplification of the junction region of single copy sequence elements originating from the maize *adh* 1S-Intron2 (IVS2) and the *pat* gene from *Streptomyces viridochromogenes*.

Characteristics of primers

IVS2-2	
Sequence	CTGGGAGGCCAAGGTATCTAAT
Length	22
Mol. weight (g/mol)	6799.5
Melting point * (G/C)	54.8

PAT-B	
Sequence	GCTGCTGTAGCTGGCCTAATCT
Length	22
Mol. weight (g/mol)	6717.4
Melting point * (G/C)	56.7

*based on a [Na^+] of 50 mM

Controls

- Positive control: DNA from reference material (Bt11 1% GM)
- Negative control: DNA from reference material (maize 0% GM)
- No-template control (NTC): negative control of the Mastermix, in which water is used instead of DNA

Mastermix preparation

The necessary reagents for a series of 10 samples (including positive/negative/no template controls) are mixed together according to Table 6.

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

Table 6. IVS2-2/PAT-B Mastermix

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

- Prepare a 1.5 ml microcentrifuge tube
- Add the reagents following the order given in Table 6
- Mix gently the P35S-af2/petu-ar1 Mastermix by pipetting and centrifuge briefly
- Divide the Mastermix into aliquots of 20 µL in 0.2 ml PCR reaction tubes
- Add 5 µL of the DNA solution to the aliquots of Mastermix (note: dilute the DNA at 10-20 ng/µL
- Vortex and centrifuge briefly
- Place the PCR reaction tubes in the thermocycler

PCR program (IVS2-2/PAT-B)

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

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

Analysis of PCR products

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

Interpretation of the results

The primer pair IVS2-2/PAT-B is used for detection of the construct present in Bt11 maize and stacked events of this GM crop. If the extracted DNA is of sufficient amplification quality and contains the target, a specific band of **189 bp** will be observed on the gel.

The positive control should amplify a band of 189 bp.

The negative control and the no-template should not display any PCR amplification.

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

If the controls give the expected results and the sample gives a band at 189 bp it can be assumed that in this sample Bt11 DNA is present.

Detection of maize MON810

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

Characteristics of primers

VW01	
Sequence	TCGAAGGACGAAGGACTCTAACG
Length	23
Mol. weight (g/mol)	7106.7
Melting point * (G/C)	57.1

VW03	
Sequence	TCCATCTTTGGGACCACTGTCG
Length	22
Mol. weight (g/mol)	6677.4
Melting point * (G/C)	56.7

*based on a [Na^+] of 50 mM

Controls

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

Mastermix preparation

The necessary reagents for one sample are mixed together according to Table 7.

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

Table 7. VW01/VW03 Mastermix

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

- Prepare a 1.5 ml microcentrifuge tube
- Add the reagents following the order given in Table 7
- Mix gently the P35S-af2/petu-ar1 Mastermix by pipetting and centrifuge briefly
- Divide the Mastermix into aliquots of 20 µL in 0.2 ml PCR reaction tubes
- Add 5 µL of the DNA solution to the aliquots of Mastermix (note: dilute the DNA at 10-20 ng/µL)
- Vortex and centrifuge briefly
- Place the PCR reaction tubes in the thermocycler

PCR program (VW01/VW03)

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

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

Analysis of PCR products

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

Interpretation of the results

The primer pair VW01/VW03 is used for detection of the construct present in MON810 maize and stacked events of this GM crop. If the extracted DNA is of sufficient amplification quality and contains the target, a specific band of **170 bp** will be observed on the gel.

The positive control should amplify a band of 170 bp.

The negative control and the no-template should not display any PCR amplification.

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

If the controls give the expected results and the sample gives a band of 170 bp it can be assumed that in this sample MON810 DNA is present.

Session 10

Real-time PCR and Quantitative Analysis of GMOs

Introduction

Once a food product has been found to be positive for one or more GM events (e.g. Roundup Ready® soybean, Bt-11 maize, MON810 maize, and T25 maize), the subsequent analytical steps consist of assessing compliance with the legislation in force, (EU Regulation (EC) 1829/2003 and Regulation (EC) 1830/2003) by measuring the amount of each GMO event present in the individual ingredients (Figure 1).

The above-mentioned Regulations establish that all products consisting of or containing GMOs, or produced from GMOs must be labelled as such, if the GM ingredient is present in a proportion higher than 0.9%. Labelling is not required for products containing materials which contain, consist of, or are produced from GMOs in a proportion no higher than 0.9% of the food ingredients considered individually, provided that this presence is adventitious or technically unavoidable.

All the ingredients (flour, grid, oil, etc.) derived from one species (e.g. maize, soybean, rapeseed, etc.) are considered collectively as one individual ingredient (e.g. maize).

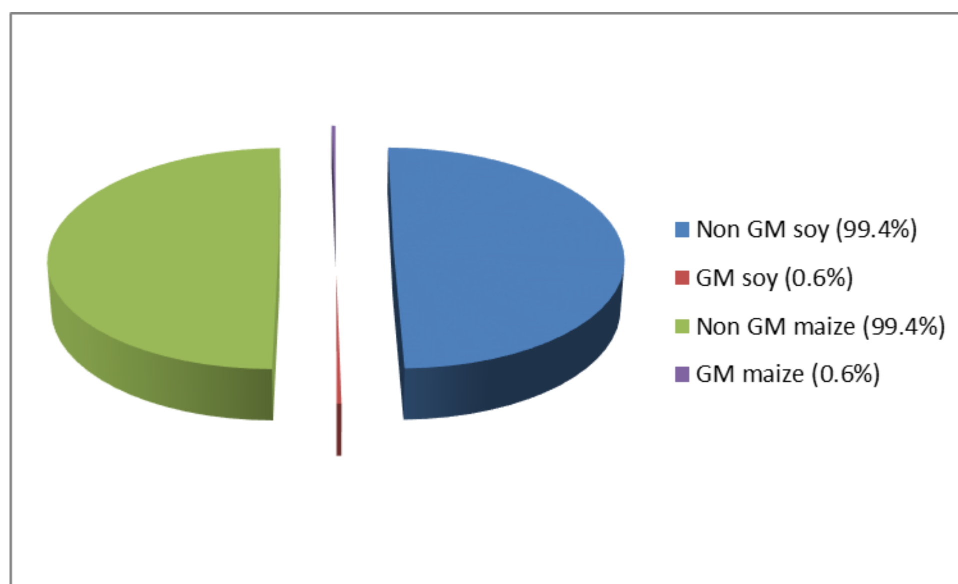


Figure 1. No labelling required. The amount of both GM soybean and GM maize is below the legal threshold.

If, for instance, an ingredient exclusively derived from maize contains less than 0.9% GM maize, no labelling is necessary for the foodstuff derived from it. If, on the other hand, it contains more than 0.9% GM maize, the derived food products must be labelled. This is also true even if in the final product, considering the sum of all the ingredients derived from different species (e.g. soy and maize), the relative amount of GM maize drops below 0.9%. If two or more different GM maize varieties/events are present, their concentrations should be summed up, and the total percentage used to determine the requirement for

labelling (Figure 2). If the resulting sum is below the 0.9% threshold, no labelling is required.

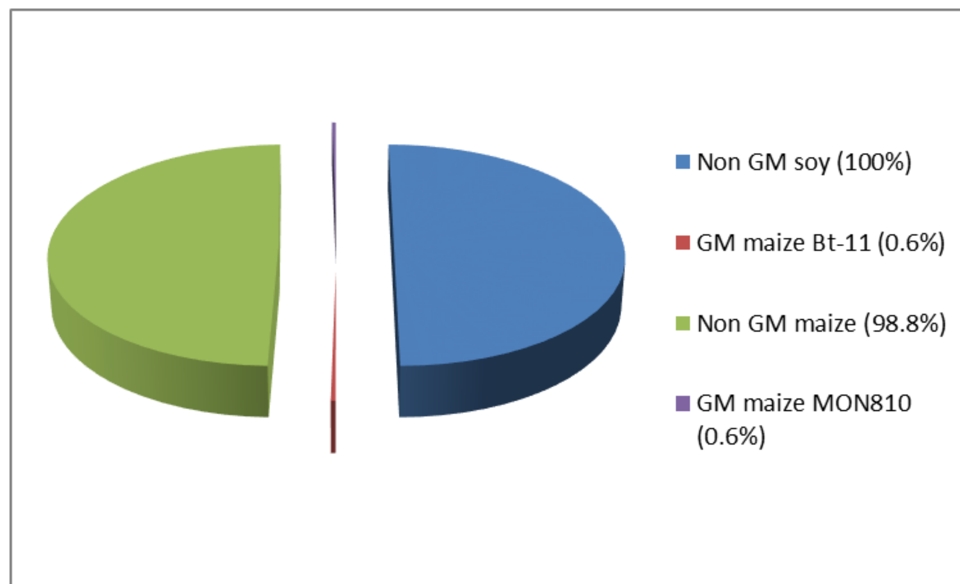


Figure 2. Labelling required for the maize ingredient. The sums of the Bt-11 (0.6%) and MON810 (0.6%) events exceed the 0.9% threshold for labelling.

The relative GMO content (percentage) is determined by normalising the amount of the GMO specific sequences against the amount of a plant specific gene (e.g. *lectin* for soybean and *invertase* for maize). The resulting GMO percentage is therefore expressed as GMO (%) = GM-DNA/reference-DNA x 100.

PCR methods for quantification

A major drawback of conventional PCR is the lack of accurate quantitative information due to amplification efficiency. If the reaction efficiency for each amplification cycle remains constant, the concentration of DNA following PCR would be directly proportional to the amount of initial DNA target. Unfortunately, the amplification efficiency varies among different reactions, as well as in subsequent cycles in a single reaction. In particular, in the later cycles of the PCR, the amplification products are formed in a non-exponential way at an unknown reaction rate.

DNA quantification based on conventional PCR relies on end-point measurements, in order to achieve the maximum sensitivity, when the amplification reaches the maximum product yield (known as the "plateau phase"). At this stage, the reaction has gone beyond the exponential phase primarily due to depletion of reagents and the gradual thermal

inactivation of the polymerase used. The resulting correlation between the final product concentration and number of initial target molecules is therefore limited.

To overcome this problem, real-time PCR method, have been developed, which address the problems of establishing a relationship between the concentration of target DNA and the amount of PCR product generated by amplification.

Real-time PCR

A more accurate and currently more widely used quantitative PCR methodology is represented by real-time PCR. In contrast to the end-point determinations, real-time PCR systems monitor the reaction as it actually occurs, in real time. In this kind of system the PCR reaction is coupled to the emission of a fluorescent signal, being proportional to the amount of PCR product produced in subsequent cycles. This signal increases proportionally to the amount of PCR product generated in each successive reaction cycle. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during its exponential phase. The first significant increase of fluorescence correlates to the initial amount of target template (Ahmed, 2002).

Real-time PCR principles

Higuchi et al. (1992, 1993) pioneered the analysis of PCR kinetics by setting-up a system able to detect PCR products as they accumulate. This "real-time" system included the intercalating molecule ethidium bromide in each reaction mix. A thermal cycler adapted to irradiate the samples with UV light, and able to detect the resulting fluorescence with a computer-controlled cooled CCD (charged coupled device) camera was used to perform the runs. As amplification occurred, increasing amounts of double-stranded DNA produced, and intercalated by ethidium bromide, resulted in an increase in fluorescence. By plotting the fluorescent light emission versus the cycle number, the system produced amplification plots providing a more complete picture of the PCR process than assaying product accumulation after a fixed number of cycles.

Today, real-time PCR is the most widely accepted method, not only for quantification, but also for identification.

The specificity of a real-time PCR method depends both on the chemistry used to generate and monitor the amplification reaction and the instrument used to monitor the signal. Various chemistries have been developed for this purpose: intercalating dyes (ethidium bromide, SYBR Green I) and hybridisation probes (TaqMan probes, Fluorescence Resonance Energy Transfer probes, Molecular Beacons, Scorpions and TaqMan Minor Groove Binder probes).

SYBR Green I dye based real-time PCR

The first real-time PCR application was directly derived from the experiments by Higuchi et al. (1992, 1993) substituting ethidium bromide with a less toxic and more specific and sensitive (from 10 to 25 times) fluorescent double stranded (ds) DNA intercalating agent, SYBR Green I (Haugland, 2002).

SYBR Green I dye binds to the minor groove of dsDNA, but not to single stranded DNA. As a consequence of binding, fluorescence (excitation approx. 488 nm and 254 nm; emission approx. 560 nm) is greatly enhanced (approx. from 800 to 1000 times). As the PCR proceeds, the increasing amount of newly synthesised DNA results in an increasing fluorescent signal (Figure 3). A limitation of the SYBR Green I based sequence detection system is represented by its non-specific DNA recognition mode. In fact, every double-stranded DNA molecule present in a PCR reaction is quantified, including therefore non-specific PCR products and primer-dimers. To overcome the problem and subtract the quantification component due to non-specific DNAs and to primer dimers on some devices, it is possible to perform a melting curve analysis. After the final stage of PCR, the products are slowly melted (melting curve analysis) and fluorescence data collected. Since every dsDNA has a specific melting temperature, it is possible to quantify the components having different melting temperatures in one single reaction mix, and therefore to eliminate the non-specific components from the quantification.

Sequence specific probes based real-time PCR methods

The problem of amplicon fluorescent detection specificity has been overcome using sequence specific probes with fluorescent labelling designed inside the PCR primers pair. The process of probe hybridisation (and eventual degradation) usually does not interfere with the exponential accumulation of the PCR product. A few different principles are now used to achieve specific real-time PCR based quantification reactions.

Fluorescence Resonance Energy Transfer (FRET) probes

Fluorescence Resonance Energy Transfer (FRET) is based on the energy transfer from a donor fluorophore to an acceptor fluorophore (Figure 3) (Haugland, 2002). Basic conditions for the FRET are:

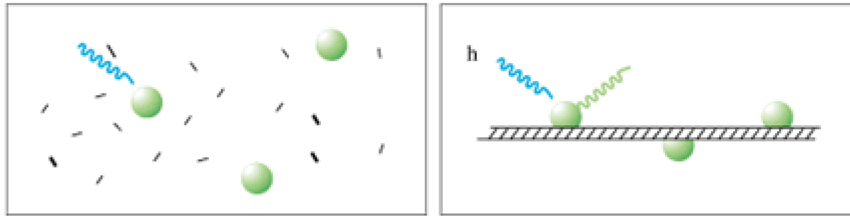
- Donor and acceptor molecules must be in close proximity (typically 10–100 Å);
- The absorption spectrum of the acceptor must overlap with the fluorescence emission spectrum of the donor;
- Donor and acceptor transition dipole orientations must be approximately parallel.

If the donor and the acceptor fluorophore are in close proximity to each other, excitation of the donor by blue light results in energy transfer to the acceptor, which can then emit light of longer wavelength. The formation of PCR products can be monitored by using two sequence specific, oligonucleotide probes with a fluorescent label, called hybridisation probes, in addition to the PCR primers. Hybridisation probes are designed as a pair of which one probe is labelled with the donor (3’-Fluorescine) and one with the acceptor (5’- Red 640 or 5’-Red 705) dye. As FRET decreases with the sixth power of distance, hybridisation probes have to be designed to hybridise to adjacent regions of the template DNA (usually they are separated by a 1-5 nucleotides gap). If both probes hybridise, the two dyes are brought close together and FRET to the acceptor dye results in a signal measurable by means of fluorometry.

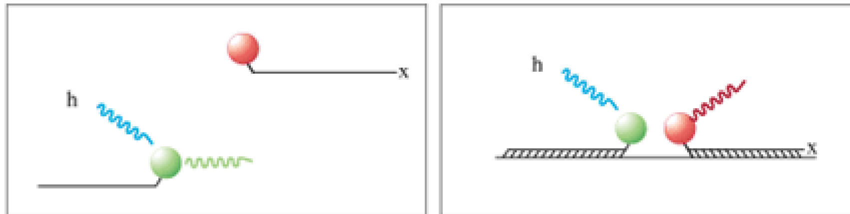
Degradation probes (TaqMan principle)

The TaqMan assay exploits the 5'-3' exonuclease activity of *Taq* DNA Polymerase to cleave a degradation probe during PCR (Lie and Petropoulos, 1998). The degradation, or TaqMan, probe is typically a 20-30 base long oligonucleotide (usually with a Tm 10°C higher than the Tm of the primers) that contains a reporter fluorescent dye at the 5' and a quenching dye at the 3' end (Figure 3). Since the 3' end is blocked, the probe cannot be extended like a primer. During the PCR reaction, in the presence of a target, the probe specifically anneals between the forward and reverse primer sites. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Forster-type energy transfer (Forster, 1948; Lakowicz, 1983). During the reaction, the 5’-3’ exonuclease activity of the *Taq* DNA Polymerase degrades the probe between the reporter and the quencher dyes only if the probe hybridises to the target. This results in an increase of the fluorescence as amplification proceeds. Accumulation of PCR product is detected by monitoring the increase in fluorescence of the reporter dye. This process occurs at every cycle and does not interfere with the exponential accumulation of product. Different from FRET probes, degradation probes release fluorochromes at each cycle adding new dye to the previous one released. As a consequence, the fluorescent signal is greatly enhanced at each cycle. TaqMan assay uses universal thermal cycling parameters and PCR reaction conditions. One specific requirement for fluorogenic probes is that there is no G at the 5' end. A 'G' adjacent to the reporter dye quenches reporter fluorescence even after cleavage.

I. SYBR Green



II. Hybridization Probes



III. TaqMan Probes

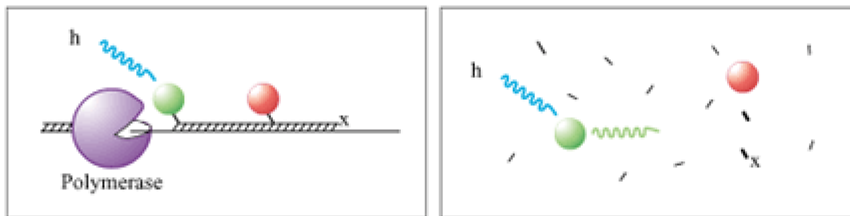


Figure 3. Different real-time PCR principles: I. SYBR green; II. FRET (Fluorescence Resonance Energy Transfer) probes; III. TaqMan 5`-3`- degradation probes[39].

TaqMan MGB probes

A Minor Groove Binder (MGB) is a small crescent-shaped molecule that fits snugly into the minor groove of duplex DNA (Kutyavin et al., 2000). In TaqMan probes, the MGB group is attached at the 3' end along with the quencher dye (Figure 4). When the TaqMan probe hybridises, the MGB stabilizes annealing by folding into the minor groove of the DNA duplex created between the probe and the target sequence. Stabilisation is much more effective when the duplexes are perfectly matched (i.e. when there are no sequence mismatches). Besides the added discriminatory power, the increased stability means TaqMan MGB Probes are very short (typically 13–20 mer) compared to standard TaqMan probes (typically 18–40 mer) while still satisfying design guidelines. TaqMan MGB Probes have several advantages for quantitative PCR, especially for multiplex assays. Improved spectral performance allows greater precision and consistency between individual assays and the greater hybridisation specificity enables enhanced target discrimination. Furthermore, the smaller probe can make it easier to design assays by providing more scope for fitting

[39] Pictures taken from http://dyes.gene-quantification.info/

probes within shorter target regions such as consensus "windows" of sequence conservation or divergence. Amplicon size can be reduced to a minimum by using shorter MGB probes that can further improve inter-assay consistency.

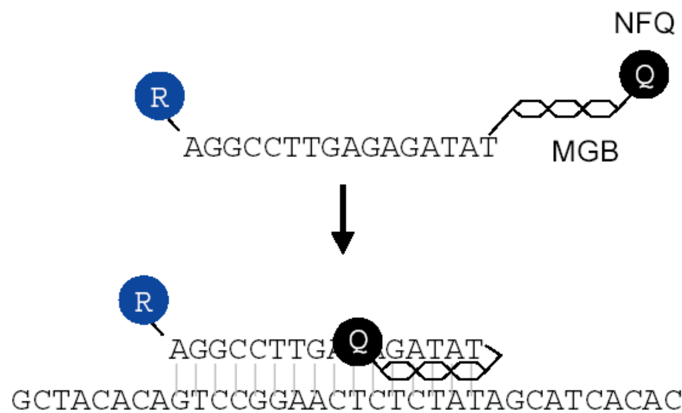


Figure 4. The principle of Minor Groove Binder (MGB) probes

Scorpion probes

A further evolution is represented by the family of probes called "Scorpions". A Scorpion consists of a specific probe sequence with a stem-loop structure (Figure 5) (Thelwell et al., 2000).

A fluorophore is attached to the 5' end giving a fluorescent signal that is quenched in the stem-loop configuration by a moiety joined to the 3' end. The stem-loop is linked to the 5' end of a primer. After the extension of the Scorpion primer, during amplification, the specific probe sequence is able to bind to its complement within the same strand of DNA. This hybridisation event opens the hairpin loop so that fluorescence is no longer quenched and an increase in signal is observed. A PCR stopper between the primer and the stem sequence prevents read-through of the hairpin loop, which could lead to the opening of the hairpin loop in the absence of the specific target sequence. The unimolecular nature of the hybridisation event gives rise to some advantages over homogeneous probe systems. Unlike Molecular Beacons, TaqMan or FRET assays, Scorpion assays do not require a separate probe besides the primers.

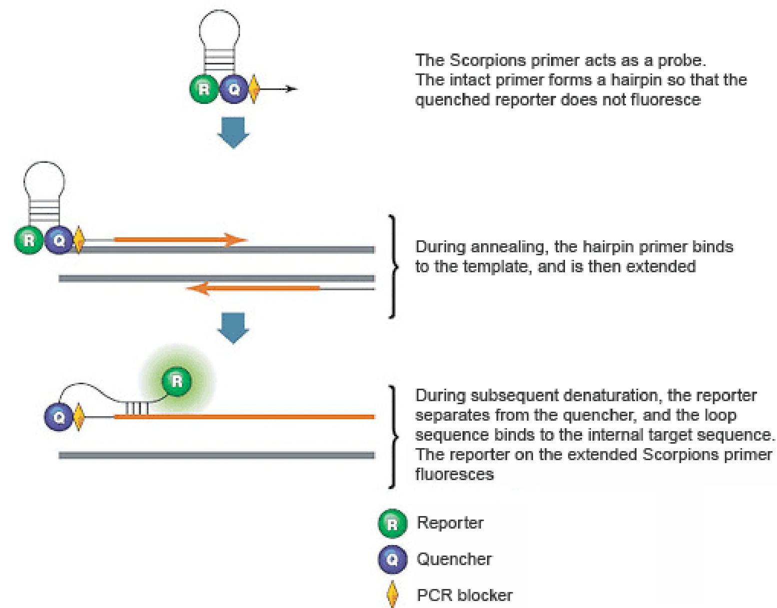


Figure 5. The principle of Scorpion probes (Bio-rad)[40].

Molecular Beacons

Molecular Beacons are DNA probes designed to contain a stem-loop structure. The loop sequence is complementary to the specific target of the probe and the stem sequences are designed to be complementary to each other (Figure 6) (Tyagi and Kramer, 1996). The 5' and 3' ends of the probe are covalently bound to a fluorophore and a quencher. When the stem-loop structure is closed the fluorophore and the quencher are close together. In this case, all photons emitted by the fluorophore are absorbed by the quencher. In the presence of a complementary sequence, the probe unfolds and hybridises to the target. The fluorophore is displaced from the quencher, and the probe starts to emit fluorescence.

Molecular beacons and scorpion probes are more sophisticated; therefore, they are usually used for research purposes and not for routine analysis.

[40] Photo taken from http://www.bio-rad.com/it-it/applications-technologies/introduction-pcr-primer-probe-chemistries?ID=LUSOJW3Q3, accessed 06/08/2019.

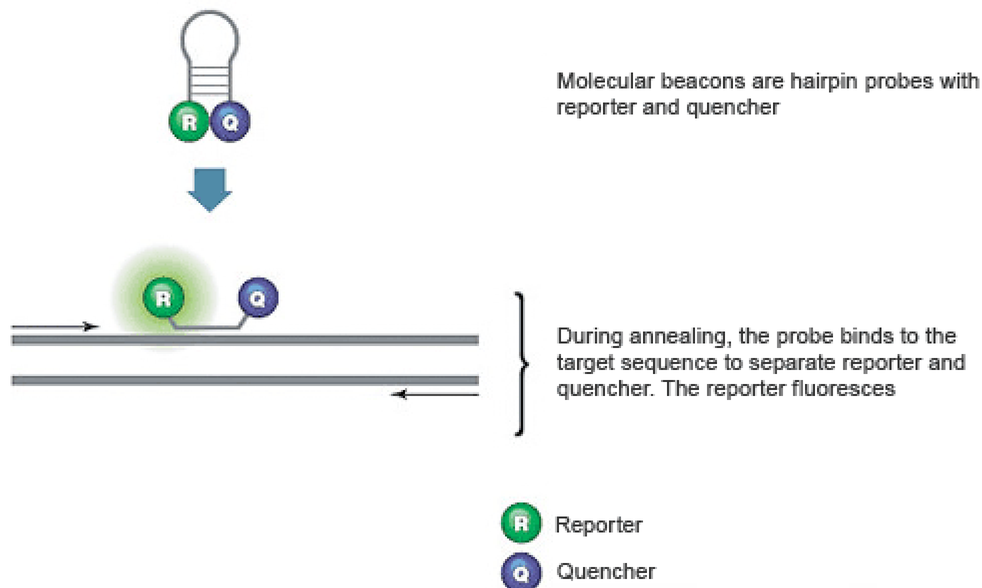


Figure 6. The principle of Molecular Beacons (Bio-rad)[41].

Quantification with real-time PCR

Relative quantification

The GMO content of a sample can be expressed as the amount of genetically modified material in the amount of the total material. In order to determine this value in a real-time PCR based system it is necessary to measure the number of GMO specific target DNA sequences as well as the number of DNA sequences of an endogenous reference gene (for use as a "normaliser"). The reference gene should be chosen in order to be species specific, present as a single copy per haploid genome, stably represented as such in different lines of the same species and as amplifiable as the GMO traits in analysis (although this is more due to a good primers-probe design). One problem in relative quantification arises from the interpretation of percentage of GMO content that is not specified in the legislation; therefore, the GM content (percentage) can be assumed as the weight of the pure modified ingredient over the total weight of the pure ingredient (e.g. weight of GM maize over total weight of entire maize contained in the sample). From the analytical point of view, it is appropriate to calculate the GMO percentage as the number of target DNA sequences per target taxon specific sequences, however this definition does not take some important characteristics of the GMO lines; therefore the following parameters need to be carefully considered in the interpretation of results:

[41] Photo taken from http://www.bio-rad.com/it-it/applications-technologies/introduction-pcr-primer-probe-chemistries?ID=LUSOJW3Q3 accessed 06/08/2019.

a) The ploidy of the event. It is possible that the GM event has a different ploidy from the wild type (wt) event (e.g. tetraploid instead of diploid);
b) The zygosity of the event. The GM trait could be homozygous or hemizygous;
c) The number of insertions per haploid genome of one single artificial construct. One construct could be inserted as a single copy per haploid genome or in more copies.

The last point can be bypassed by designing the primer-probe system on the border of the insertion of the construct in the genome. Since border sequences are unique this will give the double advantage to the system of being event-specific and of excluding multiple insertions of the same construct from the quantification. Point a) and b) are bypassed empirically by the use of reference materials as quantification is performed in relation to the reference material of the same origin (e.g. maize flour reference material to quantify maize flour). Alternatively, quantification standards different from certified reference materials (e.g. cloned DNA sequences or genomic DNA mixtures) can be calibrated against certified reference materials (CRMs) in order to correct molecular discrepancies in quantification. A widely accepted way to solve problems related to points a) and b) is expressing the GMO percentage in terms of haploid genome equivalents.

In every case, this aspect of quantification should be taken into account when a method is developed, since the limit of detection (LOD) and the limit of quantification (LOQ) are influenced by the real number of copies being quantified.

Design of a real-time GMO quantification experiment

The design of a real-time PCR analysis must include the following components:

- A PCR system to detect a specific GM event.
- A PCR system that is taxon specific; designed to detect and quantify a species specific gene. This is a very important part of the PCR set up because it will allow us to calculate the GMO percentage over the total ingredient (used as a "normalizer" in the calculation of the GM relative concentrations).
- Standard curves for both the target and endogenous reference. For each experimental sample, the amount of target and endogenous reference is determined from the appropriate standard curve. The amount of target is normalised with the endogenous reference quantity to obtain the relative concentration of the target. To meet statistical requirements, the standard curves should include at least 4 different concentration points. Each point of the standard curve, and the sample, should be loaded at least in triplicate.
- In addition to that, a negative control (NTC – no template control) has to be added for both the reference gene and the GMO quantifications.

- Extraction blank control, environmental controls and positive extraction controls (CRMs) are usually used, as previously stated (session 6) to check the efficiency of the procedure and to check for contamination. Finally, the reference gene quantification and the GMO specific sequence quantification should occur in the same PCR run (co-amplification), and not in different runs, to avoid possible statistical fluctuations between different experiments.

Real-time PCR data

The output of the real-time PCR is a ΔRn, being the difference between Rn^+ (the fluorescence signal including all components) and Rn^- (the background signal of the reaction baseline or reading of a NTC sample).

Graphical analysis of real-time PCR data

As real-time PCR is proceeding, fluorescence data (Rn values) are collected to build up a plot of the amount of signal versus the cycle number (or the time). Usually the plot is constructed on a semi-logarithmic scale. In real-time PCR it is possible to distinguish three different phases: a first "lag" stage with slight fluctuations of the plots corresponding to background signal; a second exponential phase with increasing parallel plots, and a third stage where the plots tend to reach a "plateau" (Figure 7).

Figure 7. A real-time PCR plot. The typical phases of a real-time PCR are highlighted.

The power of real-time PCR resides in the fact that quantification occurs not at the endpoint stage of the PCR reaction (plateau), but at the stage where the exponential growth of the amount of amplified DNA (Rn value) reaches a point significantly greater than background signal. This way of measuring significantly enhances the accuracy of quantification since there is a direct correlation between the starting amount of template and the stage at which the amplification starts to become exponential. In real-time PCR a threshold cycle (C_T), also called quantification cycle (C_q), is experimentally defined as the cycle in which the fluorescence signal reaches the mean of fluorescence signals measured between the third and the fifteenth cycle plus ten standard deviations. The higher the initial amount of genomic DNA, the sooner accumulated product is detected in the PCR process, and the lower the C_q value is. In practice, the choice of the threshold line determining the C_q value is often up to the operator, representing one of the subjective elements in real-time PCR. The threshold line should be placed above any baseline activity and within the exponential increase phase, which looks linear in the log transformation (all plots are parallel). In any case the threshold line should be placed at the level where the plots of the replicas start to coincide the most. In fact, sometimes the replicas happen to have, in the very first part of the exponential phase, a slight divergence diminishing or totally disappearing as the reaction goes on.

Calculation of the GMO content

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

Comparative C_q method ($\Delta\Delta C_q$): This method uses no known amount of standards but it compares the relative amount of the GMO target sequence to the reference gene sequence. The standard curve is obtained by loading a series of samples at different known concentrations of GMO content (e.g. certified reference materials). The result is one standard curve of $\Delta\Delta C_q$ ($\Delta C_q = C_{q\ \text{reference gene}} - C_{q\ \text{GMO}}$) values. The GMO content value is obtained by calculating the ΔC_q value of the sample and comparing it with the values obtained with the standards.

For this method to be successful, the amplification efficiencies of both the target and reference PCR systems should be similar. A sensitive method to control this is to look at how ΔC_q (the difference between the two C_q values of two PCRs for the same initial template amount) varies with template dilution. If the efficiencies of the two amplicons are approximately equal, the plot of log input amount versus ΔC_q would have a nearly horizontal line (a slope of <0.10). This means that both PCRs perform equally efficiently across the range of initial template amounts. If the plot shows unequal efficiency, the standard curve method should be used for GMO quantification. The dynamic range should be determined for both (1) minimum and maximum concentrations of the targets for which

the results are accurate and (2) minimum and maximum ratios of two gene quantities for which the results are accurate.

Standard curve method: two standard curves, based on different amounts of DNA, are plotted:

- The first curve with a quantification system specific for the GM target;
- The second curve with a quantification system specific for the reference gene.

For each sample, the amounts of the specific target and of the reference gene are determined by interpolation with the corresponding standard curve. The GM DNA content (percentage) is then calculated as the ratio between the GM target sequence amount and the reference gene sequence amount (GM/reference x 100).

It is worth considering that, necessarily, the samples in analysis must fall within the upper and lower limits of both standard curves. Outliers must be excluded since they are prone to quantification errors.

The standard curve method is recently the most applied in routine analysis.

Stacked events

Stacked events are becoming more and more popular because they combine in one product characteristics inserted in single GM lines, like for example, both resistance to parasites and tolerance to pesticides at the same time.

They characterize GMOs that have been produced crossing different GM lines. For instance, MON810 and NK603 are both GM maize lines authorized for marketing in the EU as single events but they are authorized also as stacked event NK603xMON810, this meaning that the plant DNA contains the constructs of both events.

More information on the characteristics of NK603xMON810 is available and can be consulted at:

http://ec.europa.eu/food/dyna/gm_register/gm_register_auth.cfm?pr_id=17).

The presence of stacked events in a product can be an issue when the GM content needs to be quantified. It is not currently possible to discriminate if e.g. a sample contains two different GM events or if it contains a stack of the two lines.

Official control laboratories, when quantifying GM content, consider stacked events as two separate GM events. In other words, if a sample is tested for MON810 and NK603 and it is quantified as being 0.6% of both over the total ingredient, the operator would need to say that he detected both NK603 and MON810, but he would not be able to discriminate if it is

a stack or not. Different GM events of the same taxon have to be summed up to calculate the amount of GM material over the total ingredient (e.g. maize). In the case shown in Figure 1 we obtain 1.2% GM presence in both cases. This means that the GM presence should be indicated in the label because the amount of GMO over the total ingredient is above the 0.9% threshold.

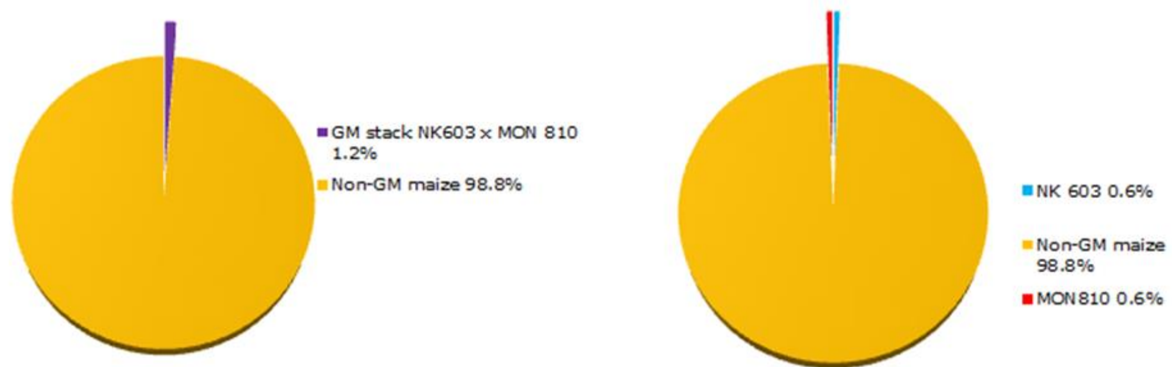


Figure 8. The total amount of GM maize is above the legal threshold: need of labelling

PCR inhibition

The previous paragraphs deal with the setup of the quantification of GMOs. However, previous to that, the extracted DNA should be checked for the presence of inhibitors. PCR inhibitors generally exert their effects through direct interaction with DNA or interference with thermostable DNA polymerases. Direct binding of agents to single stranded or double-stranded DNA can prevent its amplification and impair the results obtained in a qPCR. Therefore, before GMO quantification, the extracted DNA is checked for PCR inhibitors, in a test called "inhibition run". If the test indicates the presence of inhibitors, the extraction has to be repeated, or, alternatively, the sample can be treated for the presence of inhibitors.

This test consists of a series of four point four-fold serial dilutions, starting with the working dilution (e.g. 40 ng/µl, used later for GMO quantitation), and at least 2 replicates for each dilution. The sample is tested for a taxon-specific sequence (the taxon-specific reference system). Amplification and quantification take place with real-time PCR and from the obtained C_q values the efficiency of the reaction (R^2 and slope of the curve) is calculated.

The protocol for inhibition run will be laid down in the next session. For more information on the procedure and the analysis of inhibition run results please consult Annex II of

"Verification of analytical methods for GMO testing when implementing interlaboratory validated methods" (found at http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

Multiplexed real-time PCR reactions

Depending upon the chemistry and the apparatus used for the quantification, it is possible to design real-time PCR reactions to perform the quantification of the reference and of the GM specific sequences separately, in distinct tubes, or in the same tubes as a "multiplexed" reaction.

Both set-ups have advantages and disadvantages: multiplexed reactions are time, sample and reagent saving (it is possible to analyse twice the number of samples in one single experiment), avoid set-up errors between the measurement of the reference and the GMO target gene since they occur in the same tube, but may be less sensitive (in terms of LOQ) because of the interference between the multiplex reactions and the different time that individual reactions may take to use up the reagents. On the other hand, separate reactions to measure the reference gene and the GMO target gene are more sensitive in terms of LOQ, but require twice the reagents, sample and wells on the real-time PCR apparatus and are more exposed to the risk of, e.g. pipetting errors. The availability of multiple reporter dyes for TaqMan probes (e.g. FAM and VIC) makes it possible to detect the amplification of more than one target in the same tube. The reporter dye FAM is distinguishable from VIC because they have different maximal emission wavelengths. The availability of multiple dyes with distinct emission wavelengths (FAM, VIC, but also TET and JOE) makes possible to perform multiplex TaqMan assays. The dye TAMRA is used as a quencher on the probe and ROX as passive reference in the reaction mix. For best results, the combination of FAM (target) and VIC (endogenous control) is recommended since they have the largest difference in the emission maximum while dyes JOE and VIC should not be combined. Multiplex TaqMan assays can be performed on any instrument with capability to detect multiple dyes with distinct emission wavelengths. An example of multiplex real-time PCR is explained in session 9 while its detailed protocol can be found in session 11.

Digital PCR

Digital Polymerase Chain Reaction (dPCR) is a rather new technology which can be used for the detection and absolute quantification of DNA targets.

There are two ways to conduct dPCR: **chamber-based methods** and **droplet-based methods**. The chamber-based dPCR (**cdPCR**) uses solid-state partitions (chambers). The reaction mixture is distributed in the different chambers with the help of a syringe, and

then the PCR amplification is performed in each chamber. A dedicated thermal cycler allows the chambers to be cycled and read. The number and size of chambers is fixed and thus highly consistent over runs (Pecoraro et al., 2019).

In droplet-based dPCR (**ddPCR**), the partitioning of the reaction mix is achieved by making water-in-oil emulsion prior to the PCR, and generating high numbers of droplets. The DNA targets in the emulsion are amplified in standard PCR wells or plates. Once the PCR is finished, a dedicated reader measures the end-point fluorescence of each droplet. The number of partitions varies between different platforms and between individual reactions.

The main difference between dPCR and real-time PCR is that in dPCR the reaction volume containing the DNA is split over a high number of small partitions. The absolute number of target DNA molecules in the original sample can be calculated using limiting dilutions and Poisson statistics (Lievens et al., 2016; Pecoraro et al., 2019). After the amplification, each partition is scrutinized and defined as positive or negative respectively, with a value assigned of one or zero (that is why it is called digital).

An example is shown in Figure 9, where the result of a cdPCR run is presented in (a) and those of ddPCR in (b). The red coloured compartments indicate positive signals containing one or two targets, while the grey ones indicate no signal and are considered negative. Similarly, Figure 9(b) shows the ddPCR result, where blue dots indicate positive signals and grey negative ones.

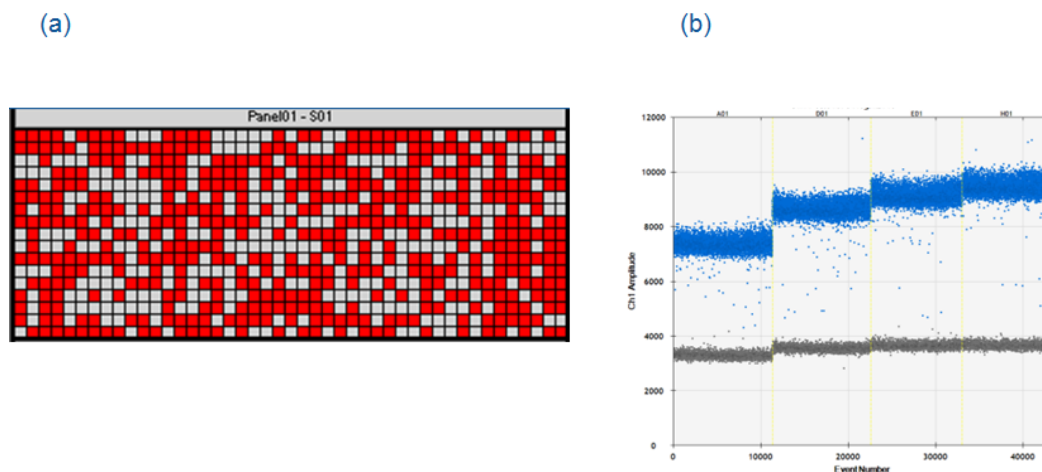


Figure 9. (a) Chamber Digital PCR, with red dots indicating positive signals and grey dots indicating negatives and (b) Droplet Digital PCR, with positive signals represented as blue dots and negative signals in grey.

The advantages of dPCR, compared with endpoint PCR and real-time PCR are summarized below:

• Absolute target quantification: no need of a calibration curve. Therefore, any matrix differences between the calibrant and test samples possibly leading to different PCR amplification efficiencies are limited;

• Very high level of sample partitioning, leading to results of very high precision (Hindson et al., 2011; Pecoraro et al., 2019);

• Ability to detect targets at very low concentrations (minority targets) even in a high background of competing non-target DNA;

• Lower effect of inhibition and better amplification rate thanks to the partitioning (Rački et al., 2014; Nixon et al., 2014; Iwobi et al., 2016; Pecoraro et al., 2019).

Digital PCR has the potential to substitute real-time PCR in the field of GMO quantification. As a general rule, any probe-based real-time PCR assay can be converted into a digital PCR assay. In fact, direct transferability of some EURL GMFF validated methods for GMOs (GMOMETHODS: EU Database of Reference Methods for GMO Analysis, https://gmo-crl.jrc.ec.europa.eu/gmomethods/) has been proven successful (Jacchia et al., 2018).

When analysing digital PCR results, the output is in copy numbers and therefore, in the case of GMOs, a conversion factor is necessary, in order to respect the legal requirements which are in mass fractions. A comprehensive approach has developed in "Recommendation for the unit of measurement and the measuring system to report traceable and comparable results expressing GM content in accordance with EU legislation" (Corbisier et al., 2017)

For more information and a detailed analysis of the recommendations of the European Network of GMO Laboratories (ENGL) for the use of digital PCR, please refer to the "Overview and recommendations for the application of digital PCR" (http://gmo-crl.jrc.ec.europa.eu/ENGL/docs/WG-dPCR-Report.pdf).

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Session 11

Real-time PCR Protocols for Qualitative and Quantitative Analysis

Experimental

Introduction

The following protocols are real-time PCR-based methods for the quantification of the specific GM event MON810 by single real-time PCR and for the detection of 35S promoter and *nos* terminator by multiplex real-time PCR (qualitative analysis).

The real-time PCRs are carried out with thermocycler ABI PRISM® 7500.

It has to be noted that both experiments should be preceded by an inhibition run to test the amplifiability of the extracted DNA.

Real-time PCR for quantitative analysis

Real-time PCR will be used to amplify an endogenous reference target DNA sequence that is unique to maize (taxon specific), plus a DNA target sequence that indicates the presence of the genetically modified crop.

The assays can encompass two independent PCR systems, or happen in the same well as a multiplex reaction (in our case they will be performed independently). Each target DNA has specific DNA primers and dye-labelled probes. One PCR system detects a GMO-specific target DNA sequence, the other is an endogenous reference system designed to serve as a quantitative reference that detects GM and non-GM maize.

Real-time PCR for qualitative analysis

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

Note: The protocols included in this manual have been chosen for didactical purposes and should be considered as basic examples of GMO analysis using the real-time PCR approach. We recommend to periodically review pertinent sources and literature to acquire information on more recently developed and validated protocols (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx). Please also note that these protocols have been selected according to the instrumentation available in our laboratory. The JRC in no way promotes the exclusive use of any particular company or brand.

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

In this paragraph the protocol for a duplex real-time PCR screening method for the detection of genetically modified (GM) plants is described.

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

Materials and equipment

- ABI PRISM® 7500 Sequence Detector System (Applied Biosystems)
- 96-Well Reaction Plates
- Optical caps/adhesion covers
- Micropipettes
- Racks for reaction tubes
- 0.5 mL and 2 mL DNase free reaction tubes
- Standard bench top centrifuge with rotor or standard microfuge fit for 0.5 mL reaction tubes, centrifuge for 96-Well reaction plates
- Vortex mixer
- 1.5 ml microcentrifuge tubes

Characteristics of primers for T-*nos*

Primer forward: 180-F	
Sequence	CATGTAATGCATGACGTTATTTATG
Length	25
Mol. weight (g/mol)	7686.1
Melting point * (G/C)	51.1

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

Primer reverse: 180-R	
Sequence	TTGTTTTCTATCGCGTATTAAATGT
Length	25
Mol. weight (g/mol)	7643.1
Melting point * (G/C)	49.5

Characteristics of primers for P 35S

Primer forward: 35S-FTM	
Sequence	GCCTCTGCCGACAGTGGT
Length	18
Mol. weight (g/mol)	5491.6
Melting point * (G/C)	54.9

Probe: 35S-TMP-FAM	
Sequence	FAM-CAAAGATGGACCCCCACCCACG-BHQ1
Length	22

Primer reverse: 35S-RTM	
Sequence	AAGACGTGGTTGGAACGTCTTC
Length	22
Mol. weight (g/mol)	6790.5
Melting point * (G/C)	54.8

Controls

Each test series shall include the controls as stated in **Table 3**; **Session 6** of this manual. If the controls do not yield the expected results, the test results shall be rejected and the analysis shall be repeated.

Experimental procedure

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

Table 1. Reaction mix for real-time PCR (ABI 7500)

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

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

2. In a 2 mL tube on ice, add the components in the order mentioned in Table 1 (<u>except DNA</u>) to prepare the reaction mix. Please prepare only the reaction mix needed for the run.
3. Mix well and centrifuge briefly.
4. Label one 0.5 mL reaction tube for each DNA sample to be tested.
5. Add into each reaction tube the amount of reaction mix needed for 3.3 repetitions (66 µL). Add into each tube the proper amount of DNA for 3.3 repetitions (16.5 µL DNA). The additional 0.3 repetition included will ensure adequate volume when loading the samples. <u>Vortex for approximately 10 sec. each tube. This step is of mandatory</u>

importance to reduce to a minimum the variability between the replicates of each sample.

6. Spin down in a micro-centrifuge. Aliquot 25 µL in each well according to the chosen plate setup loading order.
7. Place an optical cover on the reaction plate and briefly centrifuge the plate.
8. Place the reaction plate into the ABI real-time PCR equipment.
9. Programme the real-time PCR equipment.
10. Set up the plate layout, as in figure 1.

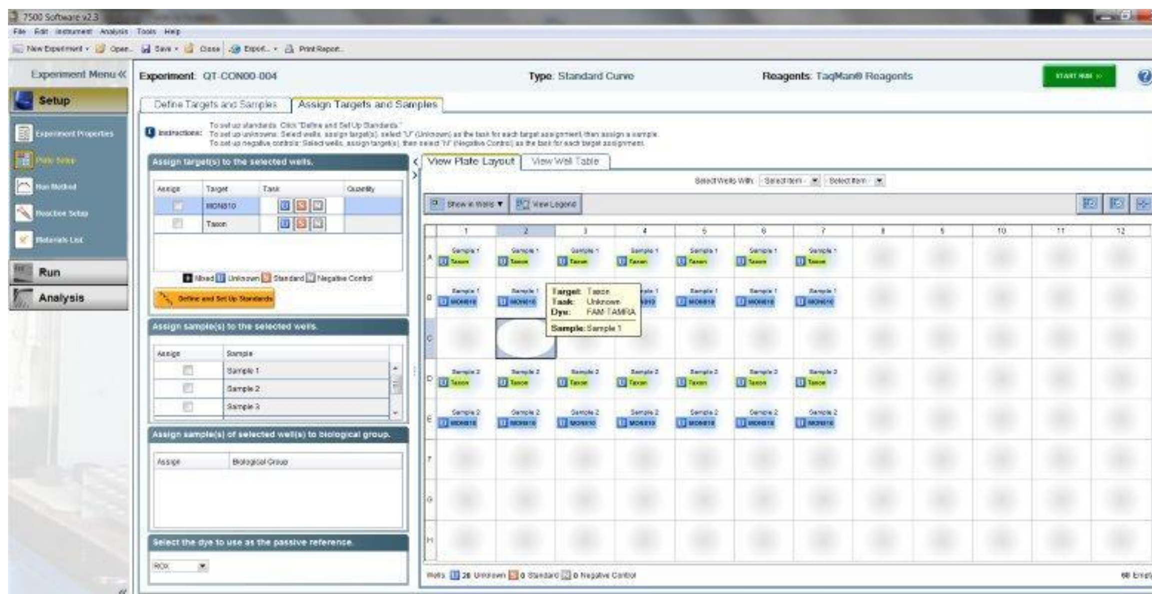

Figure 1. Software layout

11. Choose the number of cycles, the reaction volume and the details of each reaction step (Table 2).
12. Start the run.

Table 2. Amplification conditions

Stage		Time	Temperature °C	No Cycles
Pre-PCR: decontamination (UNG)		2 min	50°C	1
Pre-PCR: activation of DNA polymerase and denaturation of template DNA		10 min	95°C	1
Step 1	Denaturation	15 sec	95°C	

Step 2	Annealing and elongation	60 sec	60°C	
TOTAL				45

Data analysis and interpretation of results

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

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

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

Protocol for a construct specific method for the quantitation of MON810 by real-time PCR.

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

Equipment and Reagents

- ABI PRISM® 7500 Sequence Detector System (Applied Biosystems)
- 96-Well Reaction Plates
- Optical caps/adhesion covers

- Micropipettes
- Racks for reaction tubes
- 0.5 mL and 2 mL DNase free reaction tubes
- Real-time PCR detection system and appropriate analysis software
- Standard bench top centrifuge with rotor or standard microfuge fit for 0.5 mL reaction tubes, centrifuge for 96-Well reaction plates
- Vortex mixer
- 1.5 ml microcentrifuge tubes.

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

Primer forward: M810 2-5'	
Sequence	TCGAAGGACGAAGGACTCTAACG
Length	22
Mol. weight (g/mol)	6692.4
Melting point * (G/C)	54.8

GMO target probe: M810-Taq	
Sequence	FAM-AGATACCAAGCGGCCATGGACAACAA-TAMRA
Length	26

Primer reverse: M810 2-3'	
Sequence	GGATGCACTCGTTGATGTTTG
Length	21
Mol. weight (g/mol)	7106.3
Melting point * (G/C)	52.4

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

SSIIb1-5'	
Sequence	CTCCCAATCCTTTGACATCTGC
Length	22
Mol. weight (g/mol)	7204.3
Melting point * (G/C)	54.8

Taxon probe: SSIIb1-Taq	
Sequence	FAM-AGCAAAGTCAGAGCGCTGCAATGCA-TAMRA
Length	25

SSIIb1-3'	
Sequence	TCGATTTCTCTCTTGGTGACAGG
Length	23
Mol. weight (g/mol)	7659.6
Melting point * (G/C)	55.3

*based on a [Na^+] of 50 mM

Standard curve

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

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

MON810 construct is divided by the copy number of the *zSSIIb* gene and the construct-specific Cf of MON810, multiplied by 100 to obtain the percentage.

Controls

Each test series shall include the controls as stated in **Table 3**; **Session 6** of this manual. If the controls do not yield the expected results, the test results shall be rejected and the analysis shall be repeated.

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

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

Mastermix preparation

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

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

Table 3. PCR reaction setup

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

Table 4. Reaction conditions

Stage		**Time**	**Temperature °C**	**No Cycles**
Pre-PCR: decontamination (UNG)		2 min	50°C	1
Pre-PCR: activation of DNA polymerase and denaturation of template DNA		10 min	95°C	1
Step 1	Denaturation	30 sec	95°C	
Step 2	Annealing and elongation	60 sec	59°C	
				40

Plate setup

Table 5. Plate setup

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

Data analysis and interpretation of results

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

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

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

Inhibition run

Preparation of DNA dilution series

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

40 µl of each dilution should be prepared as follows:

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

Table 6. preparation of the dilution series

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

The test is conducted at least with the taxon specific reference system. To assess the presence of inhibitors, the C_q values of the diluted samples are plotted against the logarithm of the dilution factor and an equation is calculated by linear regression. Three

criteria have to be met: the slope of the regression line should be within -3.6 and 3.1 and the linearity should be above 0.98, finally the C_q value for the "undiluted" sample extrapolated from the linear regression is compared with the measured C_q for the same sample. The difference (ΔC_q), average between the measured C_q and the extrapolated C_q value, should be within 0.5. Therefore, one of the criteria, as defined in the Minimum Performance Requirements for Analytical Methods of GMO Methods, is dedicated to the evaluation of the expected vs measured DNA content in the most concentrated sample which can, in case of inhibition, result in underestimation (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

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Session 12

The Protein-based Approach

Introduction

Immunoassays are analytical measurement systems that use antibodies as test reagents.

The most widely used immunological detection method is the Enzyme-Linked Immunosorbent Assay (ELISA) (Clark and Adams, 1977), which relies on the specific interaction between **antibodies** and **antigens**. As in all immunological approaches, the key reagents in ELISA are the antibodies, which are soluble proteins produced by the immune system in response to infection by a foreign substance, called an "antigen" (Querci et al., 2007). When applied to GMOs, the antigens are the newly synthesised proteins.

Antibodies are specific proteins isolated from the serum of animals that physically bind only to the substance that elicited their production. Antibodies are made by injecting the substance to be detected into animals, such as rabbits and mice, where cells of the body recognise the substance as "foreign" and respond by producing antibodies against it. The antibodies are purified, attached to a detectable label and then used as reagents to detect the substance of interest. A prerequisite for the development of immunological detection methods is that highly specific antibodies directed against the new protein to be detected should be available (Ahmed, 2002). All immunoassays are based on the specific binding of antibody to antigen.

These characteristics make specific immunology, a suitable method of detection of a novel protein, synthesised by a gene introduced during transformation and constitutes an alternative approach for the identification of genetically modified plants. It should be noted, however, that genetic modification is not always specifically directed at the production of a new protein and does not always result in protein expression levels sufficient for detection purposes. In addition, certain proteins may be expressed only in specific parts of the plant (tissue-specific promoters are already being used for specific purposes) or expressed at different levels in distinct parts or during different phases of physiological development. The sample or the proteins of interest should not be significantly degraded because detectability of certain proteins is linked to their level of denaturation.

Antibodies properties

Substances external to the body, such as disease-causing bacteria, viruses and other infectious agents known as antigens, are recognised by the body's immune system as invaders. The natural defences against these agents are **antibodies**: proteins that seek out the antigens and help destroy them.

Antibodies have two very useful characteristics. They are extremely specific meaning that each antibody binds to and attacks one particular antigen. Second, some antibodies, once activated by the occurrence of a disease, continue to confer resistance against that disease.

Antibodies' specificity makes antibody technology very valuable not only therapeutically to protect against diseases, but also in the detection of drugs, allergens, viral and bacterial products.

This property of antibodies can thus be exploited for the detection of proteins in laboratory applications, one of which is GMO detection.

The utility of antibodies is also greatly enhanced by their relative stability in various chemical modification reactions, which alter antibody structure without destroying their capacity to bind antigens. Antibodies have been chemically tagged with fluorescent, magnetic, radioactive and assorted other compounds as a way of facilitating antigen detection or isolation under a variety of experimental conditions.

Monoclonal antibody production

Monoclonal antibody technology is mostly used for the production of large amounts of pure antibodies by using cells that naturally produce antibodies and a class of cells that can grow continuously in cell culture.

These antibodies are called monoclonal because they come from only one type of cells, the hybridoma cell; on the other hand, antibodies produced by conventional methods are derived from preparations containing many kinds of cells and hence are called polyclonal.

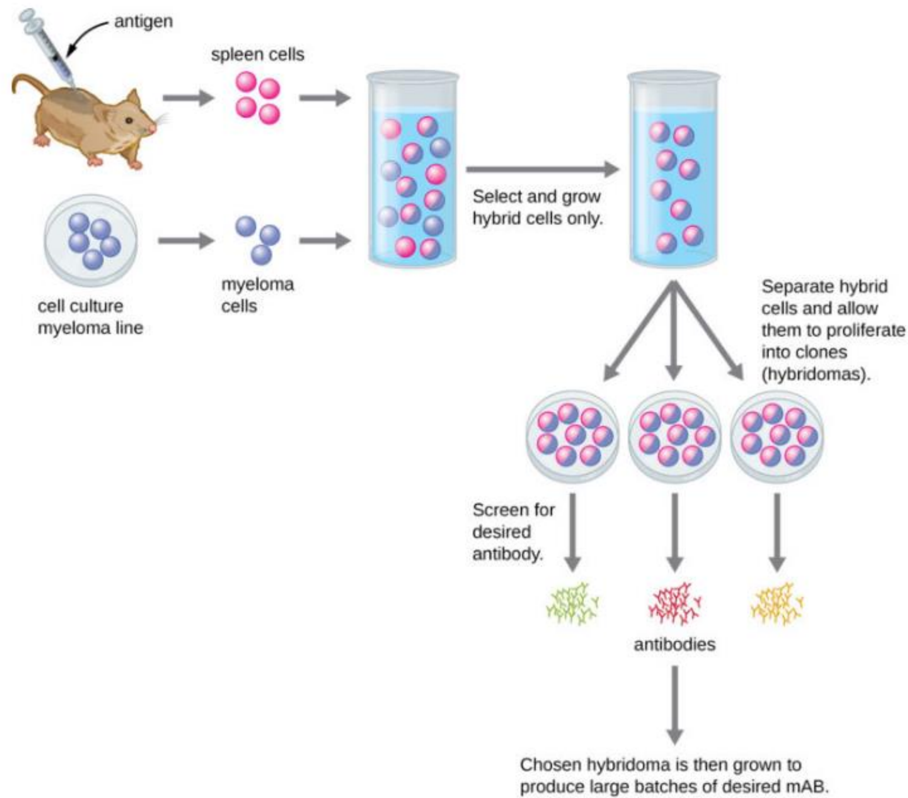

Figure 1. Monoclonal antibody production. Picture taken from Lumen Courses Microbiology (https://courses.lumenlearning.com/microbiology/chapter/polyclonal-and-monoclonal-antibody-production/ accessed 25/02/2020).

Monoclonal antibodies attack the target molecule only with no or greatly diminished side effects on the patient compared to polyclonal antibodies, leading to higher specificity.

The ELISA technique

Definition

Enzyme-**L**inked **I**mmuno**S**orbent **A**ssay: any enzyme immunoassay utilising an enzyme-labelled immunoreactant (antigen or antibody) and an immunosorbent (antigen or antibody bound to a solid support). A variety of methods (e.g. competitive binding between the labelled reactant and unlabelled unknown) may be used to measure the unknown concentration.

ELISA technique has been extensively used as a tool to confirm or follow the success of plant transformation by allowing a direct estimate of the expression level of the protein(s) synthesised by the newly introduced gene. As a consequence, information regarding the production and use of specific antibodies can be found in many articles describing the developments of transgenic plants (Mohapatra et al., 1999). However, only a few specific

antibodies directed against proteins that are the products of transgenes used in approved genetically engineered crops are commercially available: among these the ones against the *npt*II gene product, NPTII or APH(3')II (Wood et al., 1995), against the product of the *gus* gene, against some variants of the *cry* genes from *Bacillus thuringensis* (Koziel et al., 1993; Ermolli et al., 2006b), or against the CP4-EPSPS protein (5-enolpyruvylshikimate-3-phosphate synthase, an enzyme from *Agrobacterium* spp. strain CP4) which confers tolerance to the herbicide RoundupTM in Roundup ReadyTM soybean (Padgette et al., 1995).

Among the different ELISA formats, the direct sandwich ELISA method, in which the analyte is 'sandwiched' between the capture and the detector antibodies, is the immunoassay most used in GMO detection (Stave, 2002). Colour development is linearly proportional to the concentration of antigen that is directly dependant upon the amount of (GM) protein originally in the sample.

A variation to the standard ELISA is called 'ELISA Reverse' and is specifically designed on solid phase which is then immersed directly into liquid samples. It can be applied to the simultaneous detection and quantification of CP4-EPSPS and Cry1A(b) proteins (Ermolli et al., 2006a).

There are 3 different methods to perform an ELISA test as shown in Figure 2.

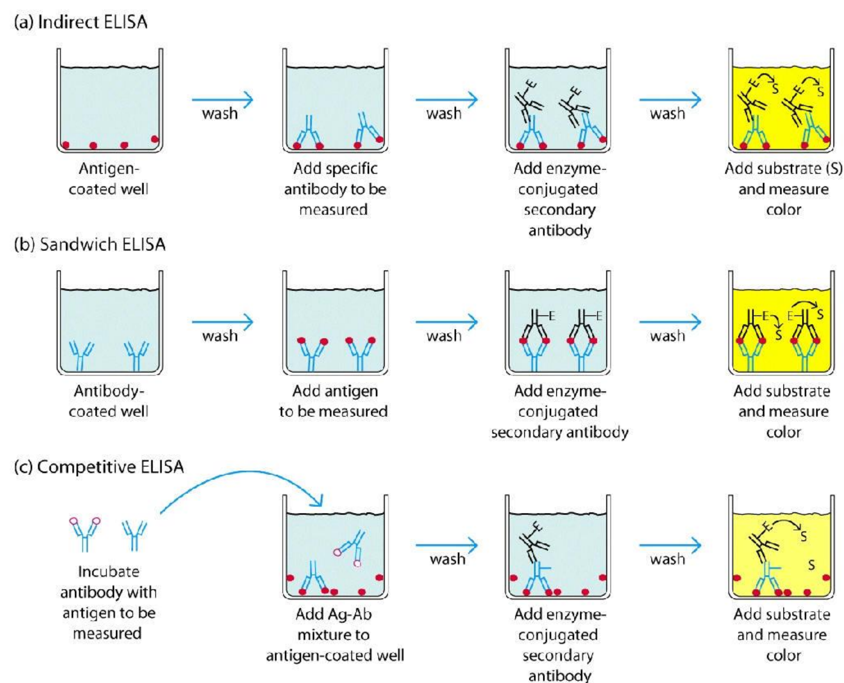

Figure 2. Schematic representation of (a) Indirect ELISA; (b) Sandwich ELISA and (c) Competitive ELISA.

Principle

A direct sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA) is used for the detection of the CP4 EPSPS protein as shown in Figure 3 below:

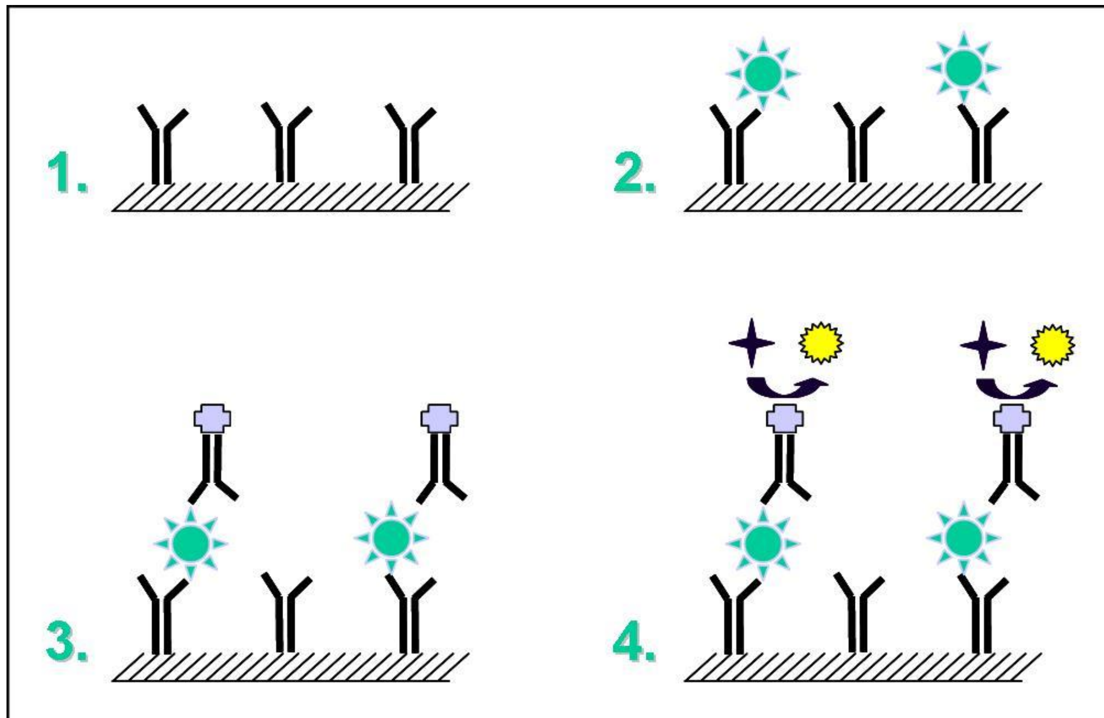

Figure 3. Schematic representation of the steps involved in direct sandwich ELISA.

1. The surface of a microtiter plate is coated with a specific monoclonal capture antibody.
2. When the sample of interest is added, the capture antibody binds the antigen. Unbound components of the sample are removed by washing.
3. After washing, a polyclonal antibody, covalently linked to horseradish peroxidase (HRP) is added, which is specific for a second antigenic site on the bound CP4 EPSPS protein.
4. After washing, a tetramethylbenzidine chromogen for horseradish peroxidase is added. The horseradish peroxidase generates a colour signal, which is proportional to the concentration of antigen in a linear range. To stop the colour development a stop solution is added. The degree of colour produced is measured at a wavelength of 450 nm.

An example of an ELISA-based method for the specific detection of Roundup Ready® soybean was validated by Lipp et al*.* (2000).

The method is based on the use of specific antibodies directed against the protein CP4-EPSPS (5-enolpyruvylshikimate-3-phosphate synthase, enzyme from the *Agrobacterium* sp. strain CP4) (Padgette et al*.*, 1995), which is the protein conferring tolerance against the herbicide Roundup in the Roundup Ready® soybean. Preliminary results indicate that the method (performed by using a commercialised ELISA kit) is able to detect the presence of GMOs in raw soybean material at concentrations ranging between 0.3% and 5%.

Lateral flow strips

Particular interest has been given to the so called immunochromatographic strip test (lateral flow test, LFT). This variation of the ELISA technique is a testing method that operates in a way similar to the one of double antibody sandwich but with an advantage: the reaction takes place on a solid support exploiting the protein solution flow through an absorbent strip resulting in very quick time of analysis and cost effectiveness. A scheme of the structure of the strip is presented in Figure 4 below.

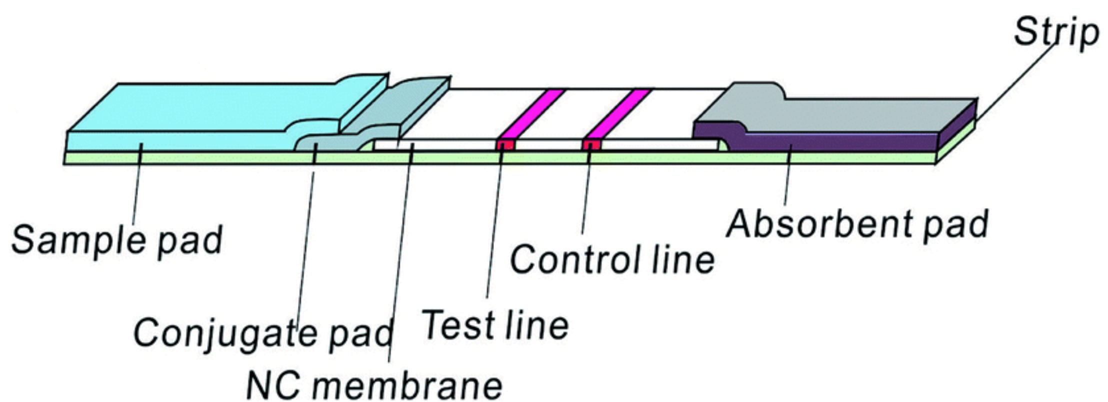

Figure 4. Schematic representation of the strip used in lateral flow tests

The strip test generally presents two antibody lines: the **test line** (specific for the transgenic protein) and the **control line** (binding the excess antibodies). The strip can be immersed into a previously homogenized solution. Compared to standard ELISA procedures, lateral flow strips have the advantage that the reaction takes place on one solid medium, exploiting the protein solution flux through the absorbent strip (Figure 5). As a consequence, results are obtained in a few minutes and the method although not quantitative is cheaper.

If the sample does not contain the transgenic protein, the fluid containing the antibodies will freely flow up to the control line, undisturbed by the presence of the test line's antibodies.

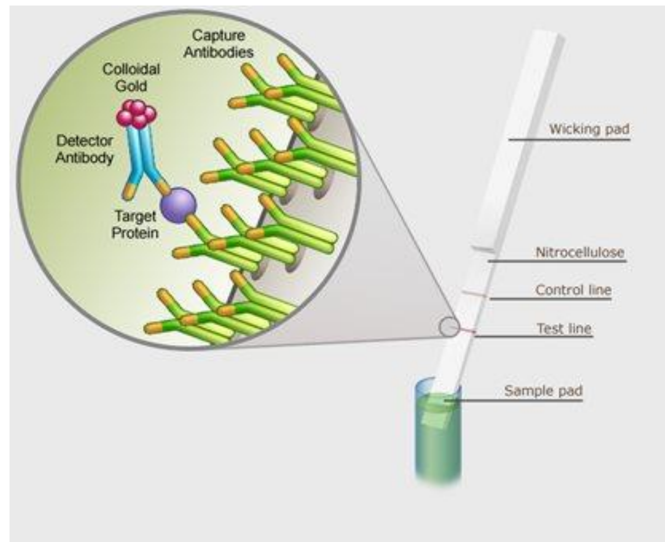

Figure 5. Schematic representation of the protein/antibody interaction in a lateral flow strip.

Otherwise, if the sample contains the transgenic protein, the antibodies will specifically bind it. Then the antibody/protein complex will only rise up to be bound by the specific antibodies of the test line (Figure 6). The remaining unbound antibodies, carried by the flow, will be caught by the control line.

In LFT, for the test to be positive, both the control and sample line has to be visible. If the control line is the only visible one, the result shall be considered negative. If no lines is observed, then the test is invalid, as at least the control line should be present. In this case, the test should be repeated.

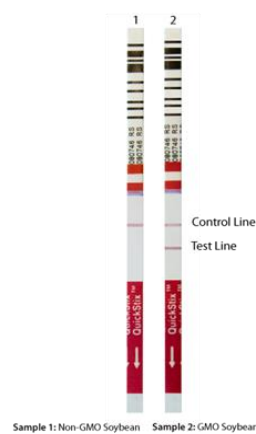

Figure 6. Two LFT's results for soybean: the left one negative for GM soybean; the right one positive.

Protein approaches give quick results and are easy to use; even though the initial cost for the development of the method is high, the cost per sample is relatively small. For this reason, they can be helpful for *on-site* fast screening.

Advantages and limitations of protein-based detection methods in routine GMO testing have been discussed in Session 2.

The prerequisite for the development of ELISA and of other immunological detection methods is the availability of highly specific antibodies (either monoclonal, more specific, or polyclonal, more sensitive) directed against the new protein to be detected. Since the production of antibodies, either monoclonal or polyclonal, is an extremely complex and expensive process and requires highly specialised setting, it is almost exclusively conducted by specialised companies and only very seldom performed at the level of individual laboratories. Consequently, in practice, the application of immunological methods for routine GMO analysis generally relies on the use of antibodies made commercially available as part of ELISA kits or integrated into lateral flow strips.

For this reason, no detailed protocols are included in this User Manual. Users are invited to refer to the specific instructions provided by the manufacturers.

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Additional Suggested Reading

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Annexes

Annex 1. Abbreviations

CTAB	Cetyltrimethyl ammonium bromide
Da	Dalton
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
dsDNA	Double stranded DNA
dTTP	Deoxythymidine triphosphate
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
ENGL	European Network of GMO Laboratories
EPSPS	5-enol-pyruvylshikimate-3-phosphate synthase
EU	European Union
EURL GMFF	European Union Reference Laboratory for GM Food and Feed
FRET	Fluorescence Resonance Energy Transfer
GMO	Genetic Modified Organism
IRMM	Institute for Reference Materials and Measurements
JRC	Joint Research Centre
LFT	Lateral Flow Strips Test
LOD	Limit of Detection
mRNA	Messenger RNA
Na2-EDTA	Ethylenediaminetetraacetic Acid, Disodium Salt
NaCl	Sodium chloride
NaOH	Sodium hydroxide
OD	Optical density
oligo(dT)	Deoxy-thymidine nucleotides
PCR	Polymerase Chain Reaction
poly(A)	Polyadenylic acid
RNA	Ribonucleic acid
rpm	Run per minute
SDS	Sodium dodecyl sulfate
ssDNA	Single stranded DNA
TAE	Tris-acetate
TBE	Tris-borate
TPE	Tris-phosphate
Tris	Tris[hydroxymethyl] aminomethane
UV	Ultraviolet
V	Voltage

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