TRAINING COURSE ON

THE ANALYSIS OF FOOD SAMPLES FOR
THE PRESENCE OF GENETICALLY
MODIFIED ORGANISMS

USER MANUAL

Edited by Maddalena Querci, Marco Jermini and Guy Van den Eede

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Foreword

The Institute for Health and Consumer Protection of the Joint Research Centre of the European Commission and the Food Safety Programme within the European Centre for Environment and Health - Rome Division (ECR) of the World Health Organization have jointly organised a series of training courses on “The Analysis of Food Samples for the Presence of Genetically Modified Organisms”.

The Joint Research Centre gives scientific and technical support to EU policies by collaborating with EC Directorates General and by interacting with European Institutions, Organizations and Industries through networking with Member State laboratories. The overall task of the WHO’s ECR is to provide support in a complete and coordinated way to both decision-makers and to European citizens in the environmental health field. These training courses are part of collaboration between both Institutions to promote food safety related issues in the WHO European Region, within and beyond actual EU borders, taking into special consideration EU Accession Countries, as well as Central and Eastern Countries with transitional economies.

The scope of the training courses is to assist staff of control laboratories to become accustomed with molecular detection 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 courses are intended to teach molecular detection techniques to laboratory personnel with a good level of analytical knowledge, but with no or little expertise in this specific domain.

The Joint Research Centre has been committed to providing training in detection and quantification of GMOs and, besides the training courses, it offers, and has offered in the past, individual training for specific needs. Training in this topic has been frequently requested due to its importance according to the increasing need to comply with the current and developing European legislative framework.

Over the years, the Molecular Biology & Genomics Unit has developed a profound knowledge of the different aspects related to GMO detection and quantification, and
has designed, adapted or validated advanced methods for their detection and quantification.

Knowledge of these techniques has been transferred to collaborating laboratories through publications, collaborative projects, individual training or specific courses. Technical details have also been provided to trainees as oral presentations or brief written outlines. Aware of the need for a permanent source of information, the Molecular Biology & Genomics Unit staff developed this manual, which describes some of the techniques used in our laboratory.

The following areas are covered throughout the courses;
- DNA extraction from raw and processed materials
- Screening of foodstuffs for the presence of GMOs by simple Polymerase Chain Reaction and by nested Polymerase Chain Reaction
- Quantification of GMOs in ingredients by real-time Polymerase Chain Reaction
- Quantification of GMOs in ingredients by the Enzyme-Linked ImmunoSorbent Assay

This Manual has been prepared at the Joint Research Centre, Institute for Health and Consumer Protection (IHCP) as background information for course participants and is intended to provide the theoretical and practical information on methodologies and protocols currently used. The subject matter covers a wide variety of techniques for GMOs detection, identification, characterisation, and quantification, and includes theoretical information considered important background information for anyone wishing to enter and work in the field of GMO detection.

It is our hope that the structure and content of this manual will help course participants (as well as other users) in the diffusion and dissemination of the acquired skills in the context of the different working environments according to needs.
In no way was there an attempt to compete with information available in textbooks or journals. This manual aims to complement existing information in the specialised literature.

To facilitate diffusion and consultation, this publication is also available online at: http://mbg.jrc.ec.europa.eu/capacitybuilding/documentation.htm.

JRC staff members who participated in the preparation of this manual were supervised by Maddalena Querci and are mentioned in the Table of Content according to their contribution.

A special recognition and acknowledgment is hereby also given to all Unit personnel who, even not individually mentioned, contributed to the successful preparation of the manual.

Thanks are also extended to Sabrina Miglierina, Elisabeth Dilger, Manuela Zingales, Stephen Langrell and Steven Price for their support and collaboration for the revision of this manual.

Maddalena Querci, PhD

Course co-ordinator

June 2004
Introductory Remarks from the World Health Organization

The World Health Organization gives high priority to the safe use and application of modern biotechnology to food production and processing. For this reason WHO has been very active in the organization of expert consultations on the safety of foods derived from biotechnology since early 90’s. In May 2000, the 53rd World Health Assembly adopted a resolution that WHO should support Member States to provide scientific basis for health-related decision regarding genetically modified foods. Additionally, the WHO Executive Board envisaged that other relevant considerations would be explored in collaboration with other agencies.

The Codex Alimentarius Commission (CAC) is an intergovernmental body set up to establish international standards on foods. Its primary objective is to protect the health of consumers and to ensure fair practices in food trade. WHO has been one of the parent organizations of the CAC since its establishment in 1963. Within the framework of the Codex Alimentarius activity WHO, and its UN sister Organization FAO, have been involved in the Ad Hoc Intergovernmental Codex Task Force on Foods Derived from Biotechnology, the Codex Committee on Food Labeling and the Codex Committee for Methods of Analysis and Sampling.

The WHO Food Safety Programme in Europe has been collaborating with the Joint Research Centre (JRC) of the European Commission since 2000 in the organization of training courses on detection techniques for Genetically Modified Organisms (GMOs) in foods. The aim of these trainings is to provide analytical biotechnology skills to food control laboratory staff and promote the use of validated and harmonized methods for detecting GMOs in Europe and internationally.

Adequate methods for analysis and sampling are essential for the appropriate labeling of foods to increase transparency on production processes and to facilitate traceability thus contributing to strengthen food safety systems. To allow broader access to these methods it has been decided that the tutorial manual used during the JRC/WHO Joint Training Courses will be published on the web. The methods presented in this manual are in line with those considered by the Codex Committee for Methods of Analysis and Sampling.
The WHO Food Safety Programme in Europe recognizes the importance of the collaboration between the European Commission Joint Research Centre, a worldwide recognized scientific institution in the field of GMOs, and will continue promoting capacity building activities in the field of detection methods of GMOs in foods in Europe and other Regions.

Cristina Tirado, DVM, PhD
WHO Food Safety Regional Adviser in Europe
TRAINING COURSE ON:
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Table of contents

<table>
<thead>
<tr>
<th>Session</th>
<th>Title</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Overview, general introduction on Genetically Modified Organisms (GMOs), EU legislation</td>
<td>M. Querci, G. Van den Eede, M. Jermini</td>
</tr>
<tr>
<td>2</td>
<td>Manual presentation, working methods and course introduction</td>
<td>M. Querci</td>
</tr>
<tr>
<td>3</td>
<td>Samples used during the course</td>
<td>M. Querci, N. Foti</td>
</tr>
<tr>
<td>4</td>
<td>Extraction and purification of DNA</td>
<td>M. Somma</td>
</tr>
<tr>
<td>5</td>
<td>Agarose gel electrophoresis</td>
<td>M. Somma, M. Querci</td>
</tr>
<tr>
<td>6</td>
<td>The Polymerase Chain Reaction (PCR)</td>
<td>M. Somma, M. Querci</td>
</tr>
<tr>
<td>7</td>
<td>Characteristics of Roundup Ready® soybean, MON810 maize, and Bt-176 maize</td>
<td>M. Querci, M. Mazzara</td>
</tr>
<tr>
<td>8</td>
<td>Characteristics of the qualitative PCR systems described in the manual</td>
<td>M. Querci, M. Mazzara</td>
</tr>
<tr>
<td>9</td>
<td>Qualitative detection of MON810 maize, Bt-176 maize and Roundup Ready® soybean by PCR</td>
<td>M. Querci, M. Maretti, M. Mazzara</td>
</tr>
<tr>
<td>10</td>
<td>Quantitative PCR for the detection of GMOs</td>
<td>F. Weighardt</td>
</tr>
<tr>
<td>11</td>
<td>Quantitative detection of Roundup Ready® soybean by real-time PCR</td>
<td>N. Foti</td>
</tr>
<tr>
<td>12</td>
<td>Quantitative detection of Roundup Ready® soybean by ELISA</td>
<td>F. Eyquem</td>
</tr>
<tr>
<td>Appendix</td>
<td>Example of work programme</td>
<td>M. Querci</td>
</tr>
</tbody>
</table>
The Analysis of Food Samples for the Presence of Genetically Modified Organisms

Session 1

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

M. Querci, G. Van den Eede, M. Jermini
Table of Contents

Session 1

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

Introduction 3

Genetic modification in plants 4
EU legislation 5
Safety and labelling of foods derived from modern biotechnology - The WHO perspective 13

Annex 1 – EU legislation governing the placing on the market of GMOs, including the related labelling and control programmes 17
Introduction

Apart from the range of genetically modified crop lines deployed across parts of the globe 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 continual crossing over time within species or between closely related, sexually compatible species groups. In recent decades it has become possible to produce not only crosses between plants that are cross compatible in nature, but also between plants that are considered as naturally cross sterile. 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 are 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 cross-breeding or natural recombination. The GMO itself must be a biological unit that is able to multiply or to 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 have been shown to 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 [http://gmoinfo.jrc.ec.europa.eu/](http://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, *Agrobacterium 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 polyethylene glycol-mediated transfer, microinjection, protoplast, and intact cell electroporation and gene gun (biolistic) technology.

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

In both cases, the cells (either those infected by *Agrobacterium* or 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.
EU legislation\textsuperscript{1,2}


Under Directive 2001/18/EC existing consents granted under Council Directive 90/220/EEC must be renewed in order to avoid disparities and to take full account of the conditions of consent of Directive 2001/18/EC. The consent (renewable) is granted for a maximum period of ten years starting from the date on which the consent is issued.

Following 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.

Directive 2001/18/EC, which is implemented in each Member State by national regulations, 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). As listed in Table 1, a total of 18 GMOs have been authorised under the former 90/220/EEC procedure (15 out of 18 concerning plants, 3 of which by Member States consent). To date, more than twenty-five applications for the placing on the market of GMOs have been submitted for authorisation under Directive 2001/18/EC (for updated information and status of the dossiers please consult \url{http://gmoinfo.jrc.ec.europa.eu/}).

\textsuperscript{1} See a near complete but non-exhaustive list of European Union Regulations/Directives pertaining GMOs in Annex 1.

\textsuperscript{2} Status on 9.06.2004
**Table 1. Genetically modified plants approved for marketing in the EU under Council Directive 90/220/EEC.**

<table>
<thead>
<tr>
<th>Product</th>
<th>Line</th>
<th>Notifier</th>
<th>Main traits</th>
<th>Commission Decision No/date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carnation</td>
<td></td>
<td>Florigene</td>
<td>Modified flower colour</td>
<td>20.10.98 (MS consent)</td>
</tr>
<tr>
<td>Carnation</td>
<td></td>
<td>Florigene</td>
<td>Modified vase life</td>
<td>20.10.98 (MS consent)</td>
</tr>
<tr>
<td>Carnation</td>
<td></td>
<td>Florigene</td>
<td>Modified flower colour</td>
<td>01/12.97 (MS consent)</td>
</tr>
<tr>
<td>Maize</td>
<td>Zea mays L. line MON 810</td>
<td>Monsanto</td>
<td>Expression of the Bt crudeA(b) gene</td>
<td>98/294/EC of 22 April 1998</td>
</tr>
<tr>
<td>Maize*</td>
<td>Zea mays L. line Bt-11</td>
<td>Novartis</td>
<td>Tolerance to glufosinate ammonium and expression of the Bt crudeA(b) gene</td>
<td>98/292/EC of 22 April 1998</td>
</tr>
<tr>
<td>Spring swede rape*</td>
<td>Brassica napus L. ssp. oleifera</td>
<td>AgrEvo</td>
<td>Tolerance to glufosinate ammonium</td>
<td>98/291/EC of 22 April 1998</td>
</tr>
<tr>
<td>Swede rape</td>
<td>Brassica napus L. oleifera Metzg. MS1, RF2</td>
<td>Plant Genetic Systems</td>
<td>Tolerance to glufosinate ammonium</td>
<td>97/393/EC of 6 June 1997</td>
</tr>
<tr>
<td>Swede rape</td>
<td>Brassica napus L. oleifera Metzg. MS1, RF1</td>
<td>Plant Genetic Systems</td>
<td>Tolerance to glufosinate ammonium</td>
<td>97/392/EC of 6 June 1997</td>
</tr>
<tr>
<td>Male sterile chicory**</td>
<td>Cichorium intybus L.</td>
<td>Bejo-Zaden BV</td>
<td>Tolerance to glufosinate ammonium</td>
<td>96/424/EC of 20 May 1996</td>
</tr>
<tr>
<td>Soybean*</td>
<td>Glycine max L. (Roundup Ready)</td>
<td>Monsanto</td>
<td>Tolerance to glyphosate</td>
<td>96/281/EC of 3 April 1996</td>
</tr>
<tr>
<td>Swede rape</td>
<td>Brassica napus L. oleifera Metzg. MS1Bn x RF1Bn</td>
<td>Plant Genetic Systems</td>
<td>Tolerance to glufosinate ammonium</td>
<td>96/158/EC of 6 February 1996</td>
</tr>
<tr>
<td>Tobacco</td>
<td>Variety ITB 1000 OX</td>
<td>SEITA</td>
<td>Tolerance to bromoxynil</td>
<td>94/385/EC of 8 June 1994</td>
</tr>
</tbody>
</table>

*Cultivation in the EU not authorised; **Only for seed production

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 that specifically concerned:
- foods and food ingredients containing or consisting of genetically modified organisms within the meaning of Directive 90/220/EEC;
- foods and food ingredients produced from, but not containing, genetically modified organisms;
- foods and food ingredients with a new or intentionally modified primary molecular structure;
- foods and food ingredients consisting of or isolated from micro-organisms, fungi or algae;
- foods and food ingredients consisting of, or isolated from, plants and food ingredients isolated from animals, except for foods and food ingredients obtained by traditional propagating or breeding practices and having a history of safe food use;
- foods and food ingredients to which a production process not currently used has been applied, where that process gives rise to significant changes in the composition or structure of the foods or food ingredients which affect their nutritional value, metabolism or level of undesirable substances.

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.

In fact, as two GMOs (Roundup Ready® soybean and Maximizer maize) had been placed on the market before Regulation (EC) 258/97 came into force, specific labelling requirements for these GMOs have been dealt with a posteriori by Council Regulation (EC) No 1139/98.

In Regulation (EC) 258/97 specific labelling requirements were established in order to ensure that the final consumer was informed of any change in the characteristic or food property such as: composition, nutritional value or nutritional effects or intended use of the food that rendered a novel food or food ingredient no longer equivalent to an existing food or
food ingredient. At present products form seventeen GM events have been approved and can be legally marketed in the EU (see Table 2). One GM soy and one GM maize were approved under Directive 90/220/EEC prior to the entering into force of the Novel Foods Regulation. The others – processed foods derived from *inter alia* 7 GM oilseed rape, 5 GM maize and oil from 2 GM cottonseeds - have all been notified as substantially equivalent in accordance with the Novel Foods Regulation and authorised via the simplified procedure.

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 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.
Table 2. Genetically modified (GM) foods authorised in the European Union

<table>
<thead>
<tr>
<th>EVENT</th>
<th>CROP</th>
<th>APPLICANT</th>
<th>TRAIT</th>
<th>POTENTIAL FOOD USES</th>
<th>DATE</th>
<th>LEGAL BASIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTS 40/3/2</td>
<td>Soybean</td>
<td>Monsanto</td>
<td>Insect protection and herbicide tolerance</td>
<td>Soy foods. Soy foods include soy beverages, tofu, soy oil, soy flour, lecithin.</td>
<td>03.04.1996</td>
<td>Dir. 90/220/EEC Art. 13</td>
</tr>
<tr>
<td>Bt 176</td>
<td>Maize</td>
<td>Ciba-Geigy</td>
<td>Insect protection and herbicide tolerance</td>
<td>Maize foods. Maize foods include kernels, oil, maize flour, sugar, syrup.</td>
<td>23.01.1997</td>
<td>Dir. 90/220/EEC Art. 13</td>
</tr>
<tr>
<td>TOPAS 19/2</td>
<td>Oilseed rape</td>
<td>AgrEvo</td>
<td>Herbicide tolerance</td>
<td>Rapeseed oil. Products made with rapeseed oil may include fried foods, baked products and snack foods.</td>
<td>24.06.1997</td>
<td>Reg. (EC) 258/97 Art. 5</td>
</tr>
<tr>
<td>MS1 / RF2</td>
<td>Oilseed rape</td>
<td>Plant Genetic Systems</td>
<td>Herbicide tolerance</td>
<td></td>
<td>24.06.1997</td>
<td>Reg. (EC) 258/97 Art. 5</td>
</tr>
<tr>
<td>MS1 / RF1</td>
<td>Oilseed rape</td>
<td>Plant Genetic Systems</td>
<td>Herbicide tolerance</td>
<td></td>
<td>24.06.1997</td>
<td>Reg. (EC) 258/97 Art. 5</td>
</tr>
<tr>
<td>GT 73</td>
<td>Oilseed rape</td>
<td>Monsanto</td>
<td>Herbicide tolerance</td>
<td></td>
<td>21.11.1997</td>
<td>Reg. (EC) 258/97 Art. 5</td>
</tr>
<tr>
<td>MON 810</td>
<td>Maize</td>
<td>Monsanto</td>
<td>Insect protection</td>
<td>Maize derivatives. These may include maize oil, maize flour, sugar and syrup. Products made with maize derivatives may include snack foods, baked foods, fried foods, confectionary and soft drinks.</td>
<td>06.02.1998</td>
<td>Reg. (EC) 258/97 Art. 5</td>
</tr>
<tr>
<td>T 25</td>
<td>Maize</td>
<td>AgrEvo</td>
<td>Herbicide tolerance</td>
<td></td>
<td>06.02.1998</td>
<td>Reg. (EC) 258/97 Art. 5</td>
</tr>
<tr>
<td>Bt 11</td>
<td>Maize</td>
<td>Novartis</td>
<td>Insect protection</td>
<td></td>
<td>06.02.1998</td>
<td>Reg. (EC) 258/97 Art. 5</td>
</tr>
<tr>
<td>MON 809</td>
<td>Maize</td>
<td>Pioneer</td>
<td>Insect protection</td>
<td></td>
<td>23.10.1998</td>
<td>Reg. (EC) 258/97 Art. 5</td>
</tr>
<tr>
<td>Falcon GS 40/90</td>
<td>Oilseed rape</td>
<td>Hoechst / AgrEvo</td>
<td>Herbicide tolerance</td>
<td>Rapeseed oil. Products made with rapeseed oil may include fried foods, baked foods and snack foods</td>
<td>08.11.1999</td>
<td>Reg. (EC) 258/97 Art. 5</td>
</tr>
<tr>
<td>Liberator L62</td>
<td>Oilseed rape</td>
<td>Hoechst / AgrEvo</td>
<td>Herbicide tolerance</td>
<td></td>
<td>08.11.1999</td>
<td>Reg. (EC) 258/97 Art. 5</td>
</tr>
<tr>
<td>1445</td>
<td>Cotton</td>
<td>Monsanto</td>
<td>Herbicide tolerance</td>
<td>Cottonseed oil. Products made with cottonseed oil may include fried foods, baked foods and snack foods.</td>
<td>19.12.2002</td>
<td>Reg. (EC) 258/97 Art. 5</td>
</tr>
<tr>
<td>pRF69/pRF93</td>
<td>Bacillus subtilis</td>
<td>F. Hoffmann - La Roche</td>
<td>Riboflavin</td>
<td>Vitamin B2</td>
<td>23.03.2000</td>
<td>Reg. (EC) 258/97 Art. 5</td>
</tr>
<tr>
<td>Bt11</td>
<td>Maize</td>
<td>Syngenta</td>
<td>Insect resistance</td>
<td>Sweet maize</td>
<td>19.05.2004</td>
<td>Reg. (EC) No. 258/97 Art. 7</td>
</tr>
</tbody>
</table>

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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 vacuum for feed products approval, whilst abandoning the simplified procedure based on the concept of “substantial equivalence”.

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, will be 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 will be evaluated and validated by the Community Reference Laboratory (Established by Regulation (EC) 1829/2003).

In the Regulation new *de minimis* thresholds for labelling are defined. The 1% threshold specified under Commission Regulation (EC) 49/2000 for the adventitious presence of approved GMOs has been lowered to 0.9%. In addition, a 0.5% threshold for the adventitious presence of non-approved GMOs, has been established, as a transitional rule, provided they have benefitted from a favourable opinion from the relevant Scientific Committee(s). The EU recognizes the consumers’ right for information and labelling as a tool to make an informed choice. Since 1997 labelling to indicate the presence of GMOs as such or in a product is mandatory. However, **Regulation (EC) 1830/2003** reinforces the current labelling rules on GM food: mandatory labelling is extended to all food and feed irrespective of detectability, and provides a definition of traceability as the ability to trace GMOs and products produced from GMOs at all stages of their placing on the market through their production and distribution chains. Methods are thus necessary, not only to detect the eventual presence of a GMO in a food 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 products, to investigate whether GMO specific compounds (DNA and/or proteins) are present. Qualitative analysis could thus be performed on products, sampled from the shelves of supermarkets, 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, a subsequent quantitative test might give a decisive answer concerning the labelling requirement.

As previously mentioned, an essential new integral component of the legislative procedure enters the statutory framework: the Community Reference Laboratory (CRL). In the context of Regulation (EC) 1829/2003, the JRC, assisted by the European Network of GMO Laboratories, has been appointed as the Community Reference Laboratory for GM Food and Feed (CRL-GMFF; http://gmo-crl.jrc.ec.europa.eu/).

The CRL-GMFF has the mandate to evaluate and validate analytical methods to ensure that they are “fit for the purpose of regulatory compliance” and to provide scientific and technical advice in case of disputes.

An integral theme of the legislation cited calls for the availability of methods of analysis that are sound, precise and robust. This calls for research activity to help ensure a harmonised and standardised approach and set of analytical procedures and performances across all EU GMO enforcement and control laboratories.

The need for harmonisation and standardization of procedures and performance within European control laboratories has already been identified by the JRC as an essential element for the success of any piece of control legislation for some years.

Indeed, The Molecular Biology & Genomics Unit of the JRC's Institute for Health and Consumer Protection proposed and promoted the formation of an enforcement network of laboratories involved in GMOs related issues since 1999.

The European Network of GMO Laboratories (ENGL; http://engl.jrc.ec.europa.eu/), formally inaugurated in December 2002, is now composed by around 100 control laboratories from all EU Member States plus Switzerland and Norway. The chairmanship and secretariat of the Network is under the responsibility of the Molecular Biology & Genomics Unit of the JRC's Institute for Health and Consumer Protection.

The scope of the European Network of GMO Laboratories is to create 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, namely:

- Method development for qualitative and quantitative analysis;
- Technology transfer, training and capacity building;
- Validation and proficiency studies of methods suitable either for screening of various matrices for the presence of GMOs, or for the estimation of the GMO quantities present;
- Reference material (the responsibility for this work package lies with the JRC's Institute for Reference Materials and Measurements);
- Sampling strategies and procedures for different GM-commodities (seeds, grains, raw material, products for final consumers or mass caterers);
- Databases and bioinformatics and requirements for unique identification of GMOs and setting up of databases that contain such molecular data.

In the framework of the network activities, training courses are to be considered as one of the major tools to achieve the above-mentioned objectives.
Safety and labelling of foods derived from modern biotechnology - The WHO perspective

The release of GMOs into the environment and the marketing of GM foods have resulted in a public debate in many parts of the world. This debate is likely to continue, probably in the broader context of other uses of biotechnology (e.g. in human medicine) and their consequences for human societies. Even though the issues under debate are usually very similar (costs and benefits, safety issues), the outcome of the debate differs from country to country. On issues such as labelling and traceability of GM foods as a way to address consumer concerns, there is no consensus to date. This has become apparent during discussions within the Codex Alimentarius Commission over the past few years. The Codex Alimentarius Commission (Codex; http://www.codexalimentarius.net/web/index_en.jsp) is the joint FAO/WHO body responsible for compiling the standards, codes of practice, guidelines and recommendations that constitute the Codex Alimentarius: the international food code. Despite the lack of consensus on these topics, significant progress has been made on the harmonization of views concerning risk assessment. The Codex is however about to adopt principles on premarket risk assessment revealing a growing understanding at the international level (see prepublication of the “Principles for the risk analysis of foods derived from modern biotechnology, CAC/GL 44-2003 at ftp://ftp.fao.org/es/esn/food/princ_gmfoods_en.pdf)

Different GM organisms include different genes inserted in different ways. This means that individual GM foods and their safety should be assessed on a case-by-case basis and that it is not possible to make general statements on the safety of all GM foods. According to the WHO, GM foods currently available on the international market have passed risk assessments and are not likely to present risks for human health. In addition, no effects on human health have been shown as a result of the consumption of such foods by the general population in the countries where they have been approved. Continuous use of risk assessments based on the Codex principles and, where appropriate, including post market monitoring, should form the basis for evaluating the safety of GM foods.

Labelling of foods produced through biotechnology may or may not be related to food safety per se, but it is being seen by the WHO as a tool to increase the transparency of food production processes. Such labelling may also foster the development of traceability

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4 For exhaustive information on the activity of WHO and other UN agencies on Foods derived from Modern Biotechnologies see http://www.who.int/foodsafety/en/.
strategies, which could be seen to contribute to improving national food safety programmes and therefore contribute indirectly to food safety in general. Thus the importance of suitable methods of analysis and sampling is clear.

The WHO has been active in the development of principles and recommendations for the safety and risk assessment of foods derived from biotechnology. The results developed in the course of various expert consultations form the basis for guidelines at the national level and are presently being incorporated into internationally recognized guidelines. The approach based on the principle of substantial equivalence was developed for the safety assessment of the first generation of genetically modified (GM) crops and is felt by many to be an adequate approach. Nevertheless, the concept is subject to ongoing criticism. Contemporary activities have to take these arguments into account and contribute to the development of science-based adjustments and improvements.

The WHO/FAO Expert Consultation on Safety Aspects of Genetically Modified Foods of Plant Origin held in 2000 recognised that the concept of substantial equivalence can be used as a comparative approach focusing on the similarities and differences between the genetically modified food and its conventional counterpart. Simultaneously, it expressed the view that the concept of substantial equivalence is not a safety assessment in itself nor an endpoint but just a starting point of the safety assessment (http://www.fao.org/ag/agn/food/risk_biotech_aspects_en.stm).

The next generation of GM foods will be crops with improved nutritional value, thus crossing the borderline to functional foods and nutraceuticals. Future food safety assessment strategies will have to cope with the more complex metabolic changes caused by the given genetic modification(s). Evaluations will increasingly have to consider the impact of a GM food on the overall nutritional status taking into account the different needs in developed and developing countries.

No specific international regulatory systems are currently in place. However, several international organizations are involved in developing protocols for GMOs. As mentioned above, Codex is developing principles for human health risk analysis of GM foods. The premise of these principles dictates a pre-market assessment, performed on a case-by-case basis and including an evaluation of both direct effects (from the inserted gene) and unintended effects (that may arise as a consequence of the insertion of a new gene). The principles are at an advanced stage of development but have not been adopted yet (see prepublication of the “Principles for the risk analysis of foods derived from modern biotechnology, CAC/GL 44-2003 at ftp://ftp.fao.org/es/esn/food/princ_gmfoods_en.pdf). These and other Codex principles under development (including Principles on Methods of
Analysis and Sampling) do not have a binding effect on national legislation, but are referred to specifically in the Sanitary and Phytosanitary Agreement of the World Trade Organization (SPS Agreement), and can be used as a reference in cases of trade disputes.

Taking into account the work delivered between 1999-2003 by the Ad Hoc Intergovernmental Codex Task Force on Foods Derived from Biotechnology (http://www.fao.org/ag/agn/agns/biotechnology_codex_en.asp), the Codex Committee on Food Labelling and the Codex Committee for Methods of Analysis and Sampling, the development of generally accepted guidelines for the safety assessment of foods derived from biotechnology, risk communication issues (e.g. labelling) and therefore the development of appropriate methods of analysis will continue to be a focus of WHO activities.

WHO continues therefore to convene expert consultations (http://www.who.int/foodsafety/biotech/consult/en/) to develop principles and methodologies for risk assessment, risk management, and risk communication associated with foods produced through biotechnology and coordinates collaboration between developed and developing countries in the field of detection methods.

The Food Safety Department within WHO is finalizing an evidence-based study of the implications of modern food biotechnology on human health and development. Impetus for the study arose from a resolution of the fifty-third World Health Assembly in May 2000 that the WHO should strengthen its capacity to support Member States establish the scientific basis for decisions on modern food biotechnology, and to ensure the transparency, excellence and independence of opinions delivered.

The study (http://www.who.int/foodsafety/biotech/who_study/en/index.html) aims to complement the efforts of other international agencies by collating already existing information and analysing it as it pertains to the WHO mandate. To enhance transparency in the process, WHO has collaborated with FAO and involved an array of stakeholders and interest groups. The primary aim is to create an accessible knowledge base to assist Member States, international standards bodies and other stakeholders to achieve transparent and inclusive consensus on the evaluation and application of biotechnology. Finally, WHO is seeking to establish an evidence-based foundation for a more holistic evaluation of biotechnology in the future.

This study was looking to place the overall contribution that modern food biotechnology can make to human health and development in context. It includes the application of modern food biotechnology to microorganisms, plants and animals. An integrated (holistic) approach was adopted to identify the key issues impacting directly or indirectly on human health and
development, and establish the available evidence. The main issues on which evidence was invited:

- Research and Development;
- Impact on human health (food safety and environmental effects);
- Food security, cost and access to the technology;
- Ethical, legal and social issues;
- Capacity-building initiatives.

Data were gathered through extensive literature, Internet and enquiry-based research supported by approximately 120 responses to a questionnaire, which was circulated to a broad range of stakeholders and experts in May 2002. The comments received from an electronic stakeholder discussion held between January and April 2003 have also been incorporated. The opinions of participants comprising representatives from government, consumer, industry, research, non-government organizations (NGOs) from developed and developing countries, who attended a stakeholder meeting on 5 and 6 June 2003 in Geneva, were also included.

The report produced from this consultation process will be used directly by WHO in planning its future activities with regard to the use and application of modern biotechnology in human health and development.
Annex 1 – EU legislation governing the placing on the market of GMOs, including the related labelling and control programmes

(OJ L 117, 8.5.1990, p. 15)
Repealed by:
(OJ L 106, 17.4.2001, p. 1)

(OJ L 117, 8.5.1990, p. 1)
Amended by:


Council Regulation (EC) 1139/98 of 26 May 1998 concerning the compulsory indication of the labelling of certain foodstuffs produced from genetically modified organisms of particulars other than those provided for in Directive 79/112/EEC.
(OJ L 159, 3.6.1998, p. 4)


Commission Regulation (EC) 50/2000 of 10 January 2000 on the labelling of foodstuffs and food ingredients containing additives and flavourings that have been genetically modified or have been produced from genetically modified organisms. (OJ L 006, 11.1.2000, p. 15)


(OJ L 268, 18.10.2003, p. 1)

(OJ L 268, 18.10.2003, p. 24)

Commission Regulation (EC) 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation
(OJ L 102, 7.4.2004, p. 14)

(OJ L 33, 8.2.1979, p. 1)

Commission Regulation (EC) 65/2004 of 14 January 2004 establishing a system for the development and assignment of unique identifiers for genetically modified organisms
(OJ L 10, 16.1.2004, p. 6)

REGULATION (EC) No 882/2004 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules
(OJ L 165, 30.4.2004, p. 141)
The Analysis of Food Samples for the Presence of Genetically Modified Organisms

Session 2

Manual Presentation, Working Methods and Course Introduction

M. Querci
Table of Contents

Session 2

Manual Presentation, Working Methods and Course Introduction

How to detect GMOs 3
Inherent advantages and limitations of DNA and protein-based approaches 4
General considerations and presentation of the manual 7

References 11
How to detect GMOs

As discussed previously, 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 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.

There are two scientific approaches generally used today for detecting genetic modification in crops such as soybeans, corn, cotton and others. One, **ELISA** (Enzyme-Linked ImmunoSorbent Assay), involves testing for the presence of specific proteins by exploiting the specificity of binding between expressed antigen and target antibody; the other, **PCR** (Polymerase Chain Reaction), is based on the detection of novel DNA sequences inserted into the crops genome. These methods show the absence or presence of the GMO in the sample but can also give some indication of quantity (percentage) in a tested sample.

The first method validated at the EU level was for a PCR-based screening method able to detect most of the GMOs presently approved for marketing (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 co-ordinated by the Food Products and Consumer Goods Unit of the IHCP, Joint Research Centre and carried out in collaboration with the JRC Institute for Reference Materials and Measurements (IRMM), which was responsible for the production of appropriate Certified Reference Materials.

As mentioned above, research efforts are also directed at the development of protein-based methods. A highly specific method for the detection of Roundup Ready® soybean using ELISA has been validated (Lipp et al., 2000) and others have been developed (http://mbg.jrc.ec.europa.eu/home/ict/methodsdatabase.htm).
Inherent advantages and limitations of DNA 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 frequency in a complex mixture of other DNA sequences. In PCR, the small complementary DNA pieces are referred to as primers and are used in pairs. These primers are designed to hybridise to complementary sequence recognition sites on opposite strands of the gene of interest. Through a series of repetitive differential thermal cycles a DNA polymerase enzyme aids the replication and exponential amplification of the sequence between the primer pair. Finally, these amplified pieces are subjected to standard gel electrophoresis so their presence can be detected based on their size determination.

Numerous PCR-based methods have been developed which can detect and quantify GMOs in agricultural food and feed crops. Moreover, the determination of genetic identity allows for segregation and traceability (identity preservation) throughout the supply chain of GM crops.

An essential 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, 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:
- It can be extremely 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 inadvertent contamination can result in false positives. Therefore, great care must be taken to prevent cross contamination.
- It requires little reagent development time compared to immunological assays (primer synthesis vs. antibody production).
- Nearly all reagents needed are commercially available and can be readily 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 to 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 also required for proper execution of the test.

Key characteristics of ELISA evaluations include:
- Less sensitive than PCR, therefore, less susceptible than PCR to ‘false positives’ caused by minor levels of contamination.
- High up front costs for assay development and generation of antibodies and protein standards.
- Low per sample cost once reagents are developed.
- It cannot discriminate between different expression patterns and modes among different transgenic events that express similar protein characteristics.
- Protein-based methods require significant lead-time for reagents and method development.
- Protein-based testing provides a practical and effective testing process when a detectable protein is produced. However, genetically modified products might be produced only during certain developmental stages or in certain plant parts and such GMOs are therefore unlikely to be readily detected with ELISA. In addition, industrial processing easily denatures proteins, which makes it problematical to use ELISA methods for processed food fractions.
Considering these facts, it should be clear that both ELISA and PCR should be regarded as complementary rather than exclusive to each other.

**Table 1.** Summarised comparison of the ELISA and PCR methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Tests for</th>
<th>Duration</th>
<th>Ease of use</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>Protein</td>
<td>2 - 8 hours</td>
<td>Moderate; requires familiarity with laboratory practices; tests are crop and variety specific</td>
<td>Confirms specific genetic modification and allows quantification.</td>
</tr>
<tr>
<td>PCR</td>
<td>DNA</td>
<td>1 - 3 days</td>
<td>Difficult; requires specialised equipment and training</td>
<td>Very sensitive; prone to false positives; confirms presence of GM DNA and allows quantification.</td>
</tr>
</tbody>
</table>
**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, sensitive and specific results. The Joint Research Centre of the European Commission was 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 then, 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://mbg.jrc.ec.europa.eu/home/ict/methodsdatabase.htm](http://mbg.jrc.ec.europa.eu/home/ict/methodsdatabase.htm). **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 question (e.g. qualitative or quantitative) of interest. 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 JRC IRMM (Trapmann *et al.*, 2002 and 2001). The characteristics and corresponding certificates are presented in Session 3. Another critical step, which will not be performed during the course, but is important to mention, is **sample homogenisation**.

Figure 1 summarises the different steps performed during the course. Optimised **DNA extraction** is fundamental to insure the presence and quality of extracted, 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 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 insure 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 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 **Polymerase Chain Reaction** 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 target gene sequence and type of genetic modification. The specific characteristics of transgenic lines MON810 maize, Bt-176 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 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 generally obtained 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 amount 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 two most common DNA-based approaches are competitive PCR and real-time PCR (Session 10). Real-time PCR is performed using specific and sophisticated instrumentation, currently available from only a few 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 serological approach for the detection of genetically modified organisms. In particular the **ELISA** technique will be explained and the protocol for conducting of a Roundup Ready® specific ELISA test will be provided.
Figure 1. Flowchart of methods performed during the course.

- **Sampling Homogenisation**
- **DNA extraction**
- **Plant DNA check by PCR**
  - **Plant DNA present**
  - **No plant DNA detectable**
- **Screening PCR**
  - **GM Plant**
  - **Non GM Plant**
- **Specific GMO detection by nested PCR**
- **DNA quantification using spectrophotometry**
- **GMO quantification by real-time PCR**
- **Lectin-PCR for soybean and zein-PCR for maize**
- **Detection of regulatory elements (35S promoter and nos terminator)**

**Not performed during the course**
References


The Analysis of Food Samples for the Presence of Genetically Modified Organisms

Session 3

Samples Used during the Course

M. Querci, N. Foti
Table of Contents

Session 3

Samples Used during the Course

Samples used during the course 3
Certified Reference Material 3
Composition of raw and processed materials distributed to the participants 4
List of samples distributed during the course 6
Expected results by PCR 6

References 7
Samples used during the course

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

- Certified Reference Materials
- Different raw and processed materials distributed to the participants

Certified Reference Material

Raw plant materials used during the course are Certified Reference Materials IRMM-410S (Roundup Ready® soybean) and IRMM-413 (MON810 maize). IRMM-410S and IRMM-413 consist of two sets of CRMs of dried soybean and maize powder, respectively, with different mass fractions (0, 0.1, 0.5, 1, 2, and 5%) of dried powder prepared from genetically modified Roundup Ready® soybean and MON810 maize. The CRMs were produced by the Institute for Reference Materials and Measurements (IRMM - http://irmm.jrc.ec.europa.eu/html/homepage.htm) on behalf of Fluka Chemie AG (Buchs, Switzerland) in the frame of a collaboration with the Institute for Health and Consumer Protection (IHCP - http://ihcp.jrc.ec.europa.eu/) of the Joint Research Centre of the European Commission (Ispra, Italy) (Trapmann et al., 2002 and 2001). They are intended for the validation of methods for the detection of genetically modified food. As DNA and/or protein quantification may depend on varieties, one has to exercise due care when drawing quantitative conclusions from measurements of unknown samples.

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 DK512 and MON810 cultivar DK513.
Composition of raw and processed materials distributed to the participants

We will test different materials: biscuit, milk powder, snack food crumb and flour.

Raw materials

Mixed Flour. In order to obtain 0.5% of each GMO event (total dry weight) 1% of RR soya (IRMM-410S) was added to 1% MON810 (IRMM-413). 50 mg of both flours were weighed and added directly to reaction tubes ready for DNA extraction. Flour MON810 1% is a Certified Reference Materials IRMM-413 (1% MON810 maize).

Snack food crumb

This sample is derived from a GMO proficiency testing cheme (GeMMA Scheme, Round 06, test material GMO-06B). The material was prepared from commercially available GM free soya-based dried snack food and GM-containing soya snack food.

<table>
<thead>
<tr>
<th>Snack food crumb</th>
<th>1372 g GM-free soya snack food, 28 g GM soya snack food</th>
</tr>
</thead>
</table>

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 material was produced at the JRC Institute for Health and Consumer Protection 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). Dry soybean and maize derived flour were weighed and mixed with the other ingredients in the proportion indicated below.

<table>
<thead>
<tr>
<th>Biscuits # 1</th>
<th>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</th>
</tr>
</thead>
</table>

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 allowed to cool to room temperature. Storage was at –20°C.

**Soya milk powder**

This sample is derived from Round 05 GeMMA proficiency testing scheme.

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 Biotechnology and GMOs Unit.

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 to room temperature. Storage was at 4°C until required.
### List of samples distributed during the course

<table>
<thead>
<tr>
<th>Sample</th>
<th>% GMO (specific ingredient)</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>RR soybean</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>MON810 maize</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Biscuits #1</td>
<td>0%</td>
<td></td>
</tr>
<tr>
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<tr>
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</tr>
<tr>
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### Expected results by PCR

<table>
<thead>
<tr>
<th>Sample</th>
<th>zein</th>
<th>lectin</th>
<th>35S</th>
<th>nos</th>
<th>E35S/hsp70(b)</th>
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<tr>
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<tr>
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<tr>
<td>IRMM- 413-1 (0.1%)</td>
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The Analysis of Food Samples for the Presence of Genetically Modified Organisms

Session 4

Extraction and Purification of DNA

M. Somma
Table of Contents

Session 4

Extraction and Purification of DNA

Introduction 3

Extraction methods 4
Purification methods 4
CTAB extraction and purification method 6
Quantification of DNA by spectrophotometry 9
Principles of spectrophotometric determination of DNA 9
Determination of the concentration of nucleic acids 11

Experimental 13

References 17
**Introduction**

Extraction and purification of nucleic acids is the first step in most molecular biology studies and in all recombinant DNA techniques. Here the objective of nucleic acid extraction methods is to obtain purified nucleic acids from various sources with the aim of conducting a GM specific analysis using the Polymerase Chain Reaction (PCR). Quality and purity of nucleic acids are some of the most critical factors for PCR analysis. In order to obtain highly purified nucleic acids free from inhibiting contaminants, suitable extraction methods should be applied. The possible contaminants that could inhibit the performance of the PCR analysis are listed in Table 1. In order to avoid the arising of a false negative result due to 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.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibiting concentration</th>
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<tbody>
<tr>
<td>SDS</td>
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</tr>
<tr>
<td>Phenol</td>
<td>&gt; 0.2%</td>
</tr>
<tr>
<td>Ethanol</td>
<td>&gt; 1%</td>
</tr>
<tr>
<td>Isopropanol</td>
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</tr>
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<td>Sodium acetate</td>
<td>&gt; 5 mM</td>
</tr>
<tr>
<td>Sodium chloride</td>
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<tr>
<td>EDTA</td>
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<tr>
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<tr>
<td>Urea</td>
<td>&gt; 20 mM</td>
</tr>
<tr>
<td>Reaction mixture</td>
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</table>

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

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

The principles of some of the most common methodologies used today for the extraction and purification of nucleic acids are described in the following sections.
Extraction methods

The extraction of nucleic acids from biological material requires cell lysis, inactivation of cellular nucleases and separation of the desired nucleic acid from cellular debris. 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 may 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 may easily be removed by filtration 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 will be given in the following paragraphs (Zimmermann et al., 1998).

Extraction/Precipitation

Solvent extraction is often used to eliminate contaminants from nucleic acids. For example, a combination of phenol and chloroform is frequently used to remove proteins. Precipitation with isopropanol or ethanol is generally 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 may be used directly in downstream applications.

Centrifugation

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

Affinity separation

In recent years, more and more purification methods have combined affinity immobilisation of nucleic acids with magnetic separation. 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 foodstuff and is particularly suitable for the elimination of polysaccharides and polyphenolic compounds otherwise affecting the 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 to adapt the method to 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 cetyltrimethylammonium bromide (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.
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 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

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. 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,

1 Pictures in current and following page: "Genetic Science Learning Center, University of Utah, http://gslc.genetics.utah.edu."
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 is performed.

**Figure 3:** Disruption of the cellular membrane and extraction of genomic DNA

**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 particularly important because of their capability 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 completely remove 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 then added to the prior extract. Once the nucleic acid complex has been purified, the last step of the procedure, precipitation, can be accomplished.

**Precipitation.** In this final stage, the nucleic acid is liberated from the detergent. For this purpose, the aqueous solution is first treated with a precipitation solution comprising a mixture of CTAB and NaCl at elevated concentration (> 0.8 M NaCl). The salt is needed for the formation of a nucleic acid precipitate. Sodium acetate may
be preferred over NaCl for its buffering capacity. Under these conditions, the detergent, which is more soluble in alcohol than in water, can be washed out, while the nucleic acid precipitates. The successive treatment with 70% ethanol allows an additional purification, or wash, of the nucleic acid from the remaining salt.

**Quantification of DNA by spectrophotometry**

DNA, RNA, oligonucleotides and even mononucleotides can be measured directly in aqueous solutions in a diluted or undiluted form 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 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 by 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.

An alternative method, the ethidium bromide agarose plate method, is useful when only small quantities of nucleic acid are available; the amount of nucleic acid can be estimated, when compared to a range of concentration standards, from the intensity of fluorescence emitted by the ethidium bromide when irradiated with UV light.

**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.

![Light Transmission Diagram](image)

**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 $A$ (also called optical density, $OD$) and the concentration of the macromolecule given by the following equation:

$$A = OD = \varepsilon lc$$

(1)

Where $\varepsilon$ is the molar extinction coefficient, $c$ is the concentration; and $l$ is the pathlength 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 the fact 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 GMOs 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 pathlength
- to set the correct factor (select between dsDNA, ssDNA, RNA)
- to measure a blank solution (set reference) constituted by either water or a buffer solution ($A_{260} = 0$)
- to ensure that the set reference is renewed periodically
- to measure a known amount of pure nucleic acid in order to check the reliability of set reference

**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). 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 because of 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/µl) = $A_{260}$/0.027
- **Double-stranded DNA:** $c$(pmol/µl) = $A_{260}$/0.020
- **Single-stranded RNA:** $c$(pmol/µl) = $A_{260}$/0.025
- **Oligonucleotide:** $c$(pmol/µl) = $A_{260}100/1.5N_A+0.71N_C+1.20N_G + 0.84N_T$
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 pathlength 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**

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<thead>
<tr>
<th>Wavelength</th>
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<th>$A_{260}/A_{280}$</th>
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<td>325</td>
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<tr>
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<td>0.28</td>
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<td>2.0</td>
<td>28</td>
</tr>
<tr>
<td>230</td>
<td>0.30</td>
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<td>-</td>
</tr>
</tbody>
</table>
Experimental

Equipment

**REMARK**
All of the equipment used 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
- Weigh boats or equivalents
- Spatulas
- Balance capable of 0.01 g measurement
- Loops
- Rack for microcentrifuge tubes
- Optional: vacuum desiccator to dry DNA pellets

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 124-03-8
- Chloroform
- Isopropanol
- Na₂EDTA CAS 6381-92-6
- Ethanol
- NaCl
• Proteinase K
• RNase A
• Tris[hydroxymethyl] aminomethane hydrochloride (Tris-HCl)
• Sterile deionised water

**CTAB-buffer**

- 20 g/l CTAB
- 1.4 M NaCl
- 0.1 M Tris-HCl
- 20 mM Na₂EDTA

- add 100 ml of deionised water
- adjust pH to a value of 8.0 with 1M NaOH
- fill up to 200 ml and autoclave
- store buffer at 4°C for max. 6 months

**CTAB-precipitation solution**

- 5 g/l CTAB
- 0.04 M NaCl

- add 100 ml of deionised water
- adjust pH to a value of 8.0 with 1 M NaOH
- fill up to 200 ml and autoclave
- store solution at 4°C for max. 6 months

**NaCl 1.2 M**

- dissolve 7.0 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.

**RNase A 10 mg/ml** store at –20°C
Proteinase K 20 mg/ml   store at –20°C

Procedure

The procedure requires sterile conditions. Contamination may be avoided during sample preparation by using single-use equipment, decontamination solutions and by avoiding the formation of dust.

- transfer 100 mg of a homogeneous sample into a sterile 1.5 ml microcentrifuge tube
- add 300 µl of sterile deionised water, mix with a loop
- add 500 µl of CTAB-buffer, mix with a loop
- Add 20 µl Proteinase K (20 mg/ml), shake and incubate at 65°C for 30-90 min *
- Add 20 µl RNase A (10 mg/ml), shake and incubate at 65°C for 5-10 min *
- centrifuge for 10 min at about 16,000 xg
- transfer supernatant to a microcentrifuge tube containing 500 µl chloroform, shake for 30 sec
- centrifuge for 10 min at 16,000 xg until phase separation occurs
- transfer 500 µl of upper layer into a new microcentrifuge tube containing 500 µl chloroform, shake
- centrifuge for 5 min at 16,000 xg
- transfer upper layer to a new microcentrifuge tube
- add 2 volumes of CTAB precipitation solution, mix by pipetting
- incubate for 60 min at room temperature
- centrifuge for 5 min at 16,000 xg
- discard supernatant
- dissolve precipitate in 350 µl NaCl (1.2 M)
- add 350 µl chloroform and shake for 30 sec
- centrifuge for 10 min at 16,000 xg until phase separation occurs
- transfer upper layer to a new microcentrifuge tube
- add 0.6 volumes of isopropanol, shake

* These additional optional steps are now commonly introduced to the CTAB extraction method to enhance the yield of genomic DNA from highly complex matrices.
• centrifuge for 10 min at 16,000 xg
• discard the supernatant
• add 500 µl of 70% ethanol solution and shake carefully
• centrifuge for 10 min at 16,000 xg
• discard supernatant
• dry pellets and re-dissolve DNA in 100 µl sterile deionised water

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


The Analysis of Food Samples for the Presence of Genetically Modified Organisms

Session 5

Agarose Gel Electrophoresis

M. Somma, M. Querci
# Table of Contents

## Session 5

### Agarose Gel Electrophoresis

*Introduction* 3

Physical principles of agarose gel electrophoresis 3
Components of agarose gel electrophoresis 6

Experimental 8

*References* 12
Introduction

Gel electrophoresis is a method that separates macromolecules on the basis of 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 gel, motivated by an electrical current. The driving force for electrophoresis is the voltage applied to electrodes at either end of the gel. The properties of a molecule determine how rapidly an electric field can move it through a gelatinous medium.

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).

Electrophoresis through agarose 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 ethidium bromide, a fluorescent intercalating dye. 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, 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.

To completely understand 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:

$$ E = \frac{V}{d} $$

(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:

$$ F = Eq $$

(2)

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

There is also a 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:

$$ v = Eq / f $$

(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:

$$ M = \frac{v}{E} $$

(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, $f$. 
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:

\[ R = \frac{V}{I} \]  

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:

\[ W = I^2R \]  

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 handling. Agarose gels have large "pore" sizes and are used primarily to separate large molecules with a molecular mass greater than 200 kDa.

Agarose gels process quickly, but with limited resolution since the bands formed in the agarose gels tend to be fuzzy/diffuse and spread apart. This is a result of pore size and cannot be controlled. Agarose gels are obtained by suspending dry powdered agarose in an aqueous buffer, then boiling the mixture until the agarose melts into a clear solution. The solution is then poured onto a gel-tray and allowed to cool to room temperature to form a rigid gel. Upon hardening, the agarose forms a matrix whose density is determined by its concentration.

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 and a significant amount of heat is generated. In the worst circumstance, the gel melts and the DNA denatures.

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 1x for agarose gel electrophoresis. However, a working solution of 0.5x 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 gels 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

<table>
<thead>
<tr>
<th>% agarose</th>
<th>DNA size range (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.75</td>
<td>10.000 - 15.000</td>
</tr>
<tr>
<td>1.0</td>
<td>500 - 10.000</td>
</tr>
<tr>
<td>1.25</td>
<td>300 - 5000</td>
</tr>
<tr>
<td>1.5</td>
<td>200 - 4000</td>
</tr>
<tr>
<td>2.0</td>
<td>100 - 2500</td>
</tr>
<tr>
<td>2.5</td>
<td>50 - 1000</td>
</tr>
</tbody>
</table>

Marker DNA

For a given voltage, agarose gel and buffer concentrations, the migration distance depends on the molecular weight of the starting material. Therefore, a marker DNA of known size should be loaded into slots on both the right and left sides of the gel. A marker generally contains a defined number of known DNA segments, which makes it easier to determine the size of the unknown DNAs if any systematic distortion of the gel should occur 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, and a dye (e.g. xylene cyanole, bromophenol blue, bromocresol green, etc.). The maximum amount of DNA that can be loaded depends on the number of fragments. The minimum amount of DNA that can be detected by photography of ethidium bromide stained gels is about 2 ng in a 0.5-cm wide band. If there is more than 500 ng of DNA in a band of this width, the slot 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
- Transilluminator (UV radiation, 312 nm)
- Instruments for documentation (e.g. Polaroid camera or a video recorder)

**Reagents**

- Agarose, suitable for DNA electrophoresis
- Tris[hydroxymethyl] aminomethane (Tris) CAS 77-68-1
- Boric acid
- Na$_2$EDTA CAS 6381-92-6
- Ethidium bromide CAS 1239-45-8
- Sucrose
- Xylene cyanole FF CAS 2650-17-1
- DNA markers:
  - Lambda DNA EcoRI/HindIII digested *(or other similar suitable marker)*
  - 100 bp DNA ladder
10x TBE buffer (1 litre)

Tris[hydroxymethyl] aminomethane (Tris) 54.0 g
Boric acid 27.5 g
Na₂EDTA 7.44 g

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

6x loading buffer (10 ml)

Xylene cyanole FF 0.025 g
Sucrose 4 g

- Add sucrose and Xylene cyanole FF to deionised water to obtain 10 ml of solution.
- Mix the solution, autoclave and store at 4°C.
Agarose Gel Electrophoresis

Procedure

- Seal the edges of a clean, dry plastic gel-tray either with tape or other means. Position the appropriate comb so that complete wells are formed when the agarose solution is added.
- Dilute 10x TBE buffer to prepare the appropriate amount of 0.5x TBE buffer to fill the electrophoresis tank and to prepare the gel.
- Weigh powdered agarose according to Table 2 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).

Table 2. Agarose gel concentrations used during the course

<table>
<thead>
<tr>
<th>Genotype</th>
<th>GMO3</th>
<th>GMO4</th>
<th>ZEIN3</th>
<th>ZEIN4</th>
<th>p35S-cf3</th>
<th>p35S-cr4</th>
<th>HA-nos1/18-r</th>
<th>HA-nos1/16-f</th>
<th>CK1/15</th>
<th>CR1/A4</th>
<th>GM07</th>
<th>mg3</th>
<th>mg4</th>
<th>Genomic DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8 - 1%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>1.5%</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>2.0%</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>

- Heat the slurry in a microwave oven or in a boiling water bath until the agarose dissolves (check the volume of the solution after heating).
- Cool the mixture to 50 - 60°C and add ethidium bromide (from a stock solution of 10 mg/ml) to a final concentration of 0.2 µg/ml and mix thoroughly.
- Pour the solution into the gel-tray and allow the gel to set. The amount of gel used should correspond to a depth of approximately 3 - 5 mm.
- After the gel is completely set, carefully remove the comb and the tape and place the gel in the electrophoresis tank.
- Add enough 0.5x 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:

<table>
<thead>
<tr>
<th>sample</th>
<th>marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>water</td>
<td>water</td>
</tr>
<tr>
<td>loading buffer</td>
<td>loading buffer</td>
</tr>
<tr>
<td>sample</td>
<td>2 µl λ DNA EcoRI / HindIII</td>
</tr>
</tbody>
</table>

10 µl                                      10 µl

Prepare samples and marker for PCR products as follows:

<table>
<thead>
<tr>
<th>sample</th>
<th>marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>loading buffer</td>
<td>2 µl 100 bp DNA ladder</td>
</tr>
<tr>
<td>sample</td>
<td>8 µl</td>
</tr>
</tbody>
</table>

10 µl

- Load 10 µl of each sample into consecutive wells and the appropriate DNA marker into the first and last lane
- Close the lid of the gel tank and attach the electrical leads so that the DNA will migrate toward the anode and apply a voltage of 5 - 10 V/cm
- Run the gel until the xylene cyanole has migrated the appropriate distance through the gel (~ 40 - 60 minutes)
- Turn off the current; remove the leads and the lid from the gel tank. Place the gel on a UV lightbox and photograph the gel
- Discard the gel into the provided ethidium bromide solid waste bin
References


The Analysis of Food Samples for the Presence of Genetically Modified Organisms

Session 6

The Polymerase Chain Reaction (PCR)

M. Somma, M. Querci
Table of Contents

Session 6
The Polymerase Chain Reaction (PCR)

*Introduction* 3

Components, structure and replication of DNA 3
Principles of PCR 9
Instrumentation and components for the PCR 12
Design of primers for PCR 17
Specialised PCR 21
PCR in practice 23

*References* 29
Introduction

The invention of Polymerase Chain Reaction (PCR) by K. Mullis and co-workers in 1985 has revolutionised molecular biology and molecular medicine (Saiki et al., 1985). The Polymerase Chain Reaction is an in vitro technique used to enzymatically amplify a specific DNA region that lies between two regions of known DNA sequence. Whereas previously only minute amounts of a specific gene could be obtained, now even a single gene copy can be amplified to a million copies within a few hours using PCR.

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. More recently, 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 eucaryotic cells, most of the DNA is contained within the nucleus and is referred to as chromosomal DNA. It is separated from the rest of the cell (cytoplasm) by a double layer membrane (nuclear envelope). In addition to this, extrachromosomal DNA can be found in the mitochondria and chloroplasts.

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 is constituted of 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 an N-glycosydic 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 (Picture: Andy 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 with one another in a very precise way. Each base in a strand will pair with only one kind of base across from it in 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.
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. In physiological conditions, double-stranded DNA helix is more stable than a single-stranded DNA helix.

**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 identical copies. This process perpetuates the genetic information. 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 (Picture: Andy 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 an exact copy of the parent molecule.

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 but it is DNA polymerase III that is 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 \textit{ex novo} on a bare 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 Mg\textsuperscript{2+} ions.

The oligonucleotides typically consist of relatively short sequences, which are different to each other and complementary to recognition sites flanking the segment of target DNA to be amplified. The steps of template denaturation, primer annealing and primer extension comprise a single "cycle" in the PCR amplification methodology. Figure 5 illustrates the three major steps in a PCR amplification process.

![Figure 5. The steps of PCR amplification (Picture: Andy Vierstraete, 1999)](image)

After each cycle, the newly synthesised DNA strands can serve as templates in the next cycle. As shown in Figure 6, the major product of this exponential reaction is a
The 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 a 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 will accumulate in an exponential fashion in later rounds of amplification and will form the dominant products of the reaction. Thus, amplification, as a final number of copies of the target sequence, is expressed by the following equation:

\[ (2^n - 2n)x \]  

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. Potentially, after 20 cycles of PCR there will be a \( 2^{20} \)-fold amplification, assuming 100% efficiency during each cycle. The efficiency of a PCR will vary 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

**Template denaturation**

During denaturation, the double strand 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 rehybridisation of the DNA strands takes place at lower temperature (usually 55 - 65°C). Once the temperature is reduced, 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 min is sufficient to get a complete extension.

Instrumentation and components for the PCR

Instruments

Two major advances 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 could last throughout numerous cycles of the protocol.

b. The development of temperature baths, which could shift their temperatures up and down rapidly and in an automated, programmed manner. These are known as thermal cyclers or PCR machines.

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.

![PCR temperature cycling profile](image)

Figure 7. PCR temperature cycling profile

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, are critical for a successful PCR.
Target DNA

In principle, PCR amplification can be performed if at least one intact copy of the target gene 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 block 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, Mg²⁺-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 repeating motifs - 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. Sequences complementary to other primers used in the PCR should also be avoided so to prevent hybridisation between primers, or primer dimer formation (particularly important for the 3’ end of the primer). If possible, 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 limiting primer concentration.

DNA polymerase

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 had 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). Even though this enzyme is probably the most widely used in PCR applications, several other DNA polymerases are commercially available. Table 1 lists the properties of some thermostable DNA polymerases currently in use for PCR (Newton and Graham, 1994).

**Table 1. Characteristics of some DNA polymerases used for PCR**

<table>
<thead>
<tr>
<th>Source</th>
<th><em>Taq</em>/AmpliTaq®</th>
<th>Vent™</th>
<th>Deep-Vent™</th>
<th>Pfu</th>
<th>Tth</th>
<th>UIrm™</th>
</tr>
</thead>
<tbody>
<tr>
<td>Application</td>
<td><em>Taq</em>: natural</td>
<td>For genetic engineering</td>
<td>For genetic engineering</td>
<td>Natural</td>
<td>For genetic engineering</td>
<td>For genetic engineering</td>
</tr>
<tr>
<td><em>Tₚₐ of activity at 95 °C (min)</em></td>
<td>40</td>
<td>1380</td>
<td>400</td>
<td>&gt;120</td>
<td>20</td>
<td>&gt;50²</td>
</tr>
<tr>
<td><em>5’ to 3’ Exonuclease activity</em></td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td><em>3’ to 5’ Exonuclease activity</em></td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Processivity</td>
<td>50-60</td>
<td>?</td>
<td>7</td>
<td>?</td>
<td>30-40</td>
<td>?</td>
</tr>
<tr>
<td>Extension rate (nt/s)</td>
<td>75</td>
<td>?</td>
<td>&gt;80</td>
<td>60</td>
<td>&gt;33</td>
<td>?</td>
</tr>
<tr>
<td>Resulting DNA ends</td>
<td>3’A</td>
<td>&gt;95% blunt</td>
<td>&gt;95% blunt</td>
<td>?</td>
<td>3’A blunt</td>
<td></td>
</tr>
<tr>
<td>MW in kDa</td>
<td>94</td>
<td>?</td>
<td>?</td>
<td>92</td>
<td>94</td>
<td>70</td>
</tr>
</tbody>
</table>

*AmpliTaq® DNA polymerase*. As already mentioned, this enzyme was isolated from the bacterium *Thermus aquaticus* 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. *AmpliTaq®* DNA polymerase is a genetically modified enzyme
expressed by *E. coli*. Since AmpliTaq® is recombinant, the purity and reproducibility of this enzyme are higher than those of the wild type. However, potential contamination might occur during the DNA amplification, with some homologous *E. coli* sequences. In this case, the use of a DNA polymerase which has not been expressed with *E. coli*, as host organism, is recommended. Both *Taq*, and AmpliTaq® DNA polymerases possess a 5’ to 3’ exonuclease activity, which removes nucleotides ahead of the growing chain.

**Vent™ -; DeepVent™-; Pfu- and UITma™- DNA polymerases.** These enzymes have a 3’- 5’ exonuclease activity which allow the removal of mismatched residues until a correctly base-paired terminus is generated. However, the 3’- 5’ exonuclease activity 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.

**AmpliTaqGold™- DNA polymerase.** This enzyme consists of an AmpliTaq DNA polymerase, inactive at room temperature, and can only be activated during an incubation period at 94°C. In this case, the program of the thermocycler should include a pre-incubation period at a temperature of 92 - 95°C. For the time-released PCR, the pre-incubation can be eliminated, but at least 10 cycles more than the classic PCR must be performed.

**Reaction buffers and MgCl₂ in PCR reactions**

In addition to the reagents directly involved in the reaction, PCR requires a suitable buffer. 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*/AmpliTaq® DNA polymerase contains:

- 10 mM Tris, pH 8.3
- 50 mM KCl
- 1.5-2.5 mM MgCl₂
The presence of divalent cations in PCR is critical. The MgCl₂ 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).

Mg²⁺ 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 Mg²⁺ concentration leads to low yields (or no yield) whereas a high Mg²⁺ 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 supplements, such as DMSO, PEG 6000, formamide, glycerol, spermidine and non-ionic detergents, used to increase the reaction specificity or efficiency (Roux, 1995).

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 misincorporation 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 a 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 $3 \times 10^5$ 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 reactants (dNTPs, enzyme), reactant depletion (primers, dNTPs – the former a problem with short products, the latter with long products), end-product inhibition (pyrophosphate formation), competition for reactants by non-specific products, 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 should 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.

**Design of primers for PCR**

Perhaps the most critical parameter for successful PCR is the design of primers. All things being equal, a poorly designed primer can result in a PCR reaction that will not work. The primer sequence determines several things such as the 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 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 is 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, however, 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 in this fashion will not be optimal and empirical experiments will have to be performed to determine the optimal temperature. 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. Both of the oligonucleotide primers should be designed so that they 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:

\[
T_{m \text{ primer}} = \Delta H + \Delta S + R \ln (c/4) - 273.15°C + 16.6 \log_{10} [K^-] \tag{2}
\]
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:

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

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

Table 2 shows 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 2.** Calculation of the Tm of the primers with Wallace’s equation

<table>
<thead>
<tr>
<th>Primer length</th>
<th>$T_m = 2(A+T) + 4(G+C)$</th>
<th>Primer length</th>
<th>$T_m = 2(A+T) + 4(G+C)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>12°C</td>
<td>22</td>
<td>66°C</td>
</tr>
<tr>
<td>6</td>
<td>18°C</td>
<td>24</td>
<td>72°C</td>
</tr>
<tr>
<td>8</td>
<td>24°C</td>
<td>26</td>
<td>78°C</td>
</tr>
<tr>
<td>10</td>
<td>30°C</td>
<td>28</td>
<td>84°C</td>
</tr>
<tr>
<td>12</td>
<td>36°C</td>
<td>30</td>
<td>90°C</td>
</tr>
<tr>
<td>14</td>
<td>42°C</td>
<td>32</td>
<td>96°C</td>
</tr>
<tr>
<td>16</td>
<td>48°C</td>
<td>34</td>
<td>102°C</td>
</tr>
<tr>
<td>18</td>
<td>54°C</td>
<td>36</td>
<td>108°C</td>
</tr>
<tr>
<td>20</td>
<td>66°C</td>
<td>38</td>
<td>114°C</td>
</tr>
</tbody>
</table>

The temperatures calculated using 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 doubt, the product $T_m$ can be calculated using the formula:
Specificity

As mentioned above, primer specificity is at least partly dependent on primer length. It is evident that there are many more unique 24 base oligos than there are 15 base oligos. 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 \textit{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 either primer, primer dimer formation will occur, which, more often than not, will prevent the formation of the desired product via competition.

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

The base composition of primers should be between 45% and 55% GC. The primer sequence must be chosen so that there is no 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 open up stretches of the primer-template complex. This can lower the efficiency of amplification. Polypyrimidine (T, C) and polypurine (A, G)
The Polymerase Chain Reaction (PCR) 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

It is well established that the 3’ terminal position in PCR primers is essential for the control of mis-priming. 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 bonding of 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 sets of primers can be used to improve PCR yield of the target DNA sequence (Newton and Graham, 1994). PCR with nested primers is performed for 15 to 30 cycles with one primer set and then for an additional 15 to 30 cycles, with a second primer set, for an internal region of the first amplified DNA product. Thus, the larger fragment produced by the first round of PCR is used as the template for the second PCR. Using the nested PCR method can dramatically increase the sensitivity and specificity of DNA amplification. The specificity is particularly enhanced because this technique almost always eliminates any spurious non-specific amplification products. This is because after the first round of PCR any non-specific products are unlikely to be sufficiently complementary to the nested primers to be able to serve as a template for further amplification, thus the desired target sequence is preferentially amplified. However, the increased risk of contamination is a drawback of this extreme
sensitivity, and great care must be taken when performing such PCRs, particularly in a diagnostic laboratory.

**Multiplex PCR**

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

For this type of PCR amplification, primers are chosen with similar annealing temperatures. The lengths of amplified products should be similar; large differences in the lengths of the target DNAs will favour the amplification of the shorter target over the longer one, resulting in differential yields of amplified products. In addition, Multiplex PCR buffers contain Taq polymerase additive, which decreases the competition among amplicons and the discrimination of longer DNA fragments during Multiplex PCR.

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

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

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

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

Physical prevention methods

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

1. Sample preparation area
   This room consists of an area where all the steps prior to amplification of the template DNA are performed (e.g. isolation and purification of DNA).

2. PCR set-up room
   This “clean” room is devoted to the procedures related to the preparation of the PCR reaction (e.g. mastermix, primers dilutions etc.).

3. Post-PCR area
   The area is dedicated to the amplification of the target DNA sequence, and the detection and analysis of the PCR products.

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

Sample handling

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

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

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

![Figure 8. Uracil-DNA Glycosylase reaction](image)

This method, of course, requires that all PCR-reactions in the lab have to be carried out with dUTP instead of dTTP.

Note the following when using dU-containing PCR products in downstream applications:

- PCR products containing dU perform as well as those containing dT when used as hybridisation targets or as templates for dideoxy sequencing.
- PCR products containing dU can be cloned directly, if they are transformed into UNG–bacterial hosts.
A dU-containing substrate is readily digested by some common restriction enzymes (e.g. EcoR I and BamH I), while others show reduced activity (e.g. Hpa I, Hind II, Hind III) on these substrates.

The use of dU-containing DNA is not recommended for protein-binding or DNA-protein interaction studies.

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

**Preparation of the mixture for the PCR reaction (Mastermix)**

The essential reagent components for PCR are water, the reaction buffer, a thermostable DNA polymerase, oligonucleotide primers, deoxynucleotides (dNTPs), template (target) DNA, and magnesium ions (Mg²⁺). 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). The mastermix is then aliquotted 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 uniformed 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,
- 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 in a defined amount of DNA, are given in Table 3.
Table 3. Comparison of genome size of some plant species and corresponding genome copies in defined amount of DNA

<table>
<thead>
<tr>
<th>Sample</th>
<th>Genome size</th>
<th>Genome copies in 1 µg DNA</th>
<th>Genome copies in 1 ng DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>5 x 10⁹ bp</td>
<td>1.85 x 10⁵</td>
<td>185</td>
</tr>
<tr>
<td>Soybean</td>
<td>1.55 x 10⁹ bp</td>
<td>5.98 x 10⁵</td>
<td>598</td>
</tr>
<tr>
<td>Tobacco</td>
<td>3.8 x 10⁹ bp</td>
<td>2.43 x 10⁵</td>
<td>245</td>
</tr>
<tr>
<td>Rice</td>
<td>4 x 10⁸ bp</td>
<td>2.31 x 10⁶</td>
<td>2310</td>
</tr>
</tbody>
</table>

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 10⁹ bp) represents approximately 0.00002% of the input DNA. Approximately 1,000,000-fold more maize genomic DNA is required to maintain 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.

Controls

As reported in the previous section, 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 be reported:
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 past sample preparation
4. Degrading products from decontamination reactions

Whereas the first three forms of contamination produce false positives the latter type causes false negatives. 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, both positive and negative controls must always be used during a PCR reaction. Table 4 indicates some of the most common controls used to ensure the performance of nucleic acid amplification procedures.

**Table 4. Controls to be inserted within the PCR based tests**

<table>
<thead>
<tr>
<th>Control</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contamination of the reagents with the target DNA</td>
<td>PCR without DNA template (only mastermix) negative control</td>
</tr>
<tr>
<td>Specificity of the reaction</td>
<td>Controls to find secondary and non-specific products</td>
</tr>
<tr>
<td>Development and sensitivity of the reaction</td>
<td>Positive/negative controls to verify that the desired conditions and yields are fulfilled</td>
</tr>
<tr>
<td>Integrity of the PCR mixture</td>
<td>PCR with a DNA positive control</td>
</tr>
</tbody>
</table>

**Positive controls**

The efficiency of the DNA extraction and its amplification has to be checked by 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. Insertion of a negative control with the amplification reaction mixture is therefore required.
References


**Additional Reading**


The Analysis of Food Samples for the Presence of Genetically Modified Organisms

Session 7

Characteristics of Roundup Ready® Soybean, MON810 Maize, and Bt-176 Maize

M. Querci, M. Mazzara
Table of Contents

Session 7

Characteristics of Roundup Ready® Soybean, MON810 Maize, and Bt-176 Maize

Characteristics of Roundup Ready® soybean 3
Characteristics of maize MON810 7
Characteristics of maize Bt-176 11

References 16
Characteristics of Roundup Ready® soybean

Brief identification

<table>
<thead>
<tr>
<th><strong>Designation</strong></th>
<th>GTS 40-3-2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Applicant</strong></td>
<td>Monsanto Canada Inc.</td>
</tr>
<tr>
<td><strong>Plant Species</strong></td>
<td><em>Glycine max</em> L. (soybean)</td>
</tr>
<tr>
<td><strong>Novel Traits</strong></td>
<td>Novel tolerance to glyphosate, the active ingredient of Roundup® herbicide</td>
</tr>
<tr>
<td><strong>Trait Introduction Method</strong></td>
<td>Particle acceleration (biolistics)</td>
</tr>
<tr>
<td><strong>Proposed Use</strong></td>
<td>Production of soybeans for animal feed (mostly defatted toasted meal and flakes) and human consumption (mostly oil, protein fractions and dietary fibre).</td>
</tr>
</tbody>
</table>

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-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*), an essential enzyme of the shikimate biochemical pathway involved in the production of the aromatic 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

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1 Extracted from the Canadian Food Inspection Agency, Decision Document DD95-05.
plants, fungi and microorganisms and is highly insensitive to glyphosate. It can therefore fulfill the aromatic amino acid 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 amino acids 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.
**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 β-glucuronidase as a selectable marker, and the nptII 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 2.** Schematic representation of the Roundup Ready® soybean gene cassette (modified from Padgette *et al.* 1995).

**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)
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 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 is, at present, the only transgenic 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). This decision 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, only.
Characteristics of maize MON810

Brief identification

**Designation**
Event MON810 maize (trade name YieldGard®)

**Applicant**
Monsanto Canada Inc.

**Plant Species**
Zea mays L. (maize)

**Novel Traits**
Resistance to European Corn Borer (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 (YieldGard®) 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, besides an endospore, it produces several

---

2 Extracted from the Canadian Food Inspection Agency, Decision Document 97-19.
insecticidal protein crystals, including the δ-endotoxin CRYIA(b) protein active against certain lepidopteran insects such as the European Corn Borer (ECB), Spruce Budworm, Tent Caterpillar, Gypsy Moth, Diamondback Moth, Cabbage Looper, 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 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 plasmid PV-ZMBK07 used in the transformation of MON810, including the enhanced CaMV 35S-promoter, the maize hsp70 intron 1 and the synthetic δ-endotoxin cryIA(b) gene followed by the nos terminator (modified from BATS, 2003).

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 nptII 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 (taken from Agbios Database on Essential Biosafety)

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 intended for human consumption (wet mill, dry mill or seed oil), and meal and silage for livestock feed.
Characteristics of maize Bt-176

Brief identification

<table>
<thead>
<tr>
<th>Description</th>
<th>Detail</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Designation</strong></td>
<td>Event 176 Bt maize</td>
</tr>
<tr>
<td><strong>Applicant</strong></td>
<td>Ciba Seeds of Ciba-Geigy and Mycogen Corporation</td>
</tr>
<tr>
<td><strong>Plant Species</strong></td>
<td>Zea mays L. (maize)</td>
</tr>
<tr>
<td><strong>Novel Traits</strong></td>
<td>Resistance to European Corn Borer <em>(Ostrinia nubilalis)</em>; tolerance to glufosinate ammonium herbicide</td>
</tr>
<tr>
<td><strong>Trait Introduction Method</strong></td>
<td>Particle acceleration (biolistics) on immature embryos</td>
</tr>
<tr>
<td><strong>Proposed Use</strong></td>
<td>For cultivation as hybrid grain maize</td>
</tr>
</tbody>
</table>

Background information

Ciba Seeds and Mycogen Corporation have developed a maize line resistant to the European Corn Borer (ECB). This maize line, designated as Event Bt-176, has been transformed by means of recombinant DNA technology and microprojectile bombardment of embryos, to produce an insecticidal protein, from *Bacillus thuringiensis* ssp. *kurstaki*, active against certain species of *Lepidoptera*, the insect order to which butterflies and moths belong, including ECB. Specifically, this protein is a truncated form of the CRYIA(b) δ-endotoxin and protects maize plants against feeding damage caused by ECB larvae. In addition, this line of maize was co-transformed with a gene that confers tolerance to the herbicide glufosinate ammonium, used to select transformed plants at very early stages of development.

Description of the novel traits

Resistance to European Corn Borer (ECB)

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

3 Extracted from the Canadian Food Inspection Agency, Decision Document DD96-09.
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). A synthetic cryIA(b) gene, derived from Bacillus thuringiensis ssp. kurstaki strain HD-1, coding for a truncated form of the CRYIA(b) δ-endotoxin, and modified to enhance its expression in maize was developed. The synthetic gene has approximately 65% homology at nucleotide level with the native gene (Koziel et al., 1993). The truncated CRYIA(b) protein contains the insecticidal region of the native CRYIA(b). 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 which disrupt the osmotic balance, resulting in cell lysis, cessation of feeding and eventual insect death.

Event Bt-176 was obtained by transformation with two synthetic cryIA(b) gene constructs. One construct is under the transcriptional control of the maize phosphoenolpyruvate-carboxylase promoter (P-PEPC), and is expressed in green tissues. The second construct is under the control of a maize calcium-dependent protein-kinase promoter (P-CDPK) and is specifically expressed in the pollen. Both constructs are terminated with a Cauliflower Mosaic Virus derived terminator (T-CaMV 35S) and also include intron 9 from the maize phosphoenolpyruvate-carboxylase gene (see Figure 6 and Figure 7).

Expression of the CRYIA(b) protein in green tissues is intended to render the plant resistant to first generation ECB larvae feeding on leaves. Expression in pollen is intended to target second-generation ECB larvae, which are known to feed on pollen. CRYIA(b) protein from Event Bt-176 leaves was subjected to in vitro digestibility studies under simulated mammalian gastric conditions and was shown to be degraded as conventional dietary protein.

![Figure 6. Schematic representation of the synthetic cryIA(b) gene under the control of the CDPK promoter (from Matsuoka et al., 2000).](image-url)
Glufosinate ammonium herbicide tolerance

The glufosinate ammonium tolerance gene (bar gene), derived from the common soil bacterium *Streptomyces hygroscopicus*, codes for a phosphinotricin acetyltransferase (PAT) under the transcriptional control of the CaMV 35S constitutive promoter, active in all plant tissues except pollen. Phosphinotricin, a glutamine-synthetase inhibitor, is the active moiety of glufosinate ammonium. The herbicidal activity of phosphinotricin is characterised by the inhibition of glutamine-synthetase resulting in the accumulation of lethal amounts of ammonia in the plant. PAT catalyses the acetylation of phosphinotricin, thus eliminating its herbicidal activity.

The L- isomer of phosphinotricin (L-PPT) is widely used as a broad-spectrum weed control agent. L-PPT is the active ingredient of the herbicide glufosinate ammonium developed by Hoechst and named BASTA. This isomer is a structural analogue of glutamate, the substrate of glutamine-synthetase (see the comparison of L-PPT and glutamate in Figure 8).

![Figure 7. Schematic representation of the synthetic cryIA(b) gene under the control of the PEPC promoter (from Matsuoka et al., 2000).](image)

![Figure 8. L-isomer of phosphinotricin (left) compared to glutamate (right)](image)
Originally L-PPT was isolated from *Streptomyces viridochromogenes*, which synthesises only the L-isomer of phosphinotricin. Synthetic glufosinate ammonium is an equimolar, racemic mixture of the D- and L-isomers of PPT (D-PPT exhibits no herbicidal activity). PAT was shown to act specifically on phosphinotricin, since no other activity was observed on other common acetyltransferase substrates, including pyruvate, choline or serine.

*In vitro* digestibility studies, under simulated mammalian gastric conditions, conducted on *E. coli* expressed PAT, revealed that this protein is digested as conventional dietary protein.

The glufosinate ammonium tolerance gene was co-introduced as a selectable marker allowing the identification of transformed embryos on selective medium and to allow tracking of introduced genes during plant breeding. As reported (FSANZ, 2000), molecular data indicated that line Bt-176 contains one copy of the *bar* gene, under transcriptional regulation of the 35S promoter and the 35S terminator from Cauliflower Mosaic Virus (P-CaMV 35S and T-CaMV 35S, respectively) (see Figure 9).

**Figure 9.** Schematic representation of the *bar* gene (derived from Matsuoka *et al.*, 2000)

**Development method**

Event Bt-176 was obtained by biolistic transformation of the inbred maize line CG00526 (*Zea mays* L.) with two plasmids. The two synthetic *crylA(b)* gene constructs were co-cloned into a single plasmid vector (pCIB4431). A second plasmid vector (pCIB3064) contained the herbicide tolerance gene (*bar*) isolated from the soil bacterium *Streptomyces hygroscopicus*. The two vectors were introduced into the maize line CG00526 by microprojectile bombardment of immature embryos. Molecular analyses of the transformed plant indicated that two or more copies of each plasmid constructs are integrated in the genome of the maize line. Assays and Northern blot analyses indicated that the ampicillin resistance gene (*bla* gene),
regulated by a bacterial promoter (used for selection of the vectors in bacterial backgrounds) was not expressed in either leaf tissues or pollen from the plant. Two independent transgenic maize events were chosen for further crossing and characterisation: Event 171 and Event 176 (Koziel et al., 1993).

Additional characterisation studies confirmed the presence in Bt-176 corn of the crylA(b) (Koziel et al., 1993), bar and bla genes (Privalle, 1994). Data, as reported by Food Standards Australia New Zealand (FSANZ, 2000) also indicate that there may be as many as six copies of the crylA(b) and bla genes present in Bt-176, and at least two of the bar gene (together with the 35S promoter), as determined by Southern analysis against Bt-176 maize DNA (Privalle, 1994).

**Stability of insertion of the traits**

As reported (FSANZ, 2000), the production of CRYIA(b) and PAT proteins in leaves and pollen of greenhouse-grown plants, was determined to be stable over four successive backcross generations. Segregation analyses indicated that the resistance to ECB and herbicide tolerance traits co-segregates as linked Mendelian traits. A study of 3240 plants indicated that only five plants (0.15%) were identified as being tolerant to glufosinate ammonium but susceptible to damage by ECB larvae.

**Regulatory decision**

In August 1995, the Environmental Protection Agency of the United States conditionally approved the commercialisation of field maize derived from Event 176, until the year 2000.

The commercialisation of this line of maize was authorised in the EU following Commission Decision 97/98/EC of 23 January 1997. This line of maize is intended for cultivation, for seed production and the production of silage and grain for animal feed and grain for industrial processing (Commission Decision 97/98/EC).
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Biotechnology Research Unit, Ciba-Geigy Corporation, Research Triangle Park,
NC, USA. Study No CAB-009-94.

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Research Technology 213, 107-112.
The Analysis of Food Samples for the Presence of Genetically Modified Organisms

Session 8

Characteristics of the Qualitative PCR Systems Described in the Manual

M. Querci, M. Mazzara
# Table of Contents

## Session 8

**Characteristics of the Qualitative PCR Systems Described in the Manual**

- Characteristics of the qualitative PCR systems described in the manual 3
- Plant specific PCR 3
- Detection of the *lectin* gene 3
- Detection of the *zein* gene 3
- Screening method: Detection of the CaMV 35S promoter and *nos* terminator 4
- Detection of the CaMV 35S promoter 4
- Detection of the *nos* terminator 5
- GMO specific PCR 7
- Specific detection of the CTP/EPSPS gene cassette in Roundup Ready® soybean 7
- Detection of the synthetic *crylA(b)* gene of maize Bt-176 7
- Specific detection of the E35S promoter/hsp70exon-intron cassette of maize MON810 9

### References 11
Characteristics of the qualitative PCR systems described in the manual

During this course different detection systems will be used: 1) plant-specific primers will be used to confirm the presence and quality (amplifiability) of the DNA extracted from the samples; 2) the so called “screening method”, based on the specific detection of the most common regulatory sequences, the 35S promoter and nos terminator. These two methods will be performed following a simple PCR protocol. Finally, GMO-specific primers will be used in “nested PCR” for the selective detection and identification of the different transgenic lines. This chapter contains a brief introduction to the different systems used for the detection and characterization of Roundup Ready® soybean, MON810 maize and the crylA(b) gene contained in Bt-176 maize. For further information on primer sequences and composition see Session 9.

Plant specific PCR

Detection of the lectin gene

For the identification of soybean DNA, primers GMO3 and GMO4 (Meyer et al., 1996), which amplify a fragment of the lectin gene (Le1), specific to soybean, will be used.

As indicated above, the purpose is to confirm the presence and quality of the DNA extracted from soybean containing samples, where DNA quality is intended here as amplifiability by PCR. The primers GMO3 and GMO4 are used as a nested PCR for the second soybean-PCR reported by Meyer and Jaccaud (1997) on the DNA extracted from processed foods. The expected product is an amplicon of 118 bp.

Detection of the zein gene

The primers ZEIN3 and ZEIN4 (Studer et al., 1997) specific to the maize zein gene (Ze1, coding for a 10-kb protein) will be used to confirm the presence and quality of DNA extracted from maize-containing samples. As for the lectin gene, the primers ZEIN3 and ZEIN4 have originally been designed as internal primers (second round PCR) in a nested PCR system for the detection of maize DNA extracted from
processed foods. If the extracted target DNA is present, intact and amplifiable, we expect the amplification of a band of 277 bp.

**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 method” for the identification of genetically modified plant-derived foodstuffs. The use of the 35S promoter and nos terminator as target sequences allows the detection of most genetically modified foodstuff since they are up-to-now present in nearly all EU approved genetically modified plants (Hemmer, 1997). The characteristics of some maize lines approved for market introduction in the EU are listed in Table 1 as an example. The 35S promoter and nos terminator specific primers used during the course were used to validate a PCR method for the detection of Roundup Ready® soybean and Maximizer maize (Bt-176) in processed food fractions (Lipp et al., 2001).

**Table 1.** Characteristics of some transgenic maize lines authorised for market introduction in the EU

<table>
<thead>
<tr>
<th>Name</th>
<th>Company</th>
<th>Character</th>
<th>Promoter</th>
<th>Gene(s) introduced</th>
<th>Terminator</th>
</tr>
</thead>
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<tr>
<td>Event 176</td>
<td>Ciba-Geigy</td>
<td>Bt, bar</td>
<td>35S</td>
<td>bar</td>
<td>35S</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PEPC</td>
<td>crylA(b)/int.9 PEPC</td>
<td>35S</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CDPK</td>
<td>crylA(b)/int.9 PEPC</td>
<td>35S</td>
</tr>
<tr>
<td>Line Bt-11</td>
<td>Novartis</td>
<td>Bt, pat</td>
<td>35S</td>
<td>crylA(b)/int. IVS6</td>
<td>nos</td>
</tr>
<tr>
<td>Line T25</td>
<td>AgrEvo</td>
<td>pat</td>
<td>35S</td>
<td>pat</td>
<td>35S</td>
</tr>
<tr>
<td>Line MON810</td>
<td>Monsanto</td>
<td>Bt</td>
<td>E35S</td>
<td>crylA(b)/int.hsp70</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>E35S</td>
<td>crylA(b)/int.hsp70</td>
<td>-</td>
</tr>
</tbody>
</table>

**Detection of the CaMV 35S promoter**

This promoter regulates the gene expression of many transgenic plants such as Roundup Ready® soybean and maize line Bt-176. For its specific detection, primers p35S-cf3 and p35S-cr4 will be used (Lipp et al., 2001). The expected amplicon is a
123 bp fragment as indicated below in Figure 1, where primers p35S-cf3 and p35S-cr4 have been positioned in the corresponding region of the CaMV 35S promoter sequence.

![Partial sequence of the 35S promoter from Cauliflower Mosaic Virus (CaMV) and hybridisation sites of primers p35S-cf3 and p35S-cr4](image)

**Figure 1.** Partial sequence of the 35S promoter from Cauliflower Mosaic Virus (CaMV) and hybridisation sites of primers p35S-cf3 and p35S-cr4

**Detection of the nos terminator**

Primers HA-nos118-f and HA-nos118-r (Lipp et al., 2001) are used for the detection of the nos terminator. The nos terminator is present in the Roundup Ready® soybean and other lines of transgenic plants (e.g. maize line Bt-11). Amplification of the nos terminator will result in the production of a DNA fragment of 118 bp. In Figure 2, primers HA-nos118-f and HA-nos118-r have been positioned inside the sequence of the transgenic part of Roundup Ready® soybean.
Figure 2. Sequence of the transgenic part of Roundup Ready® soybean from Monsanto according to Patent WO 92/04449
**GMO specific PCR**

The amplification primers that are used for the identification of Roundup Ready® soybean, Bt-176 maize and MON810 maize have been chosen for their capacity to detect, in a specific way, the genetic structure inserted into the Roundup Ready® soybean, Bt-176 and MON810 maize genomes, respectively.

**Specific detection of the CTP/EPSPS gene cassette in Roundup Ready® soybean**

The primer pairs GMO9/GMO5 and GMO8/GMO7 were designed for the specific detection of the transgene of Roundup Ready® soybean by nested PCR (Meyer and Jaccaud, 1997). The external primers GMO9 and GMO5 are complementary to the DNA sequence corresponding to the CP4 EPSPS gene and to the CaMV 35S promoter. The amplification of DNA with these two primers results in an amplicon of 447 bp. The internal primers, GMO8 and GMO7, are complementary to the epsps petunia gene and to the CaMV 35S promoter. The amplification of DNA with these internal primers results in a fragment of 169 bp, as shown in Figure 2.

**Detection of the synthetic cryIA(b) gene of maize Bt-176**

The primer pairs CRYIA1/CRYIA2 and CRYIA3/CRYIA4 were designed for the specific detection of the synthetic cryIA(b) gene by nested PCR (Studer et al., 1997). The external primers, CRYIA1 and CRYIA2, and the internal ones, CRYIA3 and CRYIA4, are complementary to the DNA sequence of the cryIA(b) gene. As shown in Figure 3, the two external primers (CRYIA1/CRYIA2) delimit a fragment of 420 bp while internal primers CRYIA3/CRYIA4 produce a fragment of 189 bp.
The Analysis of Food Samples for the Presence of Genetically Modified Organisms

Characteristics of the Qualitative PCR Systems Described in the Manual

ID   I41419     standard; DNA; UNC; 1947 BP.
AC   I41419;
DE   Sequence 3 from patent US 5625136.
RA   Koziel M.G., Desai N.M., Lewis K.S., Kramer V.C., Warren G.W., Evola S.V.,
RA   Crossland L.D., Wright M.S., Merlin E.J., Launis K.L., Rothstein S.J.,
RT   "Synthetic DNA sequence having enhanced insecticidal activity in maize";
FT   source 1..1947
FT   /db_xref="taxon:12908"
FT   /organism="unclassified"
SQ   Sequence 1947 BP; 412 A; 729 C; 528 G; 278 T; 0 other;
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120  agcctgaccc atgtcctcctg gcggagtctc gtgccggcggc ccggctcctg gctgggcttg
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720  ctggacctcg gcgtactcgt cccgacgact ccgggtgctgc gcgggctggc cggcgcccgag
780  aacacgctag ccatctacac gcacggccgac ccgcggtcgag cggcgcccgag gcacccggag
840  atcatgccg gcccgtgctg ctgcagcgcc gccggggttc ccgccaggtc gccggggttc
cggccgag ccggggcgag tcggggcgag tcggggcgag gcacccggag gcacccggag
900  cggcgccgag tcggggcgag tcggggcgag tcggggcgag gcacccggag gcacccggag
960  CRYIA1 forward primer outer PCR
1020  atgggaacag ctggaccaacct cacgacacact gcgggccgag gcggcaggtta ctgcgccggt
cggccgag tcggggcgag tcggggcgag gcacccggag gcacccggag gcacccggag
1080  acctgagga cgcacctgtg ccctgacacgc caggtactgc gcgtgactgc ccggccgag
1140  CRYIA3 forward primer inner (nested) PCR
1200  agggtgtgcttc cgggacaccgc ctgggcatcgc gcgggctcgc gcgggctcgc gcgggctcgc
1260  taccggcaag gcgggacaccgc gcgggctcgc gcgggctcgc gcgggctcgc gcgggctcgc
cggccgag ccggggcgag tcggggcgag tcggggcgag gcacccggag gcacccggag
1320  CRYIA4 reverse primer inner (nested) PCR
Specific detection of the E35S promoter/hsp70 exon-intron cassette of maize MON810

The primer pairs mg1/mg2 and mg3/mg4 were designed for the specific detection of the E35S/hsp70 exon-intron 1 cassette by nested PCR (Zimmermann et al., 1998). This gene construction is specific of maize MON810. The external primers, mg1 and mg2 anneal to the E35S promoter sequence and to the hsp70 intron 1 region, respectively, while the internal primers are complementary to the DNA sequence of the E35S promoter and the hsp70 exon 1 region, respectively. As shown in Figure 4, the two external primers (mg1/mg2) produce a fragment of 401 bp while mg3/mg4 produce a fragment of 149 bp.
Figure 4. Schematic representation of part of the MON810 maize cassette including the enhanced CaMV 35S-promoter and the maize hsp70 intron, and relative position of primers mg1, mg2, mg3, and mg4 (modified from Zimmermann et al., 1998)
References


The Analysis of Food Samples for the Presence of Genetically Modified Organisms

Session 9

Qualitative Detection of MON810 Maize, Bt-176 Maize and Roundup Ready® Soybean by PCR

M. Querci, M. Maretti, M. Mazzara
Table of Contents

Session 9

Qualitative Detection of MON810 Maize, Bt-176 Maize and Roundup Ready® Soybean by PCR

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental</td>
<td>3</td>
</tr>
<tr>
<td>Introduction</td>
<td>3</td>
</tr>
<tr>
<td>Plant specific PCR: soybean-lectin</td>
<td>6</td>
</tr>
<tr>
<td>Plant specific PCR: maize-zein</td>
<td>9</td>
</tr>
<tr>
<td>Screening method for the detection of Genetically Modified Plants</td>
<td>12</td>
</tr>
<tr>
<td>Detection of the 35S promoter</td>
<td>12</td>
</tr>
<tr>
<td>Detection of the nos terminator</td>
<td>14</td>
</tr>
<tr>
<td>Specific detection of MON810 maize, Bt-176 maize and Roundup Ready® soybean by nested PCR</td>
<td>17</td>
</tr>
<tr>
<td>Detection of MON810 maize</td>
<td>17</td>
</tr>
<tr>
<td>Detection of Bt-176 maize</td>
<td>22</td>
</tr>
<tr>
<td>Detection of Roundup Ready® soybean</td>
<td>26</td>
</tr>
</tbody>
</table>
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 Bt-176 maize) in raw and processed material, by comparison with corresponding non-GM samples (soybean and maize).

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

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 a UV hood

REMARK

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

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

Reagents

- dATP CAS1923-31-7
- dCTP CAS102783-51-7
- dGTP CAS93919-41-6
- dTTP CAS18423-43-3
- 10x PCR buffer (usually delivered from the same supplier as the Taq DNA polymerase)
• 25 mM MgCl₂
• Taq DNA polymerase
• Upstream and downstream oligonucleotides
• Mineral oil (needed in case a thermocycler without hot lid is used)
• Nuclease-free water

4 mM dNTP stock solution

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

20 µM primer solution

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
  - Xnmol 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 \( \land \) Prepare 1 microcentrifuge tube with 400 µl sterile water and add 100 µl of the primer solution (100 µM) \( \land \) 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.

10x PCR buffer

• Usually the 10x PCR buffer, containing 500 mM KCl, 100 mM Tris-HCl (pH 9.0 at 25°C) and 1% Triton X-100 is provided together with the Taq DNA polymerase and is ready to use. The buffer should be mixed and briefly centrifuged before each use.
• Aliquots are stored at -20°C and are stable for several months.
25 mM MgCl₂ solution

“PCR grade” MgCl₂ solution is generally supplied together with the Taq DNA polymerase and is ready to use. The solution should be mixed (vortex) before each use and briefly centrifuged (destruction of the concentration gradient which can be formed in the case of a prolonged conservation). Store at -20°C.

Nuclease-free water aliquots

Sterile nuclease-free, 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.
Plant specific PCR: soybean-lectin

The identification of soybean DNA is performed targeting the *lectin* gene. The PCR with the primers GMO3/GMO4 determines if amplifiable soybean DNA is present in the sample.

**Characteristics of primers GMO3 and GMO4**

<table>
<thead>
<tr>
<th></th>
<th>GMO3</th>
<th>GMO4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence</td>
<td>GCCCTCTACTCCACCCCATCC</td>
<td>GCCCATCTGCAAGCCTTTTTGTG</td>
</tr>
<tr>
<td>Length</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>Mol. Weight (g/mol)</td>
<td>6471.6</td>
<td>6981.1</td>
</tr>
<tr>
<td>Melting point * (G/C)</td>
<td>65.1</td>
<td>59.6</td>
</tr>
</tbody>
</table>

*based on a [Na⁺] of 50 mM

**Controls**

It is important to perform control experiments with every PCR analysis. Negative controls are designed to check if the PCR reagents are contaminated with DNA. Positive controls with characterised samples are also critical in determining the efficiency and specificity of PCR. The following controls must be introduced in analysis performed with PCR:

- **Positive control**: pure DNA, isolated from the conventional soybean
- **Negative control**: pure DNA, isolated from another species, not containing the *lectin* gene
- **No-template**: 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 48 µl of GMO3/GMO4 Mastermix and 2 µl of DNA solution. All solutions are stored on ice during the preparation of the Mastermix.

**Table 1. GMO3/GMO4 Mastermix**

<table>
<thead>
<tr>
<th></th>
<th>Final concentration</th>
<th>Mastermix for one sample</th>
<th>Mastermix for 10 samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile, deionised water</td>
<td>32.75 µl</td>
<td>327.5 µl</td>
<td></td>
</tr>
<tr>
<td>10x PCR Buffer</td>
<td>1x</td>
<td>5 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>2.5 mM</td>
<td>5 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>4 mM dNTPs</td>
<td>0.2 mM</td>
<td>2.5 µl</td>
<td>25 µl</td>
</tr>
<tr>
<td>20 µM oligonucleotide GMO3</td>
<td>0.5 µM</td>
<td>1.25 µl</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>20 µM oligonucleotide GMO4</td>
<td>0.5 µM</td>
<td>1.25 µl</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.025 U/µl</td>
<td>0.25 µl</td>
<td>2.5 µl</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>48 µl</strong></td>
<td><strong>480 µl</strong></td>
<td></td>
</tr>
</tbody>
</table>

- Prepare a 1.5 ml microcentrifuge tube
- Add the reagents following the order given in Table 1
- Mix gently the GMO3/GMO4 Mastermix by pipetting and centrifuge briefly
- Divide the Mastermix into aliquots of 48 µl in 0.2 ml PCR reaction tubes
- Add 2 µl of the DNA solution to the previous aliquots
- Shake gently and centrifuge briefly
- Place the PCR reaction tubes in the thermocycler

**PCR program* (GMO3/GMO4)**

<table>
<thead>
<tr>
<th></th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>3 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>63°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>3 min</td>
</tr>
<tr>
<td></td>
<td>4°C</td>
<td>∞</td>
</tr>
</tbody>
</table>
Following amplification, the samples are centrifuged briefly and put on ice.

* **Note:** During the course, *Perkin Elmer Gene Amp PCR system 9600, ABI 9700* thermocyclers will be used. The use of a different thermocycler models or brand leads to the same results provided that PCR programmes are adapted and tested accordingly.

## Analysis of PCR products

After amplification of the target sequence, the PCR products are analysed by agarose gel electrophoresis in the presence of ethidium bromide. 8 µl of a PCR reaction is mixed with 2 µl loading buffer; samples are then loaded onto the agarose gel (1.5%). 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, ultraviolet transillumination allows visualisation of the DNA in the gel. The gel may be photographed to provide a permanent record of the result of the experiment.

## Interpretation of the results

The primer pair GMO3/GMO4 for the detection of the native *lectin* gene is used as a system control check; a *lectin* specific band at 118 bp confirms if the extracted DNA is of appropriate amplifiable quality.

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

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

If the controls give the expected results and the sample does not have a band at 118 bp it means that in this sample no amplifiable soybean DNA is present. It should be noted that this as well as other protocols in this session are qualitative methods, therefore allowing only a qualitative (yes/no) result.

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1 The JRC and the WHO do 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.
Plant specific PCR: maize-zein

The identification of maize DNA is performed targeting the *zein* gene. The PCR with the primers ZEIN3/ZEIN4 determines if maize DNA of suitable amplification quality is present in the sample.

**Characteristics of primers ZEIN3 and ZEIN4**

<table>
<thead>
<tr>
<th></th>
<th>ZEIN3</th>
<th>ZEIN4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence</td>
<td>AGTGCGACCCATATTCCAG</td>
<td>GACATTGTGGCATCATCATT</td>
</tr>
<tr>
<td>Length</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>Mol. Weight (g/mol)</td>
<td>5772.3</td>
<td>6410.9</td>
</tr>
<tr>
<td>Melting point * (G/C)</td>
<td>55.2</td>
<td>51.7</td>
</tr>
</tbody>
</table>

*based on a [Na⁺] of 50 mM

**Controls**

- **Positive control**: pure DNA, isolated from the conventional maize
- **Negative control**: pure DNA, isolated from another species, not containing the *zein* gene
- **No-template**: 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 according to the instructions given in Table 2. The following procedure applies to a sample containing 48 µl of ZEIN3/ZEIN4 Mastermix and 2 µl of DNA solution. All solutions are stored on ice during preparation of the Mastermix.
Table 2. ZEIN3/ZEIN4 Mastermix

<table>
<thead>
<tr>
<th></th>
<th>Final concentration</th>
<th>Mastermix for one sample</th>
<th>Mastermix for 10 samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile, deionised water</td>
<td>32.75 µl</td>
<td>327.5 µl</td>
<td></td>
</tr>
<tr>
<td>10x PCR Buffer</td>
<td>5 µl</td>
<td>50 µl</td>
<td></td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>2.5 mM</td>
<td>5 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>4 mM dNTPs</td>
<td>0.2 mM</td>
<td>2.5 µl</td>
<td>25 µl</td>
</tr>
<tr>
<td>20 µM oligonucleotide ZEIN3</td>
<td>0.5 µM</td>
<td>1.25 µl</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>20 µM oligonucleotide ZEIN4</td>
<td>0.5 µM</td>
<td>1.25 µl</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.025 U/µl</td>
<td>0.25 µl</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>TOTAL</td>
<td>48 µl</td>
<td>480 µl</td>
<td></td>
</tr>
</tbody>
</table>

- Prepare a 1.5 ml microcentrifuge tube
- Add the reagents following the order given in Table 2
- Mix gently the ZEIN3/ZEIN4 Mastermix by pipetting and centrifuge briefly
- Divide the Mastermix into aliquots of 48 µl in 0.2 ml PCR reaction tubes
- Add 2 µl of the DNA solution to the previous aliquots
- Shake gently and centrifuge briefly
- Place the PCR reaction tubes in the thermocycler

**PCR program (ZEIN3/ZEIN4)**

<table>
<thead>
<tr>
<th></th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>3 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>96°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Annealing/Extension</td>
<td>60°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>3 min</td>
</tr>
<tr>
<td></td>
<td>4°C</td>
<td>∞</td>
</tr>
</tbody>
</table>

*Following amplification, the samples are centrifuged briefly and put on ice.*
Analysis of PCR products

After amplification of the DNA, the PCR products are analysed using agarose gel electrophoresis with ethidium bromide. 8 µl of the solution is mixed with 2 µl of loading buffer. The solution mixture is then loaded onto an agarose gel (1.5%). 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 to provide a permanent record of the result of the experiment.

Interpretation of the results

The primer pair ZEIN3/ZEIN4 is used for detection of the native maize zein gene as a control check on the amplification quality of the extracted DNA. If the extracted DNA is of sufficient amplification quality a zein specific band of 277 bp will be observed on the gel. The positive control should also amplify showing a band size of 277 bp. The negative control and the no-template should not give a visible band. If the positive/negative controls do not give the expected results, the PCR analysis of the selected samples is not valid. If the controls give the expected results and the sample does not have a band at 277 bp, it means that in this sample no amplifiable maize DNA is present.
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 \textit{nos} terminator (derived from \textit{Agrobacterium tumefaciens}). The identification of one of these regulatory sequences in the soybean and/or maize containing sample under examination indicates GMO presence. In Roundup Ready® soybean, the identification of both the 35S promoter and the \textit{nos} terminator is possible, whereas only the 35S promoter is present in the Bt-176 and MON810 maize lines.

Detection of the 35S promoter

\textit{Characteristics of primers} \textit{p35S-cf3} and \textit{p35S-cr4}

\begin{table}[h]
\begin{tabular}{l|l}
\hline
\textbf{p35S-cf3} & \\
Sequence & CCACGTCTTCAAAGCAAGTGG \\
Length & 21 \\
Mol. weight (g/mol) & 6414.5 \\
Melting point $^\circ$ (G/C) & 57.4 \\
\hline
\textbf{p35S-cr4} & \\
Sequence & TCCTCTCCAAATGAAATGAACCTTCC \\
Length & 25 \\
Mol. weight (g/mol) & 7544.2 \\
Melting point $^\circ$ (G/C) & 56.3 \\
\hline
\end{tabular}
\end{table}

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

\textit{Controls}

- \textbf{Positive control}: DNA from reference material (maize 0.5\% GM)
- \textbf{Negative control}: DNA from reference material (maize 0\% GM)
- \textbf{No-template}: negative control of the Mastermix, in which water is used instead of DNA

\textit{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 48 µl of p35S-cf3/p35S-cr4 Mastermix and 2 µl of DNA solution. All solutions are stored on ice during the preparation of the Mastermix.

**Table 3. p35S-cf3/p35S-cr4 Mastermix**

<table>
<thead>
<tr>
<th></th>
<th>Final concentration</th>
<th>Mastermix for one sample</th>
<th>Mastermix for 10 samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile, deionised water</td>
<td>32.75 µl</td>
<td>327.5 µl</td>
<td></td>
</tr>
<tr>
<td>10x PCR Buffer</td>
<td>1x</td>
<td>5 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>2.5 mM</td>
<td>5 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>4 mM dNTPs</td>
<td>0.2 mM</td>
<td>2.5 µl</td>
<td>25 µl</td>
</tr>
<tr>
<td>20 µM oligonucleotide p35S-cf3</td>
<td>0.5 µM</td>
<td>1.25 µl</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>20 µM oligonucleotide p35S-cr4</td>
<td>0.5 µM</td>
<td>1.25 µl</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.025 U/µl</td>
<td>0.25 µl</td>
<td>2.5 µl</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>48 µl</td>
<td>480 µl</td>
<td></td>
</tr>
</tbody>
</table>

- 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 48 µl in 0.2 ml PCR reaction tubes
- Add 2 µl of the DNA solution to the previous aliquots
- Shake gently and centrifuge briefly
- Place the PCR reaction tubes in the thermocycler

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

<table>
<thead>
<tr>
<th></th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>3 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>25 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>62°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>45 sec</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>7 min</td>
</tr>
<tr>
<td></td>
<td>4°C</td>
<td>∞</td>
</tr>
</tbody>
</table>
Following amplification, the samples are centrifuged briefly and put on ice.

**Analysis of PCR products**

Following amplification, the PCR products are analysed by agarose gel electrophoresis with ethidium bromide. 8 µl of the solution is mixed with 2 µl of loading buffer; the solution is then loaded onto the agarose gel (2.5%). 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 to provide a permanent record of the result of the experiment.

**Interpretation of the results**

The primer pair p35S-cf3/p35S-cr4 is used for detection of the CaMV 35S promoter, yielding a 123 bp fragment. This promoter regulates the gene expression of many transgenic plants such as Roundup Ready® soybean and maize line Bt-176. The positive control will amplify showing a band at 123 bp. The negative control and the no-template should not give a visible band. If the positive/negative controls do not give the expected results, the PCR analysis of the selected samples is not valid. If the controls give the expected results and the sample gives a band at 123 bp, it means that in this sample modified DNA is present.

**Detection of the nos terminator**

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

<table>
<thead>
<tr>
<th></th>
<th>HA-nos 118-f</th>
<th></th>
<th>HA-nos 118-r</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence</td>
<td>GCATGACGTATTTATGAGATGGG</td>
<td></td>
<td>GACACCGCGCGGATAATTATCC</td>
<td></td>
</tr>
<tr>
<td>Length</td>
<td>24</td>
<td></td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Mol. weight (g/mol)</td>
<td>7462.8</td>
<td></td>
<td>7296.9</td>
<td></td>
</tr>
<tr>
<td>Melting point * (G/C)</td>
<td>56.2</td>
<td></td>
<td>61.2</td>
<td></td>
</tr>
</tbody>
</table>

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

- **Positive control**: DNA from reference material (RRS 0.5% GM)
- **Negative control**: DNA from reference material (soybean 0% GM)
- **No-template**: 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 48 µl of HA-nos118-f/HA-nos118-r Mastermix and 2 µl of DNA solution. All solutions are stored on ice during the preparation of the Mastermix.

**Table 4. HA-nos118-f/HA-nos118-r Mastermix**

<table>
<thead>
<tr>
<th></th>
<th>Final concentration</th>
<th>Mastermix for one sample</th>
<th>Mastermix for 10 samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile, deionised water</td>
<td></td>
<td>32.75 µl</td>
<td>327.5 µl</td>
</tr>
<tr>
<td>10x PCR Buffer</td>
<td>1x</td>
<td>5 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>2.5 mM</td>
<td>5 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>4 mM dNTPs</td>
<td>0.2 mM</td>
<td>2.5 µl</td>
<td>25 µl</td>
</tr>
<tr>
<td>20 µM oligonucleotide HA-nos118-f</td>
<td>0.5 µM</td>
<td>1.25 µl</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>20 µM oligonucleotide HA-nos118-r</td>
<td>0.5 µM</td>
<td>1.25 µl</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.025 U/µl</td>
<td>0.25 µl</td>
<td>2.5 µl</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td></td>
<td><strong>48 µl</strong></td>
<td><strong>480 µl</strong></td>
</tr>
</tbody>
</table>

- Prepare a 1.5 ml microcentrifuge tube
- Add the reagents following the order given in Table 4
- Mix gently the HA-nos118-f/HA-nos118-r Mastermix by pipetting and centrifuge briefly
- Divide the Mastermix into aliquots of 48 µl in 0.2 ml PCR reaction tubes
- Add 2 µl of the DNA solution to the previous aliquots
- Shake gently and centrifuge briefly
- Place the PCR reaction tubes in the thermocycler
**PCR Program (HA-nos118-f/HAnos118-r)**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
</tr>
<tr>
<td>Annealing</td>
<td>62°C</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>50</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
</tr>
<tr>
<td></td>
<td>4°C</td>
</tr>
</tbody>
</table>

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

**Analysis of PCR products**

Following amplification, the PCR products are analysed using agarose gel electrophoresis with ethidium bromide. 8 µl of the solution is mixed with 2 µl of loading buffer; the solution is then loaded onto an agarose gel (2.5%). 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 to provide a permanent record of the result of the experiment.

**Interpretation of the results**

The primer pair HA-nos118-f/HAnos118-r is used for detection of the nos terminator, yielding a 118 bp fragment. This terminator is present in the Roundup Ready® soybean and other lines of transgenic plants (e.g. Maize line Bt-11). The positive control will amplify showing a band at 118 bp. The negative control and the no-template should not give a visible band. If the positive/negative controls do not give the expected results, the PCR analysis of the selected samples is not valid. If the controls give the expected results and the sample gives a band at 118 bp, this means that in this sample modified DNA is present.
Specific detection of MON810 maize, Bt-176 maize and Roundup Ready® soybean by nested PCR

Detection of MON810 maize

The detection system is specific for MON810 maize. Target elements are the CaMV 35S promoter and the hsp70 exon1/intron1 region, which are a constitutive regulatory sequence and heat shock protein gene for an increased level of transcription, respectively.

**Characteristics of primers mg1, mg2, mg3 and mg4**

<table>
<thead>
<tr>
<th></th>
<th>Sequence</th>
<th>Length</th>
<th>Mol. weight (g/mol)</th>
<th>Melting point * (G/C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg1</td>
<td>TATCTCCACTGACGTAAGGGATGAC</td>
<td>25</td>
<td>7665.1</td>
<td>59.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg2</td>
<td>TGCCCTATAACACCAACATGTGCTT</td>
<td>25</td>
<td>7560.2</td>
<td>57.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg3</td>
<td>ACTATCCTTCGCAAGACCCCTTCTC</td>
<td>25</td>
<td>7472.2</td>
<td>61.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg4</td>
<td>GCATTCAGAGAAACGTGGCAGTAAC</td>
<td>25</td>
<td>7722.9</td>
<td>59.6</td>
</tr>
</tbody>
</table>

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

- **Positive control**: DNA from reference material (MON810 0.1%)
- **Negative control**: DNA from reference material (maize 0% GM)
- **No-template**: negative control of the Mastermix, in which water is used instead of DNA

Mastermix preparation 1

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 5. The following procedure applies to a sample containing 48 µl of mg1/mg2 Mastermix and 2 µl of DNA solution. All solutions are stored in ice during the preparation of the Mastermix.

Table 5. mg1/mg2 Mastermix

<table>
<thead>
<tr>
<th>Final concentration</th>
<th>Mastermix for one sample</th>
<th>Mastermix for 10 samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile, deionised water</td>
<td>32.75 µl</td>
<td>327.5 µl</td>
</tr>
<tr>
<td>10x PCR Buffer</td>
<td>1x</td>
<td>5 µl</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>2.5 mM</td>
<td>5 µl</td>
</tr>
<tr>
<td>4 mM dNTPs</td>
<td>0.2 mM</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>20 µM oligonucleotide mg1</td>
<td>0.5 µM</td>
<td>1.25 µl</td>
</tr>
<tr>
<td>20 µM oligonucleotide mg2</td>
<td>0.5 µM</td>
<td>1.25 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.025 U/µl</td>
<td>0.25 µl</td>
</tr>
<tr>
<td>TOTAL</td>
<td>48 µl</td>
<td>480 µl</td>
</tr>
</tbody>
</table>

- Prepare a 1.5 ml microcentrifuge tube
- Add the reagents following the order given in Table 5
- Mix gently the mg1/mg2 Mastermix by pipetting and centrifuge briefly
- Divide the Mastermix into aliquots of 48 µl in 0.2 ml PCR reaction tubes
- Add 2 µl of the DNA solution to the previous aliquots
- Shake gently and centrifuge briefly
- Place the PCR reaction tubes in the thermocycler
**PCR program (mg1/mg2)**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>35</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
</tr>
<tr>
<td></td>
<td>4°C</td>
</tr>
</tbody>
</table>

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

**Mastermix preparation 2**

The necessary reagents for a series of 10 samples are mixed together according to the instructions given in Table 6. The following procedure applies to a sample containing 49 µl of mg3/mg4 Mastermix and 1 µl of pre-amplified DNA solution of the first PCR. All solutions are stored on ice during the preparation of the Mastermix.

**Table 6. mg3/mg4 Mastermix**

<table>
<thead>
<tr>
<th></th>
<th>Final concentration</th>
<th>Mastermix for one sample</th>
<th>Mastermix for 10 samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile, deionised water</td>
<td>33.75 µl</td>
<td>337.5 µl</td>
<td></td>
</tr>
<tr>
<td>10x PCR Buffer</td>
<td>1x</td>
<td>5 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>2.5 mM</td>
<td>5 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>4 mM dNTPs</td>
<td>0.2 mM</td>
<td>2.5 µl</td>
<td>25 µl</td>
</tr>
<tr>
<td>20 µM oligonucleotide mg3</td>
<td>0.5 µM</td>
<td>1.25 µl</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>20 µM oligonucleotide mg4</td>
<td>0.5 µM</td>
<td>1.25 µl</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.025 U/µl</td>
<td>0.25 µl</td>
<td>2.5 µl</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>49 µl</td>
<td>490 µl</td>
<td></td>
</tr>
</tbody>
</table>

- Prepare a 1.5 ml microcentrifuge tube
- Add the reagents following the order given in Table 6
- Mix gently the mg3/mg4 Mastermix by pipetting and centrifuge briefly
• Divide the Mastermix into aliquots of 49 µl in 0.2 ml PCR reaction tubes
• Add 1 µl of the DNA solution to the previous aliquots
• Shake gently and centrifuge briefly
• Place the PCR reaction tubes in the thermocycler

**Program for the nested PCR (mg3-mg4)**

<table>
<thead>
<tr>
<th></th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>3 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>45 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>50 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>50 sec</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>3 min</td>
</tr>
<tr>
<td></td>
<td>4°C</td>
<td>∞</td>
</tr>
</tbody>
</table>

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

**Analysis of PCR products**

Following amplification, the PCR products are analysed using agarose gel electrophoresis with ethidium bromide. 8 µl of the solution is mixed with 2 µl of loading buffer; the solution is then loaded onto an agarose gel (2.5%). Migration should take place at 100 V over a period of 1 hour. Size markers (15 µl of 100 bp ladder) are run 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 to provide a permanent record of the result of the experiment.

**Interpretation of the results**

The primer pairs mg1/mg2 and mg3/mg4 were designed for the specific detection of the MON810 event by nested PCR, yielding a final nested PCR fragment of 149 bp. The specificity is given by the fact that primers are designed on the region spanning the E-35S promoter and the hsp70 exon/intron gene. The positive control will amplify showing a band at 149 bp. The negative control and the no-template should not give a visible band.
If the positive/negative controls do not give the expected results, the PCR analysis of the selected samples is not valid.
If the controls give the expected results and the sample gives a band at 149 bp, this means that in this sample MON810 maize DNA is present.
Detection of Bt-176 maize

The target gene is the *cryIA(b)* gene, which protects the plant from insects such as the European Corn Borer.

**Characteristics of primers CRYIA1, CRYIA2, CRYIA3 and CRYIA4**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Length</th>
<th>Mol. weight (g/mol)</th>
<th>Melting point * (G/C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRYIA1</td>
<td>CGGCCCGAGTTCACCTT</td>
<td>18</td>
<td>5394.6</td>
<td>59.5</td>
</tr>
<tr>
<td>CRYIA2</td>
<td>CTGCTGGGGATGATGTGTGT</td>
<td>21</td>
<td>6519.2</td>
<td>57.6</td>
</tr>
<tr>
<td>CRYIA3</td>
<td>CCGCACCCTGAGCAGCAC</td>
<td>18</td>
<td>5397.6</td>
<td>61.7</td>
</tr>
<tr>
<td>CRYIA4</td>
<td>GGTGGCACGTTGTTCTG</td>
<td>21</td>
<td>6479.2</td>
<td>57.6</td>
</tr>
</tbody>
</table>

*based on a [Na⁺] of 50 mM

**Controls**

- **Positive control**: DNA from reference material (Bt-176 0.1%)
- **Negative control**: DNA from reference material (maize 0% GM)
- **No-template**: negative control of the Mastermix, in which water is used instead of DNA

**Mastermix preparation 1**
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 5. The following procedure applies to a sample containing 48 µl of CRYIA1/CRYIA2 Mastermix and 2 µl of DNA solution. All solutions are stored on ice during the preparation of the Mastermix.

**Table 5. CRYIA1/CRYIA2 Mastermix**

<table>
<thead>
<tr>
<th></th>
<th>Final concentration</th>
<th>Mastermix for one sample</th>
<th>Mastermix for 10 samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile, deionised water</td>
<td>32.75 µl</td>
<td>327.5 µl</td>
<td></td>
</tr>
<tr>
<td>10x PCR Buffer</td>
<td>1x</td>
<td>5 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>2.5 mM</td>
<td>5 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>4 mM dNTPs</td>
<td>0.2 mM</td>
<td>2.5 µl</td>
<td>25 µl</td>
</tr>
<tr>
<td>20 µM oligonucleotide CRYIA1</td>
<td>0.5 µM</td>
<td>1.25 µl</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>20 µM oligonucleotide CRYIA2</td>
<td>0.5 µM</td>
<td>1.25 µl</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.025 U/µl</td>
<td>0.25 µl</td>
<td>2.5 µl</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>48 µl</strong></td>
<td><strong>480 µl</strong></td>
<td></td>
</tr>
</tbody>
</table>

- Prepare a 1.5 ml microcentrifuge tube
- Add the reagents following the order given in Table 5
- Mix gently the CRYIA1/CRYIA2 Mastermix by pipetting and centrifuge briefly
- Divide the Mastermix into aliquots of 48 µl in 0.2 ml PCR reaction tubes
- Add 2 µl of the DNA solution to the previous aliquots
- Shake gently and centrifuge briefly
- Place the PCR reaction tubes in the thermocycler

**PCR program (CRYIA1/CRYIA2)**

<table>
<thead>
<tr>
<th></th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>3 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>40 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>40 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>40 sec</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>3 min</td>
</tr>
<tr>
<td></td>
<td>4°C</td>
<td>∞</td>
</tr>
</tbody>
</table>
Following amplification, the samples are centrifuged briefly and put on ice.

**Mastermix preparation 2**

The necessary reagents for a series of 10 samples are mixed together according to the instructions given in Table 6.

The following procedure applies to a sample containing 49 µl of CRYIA3/CRYIA4 Mastermix and 1 µl of pre-amplified DNA solution of the first PCR. All solutions are stored on ice during the preparation of the Mastermix.

**Table 6. CRYIA3/CRYIA4 Mastermix**

<table>
<thead>
<tr>
<th></th>
<th>Final concentration</th>
<th>Mastermix for one sample</th>
<th>Mastermix for 10 samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile, deionised water</td>
<td>33.75 µl</td>
<td>337.5 µl</td>
<td></td>
</tr>
<tr>
<td>10x PCR Buffer</td>
<td>1x</td>
<td>5 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>2.5 mM</td>
<td>5 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>4 mM dNTPs</td>
<td>0.2 mM</td>
<td>2.5 µl</td>
<td>25 µl</td>
</tr>
<tr>
<td>20 µM oligonucleotide CRYIA3</td>
<td>0.5 µM</td>
<td>1.25 µl</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>20 µM oligonucleotide CRYIA4</td>
<td>0.5 µM</td>
<td>1.25 µl</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.025 U/µl</td>
<td>0.25 µl</td>
<td>2.5 µl</td>
</tr>
</tbody>
</table>

**TOTAL** 49 µl 490 µl

- Prepare a 1.5 ml microcentrifuge tube
- Add the reagents following the order given in Table 6
- Mix gently the CRYIA3/CRYIA4 Mastermix by pipetting and centrifuge briefly
- Divide the Mastermix into aliquots of 49 µl in 0.2 ml PCR reaction tubes
- Add 1 µl of the DNA solution to the previous aliquots
- Shake gently and centrifuge briefly
- Place the PCR reaction tubes in the thermocycler
Program for the nested PCR (CRYIA3/CRYIA4)

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>35</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
</tr>
<tr>
<td></td>
<td>4°C</td>
</tr>
</tbody>
</table>

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

Analysis of PCR products

Following amplification, the PCR products are analysed by agarose gel electrophoresis with ethidium bromide. 8 µl of the solution is mixed with 2 µl of loading buffer; the solution is then loaded onto an agarose gel (2.5%). 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 to provide a permanent record of the result of the experiment.

Interpretation of the results

The primer pairs CRYIA1/CRYIA2 and CRYIA3/CRYIA4 were designed for the specific detection of the synthetic crylA(b) gene by nested PCR, yielding a nested PCR fragment of 189 bp. This gene is present in maize line Bt-176 and other lines (e.g. Bt-11). The positive control will amplify showing a band at 189 bp.

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

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

If the controls give the expected results and the sample gives a band at 189 bp, it means that in this sample Bt-176 maize DNA is present.
Detection of Roundup Ready® soybean

The target is the CP4 \textit{EPSPS} gene, which confers resistance to the herbicide Roundup®.

\textit{Characteristics of primers GMO5, GMO9, GMO7 and GMO8}

\begin{center}
\begin{tabular}{l l l l}

\hline
 & GMO5 & GMO9 & GMO7 & GMO8 \\
\hline
Sequence & CCACTGACGTAAGGGATGACG & CATGAAGGACCGGTGGGAGAT & ATCCCACTATCCTTCGCAAGA & TGGGGTTTATGGAAATTGGAA \\
Length (bp) & 21 & 21 & 21 & 21 \\
Mol. weight (g/mol) & 6479.4 & 6559.4 & 6309.6 & 6579.8 \\
Melting point * (G/C) & 59.5 & 59.5 & 55.8 & 51.7 \\
\hline
\end{tabular}
\end{center}

*based on a [\text{Na}^+] of 50 mM

\textit{Controls}

- \textbf{Positive control}: DNA from reference material (RRS 0.1\% GM)
- \textbf{Negative control}: DNA from reference material (soybean 0\% GM)
- \textbf{No-template}: negative control of the Mastermix, in which water is used instead of DNA
**Mastermix preparation 1**

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 7. The following procedure applies to a sample containing 48 µl of GMO5/GMO9 Mastermix and 2 µl of DNA solution. All solutions are stored on ice during the preparation of the Mastermix.

**Table 7. GMO5/GMO9 Mastermix**

<table>
<thead>
<tr>
<th></th>
<th>Final concentration</th>
<th>Mastermix for one sample</th>
<th>Mastermix for 10 samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile, deionised water</td>
<td>32.75 µl</td>
<td>327.5 µl</td>
<td></td>
</tr>
<tr>
<td>10x PCR Buffer</td>
<td>1x</td>
<td>5 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>2.5 mM</td>
<td>5 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>4 mM dNTPs</td>
<td>0.2 mM</td>
<td>2.5 µl</td>
<td>25 µl</td>
</tr>
<tr>
<td>20 µM oligonucleotide GMO5</td>
<td>0.5 µM</td>
<td>1.25 µl</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>20 µM oligonucleotide GMO9</td>
<td>0.5 µM</td>
<td>1.25 µl</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.025 U/µl</td>
<td>0.25 µl</td>
<td>2.5 µl</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>48 µl</strong></td>
<td><strong>480 µl</strong></td>
<td></td>
</tr>
</tbody>
</table>

- Prepare a 1.5 ml microcentrifuge tube
- Add the reagents following the order given in Table 7
- Mix gently the GMO5/GMO9 Mastermix by pipetting and centrifuge briefly
- Divide the Mastermix into aliquots of 48 µl in 0.2 ml PCR reaction tubes
- Add 2 µl of the DNA solution to the previous aliquots
- Shake gently and centrifuge briefly
- Place the PCR reaction tubes in the thermocycler
**PCR program (GMO5/GMO9)**

<table>
<thead>
<tr>
<th></th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>3 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>40 sec</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>3 min</td>
</tr>
<tr>
<td></td>
<td>4°C</td>
<td>∞</td>
</tr>
</tbody>
</table>

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

**Mastermix preparation 2**

The necessary reagents for a series of 10 samples are mixed together according to the instructions given in Table 8.

The following procedure applies to a sample containing 49 µl of GMO7/GMO8 Mastermix and 1 µl of pre-amplified DNA solution of the first PCR. All solutions are stored on ice during the preparation of the Mastermix.

**Table 8. GMO7/GMO8 Mastermix**

<table>
<thead>
<tr>
<th></th>
<th>Final concentration</th>
<th>Mastermix for one sample</th>
<th>Mastermix for 10 samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile, deionised water</td>
<td>33.75 µl</td>
<td>337.5 µl</td>
<td></td>
</tr>
<tr>
<td>10x PCR Buffer</td>
<td>1x</td>
<td>5 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>2.5 mM</td>
<td>5 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>4 mM dNTPs</td>
<td>0.2 mM</td>
<td>2.5 µl</td>
<td>25 µl</td>
</tr>
<tr>
<td>20 µM oligonucleotide GMO7</td>
<td>0.5 µM</td>
<td>1.25 µl</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>20 µM oligonucleotide GMO8</td>
<td>0.5 µM</td>
<td>1.25 µl</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>0.025 U/µl</td>
<td>0.25 µl</td>
<td>2.5 µl</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>49 µl</strong></td>
<td><strong>490 µl</strong></td>
<td></td>
</tr>
</tbody>
</table>

- Prepare a 1.5 ml microcentrifuge tube
- Add the reagents following the order given in Table 8
- Mix gently the GMO7/GMO8 Mastermix by pipetting and centrifuge briefly
- Divide the Mastermix into aliquots of 49 µl in 0.2 ml PCR reaction tubes
- Add 1 µl of the DNA solution to the previous aliquots
- Shake gently and centrifuge briefly
- Place the PCR reaction tubes in the thermocycler

**Program for the nested PCR (GMO7/GMO8)**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C 3 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C 30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C 30 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C 40 sec</td>
</tr>
<tr>
<td>Number of cycles</td>
<td>35</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C 3 min</td>
</tr>
<tr>
<td></td>
<td>4°C ∞</td>
</tr>
</tbody>
</table>

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

**Analysis of PCR products**

Following amplification, the PCR products are analysed by agarose gel electrophoresis with ethidium bromide. 8 µl of the solution is mixed with 2 µl of loading buffer; the solution is then loaded onto an agarose gel (2.5%). 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 to provide a permanent record of the result of the experiment.

**Interpretation of the results**

The primer pairs GMO5/GMO9 and GMO7/GMO8 were designed for the specific detection of the gene construct of Roundup Ready® soybean by nested PCR, yielding a nested PCR fragment of 169 bp. The primers GMO5 and GMO7 are complementary to the CaMV 35S promoter, GMO9 hybridises with the CP4 EPSPS gene of Agrobacterium sp. strain CP4 and GMO8 with the CTP EPSPS gene.
The positive control will amplify showing a band at 169 bp. The negative control and the no-template should not give a visible band. If the positive/negative controls do not give the expected results, the PCR analysis of the selected samples is not valid. If the controls give the expected results and the sample gives a band at 169 bp, it means that in this sample Roundup Ready® soybean DNA is present.
The Analysis of Food Samples for the Presence of Genetically Modified Organisms

Session 10

Quantitative PCR for the Detection of GMOs

F. Weighardt
Table of Contents

Session 10

Quantitative PCR for the Detection of GMOs

Introduction 3
PCR methods for quantification 4
History of real-time PCR techniques 6
Real-time PCR principles 7
Principles of quantification with real-time PCR 13

References 18
Introduction

Once a food product has been found to be positive for one or more GM events (Roundup Ready® soybean, Bt-176 maize, Bt-11 maize, MON810 maize, and T25 maize), the subsequent analytical steps consist of assessing compliance with the Legislation in force, (Regulation (EC) 1829/2003, 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. 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 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 Bt-176 (0.6%) events exceed the 0.9% threshold for labelling.](image)

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, or zein 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 fashion 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, techniques such as quantitative-competitive PCR (QC-PCR) and real-time PCR, 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.

**Quantitative competitive PCR**

One of the first quantitative PCR methods developed is the quantitative-competitive PCR (Giacca *et al.*, 1994; Studer *et al.*, 1998; Hardegger *et al.*, 1999). This method is based on the co-amplification of target DNA template and defined amounts of an internal DNA standard (competitor) carrying the same primer binding sites. Since the initial amount of the competitor is known, and given that the amplification efficiencies of the target and competitor DNA are the same, the ratio of the amounts of the two PCR products, determined, e.g. by gel electrophoresis, is representative of the ratio of target DNA and competitor present in the reaction mix prior to amplification.

Typically, a competitor is a linearised plasmid bearing the same nucleotide sequence as the target DNA except for a deletion or an insertion in order to have, once co-amplified, two distinct sized bands following standard gel electrophoresis.

![Figure 3. Co-amplification of a fixed amount of target DNA with different amounts of competitor DNA.](image)

Competitive PCR has been used successfully to quantify both DNA and RNA, but its dynamic range is limited to a target-to-competitor ratio between approximately 1:10 to 10:1. In fact, the best accuracy is obtained by finding the equivalence point at which the ratio of target to competitor is 1:1 (Figure 3). To accomplish this, several dilutions must be tested in order to achieve a suitable ratio of target to competitor.
Another drawback of this approach is the need to construct and characterise a
different competitor for every target to be quantified. In fact, even a slight difference
in amplification efficiency will severely compromise the accuracy of quantification by
means of quantitative competitive PCR. Finally, at the end of the reaction,
competitive PCR requires accurate quantification of the target and competitor
amplicons, which usually entails laborious post-PCR processing steps.
The competitive PCR is a semi-quantitative method requiring a standard (the
competitor) to be compared to the sample. The results can only indicate a value
below, equal or above a defined standard concentration.
The competitive PCR system has, however, the advantage that no specialised
equipment has to be acquired by the laboratories, since it is performed on generally
standard PCR and molecular biology laboratory equipment.

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)

History of real-time PCR techniques
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.

**Real-time PCR principles**

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 ssDNA. 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 begins, the increasing amount of newly synthesised DNA results in an increasing fluorescent signal (Figure 4). 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 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 a 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 fluorophor to an acceptor fluorophor (Figure 4) (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 fluorophor 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’-Fluorescein) 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 4). 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 in 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 be 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 4. Different real-time PCR principles. I. SYBR green I. II. FRET (Fluorescence Resonance Energy Transfer) probes. III. TaqMan 5'-3'-degradation probes.
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 5) (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 fluoresce.

![Figure 5. The principle of Molecular Beacons.](image)
Scorpions

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 6) (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 6. The principle of Scorpion probes.
**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 7). 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 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 7. The principle of Minor Groove Binder (MGB) probes.](image)
Principles of 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 total material amount. In order to determine this value in a real-time PCR based system it is necessary to measure the number of DNA sequences of an endogenous reference gene (for use as a “normaliser”) as well as the number of GMO specific target DNA sequences. The reference gene should be chosen in order to be species specific, being present as a single copy per haploid genome, being stably represented as such in different lines of the same species and being 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 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:

a. The ploidy of the event. It is possible that the GM event has a different ploidy from the wt event (e.g. tetraploid instead of diploid);
b. The zygosity of the event. The GM trait could be homozygous or heterozygous;
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.

Point c. 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 excluding multiple insertions of the same construct from the quantification. Point a. and b. are bypassed empirically by the use of reference materials being homogeneous with the sample (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 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 genomes.

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:
- One PCR system designed to detect a GMO-specific target DNA sequence.
- A second PCR system designed to detect an endogenous reference sequence, possibly being species specific, but apt for use as a “normaliser” in the calculations of the GM relative concentration(s).
- 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) have to be added for both the reference gene and the GMO quantifications. Other controls may be used (e.g. negative DNA target control, positive DNA target control).

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 a possible statistical fluctuation between different experiments.

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 the GMO 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 and reagent saving (it is possible to analyse twice the number of samples in one single experiment), avoid set-up errors between the measure of the reference and the GMO target gene since they occur in the same tube, but are less sensitive (in
terms of LOQ) because of the interference between the two reactions and the differing consumptions of reagents of the two reactions. 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 and wells on the real-time PCR apparatus and are more exposed to the risk of, e.g. pipetting errors, when measuring one sample. The availability of multiple reporter dyes for TaqMan probes makes it possible to detect the amplification of more than one target in the same tube. The reporter dye (FAM) is distinguishable from the other (VIC) because they have different maximal emission wavelengths. As an example, the availability of multiple dyes with distinct emission wavelengths (FAM, TET, VIC and JOE) makes it 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. On the other hand, JOE and VIC should not be combined. Multiplex TaqMan assays can be performed on the ABI PRISM 7700, 7900, 7500, 7300 and 7000 Sequence Detection Systems due to their capability to detect multiple dyes with distinct emission wavelengths.

**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 8).
Figure 8. 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) 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_T value is. In practice, the choice of the threshold line determining the C_T 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 output of the real-time PCR is a $\Delta Rn$, 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).

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

1. Two standard curves, based on different amounts of DNA, are plotted:
   - The first curve with a quantification system specific for the reference gene;
   - The second curve with a quantification system specific for the GM target.

   For each sample the amount of the specific target and the reference gene are determined by interpolation with the standard curve. The GMO DNA content (percentage) is then calculated as the ratio between the GM target sequence amount and the reference gene sequence amount ($\text{GM/reference} \times 100$).

   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.

2. Comparative $C_T$ method ($\Delta\Delta C_T$): 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 from the IRMM). The result is one standard curve of $\Delta\Delta C_T$ ($\Delta C_T = C_T \text{reference gene} - C_T \text{GMO}$) values. The GMO content value is obtained by calculating the $\Delta C_T$ 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 $\Delta C_T$ (the difference between the two $C_T$ 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 $\Delta C_T$ 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. In conventional competitive RT-PCR, the dynamic range is limited to a target-to-competitor ratio of about 10:1 to 1:10 (the best accuracy is obtained for 1:1 ratio). The real-time PCR is able to achieve a much wider dynamic range.
References


The Analysis of Food Samples for the Presence of Genetically Modified Organisms

Session 11

Quantitative Detection of Roundup Ready® Soybean by Real-time PCR

N. Foti
Table of Contents

Session 11

Quantitative Detection of Roundup Ready® Soybean by Real-time PCR

Experimental 3

Introduction 3

Real-time PCR using the ABI PRISM® 7700 3
Real-time PCR using the LightCycler® (Roche) 8

References 14
Experimental

Introduction

The following protocols are real-time PCR-based methods for the quantification of a specific GM soybean line (Roundup Ready® soybean) in raw and processed material. The real-time PCR quantifications are carried out with two different PCR thermocyclers equipped to detect amplification in real-time by measuring fluorescence: the ABI PRISM® 7700 SDS (Applied Biosystems) and LightCycler® (Roche).

Real-time PCR is used to amplify an endogenous reference target DNA sequence (that is unique to soybean), plus a DNA target sequence that indicates the presence of genetically modified soybean (Roundup Ready® soybean).

Both assays encompass two independent PCR systems, each with 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 soybean.

Note: The protocols included in this manual have been chosen for didactical purposes and should be considered as basic examples of GMO quantification using the real-time PCR approach. We recommend to periodically reviewing pertinent sources and literature to acquire information on more recently developed and validated protocols. Please also note that these protocols have been selected according to the instrumentation available in our laboratory. The JRC and the WHO in no way promote the exclusive use of any particular company or brand.

Real-time PCR using the ABI PRISM® 7700

Protocol for RR-soybean specific real-time PCR: a multiplex PCR method

This method consists of an amplification/quantification of the lectin reference gene and a part of the RR soybean inserted cassette using a multiplex PCR assay (two PCR reactions in the same tube) (Foti et al., 2006). The TaqMan lectin and RR probes are labelled with the VIC dye and the FAM dye, respectively, making it possible to detect amplification of more than one target in the same tube. The reporter dye (FAM) is distinguishable from the VIC as a consequence of their
different maximal emission wavelengths. The ABI PRISM® 7700 SDS is able to
detect multiple dyes with distinct emission wavelengths.
The amount of RR-soybean (FAM) dye is normalised to the amount of plant material
(VIC) dye detected in each sample. This produces a ΔC_T value, which is averaged for
replicate samples. These values are compared to a calibration curve produced from
the ΔC_T of the known RR-soybean concentration standards (Comparative C_T method,
or ΔΔC_T).
This procedure was successfully applied to various raw materials, ingredients and
foods containing soybean (e.g. feed, soy drink, yoghurt, flour, lecithin etc.).
The analytical performance of the method has been successfully monitored during
several proficiency-testing schemes (e.g. FAPAS®, GIPSA).

Equipment and Reagents

- ABI PRISM® 7700 Sequence Detector System (Applied Biosystems)
- MicroAmp Optical 96-Well Reaction Plates (Cat No. N801-0560)
- MicroAmp Optical caps (Cat No. N801-0935)
- TaqMan® Universal PCR Mastermix (Cat No. 4304437) 2X containing: TaqMan
  Buffer 2x AmpliTaq Gold® DNA Polymerase (5U/µl), AmpErase® UNG (1U/ml),
  dNTPs 200 µM with dUTP, Passive Reference 1
- Microcentrifuge
- Refrigerated centrifuge for 15 ml conical tubes
- Micropipettes
- Vortex mixer
- Rack for reaction tubes
- 1.5 ml microcentrifuge tubes
- 15 ml polypropylene conical centrifuge tubes
- Nuclease-free water
- Reference gene specific primers (Le-F and Le-R) and probe (Le-Probe)
- Transgene specific primers (RR-F and RR-R) and probe (RR-Probe)

<table>
<thead>
<tr>
<th>Characteristics of primers and probe for the reference gene</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Le-F</strong></td>
</tr>
<tr>
<td>Sequence</td>
</tr>
<tr>
<td>Length (bp)</td>
</tr>
<tr>
<td>Mol. weight</td>
</tr>
</tbody>
</table>
Characteristics of primers and probe for the transgene

**RR-F**

<table>
<thead>
<tr>
<th>Sequence</th>
<th>GCC ATG TTG TTA ATT TGT GCC AT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (bp)</td>
<td>23</td>
</tr>
<tr>
<td>Mol. Weight</td>
<td>7014</td>
</tr>
</tbody>
</table>

**RR-R**

<table>
<thead>
<tr>
<th>Sequence</th>
<th>GAA GTT CAT TTC ATT TGG AGA GGA C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (bp)</td>
<td>25</td>
</tr>
<tr>
<td>Mol. Weight</td>
<td>7712</td>
</tr>
</tbody>
</table>

**RR-Probe**

<table>
<thead>
<tr>
<th>Sequence</th>
<th>5’-(FAM)-CTT GAA AGA TCT GCT AGA GTC AGC TTG TCA GCG-(TAMRA)-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (bp)</td>
<td>33</td>
</tr>
<tr>
<td>Mol. Weight</td>
<td>10137</td>
</tr>
</tbody>
</table>

**Le-R**

<table>
<thead>
<tr>
<th>Sequence</th>
<th>GGC ATA GAA GGT GAA GTT GAA GGA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (bp)</td>
<td>24</td>
</tr>
<tr>
<td>Mol. Weight</td>
<td>7532</td>
</tr>
</tbody>
</table>

**Le-Probe**

<table>
<thead>
<tr>
<th>Sequence</th>
<th>5’-(VIC)-AAC CGG TAG CGT TGC CAG CTT CG-(TAMRA)-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (bp)</td>
<td>23</td>
</tr>
<tr>
<td>Mol. weight</td>
<td>7019.0</td>
</tr>
</tbody>
</table>

Mastermix preparation

- Thaw, mix gently and centrifuge the required amount of components required for the reaction. Keep thawed reagents on ice.

- In one 15 ml centrifuge tube kept on ice, add the following components in the order mentioned below (except DNA) to prepare the mastermixes. Each DNA extract is analysed in triplicate. Please Note that four more reaction mixes are prepared to help calculate pipetting errors due to solution viscosity.
Table 1. Preparation of the mastermix for one plate of multiplex PCR assay.

<table>
<thead>
<tr>
<th></th>
<th>Concentration in PCR</th>
<th>Mastermix for reaction vessel (µl)</th>
<th>Mastermix for one sample (3 repetitions)</th>
<th>Mastermix for one plate (32+4 samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile, deionised water</td>
<td></td>
<td>18.3</td>
<td>54.9</td>
<td>1976.4</td>
</tr>
<tr>
<td>TaqMan Universal Mastermix 2X</td>
<td>1x</td>
<td>25</td>
<td>75</td>
<td>2700</td>
</tr>
<tr>
<td>Primer Le-F (20 µM)</td>
<td>40 nM</td>
<td>0.1</td>
<td>0.3</td>
<td>10.8</td>
</tr>
<tr>
<td>Primer Le-R (20 µM)</td>
<td>40 nM</td>
<td>0.1</td>
<td>0.3</td>
<td>10.8</td>
</tr>
<tr>
<td>Primer RR-F (20 µM)</td>
<td>100 nM</td>
<td>0.25</td>
<td>0.75</td>
<td>27</td>
</tr>
<tr>
<td>Primer RR-R (20 µM)</td>
<td>100 nM</td>
<td>0.25</td>
<td>0.75</td>
<td>27</td>
</tr>
<tr>
<td>Le-Probe (10 µM)</td>
<td>100 nM</td>
<td>0.5</td>
<td>1.5</td>
<td>54</td>
</tr>
<tr>
<td>RR-Probe (10 µM)</td>
<td>100 nM</td>
<td>0.5</td>
<td>1.5</td>
<td>54</td>
</tr>
<tr>
<td>DNA</td>
<td>50-250 ng</td>
<td>5</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td>50</td>
<td>150</td>
<td></td>
</tr>
</tbody>
</table>

- Mix gently and centrifuge briefly.
- Prepare one 1.5 ml microcentrifuge tube for each DNA sample to be tested: standard curve samples (CRM - RR soybean at 0.1, 0.5, 1, 2, and 5%) unknown samples and control samples (0% RR-soybean, DNA from RR-soybean and No-Template Control).
- Add to each microcentrifuge tube the amount of mastermix needed for 3 repetitions (135 µl mastermix). Add to each tube the required amount of DNA for 3 repetitions (i.e. 15 µl DNA). Vortex each tube at least three times for approx 10 sec. This step is of particular importance as it helps reduce to a minimum the variability between the three replicas of each sample.
- Spin briefly in a microcentrifuge. Place the 96-well reaction plate in a base and aliquot 50 µl in each well horizontally from left to right. After adding mastermixes to one vertical row of wells, cover the wells with optical caps using the cap-installing tool.

*Note: do not write on the optical plate and do not touch the cap*

- Ensure that the loaded mastermix aliquots are in the bottom of the wells, with no splashes or bubbles on the side or in the caps.
- Place the plate into the ABI PRISM® 7700 instrument; open a new plate setup window to assign the sample type to each well: the IPC sample type is associated with the VIC dye and the UNKN on the FAM dye layer.
- Cycle the samples as described in Table 2.
Table 2. Cycling program for the RR-soybean multiplex assay on ABI PRISM® 7700 SDS

Set: Real time PCR modus

50 µl reaction volume

<table>
<thead>
<tr>
<th>Step</th>
<th>Stage</th>
<th>T°C</th>
<th>Time</th>
<th>Acquisition</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>UNG</td>
<td>50°C</td>
<td>120 sec</td>
<td>no</td>
<td>1x</td>
</tr>
<tr>
<td>2</td>
<td>Initial denaturation</td>
<td>95°C</td>
<td>600 sec</td>
<td>no</td>
<td>1x</td>
</tr>
<tr>
<td>3</td>
<td>Amplification</td>
<td>Denaturation</td>
<td>95°C</td>
<td>15 sec</td>
<td>no</td>
</tr>
<tr>
<td>4</td>
<td>Annealing &amp; Extension</td>
<td>60°C</td>
<td>60 sec</td>
<td>Measure</td>
<td></td>
</tr>
</tbody>
</table>

Data analysis and interpretation of results

After the run is completed data are analysed using the ABI PRISM® 7700 SDS software to produce C_T values of each reporter dye for each sample.

It is important to properly number samples in the SDS software Plate Setup window. Each well should be given a unique number in the “Replicate” field; replicates of the same sample should be given the same number.

Analyse the run by selecting “analyse” from the Analysis menu to automatically access the amplification plot window; adjust the threshold value for the FAM and VIC layers.

After analysing the run export the results on an excel file Opening the exported results file in Microsoft® Excel, a table containing two sets of data corresponding to the FAM and VIC dye layers appears with C_T values for each well.

Calculate the C_T (FAM) and C_T (VIC) average of each group of replicate to calculate the ΔC_T values (C_T,FAM – C_T,VIC).

For each sample, %GMO is calculated by analysing the sample’s ΔC_T, comparing it to the set of log (% GMO) and ΔC_T values obtained from the concentration standards set.
Real-time PCR using the LightCycler® (Roche)

Protocol for RR-soybean specific real-time PCR

The method consists of an amplification/quantification of the lectin reference gene and a part of the RR soybean inserted cassette, performed in two independent PCR reactions (BgVV, EU Tender Report, 2000). The two PCR systems use FAM labelled probes.

For each sample the amount of GMO soybean specific sequences and the reference gene (lectin gene) sequence is determined from the appropriate standard curves prepared for both the transgene and the reference gene. The GMO soybean content is divided by the reference gene amount to obtain a normalized GMO soybean value.

Equipment and Reagents

- Roche LightCycler® system
- LightCycler® Sample Capillaries. Roche, Cat No 1909339
- LightCycler® Capillaries Adapter. Roche, Cat No 1909312
- LightCycler® FastStart DNA Master Hybridization Probes. Roche, Cat No 3003248 containing: FastStart Taq DNA Polymerase, dNTP mix (with dUTP instead of dTTP) and reaction buffer, 10x conc.; sterile water, PCR-grade
- Bovine serum albumin (BSA), nuclease free (DNase & RNase free) e.g. Promega Cat No R9461 (1 µg/µl)
- Platinum Taq DNA Polymerase 5U/µl (together with the original 10x buffer and 50 mM MgCl₂). Invitrogen – Life Technologies Cat No 10966026
- Microcentrifuge
- Micropipettes
- Vortex mixer
- Rack for reaction tubes
- 1.5 ml microcentrifuge tubes
- Nuclease-free water
- Reference gene specific primers (GM1-F and GM1-R) and probe (GM1-Probe)
- Transgene specific primers (GM2–F, GM2-R) and probe (GM2-Probe)
**Characteristics of primers and probe for the reference gene**

<table>
<thead>
<tr>
<th><strong>GM1-F</strong></th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5'-CCA GCT TCG CCG CTT CCT TC-3'</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>GM1-R</strong></th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5'-GAA GGC AAG CCC ATC TGC AAG CC-3'</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>GM1-Probe</strong></th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5'-(FAM)-CTT CAC CTT CTA TGC CCC TGA CAC -(TAMRA)-3'</td>
</tr>
</tbody>
</table>

**Characteristics of primers and probe for the transgene**

<table>
<thead>
<tr>
<th><strong>GM2-F</strong></th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5'-CAT TTG GAG AGG ACA CGC TGA-3'</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>GM2-R</strong></th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5'-GAG CCA TGT TGT TAA TTT GTG CC-3'</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>GM2-Probe</strong></th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5'-(FAM)-CAA GCT GAC TCT AGC AGA TCT TTC (TAMRA)-3'</td>
</tr>
</tbody>
</table>

**Standard curves**

In each run the two standard curves are generated with 5 different DNA standard dilutions. Single reactions are performed with each calibration standard DNA with both mastermixes.

Standard curves for total soybean DNA and for GMO sequence quantification are made with standard DNA solutions at decreasing concentration values starting from 2% RR soybean DNA with TE buffer (0.1 M, pH 8.0). Approximately 100 ng DNA is used for the first point of the standard curves. Dilutions and concentrations of the standard curves are summarised in Table 3.
Table 3. Quantity of DNA and dilutions of the standard curves.

<table>
<thead>
<tr>
<th>DNA amount (ng)/reaction</th>
<th>Soybean DNA %</th>
<th>GM DNA %</th>
<th>Dilution Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>STD 1</td>
<td>100</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>STD 2</td>
<td>50</td>
<td>50</td>
<td>1:2</td>
</tr>
<tr>
<td>STD 3</td>
<td>25</td>
<td>25</td>
<td>1:4</td>
</tr>
<tr>
<td>STD 4</td>
<td>12.5</td>
<td>12.5</td>
<td>1:8</td>
</tr>
<tr>
<td>STD 5</td>
<td>6.25</td>
<td>6.25</td>
<td>1:16</td>
</tr>
</tbody>
</table>

**Mastermix preparation**

- Thaw, mix gently and centrifuge the required amount of components needed for the run. Keep thawed reagents on ice.
- For each system, add the following components in the order mentioned below (except DNA) to a 1.5 ml microcentrifuge tube on ice to prepare the mastermixes. Please note that two reaction mixes include an excess volume to considering pipetting errors due to solution viscosity.

Table 4. Preparation of the mastermix for the GM1 system.

<table>
<thead>
<tr>
<th>Component</th>
<th>concentration in PCR</th>
<th>µl/reaction</th>
<th>Total µl (for 18 reactions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platinum Taq pol.ase Buffer 10x</td>
<td>1x</td>
<td>2</td>
<td>36</td>
</tr>
<tr>
<td>MgCl₂ (50 mM)</td>
<td>4 mM</td>
<td>1.6</td>
<td>28.8</td>
</tr>
<tr>
<td>dATP, dGTP, dCTP, dTTP (4 mM)</td>
<td>0.8 mM</td>
<td>4</td>
<td>72</td>
</tr>
<tr>
<td>GM1-F primer (20 µM)</td>
<td>500 mM</td>
<td>0.5</td>
<td>9</td>
</tr>
<tr>
<td>GM1-R primer (20 µM)</td>
<td>500 nM</td>
<td>0.5</td>
<td>9</td>
</tr>
<tr>
<td>GM1-Probe (10 µM)</td>
<td>200 nM</td>
<td>0.4</td>
<td>7.2</td>
</tr>
<tr>
<td>BSA nuclease free (1 µg/µl)</td>
<td>0.1 mg/ml</td>
<td>2</td>
<td>36</td>
</tr>
<tr>
<td>Platinum Taq pol.ase (5 U/µl)</td>
<td>0.8 U</td>
<td>0.16</td>
<td>2.9</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>#</td>
<td>6.85</td>
<td>123.5</td>
</tr>
<tr>
<td>DNA</td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Total volume:</td>
<td></td>
<td>18 µl+2 µl DNA</td>
<td>324.2 µl (w/o DNA)</td>
</tr>
</tbody>
</table>
Table 5. Preparation of the mastermix for the GM2 system

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration in PCR</th>
<th>µl/reaction</th>
<th>Total µl (for 18 reactions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platinum Taq pol.ase Buffer 10x</td>
<td>1x</td>
<td>2</td>
<td>36</td>
</tr>
<tr>
<td>MgCl₂ (50 mM)</td>
<td>4 mM</td>
<td>1.6</td>
<td>28.8</td>
</tr>
<tr>
<td>dATP, dGTP, dCTP, dTTP (4 mM)</td>
<td>0.8 mM</td>
<td>4</td>
<td>72</td>
</tr>
<tr>
<td>GM2-F primer (20 µM)</td>
<td>500 nM</td>
<td>0.5</td>
<td>9</td>
</tr>
<tr>
<td>GM2-R primer (20 µM)</td>
<td>500 nM</td>
<td>0.5</td>
<td>9</td>
</tr>
<tr>
<td>GM2 Probe (10 µM)</td>
<td>200 nM</td>
<td>0.4</td>
<td>7.2</td>
</tr>
<tr>
<td>BSA nuclease free (1 µg/µl)</td>
<td>0.1 mg/ml</td>
<td>2</td>
<td>36</td>
</tr>
<tr>
<td>Platinum Taq pol.ase (5 U/µl)</td>
<td>0.8 U</td>
<td>0.16</td>
<td>2.9</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>#</td>
<td>6.85</td>
<td>123.5</td>
</tr>
<tr>
<td>DNA</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total volume:</td>
<td>18 µl+2 µl DNA</td>
<td>324.2 µl (w/o DNA)</td>
<td></td>
</tr>
</tbody>
</table>

- Mix gently and centrifuge briefly
- Prepare two (one for GM1 system and one for GM2 system) 0.5 ml reaction tubes for each DNA sample to be tested (standard curve samples and unknown samples)
- Add to each reaction tube the amount of mastermix needed for 2 repetitions (36 µl mastermix). Add to each tube the proper amount of DNA for 2 repetitions (i.e. 4 µl DNA). Vortex at least three times for approx. 10 sec. each tube. This step is important in order to reduce to a minimum the variability between the replicates of each sample
- Place the capillaries in the pre-cooled centrifuge adapter
- Pipette 20 µl of the mastermix to each capillary according to the scheme provided. (see Table 6 “Plate set up – loading order” - Standard LightCycler® carousel)
- Spin down in a microcentrifuge at low speed (ca. 1150 rpm). This step ensures the concentration of the entire volume of the reaction mix into the tip of the capillary
- Transfer the capillaries into the LightCycler® (Roche) carousel
- Cycle the samples as described in Table 7
Table 6. Plate set up – LightCycler® carousel-loading order: positions 1 to 16 = GM1 system, positions 17 to 32 = GM2 system. Each DNA sample in duplicate for the reference gene and for the transgene (GMO) systems

<table>
<thead>
<tr>
<th>Position</th>
<th>Sample (%)</th>
<th>Position</th>
<th>Sample (%)</th>
<th>Position</th>
<th>Sample (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>STD (100)</td>
<td>13</td>
<td>U2</td>
<td>25</td>
<td>STD (0.125)</td>
</tr>
<tr>
<td>2</td>
<td>STD (100)</td>
<td>14</td>
<td>U2</td>
<td>26</td>
<td>STD (0.125)</td>
</tr>
<tr>
<td>3</td>
<td>STD (50)</td>
<td>15</td>
<td>NTC</td>
<td>27</td>
<td>U1</td>
</tr>
<tr>
<td>4</td>
<td>STD (50)</td>
<td>16</td>
<td>NTC</td>
<td>28</td>
<td>U1</td>
</tr>
<tr>
<td>5</td>
<td>STD (25)</td>
<td>17</td>
<td>STD (2)</td>
<td>29</td>
<td>U2</td>
</tr>
<tr>
<td>6</td>
<td>STD (25)</td>
<td>18</td>
<td>STD (2)</td>
<td>30</td>
<td>U2</td>
</tr>
<tr>
<td>7</td>
<td>STD (12.5)</td>
<td>19</td>
<td>STD (1)</td>
<td>31</td>
<td>NTC</td>
</tr>
<tr>
<td>8</td>
<td>STD (12.5)</td>
<td>20</td>
<td>STD (1)</td>
<td>32</td>
<td>NTC</td>
</tr>
<tr>
<td>9</td>
<td>STD (6.25)</td>
<td>21</td>
<td>STD (0.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>STD (6.25)</td>
<td>22</td>
<td>STD (0.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>U1</td>
<td>23</td>
<td>STD (0.25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>U1</td>
<td>24</td>
<td>STD (0.25)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7. Cycling program for the LightCycler® system

Set: slope 20°C/sec in all steps.

Fluorescent display mode: F1/1

Denaturation:

<table>
<thead>
<tr>
<th>Cycles:</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type:</td>
<td>None</td>
</tr>
<tr>
<td>Fluorescence Display Mode:</td>
<td>F1/1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Segment Number</th>
<th>Temp.</th>
<th>Time</th>
<th>Slope</th>
<th>2° Target Temp.</th>
<th>Step Size</th>
<th>Step Delay (Cycles)</th>
<th>Acquisition Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>96 °C</td>
<td>120 sec</td>
<td>20 °C/sec</td>
<td>0 °C</td>
<td>0 °C</td>
<td>0</td>
<td>None</td>
</tr>
</tbody>
</table>

Cycling:

<table>
<thead>
<tr>
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During the run, click on the **EDIT SAMPLE** button and enter the sample names in the loading screen. Define all positions (including the calibrator DNA) as "Unknowns".

**Data analysis and interpretation of results**

- For data analysis select ‘Fluorescence’ F1/F2 in the data analysis front window.
- For quantification click the quantification button.
- The LightCycler® software offers two different methods for quantification: The ‘Second Derivative Maximum’ method and the ‘Fit Points’ method. For quantification with the RR-soybean DNA detection kit preferably use the ‘Fit Points’ method. Use the following settings: baseline adjustment = ‘Proportional’; number of points = 2.
- Highlight the standard curve together with all corresponding unknown reactions.
- Open the folder ‘Step 2: Noise Band’.
- Move the noise band to a position of 0,1. The noise band can be moved either manually using the mouse or by pressing the button below the ‘Chance Graph Settings’ button. Using this button the ‘Manual Cursor Adjustment’ window appears and the cursor value can be defined as 0.1.
- Open the folder ‘Step 3: Analysis’.
- In ‘Step 3: Analysis’ the crossing points and the corresponding concentrations of calibration standards and unknown DNA templates are calculated.
- Control the r-value of the standard curve. Values of $r \geq -0.98$ are acceptable; optimal r-value = -1.
References


LightCycler® Operator’s Manual, version 3.0 (Roche Molecular Biochemicals).


The Analysis of Food Samples for the Presence of Genetically Modified Organisms

Session 12

Quantitative Detection of Roundup Ready® Soybean by ELISA

F. Eyquem
Table of Contents

Session 12

Quantitative Detection of Roundup Ready® Soybean by ELISA

Introduction 3

The ELISA technique 7

Experimental 11

References 20
Introduction

The specific immunological detection of a novel protein synthesised by a gene introduced during transformation 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 expression levels of transgene products in plants were reported to be in the range of 0 to 2% of the total soluble protein, even when strong constitutive promoters were used to drive expression (Longstaff, 1995). In most cases, however, the expression levels reported (e.g. for approved GM crops) are lower than the upper limit of 2% (Hemmer, 1997).

Immunoassays are analytical measurement systems that use antibodies as test reagents. 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 (e.g. CP4 EPSPS, the protein that confers resistance to the herbicide Roundup) 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. In addition, the sample or the proteins of interest should not be significantly degraded.

Antibodies are powerful tools for the biologist in the detection and quantification of antigens in complex mixtures. All immunoassays are based on the specific binding of antibody to antigen.

Antibody-antigen interactions

When immunologists describe the properties of antibodies as proteins, most would include a description of the capacity of these molecules to precipitate antigens from solution, even though antibody precipitation is seldom used any more to isolate or detect antigens experimentally and antibodies rarely precipitate antigens in vivo,
except in some autoimmune diseases. The instructional value of the antibody precipitation reaction, as illustrated in Figure 1, is that it neatly embodies so many of the fundamental and universal properties of antibody molecules. This technique demonstrates, among other things, that:

- serum (IgG) antibodies are bivalent in their reactions with antigen and have the capacity to cross-link antigens;
- antigens are often multivalent in their interactions with antibodies;
- serum antibodies are typically polyclonal in nature; and
- antibodies are highly specific in terms of the structures they recognise on antigenic molecules.

Figure 1. A precipitation curve for a system of one antigen and its antibodies

The plot of the amount of antibody precipitated versus increasing antigen concentration (at constant total antibody) reveals three zones:

- an ‘Antibody-excess zone’ in which precipitation is inhibited and excess antibody can be detected in the supernatant.
- an ‘Equivalence zone’ of maximal precipitation in which antibody and antigen form large insoluble complexes (shaded in blue) and neither antibody nor antigen can be detected in the supernatant.
an ‘Antigen-excess zone’ in which precipitation is inhibited and excess antigen can be detected in the supernatant.

While the precipitation reaction is particularly well suited for characterising the interactions between polyclonal antibodies and multivalent antigens, it is generally not very useful for characterising the interactions between antigens and monoclonal antibodies, unless the antigens exhibit multiple identical epitopes (like the repeating carbohydrate structures found in polysaccharides, for example). Only then will a mono-specific monoclonal antibody be able to cross-link and precipitate antigen. However, monoclonal antibodies are typically characterised by more refined types of measurements that provide detailed information regarding the antibody-antigen interaction, such as the equilibrium constant and the kinetic off- and on-rates.

The utility of antibodies for detecting and isolating antigens can hardly be overstated, given the wide spectrum of extremely powerful antibody applications developed over the years. 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.

How can monoclonal antibodies be prepared

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

Antibodies have two very useful characteristics. First, they are extremely specific; that is, 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.

The second characteristic of antibodies makes it possible to develop vaccines. A vaccine is a preparation of killed or weakened bacteria or viruses that, when introduced into the body, stimulates the production of antibodies against the antigens it contains.

It is the first trait of antibodies, their specificity, which makes monoclonal antibody technology so valuable. Not only can antibodies be used therapeutically, to protect against disease; they can also help to diagnose a wide variety of illnesses and can detect the presence of drugs, viral and bacterial products and other unusual or abnormal substances in the blood.
Given such a diversity of uses for these disease-fighting substances, their production in pure quantities has long been the focus of scientific investigation. The conventional method was to inject a laboratory animal with an antigen and then, after antibodies had been formed, collect those antibodies from the blood serum (antibody-containing blood serum is called antiserum). There are two problems with this method: it yields antiserum that contains undesired substances and it provides a very small amount of usable antibody.

Monoclonal antibody technology allows the production of large amounts of pure antibodies by using cells that produce antibodies naturally and a class of cells that can grow continually in cell culture. If we form a hybrid that combines the characteristic of "immortality" with the ability to produce the desired substance, we have, in effect, a factory to produce antibodies that works around the clock.

In monoclonal antibody technology, tumour cells that can replicate endlessly are fused with mammalian cells that produce an antibody. The result of this cell fusion is a "hybridoma," which will continually produce antibodies. These antibodies are called monoclonal because they come from only one type of cell, the hybridoma cell; antibodies produced by conventional methods, on the other hand, are derived from preparations containing many kinds of cells and hence are called polyclonal. An example of how monoclonal antibodies are derived is described below.

**Monoclonal antibody production**

A myeloma is a tumour of the bone marrow that can be adapted to grow permanently in cell culture. When myeloma cells were fused with antibody-producing mammalian spleen cells, it was found that the resulting hybrid cells, or hybridomas, produced large amounts of monoclonal antibody. This product of cell fusion combined the desired qualities of the two different types of cells: the ability to grow continually and the ability to produce large amounts of pure antibody.

Because selected hybrid cells produce only one specific antibody, they are more pure than the polyclonal antibodies produced by conventional techniques. They are potentially more effective than conventional drugs in fighting disease, since drugs attack not only the foreign substance but the body's own cells as well, sometimes producing undesirable side effects such as allergic reactions. Monoclonal antibodies attack the target molecule and only the target molecule, with no or greatly diminished side effects.
The ELISA technique

Definition

**Enzyme-Linked Immuno**Sorbent Assay: 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 (Clark and Adams, 1977) relies on specific interactions between antibodies and antigens. 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 “antigen”). In the case of detection of GMOs, the antigen can be the newly synthesised protein.

ELISA has been widely applied to evaluate, at experimental stage, the expression level of the protein(s) synthesised by the newly introduced gene. Information regarding production and use of specific antibodies can be therefore found in many articles describing the developments of transgenic plants (Mohapatra *et al.*, 1999).
Only a few specific antibodies, directed against proteins that are the products of transgenes used in approved genetically engineered crops, are commercially available: some examples are the antibodies against the \( nptI \)-gene product, NPTII, or APH(3')II and against the product of the \( gus \) gene.

There are 3 different methods to perform an ELISA Test:

(a) Indirect ELISA

(b) Sandwich ELISA

(c) Competitive ELISA

An ELISA-based method for the specific detection of Roundup Ready® soybean was developed, tested and 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%.
**Principle**

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

The surface of a microtiter plate is coated with a specific monoclonal capture antibody.

When the sample of interest is added, the capture antibody binds the antigen. Unbound components of the sample are removed by washing.

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.
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.
Experimental


Introduction

The following protocol specifies an ELISA-method for the determination of the CP4 EPSPS protein expressed in Roundup Ready® Soybean in raw agricultural commodities and products like soybean flours and soy protein isolates. The method is applicable to samples where little or no treatment has been carried out and thus the CP4 EPSPS protein is not denatured. For example, the temperature at which food ingredients are processed may impact the ability of protein detection. Data indicate that processing temperatures of no more than 65°C for no longer than 60 min, allow reliable protein detection.

The method has been validated for the detection of CP4 EPSPS protein and can be used for the determination of the concentration of the protein in the sample over the range of 0.3% to 5% (w/w) using specific reference material. The kit can also be run at lower detection levels of 0.05% to 0.3% using a modified protocol.

Equipment

- 15 ml polypropylene conical centrifuge tubes
- 12 X 75 mm glass test tubes
- Plastic wrap or aluminium foil
- Plastic tape (for manual plate washing) or automatic plate washer
- Wash bottles, e.g. of 500 ml
- Precision pipettes capable of delivering 20 µl to 500 µl
- Vortex mixer
- Weigh boats or equivalent
- Spatulas
- Balance capable of 0.01 g measurement

¹ The kit described in this Session was the only validated protocol commercially available at the time the training courses were organized and this Manual prepared. The JRC and the WHO do not promote any particular brand of commercially available kits.
• Centrifuge capable of 5000 to 10000 rpm
• Microtiter plate reader capable of reading absorbance at 450 nm
• Incubator oven capable of maintaining 37°C
• Sieve of aperture size of 450 µm, or equivalent
• Sieve of aperture size of 150 µm (100 mesh), or equivalent
• Multi-channel pipette, e.g. of 50 µl to 300 µl (optional)
• Reagent reservoirs for multi-channel dispensing (optional)
• Automated plate washer (optional)
• Test tube rack for 15 ml centrifuge tubes (optional)

Reagents

General

During the analysis, unless otherwise stated, use only reagents of recognised analytical grade and deionised or distilled water.

Any deviation from the defined performance criteria may indicate a lack of reagent stability. If the substrate components have already changed colour from clear to blue, this reagent should be discarded. Turbid buffer solutions should not be used.

All kit components should be stored at approximately 2°C to 8°C. The shelf life of the kit components is indicated by the expiry date. Based on accelerated stability testing, the expiry date for the test kit has been set as 9 months at approximately 2°C to 8°C. The antibody conjugate “soya conjugate” stock solution and the antibody conjugate working solution should be stored at 2°C to 8°C until the expiry date of the kit. The diluted working wash buffer should be stored at approximately 2°C to 8°C and not longer than the expiry date of the kit.

Reagents usually provided with the test kit

• Soya extraction buffer, sodium borate buffer, pH 7.5
• Soya assay buffer, PBS, Tween 20, bovine serum albumin, pH 7.4
• Coated strip wells (12 strips, each containing 8 wells coated with the monoclonal capture antibodies and 1 strip holder)
• Soya conjugate, rabbit anti-CP4 EPSPS protein, lyophilised
• Soya conjugate diluent, 10% heat inactivated mouse serum
• Chromogenic substrate, K-Blue™, tetramethylbenzidine (TMB), hydrogen peroxide, 5% dimethylformamide as base solvent
• Stop solution, 0.5% sulfuric acid
• 10 fold wash buffer concentrate, PBS, Tween 20, pH 7.1
• Negative and positive reference standards

Procedure

Limitation of the procedure

This ELISA GM-test system is limited to samples where the expression of the CP4 EPSPS protein can be correlated with the level of GM material present in the reference standards used.

The ELISA test kit is designed to give optimum performance at ambient temperatures of between 15°C to 30°C. The absorbance of the highest reference standard should be greater than 0.8 and should not fall outside the linear range of the spectrometer (the upper limit varies from spectrometer to spectrometer). It is important to consider that the OD values rise more rapidly at temperature higher than 30°C. Therefore, if temperatures are high, a reduced substrate incubation time will be necessary. At low temperatures (less than 15°C) the substrate incubation time should be increased.

Measures to avoid contamination during sample preparation

General. The ELISA GMO test system is an extremely sensitive technique capable of detecting very small quantities of GM contamination. For this reason it is imperative that all equipment used to process soya samples be thoroughly cleaned between sample batches. The following procedures involve a first step of physical removal of as much particulate material as possible. The second step, a wash with alcohol, is to denature the sample and thus render it unreactive in the assay of any GM protein that might have remained on the equipment.

Grinder or blender cleaning. Brush clean with a soft bristle brush. Periodically wash brush and soak in an alcohol solution. Dry brush before subsequent use. Wipe with a soft cloth or laboratory towel.
Rinse with alcohol, this can be stored and dispensed from a spray or squirt bottle. Two rinses or sprays are recommended. Then rinse thoroughly with water. Air dry or, if rapid reuse is required, dry using commercial hair dryer.

Sieve cleaning. Sieves tend to become caked with soya powder. Sharply tap sieve on hard surface to dislodge caked material.
Brush with clean soft bristle brush. Periodically wash brush and soak in an alcohol solution for at least 1 min. Dry brush before subsequent use. Wipe with soft cloth. Soak in alcohol for at least 5 min and rinse thoroughly with water. Air dry or, if rapid reuse is required, dry using commercial hair dryer. An alternative method would be to use an ultrasonic bath followed by air-drying.

Cleanliness of work area. Cleanliness of the work area is also important. Since the assay is very sensitive, a tiny GM positive dust contamination of the assay tube might render a false positive result. Avoid soya dust contamination in the work area. Do not allow soya dust from one processing to contaminate equipment to be used in a subsequent processing. For optimum performance run assay in a room separated from the facility where sample grinding and preparation is conducted to avoid potential dust contamination.

Sample preparation

A homogeneous incremental sub-sample should be taken from the laboratory sample. If the sample is raw soya beans, then at least 500 g should be placed in an appropriate blender and ground for approx. 3 min or until fine enough to pass through the sieves. For quantitative analysis, a particle size of less than 150 µm should be obtained and of less than 450 µm for qualitative analysis. To avoid contamination, care should be taken during the sieving step. Furthermore, care should be taken to avoid excessive heating. The actions of the blender will both mix and grind the sample. From the ground material a sub-sample of approximately 100 g should be taken and passed through a sieve of 450-µm-pore size (40 mesh). At least 90% of this sub-sample should pass through the 450-µm sieve. For a qualitative assay, this material can be used directly, for a quantitative assay the sieved material should be further sieved using a sieve of 150-µm-pore size. The material passing through the 450-µm sieve has been shown to be homogeneous and so it is necessary to sieve only enough material to provide a final sample through the 150-µm sieve. It is sufficient to pass only the amount of material that is necessary for the final sample. Other types of samples should be treated equivalently although smaller sample sizes may be used.

Assay procedure

Allow all reagents to warm to room temperature before using.
Remove the coated strips and strip holder from the foil bag. Always reseal the foil bag each time after removing the appropriate number of strips. Ten wells are required for reference standards and assay blanks. Each plate shall have its own standards and controls. If using manual washing, tape the edges of all strips needed for a run to the strip holder to prevent strips from accidentally falling out of the strip holder during the washing steps.

**Preparation of antibody conjugate**

**Antibody conjugate stock solution.** Pipette 1 ml Soya Conjugate Diluent from Soya Conjugate bottle into the Soya Conjugate vial. Vortex for approximately 10 s.

**Antibody conjugate working solution.** Transfer 240 µl of reconstituted Soya Conjugate back into the Soya Conjugate Diluent bottle. Label the bottle with current date and mix by inverting 20 times.

**Preparation of wash buffer**

Allow the 10X Wash Buffer Concentrate to come to room temperature. Dilute the 10X Wash Buffer Concentrate in deionised water to prepare the working wash buffer (e.g. 50 ml 10X Wash Buffer Concentrate into 450 ml deionised water). Add to Automatic Plate Washer or Wash Bottle.

**Extraction of samples and reference standards**

The samples and negative and positive reference standards are extracted under the same conditions in duplicate, described as follows. When weighing samples, weigh out each reference standard in increasing order of concentration and then the samples. Weigh out 0.5 g ± 0.01 g of each reference standard and the sample into individual 15 ml polypropylene centrifuge tubes. To avoid contamination clean the spatula between each weighing.

Add 4.5 ml of soya extraction buffer into each tube containing 0.5 g sample and vortex for 10 s. Centrifuge the mixtures at approximately 5000 rpm for 15 min.

Using a transfer pipette, carefully remove supernatant without aspirating particulate matter and place supernatant into a clean, labelled 15 ml centrifuge tube. Prior to starting the assay, dilute the sample extracts and the reference standard extracts with soya assay buffer according to Table 1.
Protein extracts should be stored at 2°C to 8°C, but not for longer than one working day.

Table 1. Dilution ranges according to the matrix

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>Soybean</td>
<td>1:300</td>
</tr>
<tr>
<td>Soy flour</td>
<td>1:300</td>
</tr>
<tr>
<td>Defatted soy flour</td>
<td>1:300</td>
</tr>
<tr>
<td>Protein isolate</td>
<td>1:10</td>
</tr>
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</table>

Dilution of sample extracts

For the extracted negative control, for each extracted positive reference and for each sample, two 12 X 75-mm test tubes are required to accomplish the dilution.

Pipette 280 µl of the Soya Assay Buffer into one of the two 12 x 75-mm test tubes.

Pipette 380 µl of the Soya Assay Buffer into the other 12 x 75-mm test tube.

Label the 280 µl tube “1:15” and label the 380 µl tube “1:300”.

Pipette 20 µl of each sample extract into the tube labelled “1:15” and vortex.

Transfer 20 µl from the mixed “1:15” tube to the tube labelled “1:300” and vortex.

Repeat steps for the negative control, each positive reference and the sample extracts.

ELISA immunoassay procedure

General

The ELISA assay kit can be run in different formats using any number of the 8-well strips. It is recommended to follow a randomised loading scheme.

All reaction wells should be run in duplicate and the mean absorbance value of each well is calculated. Each run consists of the assay buffer blank, the negative control and the positive reference standards.

When an assay has been started, all steps should be completed without interruption.

Sample addition

Add 100 µl of diluted extracts and the assay blank to the appropriate wells.

Use separate disposable tips for each pipetting step to avoid cross-contamination.

Cover plate with plastic wrap or aluminium foil to prevent contamination and evaporation.
Sample incubation

Incubate microtiter plate at 37°C for 1 h.

Washing

Wash 3 times with 300 µl wash buffer.

**Manual washing.** Empty the wells by inverting over a sink or suitable waste container. Using a 500 ml wash bottle containing working wash solution, fill each well to the top, allow to stand for 60 s, then empty the plate as described above. Repeat the washing step for a total of 3 times. Remove residual liquid and bubbles by tapping upside down on several layers of paper towels.

Prevent the strips from falling out of the frame by securing with adhesive tape.

**Automatic washing.** At the end of the incubation period aspirate the contents of all wells using a Microwell washer, then fill wells with working wash buffer. Repeat the aspiration/fill step for a total of 3 times. Finally, use the Microwell washer to aspirate all wells, and then tap the inverted plate onto a stack of paper towels to remove residual droplets of wash buffer and bubbles.

Do not let well dry out, as it may affect assay performance.

Inadequate washing will cause erroneous results. Whether using manual or automated washer it is important to ascertain that each assay well is washed with identical volumes to all other wells.

Addition of antibody conjugate

Add 100 µl of antibody conjugate working solution to each well.

Cover the plate to prevent contamination and evaporation.

Incubation

Incubate the microtiter plate at 37°C for 1 h.

Washing

At the end of the incubation period, repeat the washing steps as described above.

Substrate addition

Add 100 µl of the colour solution to each well.

Gently mix the plate and incubate for 10 min at ambient temperature.

The addition of chromogenic substrate should be completed without interruption.

Maintain the same sequence and time interval during the pipette step. Protect the microtiter plate from sunlight, otherwise colour intensity is influenced.
Stop solution addition

At the end of the incubation period, add 100 µl of stop solution to each well, pipette the stop solution in the same sequence as the colour reagent was added. The addition of stop solution should be completed without interruption. Protect the microtiter plate from sunlight, otherwise colour intensity is influenced.

Absorbance reading

Using a microplate reader fitted with a convenient filter for reading at 450 nm, measure the absorbance of each assay well. All readings should be completed within 30 min of adding the stop solution. Record the results obtained and calculate the mean absorbance values or use a computer programme.

Evaluation

Standard values should be used to develop a standard curve. The value from the assay blank should be subtracted from all values for samples and reference standards. The average corrected values from each duplicate reference point should be used to create a standard curve. The average data from each duplicate sample should then be used to insert a concentration from this curve.

Run accept/reject criteria

Each run shall meet the accept/reject criteria in the procedure to be valid. The run consists of the following: assay blank, the extraction of each GM positive reference standard, the negative control and each unknown sample. All protein extracts and the assay blank will be run in duplicates. If a run does not meet the assay acceptance criteria, the entire run shall be repeated.

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<th>Criteria</th>
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</thead>
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<td>A450nm &lt; 0,30</td>
</tr>
<tr>
<td>0% GM Standard</td>
<td>A450nm &lt; 0,30</td>
</tr>
<tr>
<td>2.5% Reference</td>
<td>A450nm ≥ 0,8</td>
</tr>
<tr>
<td>All positive standards</td>
<td>CV of duplicates ≤ 15%</td>
</tr>
<tr>
<td>Unknown samples</td>
<td>CV of duplicates ≤ 20%</td>
</tr>
</tbody>
</table>
Flowchart

Flowchart Sample and Standard Reference Extraction

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Volume/Weight</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weigh out</td>
<td>0.5 g</td>
<td>Weigh out samples, assay blank, reference standards</td>
</tr>
<tr>
<td>Addition</td>
<td>4.5 ml</td>
<td>Addition of extraction buffer</td>
</tr>
<tr>
<td>Mixing</td>
<td></td>
<td>Mix the sample with extraction buffer until it becomes homogeneous</td>
</tr>
<tr>
<td>Centrifugation</td>
<td>5000 rpm</td>
<td>Centrifuge the sample at 5000 rpm for 15 min</td>
</tr>
<tr>
<td>Dilution</td>
<td>1:300 or 1:10</td>
<td>according to the material investigated</td>
</tr>
<tr>
<td></td>
<td>according to</td>
<td>Dilute the samples and reference standards</td>
</tr>
<tr>
<td></td>
<td>the material</td>
<td></td>
</tr>
<tr>
<td></td>
<td>investigated</td>
<td></td>
</tr>
</tbody>
</table>

Flowchart ELISA Immunoassay Procedure

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Volume</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Addition</td>
<td>100 µl</td>
<td>Pipette diluted extracts of samples, reference standards and assay buffer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>blank into appropriate assay well</td>
</tr>
<tr>
<td>Incubation</td>
<td></td>
<td>Incubate 1 h at 37°C</td>
</tr>
<tr>
<td>Washing</td>
<td></td>
<td>Wash 3 times with wash buffer</td>
</tr>
<tr>
<td>Addition</td>
<td>100 µl</td>
<td>Dispense antibody conjugate into each assay well</td>
</tr>
<tr>
<td>Incubation</td>
<td></td>
<td>Incubate 1 h at 37°C</td>
</tr>
<tr>
<td>Washing</td>
<td></td>
<td>Wash 3 times with wash buffer</td>
</tr>
<tr>
<td>Addition</td>
<td>100 µl</td>
<td>Dispense colour solution into each well</td>
</tr>
<tr>
<td>Incubation</td>
<td></td>
<td>Incubate for 10 min at ambient temperature</td>
</tr>
<tr>
<td>Addition</td>
<td>100 µl</td>
<td>Dispense stop solution into each assay well</td>
</tr>
<tr>
<td>Absorbance</td>
<td></td>
<td>Measure absorbance value of each assay well in plate reader at 450 nm</td>
</tr>
</tbody>
</table>
References


Additional Reading


The Analysis of Food Samples for the Presence of Genetically Modified Organisms

Appendix

Example of work programme
(Base for a one-week course)

M. Querci


1ST DAY – MONDAY

9:00 am  Introduction to the course, presentation of the organizers and participants

9:30 am  **Theory:** Introduction on the general procedures for GMO detection and course content

**PREPARATION OF SAMPLES: DNA EXTRACTION**

10:00 am  **Experimental:** DNA extraction following the CTAB method (1st part)

10:30 am  Coffee break

11:00 am  **Theory:** Gel electrophoresis for nucleic acids analysis

                                 **Experimental:** Preparation of agarose gels

12:00 pm  **Experimental:** DNA extraction following the CTAB method (2nd part)

1:00 pm  Lunch

2:00 pm  **Experimental:** DNA extraction following the CTAB method (3rd part)

3:15 pm  **Experimental:** Sample loading of agarose gels

4:00 pm  Coffee break

4:20 pm  **Theory:** General consideration on PCR lab set up. Troubleshooting, etc...

5:20 pm  **Experimental:** Interpretation of agarose gels
2ND DAY – TUESDAY

QUALITATIVE PCR

9:00 am  **Theory:** Introduction to the Polymerase Chain Reaction (PCR) and to the use of PCR for the detection of transgenic maize and soybean

9:30 am  **Experimental:** PCR for transgenic MON810 maize and Roundup Ready® soybean
  - Plant specific: detection of the zein and lectin genes

10:15 am  Coffee break

10:40 am  Preparation of agarose gels

11:00 am  **Seminar:** Sampling and validation: basic concepts

12:30 pm  Lunch

2:00 pm  Sample loading of agarose gels

2:30 pm  **Theory:** Characteristics of Roundup Ready® soybean and MON810 maize and introduction to GMO-specific nested PCR

3:15 pm  Interpretation of agarose gels (zein and lectin specific PCR)

4:00 pm  Coffee break

4:15 pm  **Experimental:** PCR for transgenic MON810 maize and Roundup Ready® soybean
  - GMO specific: detection of the 35S promoter and nos terminator.

5:00 pm  **Seminar:** Introduction to European legislation on GMOs
3\textsuperscript{RD} DAY – WEDNESDAY

QUALITATIVE PCR

9:00 am \textbf{Experimental}: Nested PCR for the specific detection of transgenic MON810 maize and Roundup Ready\textsuperscript{®} soybean (1\textsuperscript{st} PCR reaction)

10:00 am Preparation of agarose gels

10:30 am Coffee break

11:00 am \textbf{Seminar}: GMO testing and analytical quality assurance.

12:00 pm Sample loading of agarose gels (35S promoter and \textit{nos} terminator PCR)

12:30 pm Lunch

1:30 pm \textbf{Experimental}: Nested PCR for the specific detection of transgenic MON810 maize and Roundup Ready\textsuperscript{®} soybean (2\textsuperscript{nd} PCR reaction)

2:00 pm Interpretation of agarose gels (35S promoter and \textit{nos} terminator PCR)

3:00 pm \textbf{Experimental}: Preparation of agarose gels

3:30 pm Coffee break

3:45 pm \textbf{Experimental}: Sample loading of agarose gels (nested PCR)

4:00 pm \textbf{Theory}: Introduction to real-time PCR (RT-PCR) for GMO detection and quantification

5:30 pm \textbf{Experimental}: Interpretation of agarose gels (transgenic MON810 maize and Roundup Ready\textsuperscript{®} soybean specific PCR)
4TH DAY – THURSDAY

QUANTITATIVE REAL-TIME PCR (RT-PCR)

9:00 am  **Experimental:** DNA quantification and preparation of samples for real-time PCR (RT-PCR)

9:30 am  **Experimental:** Real-time PCR (RT-PCR) for the specific detection of transgenic Roundup Ready® soybean using
- The LightCycler (Roche) *(Group 1)*
- The ABI PRISM 7700 (Applied Biosystems) *(Group 2)*

Coffee break

1:00 pm  Lunch

2:00 pm  **Experimental:** Real-time PCR (RT-PCR) for the specific detection of Roundup Ready® soybean using
- The LightCycler (Roche) *(Group 2)*
- The ABI PRISM 7700 (Applied Biosystems) *(Group 1)*

3:30 pm  **Theory:** Data analysis: Introduction to some statistical means

4:30 pm  Coffee break

5:00 pm  **Experimental:** Continuation of real-time PCR (RT-PCR) for the specific detection of transgenic Roundup Ready® soybean
5TH DAY – FRIDAY

QUANTITATIVE DETECTION OF ROUNDUP READY® SOYBEAN USING ELISA

9:00 am  **Theory:** Serological approach for the detection of GMOs

9:20 am  **Experimental:** ELISA 1st part

10:00 am  Coffee break

10:30 am  **Seminar:** Serological approach for GMO detection.

11:30 am  **Experimental:** ELISA 2nd part

12:00 pm  Lunch

1:00 pm  **Experimental:** ELISA 3rd part

2:00 pm  Interpretation of results

3:00 pm  **Seminar:** Areas of international negotiations on the safe use of Genetically Modified Organisms

4:00 pm  General discussion and conclusions
Abstract

The Institute for Health and Consumer Protection of the Joint Research Centre of the European Commission and the Food Safety Programme within the European Centre for Environment and Health - Rome Division (ECR) of the World Health Organization have jointly organised a series of training courses on “The Analysis of Food Samples for the Presence of Genetically Modified Organisms”.

The Joint Research Centre gives scientific and technical support to EU policies by collaborating with EC Directorates General and by interacting with European Institutions, Organizations and Industries through networking with Member State laboratories. The overall task of the WHO’s ECR is to provide support in a complete and coordinated way to both decision-makers and to European citizens in the environmental health field. These training courses are part of collaboration between both Institutions to promote food safety related issues in the WHO European Region, within and beyond actual EU borders, taking into special consideration EU Accession Countries, as well as Central and Eastern Countries with transitional economies.

The scope of the training courses is to assist staff of control laboratories to become accustomed with molecular detection 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 courses are intended to teach molecular detection techniques to laboratory personnel with a good level of analytical knowledge, but with no or little expertise in this specific domain.

The Joint Research Centre has been committed to providing training in detection and quantification of GMOs and, besides the training courses, it offers, and has offered in the past, individual training for specific needs. Training in this topic has been frequently requested due to its importance according to the increasing need to comply with the current and developing European legislative framework. Over the years, the Biotechnology and GMOs Unit has developed a profound knowledge of the different aspects related to GMO detection and quantification, and has designed, adapted or validated advanced methods for their detection and quantification.

Knowledge of these techniques has been transferred to collaborating laboratories through publications, collaborative projects, individual training or specific courses. Technical details have also been provided to trainees as oral presentations or brief written outlines. Aware of the need for a permanent source of information, the Biotechnology and GMOs Unit staff developed this manual, which describes some of the techniques used in our laboratory.

The following areas are covered throughout the courses:
- DNA extraction from raw and processed materials
- Screening of foodstuffs for the presence of GMOs by simple Polymerase Chain Reaction and by nested Polymerase Chain Reaction
- Quantification of GMOs in ingredients by real-time Polymerase Chain Reaction
- Quantification of GMOs in ingredients by the Enzyme-Linked ImmunoSorbent Assay

This Manual has been prepared at the Joint Research Centre, Institute for Health and Consumer Protection (IHCP) as background information for course participants and is intended to provide the theoretical and practical information on methodologies and protocols currently used. The subject matter covers a wide variety of techniques for GMOs detection, identification, characterisation, and quantification, and includes theoretical information considered important background information for anyone wishing to enter and work in the field of GMO detection.
Mission of the JRC

The mission of the JRC is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of EU policies. As a service of the European Commission, the JRC functions as a reference centre of science and technology for the Union. Close to the policy-making process, it serves the common interest of the Member States, while being independent of special interests, whether private or national.