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Scientific and technical contribution to the development of an overall health strategy in the area of GMOs

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The present study is intended to contribute to an open debate with a broad range of stakeholders on the potential health impact associated with the consumption of GMOs by providing up-to-date opinions of experts in this field. For this purpose, the Joint Research Centre has collaborated with international experts and, particularly for Annex I and II, it has closely worked with the European Food Safety Authority. The JRC is grateful for the high quality of the input provided by these colleagues.

However, the documents provided here do not necessarily represent the agreed views of the collaborating experts nor do they necessarily represent the official position of the European Commission or the European Food Safety Authority on this matter.

Acknowledgements

For the study reported in the present document the Joint Research Centre has collaborated with international experts and has closely worked with the European Food Safety Authority (EFSA).

The authors are particularly grateful to Harry Kuiper and Esther Kok from RIKILT - Institute of Food Safety and to Claudia Paoletti, Reinhilde Schoonjans, Karine Lheureux and Herman Koëter from EFSA for their important contribution.

The authors also wish to thank all participants to the '*International workshop on overall approaches to assess and monitor potential short, medium and long term effects in relation to consumption of Genetically Modified Organisms (GMOs) and products derived thereof*' organised on 26-27 November 2007 (see list).

Finally, special recognition goes to Nicoletta Foti and Alessia Bogni for the excellent technical support in the establishment of the "Real-time PCR based ready-to-use multi-target analytical system".

List of participants to the "*International workshop on overall approaches to assess and monitor potential short, medium and long term effects in relation to consumption of Genetically Modified Organisms (GMOs) and products derived thereof*".

Joint Research Centre, Ispra (Va), 26-27 November 2007

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1 Presentation of the project and report outline

Addressing the potential health impact of food derived from genetically modified plant and animal materials forms the basis of a coherent consumer protection policy. Dealing with the possible (positive and/or negative) impact of genetically modified (GM) food on human and animal health is the subject of intensive research and is closely surveyed by the scientific community and the regulatory bodies yet, as in many food related fields, associated issues are complex and often fraught with uncertainties and misconceptions.

The present study is intended to assess the current state of expertise in this field and to define possible areas of improvement. It aims at offering substantial material for further discussion with a broad range of stakeholders.

The report is the outcome of the project '*Scientific and technical contribution to the development of an overall health strategy in the area of GMOs*' carried out by the Biotechnology & GMOs Unit of the Institute for Health and Consumer Protection (Joint Research Centre, JRC), in the frame of study contract CT 30249, requested by the European Parliament Committee on Industry, Research and Energy.

As from the project proposal presented to and approved by the European Parliament at the beginning of 2007, the study intended to focus in the first instance on the assessment of short, medium and long term impact of the health effects in relation to consumption of genetically modified organisms (GMOs). This part of the study consists of a background document emphasising the state of current knowledge, the areas of possible further improvement and the possible ways in which new scientific tools may be applied to complement the ongoing safety evaluation work. In addition it contains an expert opinion in the format of a report of an international workshop with experts on assessment and monitoring of health effects of GMOs (workshop held in Ispra 26-27 November 2007).

In addition, since the first step in addressing health impact is the estimation of exposure to (the various sources of) GMOs, and the first step in risk management is providing assurance that no unauthorised GMOs enter the market, the study involved scientific research that led to the development of a unique tool to detect and identify any of the approved and non-approved GMOs known to the JRC, and in particular its Community Reference Laboratory for GM Food and Feed (CRL-GMFF).

The present document includes the items identified as key findings of the activities above. The full documents are given in Annexes I-III.

It is important to note that the analyses and discussions which have led to the present report have concentrated on the current approaches to assess the potential health effects of GM food and feed products and not on the nature of those effects themselves. In such context, there has been agreement to follow the classical risk assessment work distinction in pre-market assessment and post-market monitoring phases.

The work undertaken by the JRC has been firmly set in the current regulatory context at the EU level notably the 2003 regulations on genetically modified food and feed and on traceability and labelling [Regulation (EC) No 1829/2003 and Regulation (EC) No 1830/2003].

The overall results of this study show that:

- There is a comprehensive body of knowledge that already adequately addresses current food safety issues including those dealing with GM products; it is considered by the experts as sufficient to assess the safety of present GM products.
- Developments in biotechnology will require even more sizeable efforts to maintain an adequate capacity to deal with novel products.
- Such R&D efforts need to be firmly inscribed in an international context.
- It is essential that a forum is created where stakeholders meet regularly to share expertise, to identify areas of improvement, to forecast upcoming developments and to anticipate needs for scientific and technical efforts.

2 Overall approaches to assess potential short, medium and long term effects in relation to consumption of GMOs and products derived thereof[1]

The first part deals with the call of the European Parliament pointing for 'the need for the JRC to coordinate the research with an overall health strategy' and to 'the need to study possible health threats coming from genetically modified organisms such as maize MON863'.

In close collaboration with the European Food Safety Authority (EFSA), and with its scientific panel on GMOs, the JRC has:

- Prepared a background document on this issue entitled '*Discussion paper on overall approaches to assess potential short, medium and long term effects in relation to consumption of Genetically Modified Organisms (GMOs) and products derived thereof*'.
- Organised an '*International workshop on overall approaches to assess and monitor potential short, medium and long term effects in relation to consumption of Genetically Modified Organisms (GMOs) and products derived thereof*' to discuss the definition and possible evolution of tools to assess the potential short, medium and long term health issues in relation to GMOs and to address the possible ways ahead to collect further information in this rapidly evolving sector.
- Prepared a document collating the views of the experts as expressed during the workshop.

In addition, the JRC developed a real-time PCR based ready-to-use multi-target analytical system for the detection of EU authorised and unauthorised GM events.

1 **Timeline:**
On 5 March a first meeting was held in Parma with EFSA in order to agree on how to proceed. The background paper has been completed and distributed to invited experts by 30 October 2007 and the International Workshop took place in Ispra on 26-27 November.

The '*Discussion paper on overall approaches to assess potential short, medium and long term effects in relation to consumption of Genetically Modified Organisms (GMOs) and products derived thereof*' provides a review of current approaches to address the area of GMO health impact in the pre- and post-market assessment context. This paper has been produced by the Joint Research Centre, in collaboration with RIKILT - Institute of Food Safety (NL) and with input from the European Food Safety Authority.

The sole aim of this document is to consider a number of scientific issues related to human health and consumption of genetically modified organisms that should serve as a basis for discussions among stakeholders and not to present a complete review of all pertinent information available. It should not be regarded as an official position of the European Commission or the Joint Research Centre or the European Food Safety Authority.

Its key messages are:

1. No demonstration of any health effect of GM food products submitted to the regulatory process has been reported so far, yet, little is known about the potential long term health effects of any food, including novel food.
2. The safety of a GMO derived product is established relative to its conventional counterpart and is, therefore, not absolute. Conventional food is often evaluated on the base of its history of safe use.
3. The assessment of potential toxicity commonly includes the search for similarities between the primary structure of the protein(s) introduced by genetic modification into the host organism and the structures of known toxic proteins using bio-informatics methods. In addition, the susceptibility of the newly introduced protein to conditions of food and feed processing, as well as digestion, can provide an indication of the likelihood that the consumer will be exposed to the intact protein.
4. Repeated-dose feeding of new proteins in a subchronic experiment (e.g. for 28 days), are recommended. However, in a number

of dossiers that have already been notified for regulatory approval in the EU subchronic 90-day whole-product feeding studies in rodents (rats) have been provided. Such studies should not be done on a routine basis, but only if there are indications to do so, such as substantial differences observed in the compositional analysis between the GM and its non-GM comparator.

5. With respect to allergenicity a weight of evidence approach is recommended combining the outcome of various assessment methods. Various studies published in scientific literature focus on the possible allergenic effects of the market-approved GM crops. Sera binding or skin reactions have not been observed for GM crops that have been allowed onto the European market.

6. Genes of bacterial origin in GM plants may theoretically be capable of being taken up by bacteria in the food chain. Horizontal gene transfer risks have been raised with respect to antibiotic resistance genes which may devolve to pathogenic micro-organisms thereby impairing antibiotic therapy. However, the chances of acquiring the same gene(s) from other bacterial species in the environment rather than from GMOs are considered much greater.

7. Two points are of paramount importance to consider possible consequences for human and animal safety in the rare cases of uptake of DNA from food by mammalian cells. First, DNA sequences of various origins (plant, animal, microbia, and virus) are always present in human food and farm animal feed. Therefore, most sequences to be found in GM crop plants will have entered the mammalian gut before present time. Second, it is clear that uptake is very much more probable for somatic cells (particularly those of the gut and immune systems) than for germ line cells. This may account for the almost complete lack of evidence for sequences of plant origin in mammalian genomes. Somatic cells of the gut lining have a rapid turnover, such that the most likely fate of most modified cells is to be lost in the faeces. These considerations make deleterious consequences improbable.

8. Unintended effects are those not directly linked to the targeted genetic modifications (disruption in the natural function of genes); this may also occur in conventional crop breeding.

9. Changes in the nutrient composition of GM product may impact on human and animal nutrition; in such case *in vivo* feeding trials may be decided depending upon the knowledge available on those nutrients.

10. GM crops which are metabolically engineered to produce nutrients (or other products) of interest are likely to be prone to unintended effects besides the modification of interest. In such case, advanced 'omics' technologies can be used to identify the substance(s) linked to the transformation. Comparison with a conventional counterpart is used, taking into account natural background variations. Generally, it is considered that the routine application of these techniques in regulatory risk assessment requires additional harmonisation and validation, as well as development of databases for the data on background variation.

11. Precaution is the reason for the comprehensive pre-market safety assessment and follow-up by post-market monitoring currently applied to GMOs, in order to reduce the uncertainty regarding any potential health effects of GM technology to a minimum. Current experience with long term testing of GMO carried out in the formal regulatory approval context, point with an appropriate degree of certainty to the absence of potential health effects. The data evaluated for submitted GM dossiers do not indicate any harm caused by these GMOs.

12. Most of the multigenerational feeding studies performed with laboratory rodents show no significant effect on testicular spermatocytes (GM soya beans), on fertility (GM potatoes), cell ultrastructure (GM soya beans) and only diet-related changes with GM canola. No uptake of transgenic DNA from gastrointestinal tract has been observed. Human experiments with GM tilapia fish showed no differences in cytological and biochemical blood composition.

To examine a number of statements cited in the background document, a workshop entitled '*Overall approaches to assess and monitor potential short, medium and long term effects in relation to consumption of Genetically Modified Organisms*

(GMOs) and products derived thereof' (JRC, Ispra, 26-27 November 2007) was attended by 22 experts in different disciplines relevant to pre-assessment and post-market evaluation of GMOs and GM derived food and feeds.

The discussions held during the workshop were organised along the following headings:

- general observations
- pre-market assessment
- post-market monitoring
- future developments and research needs.

The full workshop report presented in Annex II has been reviewed and approved by the participants

Main findings as regards the pre-authorisation/ pre-market phase

1. GMOs pre- and post-market assessment activities need to be ahead of the technology and take proactive instead of reactive approaches. Regular stakeholder *fora* should be organised to increase confidence in and effectiveness of the whole risk assessment and management process.

2. Presently, the comparative approach is internationally recognised as the appropriate principle for GMOs safety assessment and guidelines have been established by EFSA, WHO, FAO and OECD. The comparative safety assessment (CSA) is based on the comparison of a GMO with an appropriate conventional counterpart (the comparator) with a history of safe use; the exercise is carried out on a case by case basis. This approach is necessary because there is a large natural variation within a plant species in terms of genetic background and environmental conditions introducing a variable comparator baseline. The weight of evidence based on the currently used toxicological, nutritional, molecular, and allergological data requirements constitutes a robust frame for the prediction of potential health effects.

3. Safety assessment of novel food can be more demanding than safety assessment of GMOs as in some cases a comparator with a history of safe use is not available.

4. The need for testing for allergenicity – a key concern in food safety – is common to GM and non-GM food products. Suitable models to address allergenicity in food in general are still missing.

5. Much more than in the case of Genetically Modified Plants, Genetically Modified Micro-organisms (GMM) raise the concern for gene transfer in the digestive tract. Codex guidelines and EFSA guidance document deal with safety evaluation procedures in such case.

6. Possible unintended effects cannot be known or defined a priori, but are subject of hazard identification and risk assessment. The probability of occurrence of unintended effects is part of the uncertainty analysis in the risk procedure per se, in which the aim is to reach the highest possible degree of scientific certainty.

7. From a scientific point of view, assessing the effect of GM food and derived products is not different than assessing safety issues associated with other types of food such as novel food and functional food types (such as those producing specific molecules for non-food purpose). Safety approaches should be coherent across the spectrum following the principle that products with similar degrees of risks should face similar degrees of scrutiny.

8. Since zero risk is inexistent (or at least science can never prove it), comparative risks analysis must be conducted and risk mitigation measures may be recommended in some cases.

9. Health effects can be positive or negative. However, benefit analysis is generally not part of the current assessment but it may become gradually more addressed.

10. More complex GM products will have to be dealt with in future safety assessment; they are those which can introduce unforeseen metabolic perturbations. These cases will require new tools to identify and characterise unforeseen effects besides the intended ones. To increase confidence in pre-market assessment, it is essential for the scientific community involved in such work to be aware of new types of GMOs under development (plants, micro-organisms and animals).

Main findings as regards the post-market monitoring phase

1. GMO identification methods are essential for traceability of GM food and feed throughout the food chain. Identification methods are available for all EU approved GMOs but more work needs to be done, for instance on the development of more robust sampling schemes. It is also noted, that it is not achievable to distinguish stacked events (i.e. the combination of two or more single events in one crop) from mixtures of crops in derived products containing the corresponding single events with the current analytical means. This may effect GM quantification.

2. Monitoring is regulated for GM derived food and feed products, as well as for cultivated GMOs, but may also be necessary in cases of contained use (e.g. non-food applications, GM micro-organisms) as an additional measure of impact assessment.

3. Case specific monitoring can address uncertainty about exposure to a specific genetic modification or potential effects thereof; in the European Union, there have been no reasons so far to undertake such action.

4. However, while no monitoring of GM products for health effects has been necessary in the European Union, EFSA is of the opinion that for the case of GM functional food (e.g. with specific health claim) a monitoring programme should be put into place.

5. As in the pre-market phase, monitoring needs of GM food and feed products would be similar to those of non-GM products; this is particularly the case for food with specific health claims.

6. Addressing the identification of unapproved GMOs must be further developed and international cooperation is essential to progress in this context.

7. Attention must be given to false negative results leading to the assumed absence of GM products when they are present in reality and various recommendations regarding certification are made in this respect.

8. Exposure assessment is central in monitoring; it requires extensive EU consumption data which are not generally available. The identity of GM products should be unambiguously traceable to facilitate the identification of target groups. This also facilitates withdrawal if necessary.

Main findings as regards future developments and research

1. There is a strong need for keeping up with scientific advances in biotechnology to address potential health effects through extensive and regular exchange between stakeholders.

2. The complex traits of future GM plants now need to be addressed when assessing health effects; the use of a wider range of organisms to introduce new traits via gene technology will also present new identification and safety assessment challenges.

3. Additional and fundamental research on new GM events or variety traits should be conducted by independent publicly funded research institutions. Insights into possible unintended changes caused by the new modifications are to be obtained. Developers, risk assessors and risk managers have to stay alert for the various scenarios which may require supplementary data in addition to the commonly employed safety tests during pre-market or post-market assessments.

4. Profiling technologies supported by bioinformatics tools and databases containing profiles of products generated under different environmental conditions and agricultural practices need to be further developed.

5. Expert groups like the one in this study should be reconvened with more stakeholders.

3 Development of a real-time PCR based ready-to-use multi-target analytical system for the detection of EU authorised and unauthorised GM events[3]

Whereas many tools are available to perform analytical tests, the conditions of post-marketing monitoring are very complex. Such monitoring is however necessary for (1) assessing the potential long term environmental impacts of GMOs, as compared with conventional crops, and (2) for monitoring of possible health effects of genetically modified food and feed as compared with conventional food and feed. Such actions are required in the context of the Commission's programme on Life Sciences and Biotechnology – A strategy for Europe [COM (2002) 27) as well as in the Commission's communication on the mid-term review of the strategy on Life Sciences and Biotechnology [COM (2007) 175].

In addition, the European market is not a closed system and GMOs and derived products from non-EU countries may enter the European Union. It can not be excluded that also unauthorised GMOs, such as in the recent cases of Bt10 maize, LL RICE 601 and Rice63, or unknown GMOs may enter on the European market.

All these elements converge on the need of high-throughput systems for the rapid and cheap screening of numerous samples allowing monitoring and tracing of GMOs, requirements to support the assessment of exposure to GMOs throughout the agricultural food and feed chain. Through its own research and chairmanship of the European Network of GMO Laboratories (ENGL), the JRC deploys, tests and implements high-throughput detection systems for the detection of GMOs (e.g. in the format of microchips) and reports here on the design, evaluation, testing and implementation of test strategies that are the basis of a decision-making process to detect GMOs and to distinguish between approved and non-approved GMOs.

Adequate monitoring and risk management rely on technologies that are able to detect the vast majority of approved GMOs as a first step in exposure assessment and of non-approved GMOs as an essential requirement to safeguard human and animal health (and of the environment, an area which is out of the context of the current study). Therefore this study was complemented with the development of an analytical high-throughput system, which is a parallel screening tool for the unequivocal simultaneous identification and evaluation of the presence of all current EU approved and of all unapproved GMOs known to the Community Reference Laboratory for GM Food and Feed (CRL-GMFF) that may be present in food and feed products.

'*Development of a real-time PCR based ready-to-use multi-target analytical system for the detection of EU authorised and unauthorised GM events*', describes thus the development, testing and production of such a system. Further it illustrates the rationale and comparative advantage of the strategy selected as well as the formulation, potentiality and flexibility of the developed analytical system; i.e. on a regular basis a new, adapted system that responds to the introduction of GMOs in the market can be readily developed. However, before applicable for regulatory purposes, extensive testing is still required. Therefore this tool can only be used for indicative purposes during its first stage. Experimental design and testing results are reported in detail, together with the timeline followed for the realisation of the project, the milestones and deliverables.

The approach taken allows the event-specific simultaneous detection of 39 single-insert GMOs, comprising all EU approved and all unapproved GM events for which a method was submitted to the CRL-GMFF and stacked events derived from them. System performance (specificity, efficiency etc) has been successfully confirmed by experimental testing (validation) conducted within the CRL-GMFF. The project has been already presented to members of the ENGL. The 'real-time PCR based ready-to-use multi-target analytical system' developed by the JRC, is the first analytical tool developed worldwide allowing the detection of so many GM events simultaneously using event-specific targets, and it is currently tested in a large variety of EU laboratories. Moreover, the use of such a tool by laboratories within the EU may guarantee a high level of harmonisation.

3 This work has been carried out throughout the year 2007 in the JRC laboratories. The final product has been presented and discussed at the 9th Plenary Meeting of ENGL where members accepted this system with interest and asked for the transferability to their laboratories provided they would report back on its effectiveness in controlling the food and feed market.

Annex I
Background paper on overall approaches to assess potential short, medium and long term effects in relation to consumption of Genetically Modified Organisms (GMOs) and products derived thereof

Disclaimer

This background paper has been produced by the Joint Research Centre, in collaboration with RIKILT - Institute of Food Safety (NL) and with input from the European Food Safety Authority.

The sole aim of this document is to consider a number of scientific issues related to human health and consumption of genetically modified organisms that should serve as a basis for discussions among stakeholders. It should not be regarded as an official position of the European Commission or the Joint Research Centre or the European Food Safety Authority.

Abbreviations

Bt, *Bacillus thuringiensis*; DNA, deoxyribonucleic acid; EC, European Commission; EFSA, European Food Safety Authority; ENGL, European Network of GMO Laboratories; EU, European Union; FAO, Food and Agriculture Organisation; FSA, Food Standards Agency; GLA, γ-linolenic acid; GM, genetically modified; GMO, genetically modified organism; JRC, Joint Research Centre; LC, liquid chromatography; MS, mass spectrometry; OECD, Organisation for Economic Cooperation and Development; UK, United Kingdom; USA, United States of America; WHO, World Health Organisation.

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1 Introduction

This document has been prepared by the Joint Research Centre (JRC) of the European Commission, following its mandate to provide an answer to queries from the European Parliament. These queries pertain to the potential health effects of genetically modified organisms (GMOs) and ways to detect these effects, including the use of advanced analytical methodologies. Emphasis is given to the data generated in the pre-marketing and post-marketing phases, in particular for the pre-marketing risk assessment and/or post-marketing monitoring of non-intended health effects.

A preliminary version of this document has served as background information for a workshop on the possible short, medium and long term health effects of genetically modified organisms (GMOs) organised by the JRC, in November 2007. The workshop addressed various questions on the nature, quality, and interpretation of the above-mentioned data generated during the pre-marketing and post-marketing phases. In particular, it was considered whether the data generated are sufficient to carry out an effective risk assessment and subsequent risk management, including post-market monitoring, if applicable.

1.1 Genetically modified food and feed

GMOs, as defined by European legislation (Directive 2001/18/EC), particularly include organisms that have undergone introduction of foreign DNA with the aid of recombinant DNA techniques. These techniques have enabled the transfer of genetic information between organisms that are not amenable to such transfer by natural means. The techniques of genetic modification therefore expand the tools available for genetic improvement of crops, animals, and micro-organisms used for production of food, medicines, and other non-food products, as well as for other purposes of utility to man.

Since the first large-scale introduction of cultivation of genetically modified (GM) crops, the area planted with these crops has continuously increased, up to 102 million hectares in 2006. In addition, most of the currently commercialised GM crops are of high economic value, including field crops such as soybean, maize, cotton, and oilseed rape. Most of these crops are cultivated outside the European Union (EU), particularly the USA, Argentina, Brazil, Canada, China, India. Only one GM maize has also been cultivated to some extent in Europe.

The major characteristics that have been introduced into GM crops are herbicide resistance and insect resistance. Herbicide resistance allows crops to survive the application of particular herbicides, thereby facilitating management of weeds growing among the crop plants. The most widely grown GM crop currently is GM herbicide-resistant soybean, which has been rendered resistant against herbicide formulations containing glyphosate, which is a 'broad-spectrum' herbicide that kills several weeds. Insect resistance renders the crop resistant against particular pest insects. Many insect-resistant GM crops have been modified with insecticidal proteins that naturally occur in the soil bacterium *Bacillus thuringiensis* (Bt), which is also used as a natural pesticide in organic agriculture. These proteins are toxic for specific insect species, such as a Bt protein toxic to European corn borer.

Besides the characteristics of agricultural importance that have been introduced into the main share of currently cultivated GM crops, crops that are currently in development and that may enter the market in the future, also contain characteristics of potential importance to consumers. A well-known example of such a crop is 'Golden Rice', which has been modified with various enzymes enabling the biosynthesis of provitamin A (β-carotene) in its kernels. The purpose of this modification is to combat malnutrition in various parts of the world where rice consumption is high and physiological levels of vitamin A are low. In addition, the modification comprises the introduction of a pathway that is not naturally active within rice kernels, and therefore is more complex than the modifications introduced into the currently commercialised GM crops. Other examples include GM crops such as soybean with modified oil composition and maize with increased levels of the essential amino acid lysine that have recently been notified for market approval as GM food and feed under Regulation (EC) No 1829/2003.

1.2 Pre-market safety assessment

Before GM crops are allowed onto the EU market, they have to be granted regulatory approval, for which they also have to pass regulatory assessment of their safety. The safety assessment is done through a centralised procedure under the auspices of the GM food and feed regulation [Regulation (EC) No 1829/2003]. In accordance with the provisions laid down in this Regulation, the European Food Safety Authority (EFSA)

conducts a comprehensive safety assessment. Based on this assessment, EFSA publishes an opinion to inform the European Commission, the EU Member States, and the public about the safety of the GM product. EFSA's advice is based on opinions issued by its GMO Panel, which consists of various experts from the EU Member States that have been elected as members based on their expertise, experience, and independence. In addition, during the regulatory safety assessment procedure, EU Member State authorities can provide comments to the dossier contents for the attention of the GMO Panel. Below, it will be discussed in further detail which data are needed to address the various potential health effects that are commonly considered during the safety assessment of GMOs.

Besides the scientific assessment of safety of GMOs, applicants submitting applications for market approval of their GM products, also have to provide a specific detection method for the pertinent GMO. This detection method usually consists of polymerase-chain-reaction-based DNA detection, which can detect DNA that is specific for the particular GMO. This method plus control samples have to be provided to the Biotechnology & GMOs Unit at the JRC, which exercises the role of Community Reference Laboratory for GM Food and Feed, as mandated in the context of Regulation (EC) No 1829/2003 (http://gmo-crl.jrc.ec.europa.eu/). The CRL-GMFF will validate the method and determine if it is suitable for regulatory purposes. This validation process includes the execution of a ring trial, which is done in collaboration with various laboratories. These laboratories are all members of the European Network of GMO Laboratories (ENGL), which is coordinated by JRC. The JRC provides a final validation report to EFSA which, together with the opinion and other particularities, forms the overall opinion according to Regulation (EC) No 1829/2003 (http://www.efsa.europa.eu).

The overall opinion will serve as input for the decision (authorisation or rejection) to be drafted by the European Commission on the market introduction of the particular GMO. This draft will be submitted to a regulatory committee, i.e. the Standing Committee on the Food Chain and Animal Health. Depending on the outcome of the Standing Committee's discussions, it may also be forwarded to the Council of Ministers, before being returned to the European Commission for the final disposition.

The pre-market assessment of the safety of the GMO thus has an important role in the EU approval procedure for market authorisation. European legislation is put in place so that the outcomes of the assessment are used as the scientific basis for the decisions by EU institutions on the management of risks following the market approval of GMOs; no GMO can be put on the market unless it receives a positive EFSA opinion. This document therefore highlights various issues surrounding potential health effects of GMOs, including (a) the current practice of pre-market assessment of potential health effects, (b) post-market monitoring for health effects, (c) scientific background of the assessment methods, and (d) potential needs for further research.

After the GM product has been introduced onto the market, there are several regulatory principles put in place to follow-up the products released, i.e. the post-market phase.

First, each GM product can be released on the market only for 10 years. After this period, the applicant must submit again a dossier for renewal of the authorisation. This dossier must contain updated scientific information and the knowledge gained from experience with the concerned GM product on the EU market. Based on this dossier, EFSA will perform its risk assessment which will be used by the EC as the scientific basis for its decision on the renewal of the market authorisation of the concerned GMO product.

Second, post-market monitoring and/or other risk management measurements of GM products can be taken if deemed necessary from a safety or economical perspective. These aspects will be further discussed in this document.

2 Current approach towards risk assessment of GMOs

Years before the first large-scale introduction of GMOs onto the market, national and international organisations discussed what approach for safety assessment of such products should be undertaken. This involved international organisations such as Food and Agricultural Organisation (FAO), World Health Organisation (WHO), Organisation for Economic Cooperation and Development (OECD), and the International Life Sciences Institute (ILSI) [Kuiper *et al.*, 2001]. For foods derived from genetically modified organisms, this resulted in the publication of the internationally harmonised guidelines for the safety assessment of foods derived from GM plants and micro-organisms by Codex Alimentarius in 2003. Codex Alimentarius is an international committee resulting from a joint collaboration of FAO and WHO, which develops protocols and standards for foods. Codex documents have to be implemented by each of its member states into national legislation because they serve as point of reference for international trade rules. At Codex meetings, the joint EU member states are represented by the European Commission with scientific support from EFSA staff and experts. The EFSA GMO Panel has published a detailed guidance document for applicants who wish to make a marketing application for GM products. This guidance extends the Codex guidelines and provides directions on the information needed to be included within the safety dossier on the pertinent GMO [EFSA, 2006a].

The internationally harmonised approach promulgated by Codex and accepted by other relevant institutions including EFSA, is that of the comparative safety assessment [Kok and Kuiper, 2003]. This approach focuses on the differences found in the comparison of a GM product with a conventional counterpart with a history of safe use. This comparison commonly includes an analysis of the molecular characteristics, the phenotypic/agronomic characteristics, and many compositional parameters in both the GMO and its counterpart. For example, in a Bt-protein-expressing GM maize, the difference with conventional maize likely includes the presence of this new protein. Other differences found may be intended or unintended. For any differences thus found, it then has to be decided if further testing for their safety is needed. In case of a new protein, this may entail the testing of its toxicity and allergenicity, for example, of which the data requirements and data collection will be further detailed below. The comparison of a GMO with its counterpart thus serves as a starting point in the safety assessment.

Following the comparative safety assessment approach, the safety of a GMO is established relative to a conventional counterpart, which implicitly presumes the safety of the latter. This is based on the fact that whilst conventional foods usually have not been tested for safety, their history of safe use indicates that a positive balance has been found between the potentially negative and positive effects of the many substances present within these foods. For example, based on experiences with breeding certain food crops, such as potato and canola (oilseed rape for human and animal consumption), threshold levels are applied for intrinsic compounds known to have adverse effects, i.e. glycoalkaloids in potato and erucic acid and glucosinolates in canola.

Items commonly addressed during the safety assessment of GMOs and GM foods and feed in particular, have been reviewed in more detail previously [Kuiper *et al.*, 2001]. For more details the guidance document published by EFSA can serve as an extensive resource [EFSA, 2006a]. An overview of these items is given in the following sections.

2.1 Molecular characteristics

The molecular characterisation of a GMO includes an analysis of the identity, organisation, location(s), and genetic stability of the DNA introduced into the host organism. In addition, the expression, function, mode of inheritance of the DNA, and the characteristics and levels of any gene product (e.g. protein) are analysed.

2.2 Comparative analysis of phenotypic, agronomic, and compositional characteristics

The comparative analysis entails a comparison of phenotypic and agronomic characteristics, including ultra-structural and physiological parameters (e.g. crop appearance, development, yield, and disease susceptibility), and an extensive range of compositional parameters, including macronutrients, micronutrients, antinutrients, toxins, and secondary metabolites. Because food organisms can differ widely from each other, the exact parameters to be measured will vary as well. The OECD Task Force on the Safety of Novel Foods and Feed has published consensus documents with recommendations for key compositional parameters to be analysed in this comparison in a range of primary crops, including soybean, canola, wheat, maize, barley, rice, potato, tomato, cotton, and forage legumes [OECD, 2007].

2.3 Potential toxicity

Genetic modification introduces proteins encoded by the transgenes into the host. These proteins may not be considered truly 'new' proteins from the perspective that they already occur in other naturally occurring organisms, such as the soil bacteria *Agrobacterium* and *Bacillus thuringiensis*. The assessment of potential toxicity commonly includes the search for similarities between the primary structure of the protein(s) introduced by genetic modification into the host organism and the structures of known toxic proteins using bio-informatics methods. In addition, the susceptibility of the newly introduced protein to conditions of food and feed processing, as well as digestion, can provide an indication of the likelihood that the consumer will be exposed to the intact protein. A common proxy for digestibility is an *in vitro* simulation model involving incubation of the newly introduced protein with simulated gastric fluid. The intactness of the new protein during incubation is subsequently measured. Additional testing may involve the dosing of the newly introduced protein to laboratory animals *in vivo* following established protocols for animal testing. The EFSA guidance document recommends the repeated-dose feeding of new proteins in a subchronic experiment (e.g. for 28 days), amongst others.

In addition, in a number of dossiers that have already been notified for regulatory approval in the EU and assessed by the EFSA GMO Panel, subchronic 90-day whole-product feeding studies in rodents (rats) have been provided. EFSA guidance does not recommend that these studies are done on a routine basis, but only if there are indications to do so, such as substantial differences observed in the compositional analysis between the GM and its non-GM comparator. The scientific basis on which this approach has been based is explained in further detail by the EFSA guidance document and by the recently adopted report on the role of animal feeding trials in the assessment of the safety and nutrition of food and feed derived from GM plants [EFSA, 2007]. This approach is also endorsed by the majority of national risk assessors convened during the special GMO meeting of the EFSA Advisory Forum in November 2007[1]. Whole-product testing has its limitations based on the fact that foods and feeds are complex mixtures. Unlike pure chemical compounds that can be added to animal diets, for example, whole foods cannot be tested in a wide dose range because of nutritional balance, palatability, and bulkiness, among other things. Interestingly, the authors of various rat feeding studies carried out with GM rice in the frame of the EU-funded SAFOTEST project recommend refinements of the 90-day protocol for testing GM products. These refinements include a consideration of the outcomes of the repeated-dose feeding studies with the purified protein, for example to establish safe doses in the 90-day study with the whole product. In addition, these authors recommended including an additional non-GM diet that has been spiked with the transgenic protein, so that effects due to this protein and other components of the GM diet can be distinguished. Besides the assays required by the internationally harmonised OECD protocol for testing chemicals in animals, these authors have also carried out additional assays, such as the profile of the intestinal microflora [Poulsen *et al.*, 2007]. Another general development that may further enhance the predictive capability of this kind of animal trial is 'toxicogenomics,' i.e. the measurement of changes in expression of toxicologically relevant genes in animals before clinical effects manifest themselves.

Any relevant effect noted in the 90-day feeding study may trigger further testing, such as chronic feeding studies [EFSA, 2006a; EFSA, 2007]. Conversely, if no effects are observed, it will be doubtful if longer-term testing will be able to detect any effects. Furthermore, longer-term testing may also conceal shorter-term effects due to adaptation of the animals.

2.4 Potential allergenicity

Allergenicity is the capacity to be an 'allergen,' i.e. a substance that is able to elicit allergy, which is a hypersensitive immune reaction. Various types of allergy exist, including respiratory, contact, and food allergies. All known food allergens are proteins and therefore the question of whether a newly expressed protein could become a potential food allergen is considered during the premarket assessment of GMOs. Codex recommends a 'weight of evidence' approach, combining the outcomes of various methods used for assessment of potential allergenicity, which is also reflected within the recommendations made by the EFSA guidance document [Codex Alimentarius, 2003; EFSA, 2006a].

1 See http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178656904823.htm

For example, the source organism of the gene for the newly expressed protein is considered, i.e. is the source an allergen in its own right. In addition, bioinformatics and digestibility analyses similar to those for toxicity are commonly carried out. The bioinformatics test outcomes can provide insight into similarities between the newly expressed protein and allergens, including the presence of antibody-binding sites in the new protein that may be recognised by sera against a known allergen.

The *in vitro* digestibility of the newly expressed protein additionally provides an indication of the likelihood that the newly expressed protein may survive digestion and reach the mucosa of the gastrointestinal tract, where it may prime the immune system for allergic reactions towards the protein during subsequent exposure. In case of positive findings, sera binding tests are carried out with sera from patients known to suffer from allergy towards the pertinent allergen with which the newly expressed protein may cross-react.

Therefore, these tests primarily focus on the potential cross-reactivity of a newly expressed protein with existing allergens and to some degree also on its potential to 'sensitise' itself, i.e. to prime the immune system to respond with allergic reactions to itself.

In summary, the 'weight of evidence' approach combines the outcomes of various tests, including a consideration of the history of allergenicity of the source of the transgene and of the host organism; *in silico* bioinformatics comparisons of the transgenic protein with known allergens; and the *in vitro* digestibility of the transgenic protein in simulated gastric fluid. In case of a positive outcome indicating potential cross-reactivity with known allergens, sera-binding tests with sera from patients allergic to the specific allergens are recommended.

Various examples exist of indications from these experiments that have triggered further testing. For example, an experimental GM soybean containing a transgenic methionine-rich protein from Brazil nut has previously been developed with the aim of imparting improved nutritional value to these soybeans. Because Brazil nut is an allergen, this soybean has been tested for potential cross-reactivity in patients allergic to this nut. It has thus been observed that the GM soybean, in contrast to non-GM soybean, indeed shows cross-reactivity with Brazil nut [Nordlee *et al.*, 1996]. Therefore the responsible company has halted its further development and this GM soybean has not been commercialised.

Another example is the Starlink™ maize, which contains the transgenic Cry9C protein. This protein has been rendered more stable towards digestion due to an amino acid mutation in its primary structure. A scientific advisory panel of the American Environmental Protection Agency has therefore considered that it has a medium likelihood of becoming an allergen. Following accidental commingling with food while it was still being allowed to be marketed as a feed in the USA, the company has initiated a major recall action and has withdrawn this from the American market [reviewed by Bucchini and Goldman, 2003].

With regard to measuring allergies post-market in a population, the French allergovigilance network has recently carried out a baseline measurement for future monitoring for respiratory allergies towards GM insect-resistant maize. This maize is currently being grown in some parts of France [Moneret-Vautrin, 2006]. Also for this kind of research, it will be important to be able to establish the GM nature of maize plants implicated in reported cases of allergy.

As noted above, EFSA's recommendations for the EU pre-market assessment of potential allergenicity of GMOs is in line with the international Codex Alimentarius guidelines.

2.5 Horizontal gene transfer

Various mechanisms exist for the 'horizontal' exchange of genetic material between non-related organisms. This is particularly the case for micro-organisms, where such transfer can take place through events such as plasmid transfer during conjugation and transfection by bacteriophages. Another mechanism, which is also possible for DNA transfer between organisms other than micro-organisms, is transformation with free DNA. This entails the uptake of DNA and subsequently its incorporation into the host genome or as a self-replicating element, as well as its stable maintenance. The latter, which is the most relevant scenario for horizontal gene transfer from GM plants to micro-organisms, is a rare event as it requires several conditions to be fulfilled.

The issue of horizontal gene transfer has been viewed so far with particular focus on antibiotic resistance genes. Besides their natural presence in

micro-organisms, several of these antibiotic resistance genes are also used in genetic modification as markers for selection of successfully transformed organisms in the initial steps of creation of the genetically modified host organism. These antibiotic resistance marker genes therefore do not serve any specific purpose in the final product. The assessment focuses on the likelihood that these genes may be transferred horizontally, particularly to disease-causing (pathogenic) micro-organisms. Such a transfer, if successful, may impair the antibiotic therapy of these pathogens with the particular antibiotic to which these genes confer resistance. Items considered during the assessment therefore also include the clinical importance of the pertinent antibiotic substance and the background level of naturally developed resistance towards the antibiotic [for a review see Van den Eede *et al.*, 2004].

Directive 2001/18/EC on the environmental release of GMOs states that particular consideration should be paid in the environmental risk assessment to GMOs containing antibiotic resistance genes in order to phase out any antibiotic resistance genes that may have an adverse effect on human health and/or the environment. Recognising the need for guidance on this issue, the EFSA GMO Panel proposed to classify antibiotic resistance marker genes into three categories, i.e. i) genes for which there is no rationale to restrict or prohibit their use; ii) genes that should be restricted to field trial purposes and not be used in GM plants to be commercialised; and iii) genes that should not be present in GM plants to be commercialised or used in field trials [EFSA, 2004]. The kanamycin resistance gene *nptII*, for example, falls within the first category of genes that could be used in commercialised crops. A recent example of a GM crop containing the *nptII* antibiotic resistance marker gene that has been assessed for its safety by the EFSA GMO Panel is a GM starch potato (EH92-527-1) with altered starch composition. The Panel has concluded that the presence of *nptII* does not pose a risk to human, animal or environmental health because of the limited use of the target antibiotics, the widespread background presence of this gene in bacteria, and the low likelihood of its horizontal transfer from GM plants to bacterial recipients [EFSA, 2006b].

As described above, the potential horizontal transfer of antibiotic resistance genes is one of the issues that is commonly considered during the premarket assessment of GM crops. Because this transfer has only been observed under particular conditions, such as the presence of highly homologous sequences in the recipient organism and the need for transfer of intact sequences, the avoidance of the use of certain antibiotic resistance genes can be regarded as a precautionary approach. The trigger for this assessment therefore is the presence of antibiotic resistance genes in the GMO under consideration.

From a broader perspective, the question can be raised as to whether the transfer of transgenes introduced into GMOs to other organisms can have an impact on the health of consumers. For example, besides antibiotic resistance, questions pertaining to the selective advantage conferred to the recipient organism and other factors influencing pathogenicity can be raised as well [e.g. Kleter *et al.*, 2005]. These considerations, which are primarily based on non-experimental data, are commonly part of the environmental risk assessment of GMOs as carried out under Directive 2001/18/EC. With regard to the transfer of transgenes to intestinal micro-organisms, Netherwood and co-workers [2004] have found indications of such a transfer of the *cp4 epsps* gene from soy ingested only by ileostomy patients having an incomplete digestion, but have been unable to further substantiate this finding or demonstrate it in healthy individuals where the ingested soy is completely digested.

Another issue is the potential transfer of fragments of digested DNA to tissues of animals and/or humans consuming the food, which can occur equally in the case of non-transgenic or transgenic DNA derived from non-GM food or GM food. Particularly in domestic animals, experiments have been carried out on the survival of DNA of GM crops during digestion and their potential uptake into animal tissues and fluids [reviewed by Alexander *et al.*, 2007].

The methodology available includes a consideration of the transgenic DNA based on current knowledge of factors facilitating gene transfer (e.g. recombination, self-replicating elements etc) and influencing pathogenicity (virulence-associated characteristics, selective advantage, background presence of gene). Horizontal transfer experiments have been carried out by scientists, both under laboratory conditions (e.g. transfer to micro-organisms) and in vivo conditions (detection of transgenic DNA in domestic animals). Whilst the uptake of plant DNA fragments into animal tissues is a normal biological process observed in many experiments, only in some cases, transfer to animal tissues has been observed also

for transgenic DNA fragments, due to the low level of presence of transgenes in the GM food.

The performance of the above type of experiments is driven by a 'low probability, high impact' scenario, particularly for antibiotic resistance genes. In addition, the above research activities should also be viewed against the wider background of development antibiotic resistance, which is associated with the intensive use of certain antibiotics in animal husbandry and in medicine.

2.6 Unintended effects

Besides the effects targeted by the genetic modification, such as the introduction of a protein of interest into the GMO, it can be envisaged that also unintended effects take place. For example, in the hypothetical case that the new DNA has been introduced into a native gene, the function of this gene may be disrupted. Unintended effects are not limited to the technique of genetic modification, but are also known to occur during conventional crop breeding.

One example is the altered glycoalkaloid content in GM and conventionally bred potatoes [Kuiper *et al.*, 2001]. Another example in which metabolic engineering has led to unexpected effects is Golden Rice. The modification of this rice has comprised the introduction of a pathway leading to beta-carotene biosynthesis. Because beta-carotene does not naturally occur in rice kernels, various enzymes have been introduced in order to convert part of a common precursor, i.e. geranylgeranyl pyrophosphate, into beta-carotene. However, it has been noted that also in the absence of one of the transgenic enzymes, GM rice kernels are still able to synthesise beta-carotene, as well as some other carotenoids that are not the target of modification [Ye *et al.*, 2000]. It is therefore assumed that the absence of beta-carotene from kernels is caused by abolishment of a specific step in its biosynthesis, but that the rest of this pathway still has been present in a latent form.

The extensive comparative analysis of a GMO and its counterpart for phenotypic, agronomic, and compositional characteristics can provide indications of the occurrence of unintended effects in the GMO. This will be in the form of differences in characteristics that are not directly linked to the targeted effects of genetic modification. In addition, the knowledge about the characteristics of the introduced DNA and gene products obtained through molecular characterisation of the GMO can also provide for the prediction of unintended effects and guide further analysis for the verification thereof.

As described above, methods currently available to measure any unintended and/or unexpected effects include a wide array of compositional, phenotypic and agronomic characteristics that are measured in a targeted fashion as part of the comparative assessment of a GM crop and its conventional counterpart. Additional analysis can be performed on the levels of compounds likely to be affected by the introduced metabolic modifications, as it has been done with carotenoids in Golden Rice. If considered appropriate for risk assessment, also less specific profiling methods may be employed to test for differences in GM crops with complicated modifications. In addition, whole-product feeding studies in laboratory animals, such as the 90-day rat feeding study, may provide additional data on the safety of a crop. It should be kept in mind though that differences do not necessarily constitute health hazards.

The issue of the choice of the appropriate comparator has an important place in the comparative safety assessment, which is also why, for example, the EFSA GMO Panel pays a lot of attention to this issue in its safety assessments of GMO applications, including a consideration of breeding pedigree of GM crops and their controls. A potential point of consideration may be that in the case of extensively modified GMOs, other comparators besides the host of the modification can be used for the comparison of compositional parameters (e.g. borage oil for oil from GM canola containing high levels of GLA, as described above).

2.7 Nutritional value

The compositional analysis of the GMO can also highlight changes in its nutrient composition that may impact the role of the GMO to human and animal nutrition. Besides changes in the level of nutrients or antinutrients, it can also be envisaged that their bio-availability during digestion can be altered, such as through a change in matrix or by the introduction of enzymes facilitating intestinal uptake. If the nutrient composition or availability has indeed been changed by the genetic modification, *in vivo* feeding trials may be decided for on a case-by-case basis, depending on the knowledge already available on these nutrients. Such feeding trials may include performance experiments, i.e. by measurement of consumption and growth, and

balance experiments, to measure bio-availability of a nutrient. Because of the differences in nutrient metabolism and requirements of humans and animals, appropriate models may differ depending upon the target animal and the affected nutrient.

Many application dossiers on GMOs contain chicken broiler feeding studies, in spite of the absence of any relevant changes in the nutrient composition. The chicken is a rapidly growing animal reaching full size within six weeks. It is therefore likely to show effects in case of nutritional differences between the tested diets fed to different groups of chicken, including diets containing the GMO and those containing its conventional counterpart.

2.8 Potential triggers for follow-up research into health effects of GMOs

Various factors that may come up during the pre-marketing and post-marketing phases are discussed below as they can be envisaged to trigger further research into the potential health effects. This is in line with the case-by-case nature of the comparative safety assessment approach according to the internationally harmonised guidelines followed by EFSA and other institutions.

2.8.1 Hazards identified during pre-market risk assessment

In this scenario, the pre-market assessment indicates that the GMO may contain particular hazards that need additional experiments for the assessment to be complete. For example, if a newly expressed protein shows specific toxic effects in a 28-day repeated-dose feeding trial, then further toxicity testing may be warranted, as recommended by EFSA guidance, such as subchronic testing or tests for specific toxic effects (e.g. neurotoxicity), if applicable. The same also holds true for non-protein substances that have been introduced or whose levels have been changed, for example as an unintended result of the genetic modification. Besides toxic effects of a substance observed in toxicity tests, also other indications for its potentially toxic properties may exist, such as data on this or similar substances from literature, databases, or computer-aided predictions. These existing data may in certain circumstances have to be complemented with the outcomes of further toxicity testing to enable a conclusion on the toxicity of the introduced or altered substance. It can be envisaged, though, that the identification of such a hazard during the pre-commercial development stage of a GMO may already be a reason for its developer to abrogate further development. It can be envisaged that 'food-grade' substances are less likely to raise concerns over their potential toxicity than certain non-food products. A dedicated working group of the EFSA GMO Panel is currently preparing recommendations complementing the current guidance document on the issues surrounding the use of GM food crops for the production of non-food/feed substances, including medicinal and industrial products.

2.8.2 Uncertainty in the current pre-market risk assessment

Various factors can be envisaged that may contribute to uncertainty over the possible occurrence of health effects caused by GMOs. Examples of this include the absence of a comparator with a history of safe use for the comparative assessment (e.g. in case of extensive modifications), the extrapolation from laboratory-scale to real-life dimensions, the use of animal models for humans, and the potential interactions between components from different foods. These scenarios do not pertain to GM products per se, but are applicable to any conventional product. So far, the data evaluated for GMO dossiers appear to have provided at least an acceptable degree of certainty of no harm and therefore little uncertainty. The level of uncertainty is an important factor for risk managers and may trigger the application of the precautionary principle. In fact, precaution is also the reason for the comprehensive pre-market safety assessment and follow-up by post-market monitoring currently applied to GMOs, in order to reduce the uncertainty regarding any potential health effects of GM technology to a minimum. It should be borne in mind that the pre-market safety assessment has to reach its conclusion within a reasonable timeframe based on sufficient conclusive evidence according to the state of the art tools and technologies.

2.8.3 Post-market verification of assumptions made during the pre-market risk assessment

Particularly assumptions about the food intake or other ways of exposure of consumers through the introduction of a new product may require post-market verification. This is because the estimated exposure and the characteristics of the hazard together are used as inputs for the risk characterisation, i.e. an estimation of the likelihood that certain

hazards will occur. This has already been practiced with various novel foods permitted under Novel Food Regulation (EC) No 258/97, such as phytosterol-containing products, of which the intake by various consumer groups has been gauged by the applicant after the market introduction. According to the internationally harmonised principles of food risk assessment, exposure assessment is an important part of the risk characterisation process and enables the provision of a quantitative risk estimate indicating the likelihood that an adverse effect associated with the presence of a given hazard in a food will indeed occur by its consumption. Other kinds of pre-market assumptions besides intake estimates can also be verified, such as, for example, the physiological levels of fat-soluble vitamins in consumers eating products containing fat substitutes that also may reduce the uptake of such vitamins from the diet.

In a hypothetical scenario, if substantive differences between the pre-market estimate and the post-market measurement occur, the risk assessment and risk management of the pertinent product may have to be revisited. The same also pertains to any unexpected effects of a marketed product reported through surveillance systems that non-discriminately record any type of effect, contrary to hypothesis-driven monitoring. In all these cases, the risk assessment has to be adjusted and further investigations may be prompted into the details of the mechanisms underlying these differences. It is important to keep in mind that science evolves over time, and may give rise to new pertinent information, which, however, does not specifically apply to modern biotechnology alone.

2.9 Current approaches for post-market monitoring

From a general perspective, article 5 of Regulation (EC) No 1829/2003 requires that a post-market monitoring plan is submitted, as appropriate for the use of food and feed. In addition, it can be envisaged that the monitoring will be facilitated by the labelling and traceability of GMO-containing foods as required by Regulation (EC) No 1830/2003. The applications that have been subject of EFSA opinions so far have contained environmental monitoring plans, but not monitoring plans for food and feed safety, though, based on the fact that no risks have been identified in the risk assessment that would require such follow-up monitoring. Monitoring plans that have been provided so far pertain to the environmental risks of GM crops, including the potential for insect resistance in crops expressing insecticidal proteins originating from *Bacillus thuringiensis*. In this context for environmental monitoring, a distinction is made between case-specific monitoring and general surveillance, being two distinct requirements under different conditions as part of the post-market monitoring plan.

In addition, applicants have provided general surveillance plans for unanticipated environmental effects, as part of the requirements of Directive 2001/18/EC on the environmental release of GMOs. These surveillance plans also include potential effects arising from animal feed use. These plans preferably should draw upon existing networks and because of their general nature, there is no specific experimental methodology, yet the statistical validity is important [Bartsch *et al.*, 2006]. The surveillance activities entail the interrogation of farmers through questionnaires, as well as the collection of information from existing networks of professionals, including veterinarians, feed processors, etc.

The Food Standards Agency (FSA) has previously commissioned research into the feasibility of the use of consumption data from household food purchase surveys and supermarket loyalty cards for post-market monitoring of health effects related to consumption of GM foods [Elliott *et al.*, 2003]. It has thus been observed that the household food purchase data are able to cover approximately 70% of the actual consumption of these households. In addition, data have been striated into various geographical regions of the UK, as well as socio-economic classes. Based on the results, FSA recommends further refinements to the collection of household food purchase data, as well as the linkage to health statistics of the national health system [Elliott *et al.*, 2003].

Various potential drivers for post-market monitoring of GM foods are considered by Hlywka and co-workers [Hlywka *et al.*, 2003], i.e. the potential for allergenicity, potential chronic health effects, confirmation of pre-market exposure estimates, and identification of changes in food intake or dietary habits. These authors also note that demonstration of causality, i.e. the link between a health effect and exposure to a given food, is important. This may entail the use of quantitative exposure assessment methods, including probabilistic methods, which also are able to discern between various consumer subgroups [Hlywka *et al.*, 2003].

In addition, the Canadian Biotechnology Advisory Committee has previously considered the possibility for post-market surveillance and monitoring of GM foods. Their recommendations have particularly focused on the potential allergenicity of these foods, given the lack of a suitable validated animal model [CBAC, 2002].

2.10 Outlook

The current approach towards the comparative analysis of the characteristics of a GMO and its conventional counterpart involves the analysis of an extensive dataset. This has been applied satisfactorily to the assessment of GM crops with modifications that are relatively minor, such as the introduction of proteins at low levels with no conspicuous impact on crop composition. It is expected that future crops will also include GM crops with more profound changes, such as in crops 'metabolically engineered' with new metabolic pathways in order to produce a nutrient of interest. These modifications are likely to be more prone to the occurrence of unintended effects besides the modification of interest.

Various European initiatives, such as those funded by the European Commission's Directorate-General for Research and the United Kingdom's (UK) Food Standards Agency (FSA), have tested the applicability and suitability of advanced analytical 'omics' technologies for the analysis of unintended effects in GM crops. These initiatives include, among others, the EU-sponsored projects GMOCARE and SAFE FOODS (Work Package 1).

Various 'omics' technologies are available, which provide 'holistic' impressions of the composition of an organism at various levels of cellular organisation. These levels include gene expression by measuring the various forms of messenger RNA ('transcriptomics'); the occurrence of the various proteins present within a sample ('proteomics'); as well as the various metabolites, i.e. chemical compounds formed by metabolism present within a biological sample ('metabolomics'). These methods are non-discriminatory in that the identity of substances linked with signals need not be known of beforehand. If the comparison between the 'omics' analysis of a GMO and its conventional counterpart show differences in a particular signal, this ideally will be traced back to the substance linked with that signal.

Any difference found should also be offset against the natural background variation for the particular parameter. For example, Lehesranta has observed in a proteomics experiment comparing a GM potato with a conventional potato that 9 out of 730 proteins occur at different levels in the GM lines. However, a comparison between non-GM genotypes, including also wild-type potato and a natural relative, has shown that 1,077 out of 1,111 proteins occur at different levels, whilst 600 additional spots do occur in particular, but not all, genotypes [Lehesranta *et al.*, 2005]. Various databases are currently being developed with the aim of providing useful background ranges for crop composition. An example is the MoTo DB database for the chemical metabolite composition of tomatoes analysed by liquid chromatography (LC) coupled to mass spectrometry (MS). In order to make chromatograms comparable, specific software is used to align peaks from different LC chromatograms and to calculate masses belonging to MS peaks. Using this method, various previously unknown metabolites have been discovered in conventional tomato [Moco *et al.*, 2006].

The potential of 'omics' technologies for application in risk assessment of GMOs have been reviewed elsewhere in more depth [e.g. Chassy *et al.*, 2004; Kok *et al.*, 2003; Kuiper *et al.*, 2003; Shewry *et al.*, 2007]. Generally, it is considered that the routine application of these techniques in regulatory risk assessment requires additional harmonisation and validation, as well as development of databases for the data on background variation. These techniques may nonetheless provide utility in the development phase for GM crops with complicated modifications as a sentinel for effects on the crop composition that warrant further testing. 'Omics' techniques can be a valuable addition, rather than a replacement, of the currently applied targeted analysis. In addition, a more targeted fashion of 'omics' can be envisaged if specific metabolic pathways are affected (e.g. carotenoid profiling in GM crops with altered carotenoid biosynthesis).

3 Experience with additional clinical and long term testing of GMOs

As described in more detail above, the tests that have to be carried out within the legal framework of regulatory approval for GMOs include an array of *in vitro*, *in silico,* and *in vivo* experiments. It should be noted that the decision for the type of tests that should be required for the safety assessment is made and adjusted for each specific GM product, taking into account the various types of genetic modifications, host organisms, and differences found in the comparative analysis. Various dossiers include, for example, results from subchronic animal trials with the whole product. These data have provided an appropriate degree of certainty regarding the absence of potential health effects.

Various reports in scientific literature also give an account of clinical and longer-term tests with GMOs, which are discussed in more detail below.

3.1 Animal testing

Animals used in testing the safety of GMOs include both laboratory rodents and domestic animals. Various types of tests that are provided with dossiers and that span much or all of the lifetime of an animal, such as nutritional studies in chicken broiler or swine, are not discussed here. The focus therefore is on studies that are not commonly included with dossiers.

3.1.1 Laboratory animal testing

Various multigenerational studies have been performed with laboratory rodents, as described in more detail below.

Glyphosate-tolerant soybeans have been tested in a multigenerational study in which each generation of mice received diets containing these soybeans (21.35%) or a conventional counterpart. Three generations of mice have been tested for possible effects on general health, litter size, and testicular spermatocytes of mice of each generation. Except for a difference in spermatocyte populations at one time point (age 26 days), which is considered to be prone to variability, differences have neither been observed for spermatocytes at other time points nor for other parameters [Brake and Evenson, 2004].

A multigenerational experiment with the same setup has been performed on GM insect-resistant Bt maize. The outcomes are similar to those of the experiment with glyphosate-resistant soybeans described above [Brake *et al.*, 2004].

Other multigenerational experiments have also been performed with experimental GM canola and potato. The GM canola has been modified so that it contains a high level of γ-linolenic acid (GLA). Diets containing oil from this GM canola and from non-GM borage oil (similar GLA content), plus an additional control diet without GLA have been fed to diets of maternal mice. The litter size and various characteristics of the offspring, including behavioural and neurotoxicity tests, body weight, brain weight and fatty acid composition, have been measured. The results show some differences between the GM canola oil and borage oil groups, including decreased body weight and altered brain lipid composition. The authors postulate that this may relate to the different forms in which GLA occurs in both oils, causing differences in its digestion and metabolism [Wainwright *et al.*, 2003].

The experimental GM herbicide-resistant potato has been fed to rats during five generations. Besides measurement of body weight and feed intake, animals have also been tested for reproductive parameters (mating, fertility, gestation, spermatocyte motility), offspring characteristics (litter size, pup gender ratio, viability, development), skeletal and visceral deformations, gross necropsy, organ weights and histopathology. In addition, organs have been checked for the presence of transgenic DNA. Besides a difference in fertility in the founder (Fo) population fed GM potato, which is still within the range of control animals, no other effects have been observed [Rhee *et al.*, 2005].

A group of researchers has also published various studies on the ultrastructure of cells of various organs (liver, spleen, testes) of mice fed glyphosate-resistant soybean for up to eight months [Vecchio *et al.*, 2004, and references therein]. Whilst these authors note that the nucleus and other organelles may show changes depending on the diet, the cause of these changes has not been established. In addition, the origin of the GM soybean is not specified in detail and the model employed is not routinely used in toxicity testing.

3.1.2 Domestic animals

A multigenerational feeding trial has been performed in quails [Flachowsky *et al.*, 2005]. Both male and female quails have been fed diets containing either insect-resistant GM Bt maize or non-GM maize. Hen eggs laid after 12 weeks have been

used to obtain each following generation. The animals have been checked for mortality, feed intake, body weight, egg laying intensity and hatchability and organ weights. In addition, tissues have been tested for the dissemination of transgenic DNA in animals that have been fed the diets during up to one year. Neither any statistically significant differences nor any uptake of transgenic DNA from the gastrointestinal tract has been observed in these animals [Flachowsky *et al.*, 2005].

3.2 Human and primate testing

An experiment involving GM fish with enhanced growth characteristics, i.e. GM tilapia expressing transgenic tilapia growth hormone, has been conducted with human volunteers [Guillen *et al.*, 1999]. Two groups of 11 healthy volunteers each have received diets containing GM or non-GM tilapia twice daily for five days. The authors mention that the subjects have shown no differences in cytological and biochemical composition of blood samples taken after termination of the experiment. The flavour of the GM tilapia was generally perceived as being better than that of non-GM tilapia [Guillen *et al.*, 1999]

In addition, in the same study, the transgenic tilapia growth hormone expressed in the GM tilapia has also been administered intravenously to non-human primates (i.e. juvenile macaque monkeys) daily during 30 days. The macaques have been tested for physiological parameters (body weight, temperature, heart and breathing rate, body dimensions), blood composition and cytology, and gross and microscopic pathology. No effects related to the growth hormone administration have been observed [Guillen *et al.*, 1999].

3.3 Post-market monitoring of allergenic potential

Various studies published in scientific literature focus on the possible allergenic effects of the market-approved GM crops. For example, a recently published study has verified whether human subjects, including allergy patients, from Europe and Korea, have developed sera responses to the transgenic CP4 EPSPS protein present in GM herbicide-resistant soybean. The underlying premise is that these subjects have had a plausible history of exposure to GM soybeans given its large-scale production. No specific reactions of allergy patients' sera with CP4 EPSPS have thus been noted [Hoff *et al.*, 2007].

Sera binding and skin prick tests with extracts from GM crops that have been allowed onto the European market, and with purified transgenic proteins that occur in these crops, have also been performed in another study. Sera binding has involved the use of SDS PAGE followed by immunoblotting. No differential effects between extracts of GM and non-GM crops have been observed. In addition, no sera binding or skin reactions have been observed against the transgenic proteins tested [Batista *et al.*, 2005]. In another study, sera from soy-allergic patients have not shown differential binding towards the individual proteins in GM and non-GM soybeans separated by two-dimensional electrophoresis [Batista *et al.*, 2007].

The screening for possible sera binding by GM maize has also been tested for Starlink™ maize, which accidentally has entered into the food supply in the USA [reviewed by Bucchini and Goldman, 2002]. Starlink™ maize contains the transgenic Bt protein Cry9C, which has become resistant towards degradation in insect intestines by one amino acid mutation. This stability towards degradation has also been observed in systems with pepsin, trypsin, and heat. Cry9C also has elicited an allergic serum reaction in Brown Norway rats, which are known to be IgE-hyperresponders, whilst also another protein without known allergenic properties tested positive in the same test. Based on these considerations, Starlink™ has previously only been allowed onto the market for feed use. Despite this, it accidentally has become commingled with human food products derived from maize, such as taco shells. This has instigated a major recall action and a request to consumers to report any allergic reactions that might have been related to the consumption of Starlink™-containing products. These self-reported cases of allergy have subsequently been verified by the American Center for Disease Control and Prevention by using sera from these subjects for binding tests with Cry9C. This center has been unable to confirm allergic reactions to Cry9C based on the negative outcomes of these binding tests [Sutton *et al.*, 2003].

4 Experiences with health effects of non-GM foods

An article that has recently been published as the outcome of an ILSI Europe activity provides a review of the experiences gained with post-market monitoring of novel foods [Hepburn *et al.*, 2007]. This review highlights various food products that have previously undergone post-market monitoring, such as the sweetener aspartame, the fat replacement olestra, and the cholesterol-lowering phytosterol esters. In addition, it also highlights the incident involving admixture of Starlink™ maize with food, although this cannot be strictly considered a post-market monitoring activity (see above).

In case of the sweetener aspartame, the monitoring has included intake surveys in various countries, after approval of this compound in various dry products and carbonated beverages. These surveys have shown that the aspartame intake is well below the acceptable daily intake for this compound. In addition, various effects were related by a passive reporting system. These anecdotal effects have not been confirmed by follow-up research in animals and humans [reviewed by Hepburn *et al.*, 2007].

Olestra is a fat replacement consisting of fatty acid esters of sucrose, which are not absorbed after consumption from the gastrointestinal tract. For olestra, the approaches followed have included post-marketing studies on the consumption of olestra-containing products including snacks and the possible effect on the status of fat-soluble vitamins and carotenoids in consumers in the USA. These monitoring activities are to verify pre-market assumptions on intake, as well as to verify the potential effect on vitamins identified during the pre-market assessment. In addition to these monitoring activities, also a reporting system for general passive surveillance has been set up [reviewed by Hepburn *et al.*, 2007].

Phytosterol esters have been approved as novel foods for the European market under Regulation (EC) No 258/97 on 'Novel Foods'. Their consumption can lower the level of serum cholesterol because of less cholesterol being taken up from the digesta. After market introduction, monitoring has been carried out to verify the assumptions made during the pre-market assessment regarding the level of consumption, differentiated over the various consumer groups. It has thus been observed by the company that the intake is actually lower than initially expected and that the target population of elderly consumers indeed constitutes the main users of the phytosterol-containing products, i.e. spread (margarine-like). In addition, general surveillance has included the registration of complaints through a dedicated telephone centre. These complaints have subsequently been assessed by health professionals, which, however, have not been able to specifically link these complaints to phytosterols [reviewed by Hepburn *et al.*, 2007].

5 Conclusions

Whilst the internationally harmonised approach towards the pre-market safety assessment of GMOs can be regarded as rigorous, biotechnological product developers, risk assessors and risk managers have to stay alert to new developments in this area. Various scenarios can be envisaged in which the commonly employed safety tests are supplemented with additional data during the pre-market or post-market assessments. The need for such additional tests may arise either from additional hazards identified during the pre-market assessment, or from uncertainties requiring a precautionary approach towards risk management. As for the commonly used methods, those that have to be applied for the additional tests should preferably be appropriate in the context of a regulatory assessment, providing results that can withstand scrutiny. In addition, certain measures may be facilitated by the current system for market approval of GMOs, such as post-market monitoring that can draw upon the mandatory labelling and traceability of GM foods and feed under Regulation (EC) No 1829/2003.

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Annex II

Overall approaches to assess and monitor potential short, medium and long term effects in relation to consumption of Genetically Modified Organisms (GMOs) and products derived thereof

Conclusions of a workshop, held at the Joint Research Centre of the European Commission in Ispra on 26-27 November 2007

Table of contents

Introduction

The present document is the main output of the '*International workshop on overall approaches to assess and monitor potential short, medium and long term effects in relation to consumption of Genetically Modified Organisms (GMOs) and products derived thereof*' organised by the Joint Research Centre (JRC) in Ispra (Italy) on 26-27 November 2007.

Experts did not define what is precisely intended with 'short', 'medium' and 'long term' health effects. Acute effects for instance are certainly 'short term' and chronic effects (eventually covering the life span of an individual) and indirect or delayed effects may be considered as 'longer-term' effects. It was perceived that such a definition would not contribute to this debate since it is acknowledged that the current pre-market assessment and post-market monitoring cover short term and long term health effects. Likewise, the observations made in this document are applicable to the three categories.

The workshop represented a key element for the accomplishment of component C 'Health issues in relation to GMOs' of the project proposal '*Scientific and technical contribution to the development of an overall health strategy in the area of GMOs*' presented by the JRC Institute for Health and Consumer Protection (IHCP) on request of the Committee on Industry, Research and Energy of the European Parliament, and approved by the European Parliament at the end of 2006[1].

Participants attending the workshop included 22 experts in different disciplines relevant to pre-assessment and post-marketing evaluation of GMOs and GM derived foods/feeds. Experts from the European Food Safety Authority (EFSA) were closely involved in this workshop together with other internationally acknowledged experts.

The workshop addressed issues raised in a 'non-paper' (see Annex I) that was circulated in advance to the participants for their consideration.

The observations and recommendations made during the two-day discussions have been grouped in the present document into four distinct chapters:

1. General observations and recommendations,
2. Pre-market assessment phase,
3. Post-market monitoring phase,
4. Future developments and research.

1 The Committee on Industry, Research and Energy also pointed out the need to study possible health threats coming from genetically modified organisms such as maize MON863. It also indicated the need for the JRC to coordinate research with an overall health strategy.

1 General observations

1. This document focuses on the assessment of possible human health effects related to the direct consumption of GMOs. In addition, when considering 'animal health', the emphasis is placed on the perspective of humans consuming animals fed with GMOs rather than animal health per se. In addition, animals serve also as good indicators for potential human health problems since their diets may contain much higher levels of GMOs than humans ever would get.

2. 'GMOs' (genetically modified organisms) comprise living organisms such as plants, micro-organisms and animals (as defined in Directive 2001/18/EC) and food and feed products derived thereof [as defined in Regulation (EC) No 1829/2003].

3. From the scientific and food safety point of view, GMOs need not necessarily be considered as a distinct group with respect to potential health effects. With the advent of more novel foods, especially functional foods, either derived from GMOs or non-GMOs, the distinction between both will become less meaningful.

4. Food safety concerns products and substances rather than the technology by which the food has been obtained. From there it follows that the approaches for pre-market assessment and post-market monitoring of GMOs, novel and functional foods (including food with health claims), should be coherent.

5. An unintended effect, possibly due to the genetic modification process, cannot be known or defined a priori, and is thus subject of the hazard identification and risk assessment. The probability of occurrence of unintended effects is part of the uncertainty analysis in the risk assessment procedure per se, in which the aim is to reach the highest possible degree of scientific certainty.

6. The distinction between what needs to be known for the (safety) evaluation per se and what might be scientifically interesting or challenging to know, but not adding significant information to the pre-market risk assessment or post-market monitoring, should be made. The degree of scrutiny should be proportional to the magnitude of risk perceived.

7. 'Health effects of food' comprises both negative and positive effects. Benefit analysis is nowadays not part of the pre-market risk assessment or of the post-market monitoring phase as performed at Community level. While zero risk is inexistent in any technological area, risk-mitigation measures could be used to minimise it. Risk-benefit analysis will become increasingly important. However, safety must continue to be the first priority with the highest possible degree of certainty.

8. Communication aspects will also have a more dominant role; the scientific community and the regulators will need to be aware of the new types of GMOs being developed. The range of organisms modified will certainly become much wider than what is currently seen and will include an ample array of plant species, animals (including fish) and micro-organisms. All experts involved in the pre-market assessment and in the post-market monitoring should be proactive and well ahead of the state of tools and technologies.

9. It is highly recommended to establish an integrated stakeholder forum in which the different experts/actors (ranging from the technology developers, the biotechnology companies, breeders, risk assessors, decision makers, retailers and other parties contributing to the successful and safe introduction of new products) can periodically interact and exchange information, in particular in relation to new developments, needs, constraints etc. These periodic meetings would serve as a dialogue forum of experts and would allow the making of an adequate inventory of new technologies and products, and the monitoring of them. The outcome of this dialogue forum would also enable proactivity in the anticipation of future legislative needs and would further contribute to increasing confidence in the effectiveness of the whole risk assessment and management process.

2 Pre-market assessment phase

As a measure of precaution, a pre-market risk assessment is performed for each individual GMO that is ready to enter the market as a commercial product. Presently, the comparative approach is internationally recognised as the appropriate principle for GMO safety assessment. The comparative safety assessment (CSA) is based on the comparison of a GMO with an appropriate conventional counterpart (the comparator) with a history of safe use [Kok and Kuiper, 2003]. This allows assessing the safety of GMOs relative to products that, even if not specifically assessed for safety, are known by experience to present no unacceptable health risks, under normal conditions of consumption. The comparison includes an extensive range of characteristics, including chemical composition, nutritional profile, phenotypic and agronomic characteristics.

1. Possible differences between GMOs and their appropriate comparators are assessed for their safety impact on a case-by-case basis, dependent on (1) the specific biological characteristics of that GMO and (2) the intended use of that GMO under assessment.

2. This approach and the necessary data requirement has been established after consensus built by various authoritative international organisations such as the United Nation Food and Agriculture Organisation (FAO), the World Health Organisation (WHO) and Organisation for Economic Cooperation and Development (OECD). International consensus has resulted in the publication of guidelines for the safety assessment of foods derived from GM plants and micro-organisms by Codex Alimentarius [Codex Alimentarius, 2003]. These documents serve as reference for international trade issues surrounding the safety of foods and must be implemented by all Codex members.

3. In agreement with this international consensus, the European Food Safety Authority (EFSA) has developed GMO guidance documents which constitute the basis for pre-market GMO risk assessment in the European Union [EFSA, 2006a]. These documents, which take into account EC legislation and the Codex guidelines, are more detailed and provide practical guidance for the compilation of the dossiers and the data requirements to be submitted by applicants in the frame of regulatory procedures for EU marketing approval of GMOs.

4. Based on the experience gained so far, the comparative approach is considered to be solid and to be the pillar for future developments in GMO risk assessment. The weight of evidence based on the currently used toxicological, nutritional, molecular, and allergological data requirements constitutes a robust frame for the prediction of potential health effects. The amount of data required for the assessment of GMOs is, in general, much larger compared to the data needed for approval of conventional or novel foods and it provides a sufficient degree of safety assurance.

5. The comparative approach implies the comparison of the GM under study with respect to an appropriate conventional counterpart. The selection of this counterpart is considered relatively straightforward for the present generation of GM plants characterised by a limited extent of genetic modifications and with a well-known genetic background. In the future, GMOs with more complex modifications, characterised by more extensive alterations, are expected. This may entail a broader selection of appropriate comparators, including possibly multiple products with a history of safe use, in order to identify possible differences that may require further investigation.

6. Extensive similarity exists between safety assessment strategies for GMOs and for novel foods (e.g. allergenicity tests, toxicity tests with animals, health claims, nutritional assessment, compositional analysis), although novel foods assessment may be more challenging since in some cases a comparator with a history of safe use is not available.

7. Statistical models and data requirements for risk assessment of GM plants and derived foods/feeds must be carefully defined upfront, i.e. as a guide for accurate experimental layout and data analysis, to ensure meaningful, reliable and comparable results. This logical approach allows maximum efficiency/accuracy during data evaluation, and it ensures full transparency with respect to uncertainties and assumptions of GMO risk assessment. When biostatistics are applied *a posteriori* this may lead to unrepresentative conclusions.

8. The European Commission and EFSA are implementing since April 2006 an action plan

aiming to improve the support of Member States in the authorisation procedure of GMOs. Following fruitful discussions with EFSA, The Commission will shortly propose new detailed rules for the assessment of GMOs for food and feed.

9. As part of the harmonisation of data requirements, for example for the statistical analysis applied later, a working group of the EFSA GMO Panel is considering an even more objective strategy for the identification of biologically relevant statistical differences between GMOs and their comparators. As briefly described above the comparison is made by measuring a number of endpoints with the objective of demonstrating biological equivalence of a GMO and its control. For each chosen endpoint, or for groups of endpoints, limit values for meaningful change have to be set a priori based on biological experience and knowledge. When this is not feasible, statistical methods can be used to calibrate the observed changes against background variability observed for commercial plant varieties already on the EU market and with a history of safe use.

10. Within a plant species there is a large natural variation (depending on variation of genetic background, epigenetics, environmental conditions and developmental conditions) and therefore the variability baseline and the link to biological relevance of observed statistically significant differences between the GM and its parental comparator is to be carefully assessed on a case-by-case basis, taking into account the variation between commercial varieties.

11. With respect to future developments, it is foreseen that, in addition to GM plants intended for food use, new GM plants producing specific molecules for non-food purposes (e.g. pharmaceuticals, vaccines) will be ready for marketing. EFSA is currently investigating the risk assessment of these new GM plants, which should consider incidental exposure of consumers in addition to risk management focused on containment of these products. In anticipation of such, a special guidance document for the risk assessment of these products is expected in 2008.

12. Other developments, driven by industrial innovation, include genetically modified micro-organisms (GMM) and genetically modified animals (GMA). For example the first application for GM fish is expected in the near future. Codex guidance has been developed for the risk assessment of edible products derived from such animals, while EFSA will concentrate on the design of guidance for the environmental risk assessment of such GM animals. Furthermore EFSA has published a guidance document on the safety evaluation of GMMs and derived products, which is in line with the Codex guidelines [EFSA, 2006b]. The EFSA document includes some additional environmental aspects, and it focuses particularly on the risk of gene transfer in the digestive tract, which is recognised as the main concern in case of GMMs use in foods.

13. The principles for the risk assessment of GM plants and GMMs are the same, i.e. the comparative safety assessment, and therefore also GMMs will be assessed on a case-by-case basis. The Codex guideline on GMA will be shortly formalised, and EFSA is expected to publish a pertinent guidance document later on. The EU regulatory system may need review and regulatory adjustment to accommodate the marketing of possible new types of GMMs and GMAs.

14. As mentioned above, some of the future GMOs may contain complex modifications such as metabolically engineered and/or nutritionally enhanced crops to which a whole new biosynthetic pathway is introduced (e.g. Golden Rice with provitamin A containing kernels). In these cases, analytical profiling techniques, once developed to full robustness and validated, may serve as complementary tools to identify and characterise any unforeseen metabolic perturbations besides those intended.

15. From a general perspective, the need for testing for potential allergenicity can derive from specific hazards identified in the pre-market assessment. In addition, this may also address the uncertainty remaining over the allergenic potential of a product based on the fact that no validated models as yet exist for some aspects of allergy, including sensitisation of laboratory animals. This has to be viewed against the background of advances in scientific research on the mechanisms and diagnosis of allergy, which will also have an impact on the detection of potential allergenic effects of non-GM products.

3 Post-marketing monitoring phase

1. Under the current EU GM food and feed regulation, it is necessary to introduce, where appropriate and on the basis of the conclusions of the risk assessment, post-market monitoring requirements for the use of genetically modified foods for human consumption and for the use of genetically modified feed for animal consumption.

2. Post-market monitoring of GM food/feed may thus have two aims: (1) confirm the assumptions and conclusions of the pre-market risk assessment, and (2) to identify the occurrence of unforeseen health effects following consumption of the GMO or its derived product. So far, i.e. for the GM products currently on the EU market, no monitoring of health effects has been necessary, but it may be required for those cases as outlined by EFSA, namely cases that include GM (functional) foods with altered nutritional composition and modified nutritional value and/or with specific health claims. This could be the case for a GM food proposed as an alternative or as a replacement for a traditional food.

3. When in certain cases (e.g. products with intended positive health effects) during the pre-market risk assessment scientific evidence is found for a potential negative health effect, and when there is uncertainty about actual consumption patterns and levels of exposure of specific segments of the population, or the extent of a potential adverse effect linked to the genetic modification, then case-specific monitoring should be carried out after placing on the market. So far, such a situation has not occurred.

4. For the specific purpose of this study, 'monitoring' is related to the surveillance of individuals/groups to observe any possible unforeseen health effect. It is carried out on a case-by-case basis and its need is identified during the process of risk assessment/management. The working group points out that post-market monitoring eventually follows a finalised pre-market safety assessment and should not (even partly) substitute for it, nor should it aim at any further data collection which was not available during the pre-market safety assessment.

5. Monitoring needs are defined under Directive 2001/18/EC and guidance is already available but the working group notes that monitoring may be necessary for products produced from GMO. This may for instance be needed to monitor effects on health of products grown under contained use conditions (Council Directive 90/219/EEC) in greenhouses, stables or ponds and not intended for consumption (e.g. medicinal plants, GM animals producing vaccines, GM fish etc.). It is important to point out that monitoring in the context of contained use is already carried out, for instance in the case of genetically modified micro-organisms, and that experience may be drawn from this area.

6. Accurate traceability (which generally is a combination of documentation and analytical testing) of GM food and feed throughout the agro-food chain is a prerequisite for surveillance. GMO identification methods are based on event-specific identification and are available for all EU-approved GMOs although particular technical problems still exist for distinction between stacked and single events in derived products (http://gmo-crl.jrc.ec.europa.eu/). Accurate traceability also comprises the application of robust sampling plans. International agreement within Codex Alimentarius will facilitate further exchange of methods and reference materials for GMO identification, in particular for GMOs approved only outside the EU.

7. Efforts are ongoing to develop methods for unapproved GMOs. However there is a further need for international cooperation and collaboration and initiatives should be taken to that end. Projects currently ongoing within the European Network of GMO Laboratories may serve as a basis for further global partnership.

8. Traceability assumes that the GMO genotype with respect to the insert is stable throughout the whole marketing phase. The Working Group points out that by all means the occurrence of possible false negative results (i.e. a test scores negative for GMO presence whereas in fact GMO is present) must be avoided. The Working Group recommends ascertaining that the detection method validated for that GMO as part of the notification procedure must be applicable to the marketed product.

9. The first step in the monitoring of health effects is the estimation of exposure. Therefore the Working Group recommends that when monitoring is considered as being required under current legislation and according to the conclusions obtained during the pre-marketing assessment, the identity of the GM should be unambiguously known. In addition, this guarantees the possibility for a complete withdrawal of specific products in cases of safety issues.

10. The potential of biomarkers as an aid for estimation of GMO exposure in humans and animals has been considered. Although this approach is generally accepted in certain areas (e.g. epidemiology of asthma), its applicability is considered only for very specific cases, such as in nutritionally improved and/or functional GM foods, currently not to be addressed.

11. Exposure assessment requires extensive EU consumption data, which are not in all cases available and current approaches are based on scenarios that assume extensive exposure.

12. The Working Group has identified the following parallels in monitoring needs between GM and non-GM crops:

13. Especially for foods with specific health claims, monitoring is equally important for GM as for non-GM foods and specific labelling may be required in order to collect data on exposure.

14. When agricultural products are approved for non-food/feed purposes, their accidental presence in the food/feed chain may need to be traced.

4 Future developments and research

1. As indicated in the previous chapters, it is foreseen that future GM plant generations will contain more complex traits, such as altered nutritional profiles, and that a wider range of organisms will be used as targets to introduce new traits via genetic engineering processes.

2. Multiple stacked GM varieties obtained by conventional breeding, carrying several GM traits within the same genome, are already on the EU market and are expected to be developed increasingly. In this respect EFSA has recently issued an opinion on the safety assessment of stacked GM plant varieties. By default these new traits would not need a different risk assessment approach to be followed; the comparative safety assessment would still be the appropriate principle for GMO safety assessment.

3. New types of GMO events or variety traits with specific nutritional and/or health claims would need additional basic and fundamental research to be conducted also by independent publicly funded research institutions/universities. Such basic research could provide insight into the possible intended and unintended changes caused by these modifications as well as the extent of background variability.

4. Profiling technologies offering the promise of a more accurate picture of the target organism with respect to integration locus, gene function, protein expression and metabolism, need to be further validated and tested for their application within the framework of a comparative safety assessment. In particular, bioinformatics tools, already considered as an integrative part of the application of these technologies, should be further developed, as well as the establishment of databases containing profiles of products produced under different external conditions and different agricultural production practices.

5. Taking all the aspects above into account, cooperation between experts/actors will become increasingly important in the EU and pre-market risk assessment and post-market monitoring should remain in line with scientifically agreed principles.

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Annex III
Development of a real-time PCR based ready-to-use multi-target analytical system for the detection of EU authorised and unauthorised GM events

Table of contents

Executive summary

The Biotechnology & GMOs Unit of the JRC Institute for Health and Consumer Protection (IHCP) has developed a real-time PCR based ready-to-use multi-target analytical system for the detection of EU authorised and unauthorised GM events. The system was established upon specific request of the European Parliament in the context of the project '*Scientific and technical contribution to the development of an overall health strategy in the area of GMOs*'. The approach allows the event-specific simultaneous detection of 39 single-insert GMOs, comprising all EU approved and all unapproved GM events for which a method was submitted to the Community Reference Laboratory for GM Food and Feed (CRL-GMFF) and stacked events derived from them. System performance (specificity, efficiency etc) has been successfully confirmed by experimental testing conducted within the CRL-GMFF. The project has already been presented to members of the European Network of GMO Laboratories (ENGL). The 'real-time PCR based ready-to-use multi-target analytical system' developed by the Biotechnology & GMOs Unit, the first analytical tool developed worldwide allowing the detection of so many GM events simultaneously using event-specific targets, could be used to conduct a survey on GMOs presence on the European territory.

1 Introduction

The project described here has been formulated by the Biotechnology & GMOs Unit of the Institute for Health and Consumer Protection (Joint Research Centre, JRC) in response to component A '*Routine high-throughput for the detection of GMOs*' in the context of the project proposal '*Scientific and technical contribution to the development of an overall health strategy in the area of GMOs*' presented to and approved by the European Parliament at the beginning of 2007. The project constitutes one of the potential analytical alternatives and, by specific request of the European Parliament, was aimed at developing and providing a fast and handy ready-to-use multi-target system for the detection of (as many as possible) GM events approved and unapproved on the European market in a single experiment.

2 Background information and strategy selection

The strategy selected for the realisation of the project presented in this document has been formulated and based on a series of considerations summarised below.

Over the past years the JRC, through the activities conducted by the Biotechnology & GMOs Unit, has developed a deep expertise in the different analytical aspects involved in quali- and quantitative GMO analysis. The established and recognised leading role in developing, optimising and validating analytical tests for the detection, identification and quantification of GMOs led to the establishment, within the Biotechnology & GMOs Unit, of the Community Reference Laboratory for GM Food and Feed (CRL-GMFF; http://gmo-crl.jrc.ec.europa.eu/) in the context of Regulation (EC) No 1829/2003.

Principal legal duties and tasks of the CRL-GMFF, as defined in Annex I of Regulation (EC) No 1829/2003, are: 1) testing and validation of detection methods for identification of the transformation event in the food or feed and 2) preparation, storage and distribution to national reference laboratories of the appropriate positive and negative control samples.

Detailed rules for the implementation of Regulation (EC) No 1829/2003, and in particular requisites to be followed by applicants when submitting a method of detection to the CRL-GMFF, as specified in Annex I of Commission Regulation (EC) No 641/2004, include information about the method as such and about the method testing carried out by the applicant and demonstration that the method fulfils, among others, the following requirements:

1. Being event-specific and therefore functional only with the GMO or GM based product considered (and not functional if applied to other events).
2. Being applicable to samples of the food or feed, to the control samples and to the reference material.

Regulation (EC) No 1829/2003 also defines labelling requirements for both food and feed (Articles 12 and 24, respectively) establishing a threshold of 0.9%, calculated at the single ingredient level, for the adventitious or technically unavoidable presence of authorised GMOs, and it therefore combines – from the applicant point of view – the need of providing an event-specific method as prerequisite for approval, with the need of quantification.

Since the introduction of mandatory labelling requirements in 1997 [Regulation (EC) No 258/97] different analytical approaches have been developed for the purpose of GM quantification: among all alternatives tested, real-time PCR [Holland *et al.*, 1991] turned out to be the most successful, accurate and powerful technique for nucleic acid quantification and, accordingly, it is now the method of choice in the EU and worldwide for GM quantification [Miraglia *et al.*, 2004].

In line with what is indicated above, all methods submitted by applicants to the CRL-GMFF for validation are, so far, also meant for quantitative purposes and are based on the real-time PCR technique.

Real-time PCR is a modification of the traditional polymerase chain reaction technique that incorporates the ability to directly measure and quantify the reaction while amplification is taking place. Among the different chemistries developed for the purpose, the most widely used in GMO quantification is the TaqMan® approach [Heid *et al.*, 1996].

The chemistry is the key to the detection system (Fig. 1). A labelled probe (i.e. TaqMan®) designed to anneal to the target sequence between the

traditional forward and reverse primers, in addition to adding specificity to the reaction, produces a fluorescent signal that is proportional to the amount of PCR product being amplified.

The TaqMan® probe is labelled at the 5' end with a reporter fluorochrome (R) and with a quencher fluorochrome (Q) at the 3' end. As long as both fluorochromes are in proximity, the quencher molecule stops all fluorescence by the reporter. However, as *Taq* polymerase extends the primer, the intrinsic 5' to 3' nuclease activity of *Taq* degrades the probe, releasing the reporter fluorochrome. The amount of fluorescence released during the amplification phase is proportional to the amount of product generated in each cycle. The detection system is so sensitive that fewer than 10 copies of target DNA can be detected.

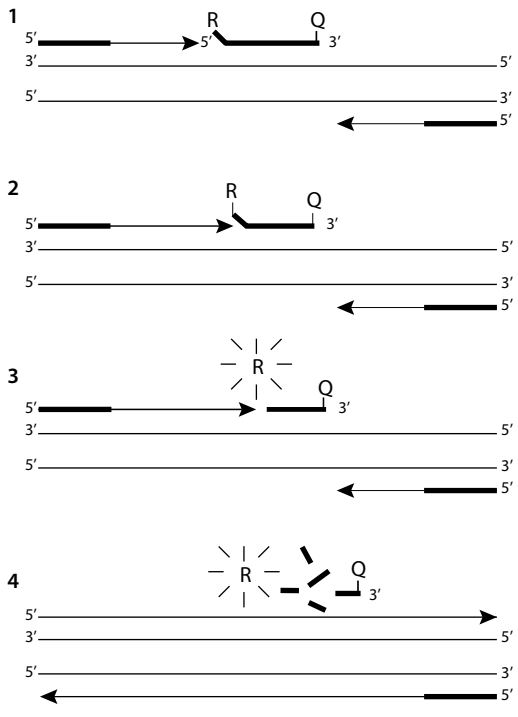

Figure 1. *TaqMan® principle in real-time PCR.* ***1)*** *Forward and reverse primers are extended by the Taq polymerase as in a traditional PCR reaction. A probe with two fluorescent dyes attached anneals to the target DNA sequence between the two primers.* ***(2)*** *As the Taq polymerase extends the primer, the probe is displaced.* ***(3)*** *The 5' nuclease activity of Taq polymerase cleaves the reporter dye from the probe.* ***(4)*** *After release of the reporter dye (R) from the quencher (Q), a fluorescent signal is generated.*

Whereas the wide diffusion and adoption of the real-time PCR approach relies on its reliability for DNA quantification, the technique is also more and more frequently used for end point analysis, for qualitative detection purposes, thanks to its increased intrinsic specificity and to the fact that it allows straight extrapolation of results directly from the instrument software avoiding analysis of PCR products by gel electrophoresis, a step that represents the main risk in terms of laboratory contamination [Reiting *et al.*, 2007].

As requested by the European Parliament, the project had the purpose of developing a fast and ready-to-use system for the detection of approved and unapproved GM events.

In the formulation of the project strategy all elements indicated above were considered and combined:

Analytical target(s): the approach is based on the detection of the different GM events by using event-specific methods. According to the mandate of Regulation (EC) No 1829/2003, the CRL-GMFF has a strategic comparative advantage, in that it is the first point of delivery, within the EU, of the information related to the molecular data of approved GMOs and in particular of the GMOs that, most probably already approved or commercialised elsewhere, are intended to be placed on the EU market. At the time of project formulation, the CRL-GMFF had received for validation dossiers containing molecular data and event-specific methods for the detection of 39 individual GM events (without considering 21 dossiers provided for the validation of methods for stacked GM lines) in 7 plant species (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

Maize	Oilseed rape	Cotton	Soybean	Rice	Sugar beet	Potato
Bt11	T45	MON1445	A2704-12	LLRICE62	H7-1	EH92-527-1
NK603	Ms8	MON88913	40-3-2	LLRice601		
GA21	Rf3	LLCotton25	MON89788	Bt63 Rice		
MON863	GT73	MON 531	DP-356043			
1507	Rf1	MON15985				
T25	Rf2	281-24-236 X				
59122	Ms1	3006-210-23				
MON810	Topas 19/2					
MIR604						
Bt176						
MON88017						
LY038						
3272						
MON89034						
Bt10						

***Table 1**. List of targets detected by the system. In addition to the 39 individual GM events included in the table, the system allows the detection of all stacked events derived from them, in 7 plant species.*

Methodological choice: the approach is based on real-time PCR. Indeed, real-time PCR, in addition to the intrinsic specificity mentioned above, has the advantage of being a technique already commonly diffused in the EU and worldwide and adopted by most if not all EU control laboratories. This choice guarantees the possibility of immediate use and integration in the laboratories working routine, without the need of acquisition of new instrumentation or of implementation of new procedures.

Format strategy: the format selected is in line with the aim of the project, i.e. to provide a rapid multi-target system (allowing the simultaneous detection of all targets in a single experiment) in a ready-to-use format, therefore reducing to the minimum the laboratory handling steps. The real-time PCR system will be delivered in the format of pre-spotted plates containing, in lyophilized format, all primers and probes for the individual detection of all 39 single-insert GM events (including both approved and non-approved) for which a method has been submitted to the CRL-GMFF, and of the corresponding 7 plants species (maize, cotton, rice, oilseed rape, soybean, sugar beet, and potato) (Table 1). To use system the operator would just need to perform a few simple steps: extract the DNA from the sample, mix it with the provided Universal PCR Master Mix, load the mixture on the plate, and start the time temperature programme. Results would then be extrapolated directly from the ad hoc computer software.

3 Timeline, milestones and deliverables

The ultimate deliverable of the project, by the end of 2007, is the production, testing, and evaluation of pre-spotted real-time PCR plates suitable for the event–specific detection of 39 GM events, approved and unapproved in the EU, and of the corresponding 7 plant species. An additional deliverable planned is their distribution to European control laboratories, members of the European Network of GMO Laboratories (ENGL), for additional testing. The project, as formulated, includes the production of a total amount of 1000 pre-spotted plates so distributed: 1) production of 50 pre-spotted plates as first delivery, intended for preliminary testing and verification of the functionality of the system and 2) production of remaining 950 plates for final method performance verification, distribution to ENGL laboratories and conduction of the survey.

Below, phases and milestones of the project:

1. Project strategy formulation (January 2007)
2. Data collection and verification (February/ March 2007)
3. Method definition (March/May 2007)
4. Delivery of methods information and result of analytical evaluation (May 2007)
5. Customisation of the 96-wells plates for pre-spotting of all 48 assays (June 2007)
6. Production of positive and negative DNA samples (June – August 2007)
7. Production and delivery (July 2007) of the first set of pre-spotted plates (No 50) for in-house testing
8. Intermediate reporting to the European Parliament (July 2007)
9. In-house testing (September – October 2007)
10. Method performance evaluation (November 2007)
11. Confirmation of suitability of experimental conditions and order confirmation for the production of the remaining 950 plates (November 2007)
12. Project and product presentation to ENGL laboratories (November 2007)
13. Distribution of plates to ENGL control laboratories for testing (Beginning 2008)
14. Data collection, obtained for ENGL laboratories participating in the testing phase and evaluation and assessment reporting (End 2008)

4 Report of activities and experimental testing

1. **Project strategy formulation.** The project strategy has been elaborated and formulated as indicated above. Practical implementation of the project implied the outsourced probes and primers synthesis and robotised spotting phases. Assessment of commercial companies providing this service was limited by the fact that some of the methods included the use of TaqMan® MGB probes, produced and distributed exclusively by the company Applied Biosystems. During this phase several meetings were held with experts of the company Applied Biosystems to discuss and clarify the different technical aspects of the project, in particular the ones related to method optimisation, robotised plate spotting, and confidentiality of data.

2. **Data collection and verification.** Molecular data (primers and probes sequences) of all methods submitted to the CRL-GMFF for the detection of single-insert GM events were retrieved from the Central Core Sequence Database of the Biotechnology & GMOs Unit, compared and manually verified with original dossiers submitted by applicants. Since for the detection of stacked GM lines applicants are required to submit one event-specific method for each parental GM event composing the stacked line, all stacked events, except one (cotton 281-24-236 X 3006-210-23) were already represented in the list of single insert events. A total of 41 different methods were selected (= all methods submitted to the CRL-GMFF for method validation represented once, including methods for emergency cases, e.g. Bt10 maize and LL601 rice) for the detection of a total of 39 GM events. This step included the ad hoc design of a real-time PCR method specific for Bt10 maize, the only event for which a quantitative method was not available. Primers and probes sequences are available upon request and according to confidentiality agreement respecting the mandate of the CRL-GMFF.

3. **Method definition.** The detection of all events in a single experiment (i.e. in the same plate) implies that all methods work and perform satisfactorily under the same experimental and cycling conditions. Evaluation of experimental compatibilities / incompatibilities among methods included primers and probes compositions, relative Tm and working concentration, PCR thermal protocols, reaction volumes and input DNA amounts.

The following are the common compatible experimental conditions selected:

- ✓ Individual reaction volume: 50 µl
- ✓ Primers and probes working concentrations: (900 nM Primers/250 nM Probes)
- ✓ Input DNA/reaction: 100 ng
- ✓ Reaction buffer: AB TaqMan® Universal PCR Master Mix
- ✓ Plate set-up: row based (Figure 2) including 48 assays. Each plate will enable the operator to analyse 2 samples in single replicate (or 1 sample in duplicate) with each assay.
- ✓ Cycling conditions:

Step	Stage	T°C	Time (sec)	Acquisition	Cycles
1	Initial denaturation	95°C	600	No	1
2	Denaturation Annealing & Extension	95°C 60°C	15 60	No Yes	45

4. Methods' details and consolidated experimental conditions were delivered at the end of May 2007 to the company Applied Biosystems (Applied Biosystems, Foster City, US) with confirmation of order for an initial amount of 50 pre-spotted plates intended for preliminary testing and verification of the functionality of the system.

5. Production of this initial amount of pre-spotted plates was preceded by the customisation and verification of the robotised system for the pre-spotting of 48 assays on 96-wells plates to guarantee absence of plate to plate variability.

6. **Production of positive and negative DNA samples:** Assay testing required the availability of DNA samples for all GM events included in the study and for the 7 wild type plants species.

   Large scale DNA extraction from the 7 wild type plants species started in June 2007. Required

	1	2	3	4	5	6	7	8	9	10	11	12	
A	HMG Maize Ref	SAH7 Cotton Ref	PLD Rice Ref	CruA Oilseed rape Ref	Lectin Soybean Ref	GS Sugarbeet Ref	UGPase Potato Ref	Bt11 Maize	NK603 Maize	GA21 Maize method 1	MON863 Maize	1507 Maize	Sample 1
B	T25 Maize	59122 Maize	H7-1 Sugarbeet	MON810 Maize	281-24-236 Cotton	3006-210-23 Cotton	LLRICE62 Rice	T45 Oilseed rape	EH92-527-1 Potato	Ms8 Oilseed rape	Rf3 Oilseed rape	GT73 Oilseed rape	
C	LLCotton25 Cotton	MON 531 Cotton	A2704-12 Soybean	MIR604 Maize	Rf1 Oilseed rape	Rf2 Oilseed rape	Ms1 Oilseed rape	Topas 19/2 Oilseed rape	MON1445 Cotton	Bt176 Maize	MON15985 Cotton	40-3-2 Soybean	
D	GA21 Maize method 2	MON88017 Maize	LY038 Maize	3272 Maize	MON89788 Soybean	MON89034 Maize	DP-356043 Soybean	MON88913 Cotton	Rice GM events P35S::bar	LLRice601 Rice	Bt63 Rice	Bt10 Maize	
E	HMG Maize Ref	SAH7 Cotton Ref	PLD Rice Ref	CruA Oilseed rape Ref	Lectin Soybean Ref	GS Sugarbeet Ref	UGPase Potato Ref	Bt11 Maize	NK603 Maize	GA21 Maize method 1	MON863 Maize	1507 Maize	Sample 2
F	T25 Maize	59122 Maize	H7-1 Sugarbeet	MON810 Maize	281-24-236 Cotton	3006-210-23 Cotton	LLRICE62 Rice	T45 Oilseed rape	EH92-527-1 Potato	Ms8 Oilseed rape	Rf3 Oilseed rape	GT73 Oilseed rape	
G	LLCotton25 Cotton	MON 531 Cotton	A2704-12 Soybean	MIR604 Maize	Rf1 Oilseed rape	Rf2 Oilseed rape	Ms1 Oilseed rape	Topas 19/2 Oilseed rape	MON1445 Cotton	Bt176 Maize	MON15985 Cotton	40-3-2 Soybean	
H	GA21 Maize method 2	MON88017 Maize	LY038 Maize	3272 Maize	MON89788 Soybean	MON89034 Maize	DP-356043 Soybean	MON88913 Cotton	Rice GM events P35S::bar	LLRice601 Rice	Bt63 Rice	Bt10 Maize	

Figure 2. *Plate set-up*

amounts were calculated according to the defined experimental design including verification of method efficiency, specificity, and LOD calculation for all assays analysed individually. Wild type plants species DNA was required both as negative control and as diluent to bring each GM event to the same GM % while guaranteeing the same amount of input DNA in each test. DNA from each plant species was extracted following the validated DNA extraction method for that species or applying the CTAB method [Murray *et al.*, 1980]. DNA extraction was followed by DNA concentration estimation by spectrophotometry and fluorimetry (Picogreen®) and inhibition test [Zel *et al.*, 2008] to assess DNA quality, absence of inhibitory compounds and optimal working concentration.

Extraction of DNA from all 39 GM events was conducted during the months of July and August 2007 using the same approach used for the wt using the corresponding validated methods. Positive control samples are available at the CRL-GMFF either as 100% pure GM material (flour), as purified GM DNA, or as 1% w/w food/feed sample. Accordingly, to standardise testing conditions for all assays, the highest GM concentration examined was 1% from which sequential dilutions were performed to estimate assays LOD.

7. The first set of 50 pre-spotted plates for in-house testing was delivered in July 2007.

8. As from project proposal timeline, an intermediate report containing structure and status of advancement of the project was delivered to the European Parliament in July 2007.

9. **In-house testing:** Verification of performance of the real-time PCR based ready-to-use analytical system included the following:

   - Confirmation of robotised spotting quality i.e. for all the 48 assays the same quality and quantity of primers and probes was delivered in each well of the plate with no cross contamination between adjacent wells

   - Verification of method performance, i.e. the specificity, reliability and efficiency was maintained for all methods, i.e. all methods were performing satisfactorily using the unique experimental conditions defined for the pre-spotted plates and no significant method performance deviation occurred in comparison with original validated conditions.

- Confirmation of specificity for each individual method. As specified in Annex I of Commission Regulation (EC) No 641/2004 each method submitted to the CRL-GMFF must be pre-validated by the notifier and it must meet defined performance criteria including test of specificity. In practice the fact that different methods are submitted by different notifiers and the differential development and submission times (spread over years) leads to the fact that that none of the methods submitted to the CRL-GMFF, and included in the present project, was tested for specificity against the whole range of GM events.

For experimental testing, individual GM DNA samples were diluted to working concentration in wild type DNA of the corresponding plant species to guarantee the same amount of DNA present in each plate well.

Individual samples for specificity tests were prepared as follows:

- **WT** = 20 ng/µl WT DNA solutions from each plant species. 5 µl/well (=100 ng total) loaded in each well.

- **GM** = WT and GM DNA stock solutions were used to prepare individual GM samples at 0.1% GM content (20 ng/µl). Each event (100 ng total DNA at 0.1% in 5 µl) was loaded in each well.

- Amplification reaction mixture in the final volume per reaction well:

Component	Final concentration	µl/ reaction
TaqMan® Universal PCR Master Mix (2x)	1x	25
Nuclease free water	#	20
Template DNA (100 ng at 20 ng/µl)	#	5.0
Total reaction volume:		50

Specificity of the system, and of each of the 48 methods included in the plate, was assessed by testing each wt plant species and each GM event individually against the whole set of methods at the cycling conditions indicated above.

In cases of doubtful results (false positives, contamination etc), tests were repeated, specifically for the method under evaluation, on normal RT-PCR plates under original method's validation conditions and under pre-spotted plates conditions.

LOD: System LOD will be defined on final batch of plates prior to distribution to ENGL laboratories. Preliminary sensitivity testing was performed by individually loading, in each well, according to the plate design, the corresponding DNA (GM or wt) sample in a final quantity of 100 ng/well.

The highest concentration tested was 0.1% w/w from which sequential dilutions have been performed. Lowest GM% tested so far 0.045%.

Absolute copy numbers equivalents detected in the different plant species (Table 2) were calculated for each event according to the nuclear content (average C1 value) of the individual plant species (Table 3) by dividing the sample DNA weight by the published average C1 value for the genome of the corresponding species.

Results obtained from the preliminary sensitivity testing are reported in Table 4. Data, reported as average Cts, are based on four repetitions. The four repetitions always fell within 1 Ct value.

The system was also tested with composite samples resembling real samples to be included in the survey to verify correspondence of results. Two examples are reported:

Sample 1: Test material GeM MU01 (GM events in Mixed Flours) from GeMMA Proficiency Scheme. As from Proficiency Scheme report, the sample was known to be: Positive for Roundup Ready soybean (not quantified but reported to be < 0.72% w/w), MON810 maize (1.29% w/w) and NK603 maize (1.33% w/w) and negative for Bt176 maize, Bt11 maize, GA21 maize, TC1507 maize and MON863 maize.

Common name	Total amount of DNA in reaction (ng/5 µl)	Species copy numbers	GM copies (0.1%)*	GM copies (0.045%)*
Maize	100	36.697	36	16.5
Cotton	100	42.918	42	19
Rice	100	222.222	222	100
Oilseed rape	100	86.956	86	39
Soybean	100	88.495	88	39.8
Sugar beet	100	80.000	80	36
Potato	100	55.555	55	25

Table 2. *Copy numbers equivalents detected for the 7 plant species.*

** GM copy numbers calculated assuming homozygous status for all GM events*

Scientific name	Common name	C1 average value
Zea mays	Maize	2.725 pg
Gossypium hirsutum (2n=4X)	Cotton	2.33 pg
Oryza sativa ssp	Rice	0.45 pg
Brassica napus	Oilseed rape	1.15 pg
Glycine max (2n=4X)	Soybean	1.13 pg
Beta vulgaris ssp. saccharifera	Sugar beet	1.25 pg
Solanum tuberosum (2n=4X)	Potato	1.8 pg

Table 3. *Nuclear DNA content of plant species included in the project (Arumuganathan and Earle, 1991).*

Method	average Ct
HGM Maize R	**23.596**
Bt11	42.115
NK603	38.669
GA21 (method 1)	37.949
GA21 (method 2)	36.504
MON863	36.447
1507	36.467
T25	32.056
59122	36.970
MON810	37.333
MIR604	32.628
Bt176	34.626
MON88017	36.441
LY038	37.328
3272	36.203
MON89034	36.720
Bt10	38.611
Lectin Soybean R	**21.337**
A2704-12	33.683
40-3-2	35.235
MON89788	33.616
DP-356043	34.446
UGPase Potato R	**16.372**
EH92-527-1	33.473
CruA Oilseed R	**23.020**
T45	34.730
Ms8	37.014
Rf3	37.076
GT73 (RT63)	34.574
Rf1	34.814
Rf2	33.809
Ms1	36.314
Topas 19/2	36.604
PLD Rice R	**21.170**
LLRICE62	33.795
LLRice601	32.416
Bt63	34.711
Rice P35S::bar	35.714
GS Sugar beet R	**21.954**
H7-1	36.037
SAH7 Cotton R	**27.359**
MON1445	38.263
MON88913	39.267
LLCotton25	40.776
MON 531	40.995
281-24-236	39.048
3006-210-23	39.766

Table 4. *Average Ct values for each method based on 4 repetitions. Input DNA = 100 ng/well. GM% = 0.045% (w/w). The four repetitions always fell within 1 Ct value.*

Qualitative results obtained with the pre-spotted plates matched at 100% with Proficiency Scheme test report.

Sample 2: Test material C4.4 (GM in corn flour) from FAPAS Proficiency Scheme. As from Proficiency Scheme report, the sample was known to be: Positive for MON810 maize (0.4%), GA21 maize (1.5%), Bt176 maize (0.8%), Bt11 (3.0%), Herculex (59122) maize (1.0%) and MON863 maize (1.5%) and negative for T25 maize, CBH351 maize and NK603 maize. Qualitative results obtained with the pre-spotted plates matched with Proficiency Scheme test report; in addition results indicated a minute contamination (Ct ~ 42-43) from GT73 oilseed rape, not tested during the Proficiency Scheme.

10. Upon evaluation of the results and verification of the suitability of experimental conditions, order confirmation was given to the company Applied Biosystems for the production of the remaining 950 plates (November 2007)

11. The formulation of the 'real-time PCR based ready-to-use multi-target analytical system' and the results so far obtained on system performance were presented in November 2007 at the Plenary Meeting of the ENGL. ENGL experts evaluated very positively both the system and the shown performance, and approximately 50 laboratories indicated their interest in further testing the system.

12. Distribution of plates to ENGL control laboratories for testing was conducted at the beginning of 2008 upon receipt of the remaining 950 pre-spotted plates and batch LOD definition.

13. The present report is prepared in fulfilment of timelines requirements, established within the project '*Scientific and technical contribution to the development of an overall health strategy in the area of GMOs*'. It is anticipated that an additional reporting will follow including assessment of presence of authorised and unauthorised GM events in the European market.

5 Conclusions

Experimental data as illustrated in the previous section, indicate that the 'real-time PCR based ready-to-use multi-target analytical system' developed by the Biotechnology & GMOs Unit in the context of the project '*Scientific and technical contribution to the development of an overall health strategy in the area of GMOs*' presented to and approved by the European Parliament early 2007 is fit for the purpose of detection of several GM events in a single experiment and, according to the purpose of the project, is a very useful tool for the conduction of a survey of the presence on the European market of authorised and unauthorised GM events. The system was developed to allow the simultaneous event-specific detection of 39 GM events belonging to 7 plant species and of the corresponding species-specific genes. Specificity of each of the methods (Figure 3) was confirmed and sensibility of the system allows the detection of even minute amounts of GMOs. The methodology and format selected allow the immediate implementation of the system since real-time PCR using the 96-well plates format is a technique commonly diffused in the EU and worldwide and adopted by most if not all EU control laboratories.

The system offers the unique opportunity to allow testing for all 39 events with minor handling required. Using the traditional approach, a series of sequential tests need to be performed on each sample to be analysed. Screening tests, based on the detection of the 35S promoter and the NOS terminator (regulatory sequences globally used in building GMOs), are generally applied at first to assay, irrespective of modification type, the presence of a GMO. Depending on the outcome of the 35S/NOS results, additional tests are performed for confirmation and identification purposes. Event-specific methods, the only ones allowing the univocal identification of each GM event, need so far to be performed one at the time.

Accordingly, testing for the presence of several GMOs in the same sample results in a huge amount of work, making it almost impossible for control laboratories to test each food/feed sample for all events. In contrast, by using the system just presented, the user would just need to perform a few simple steps: extract the DNA from the sample, mix it with the provided Universal PCR Master Mix, load the mixture on the plate and start the time temperature programme. Straight and immediate extrapolation of the results directly from the ad hoc instrument software imparts additional value to the system. A few examples are reported below, showing how results are visualised (Figures 4-7).

The developed system has the additional advantage of guaranteeing comparable results since plates are pre-developed and pre-tested to guarantee absence of plate to plate variability within the same batch.

This is an additional step towards harmonisation:

- The implementation of the ready-to-use system as described above will be a major step towards harmonisation throughout the European Community.
- The flexibility of the system allows the rapid inclusion of new methods targeting GM events for which a method becomes available.
- If all control laboratories use this system, results will be comparable between the laboratories. Double-checking of samples can be avoided.
- The laboratories will save time and reduce costs, because several individual steps are eliminated when using the system.
- Since only one source (e.g. JRC) for the deliverable of the ready-to-use system would be considered, laboratories don't need to test the reliability of the components individually.

UPPER 1/2 PLATE	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
LOWER 1/2 PLATE	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
METHOD vs SAMPLE	HGM Maize Ref	SAH7 Cotton Ref	PLD Rice Ref	CruA Oilseed rape Ref	Lectin Soybean Ref	GS Sugar beet Ref	UGPase Potato Ref	Bt11 Maize	NK603 Maize	GA21 Maize method 1	MON863 Maize	1507 Maize	T25 Maize	59122 Maize	H7-1 Sugar beet	MON810 Maize	281-24-236 Cotton	3006-210-23 Cotton	LLRICE62 Rice	T45 Oilseed rape	EH92-527-1 Potato	Ms8 Oilseed rape	Rf3 Oilseed rape	GT73 Oilseed rape
Maize	■																							
Cotton		■																						
Rice			■																					
Oilseed rape				■																				
Soybean					■																			
Sugar beet						■																		
Potato							■																	
Bt11 Maize	■							■																
NK603 Maize	■								■															
GA21 Maize (1)	■									■														
MON863 Maize	■										■													
1507 Maize	■											■												
T25 Maize	■												■											
59122 Maize	■													■										
H7-1 Sugar beet						■									■									
MON810 Maize	■															■								
281-24-236X3006-210-23		■															■	■						
LLRICE62 Rice			■																■					
T45 Oilseed rape				■																■				
EH92-527-1 Potato							■														■			
Ms8 Oilseed rape				■																		■		
Rf3 Oilseed rape				■																			■	
GT73 Oilseed rape		■		■																				■
LLCotton25 Cotton		■																						
MON 531 Cotton		■																						
A2704-12 Soybean					■																			
MIR604 Maize	■																							
Rf1 Oilseed rape				■																				
Rf2 Oilseed rape				■																				
Ms1 Oilseed rape				■																				
Topas 19/2 Oilseed rape				■																				
MON1445 Cotton		■																						?
Bt176 Maize	■																							
MON15985 Cotton		■																						
40-3-2 Soybean					■																			
GA21 Maize (2)	■									■														
MON88017 Maize	■																							
LY038 Maize	■																							
3272 Maize	■																							
MON89788 Soybean					■																			
MON89034 Maize	■																							?
DP-356043 Soybean					■																			
MON88913 Cotton		■																						
LLRice601 Rice			■																					
Bt63 Rice			■																					
Bt10 Maize	■																							

UPPER 1/2 PLATE	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
LOWER 1/2 PLATE	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
METHOD vs SAMPLE	LLCotton25 Cotton	MON 531 Cotton	A2704-12 Soybean	MIR604 Maize	Rf1 Oilseed rape	Rf2 Oilseed rape	Ms1 Oilseed rape	Topas 19/2 Oilseed rape	MON1445 Cotton	Bt176 Maize	MON15985 Cotton	40-3-2 Soybean	GA21 Maize method 2	MON88017Maize	LY038 Maize	3272 Maize	MON89788 Soybean	MON89034	DP-356043 Soybean	MON88913 Cotton	Rice P35S::bar	LLRice601 Rice	Bt63 Rice	Bt10 Maize
Maize																								
Cotton																								
Rice																								
Oilseed rape																								
Soybean																								
Sugar beet																								
Potato																								
Bt11 Maize																								
NK603 Maize																								
GA21 Maize (1)													■											
MON863 Maize																								
1507 Maize																								
T25 Maize																								
59122 Maize																								
H7-1 Sugar beet																								
MON810 Maize																								
281-24-236X3006-210-23																								
LLRICE62 Rice																					■			
T45 Oilseed rape																								
EH92-527-1 Potato																								
Ms8 Oilseed rape																								
Rf3 Oilseed rape																								
GT73 Oilseed rape									?															
LLCotton25 Cotton	■																							
MON 531 Cotton		■																						
A2704-12 Soybean			■																					
MIR604 Maize				■																				
Rf1 Oilseed rape					■																			
Rf2 Oilseed rape						■																		
Ms1 Oilseed rape							■																	
Topas 19/2 Oilseed rape								■																
MON1445 Cotton									■															
Bt176 Maize										■														
MON15985 Cotton		■									■													
40-3-2 Soybean												■												
GA21 Maize (2)													■											
MON88017 Maize														■										
LY038 Maize															■									
3272 Maize																■								
MON89788 Soybean																	■							
MON89034 Maize																		■						
DP-356043 Soybean																			■					
MON88913 Cotton																				■				
LLRice601 Rice																					■	■		
Bt63 Rice																							■	
Bt10 Maize																								■

Figure 3. *Summary of results from specificity tests performed on each method included in the system. Columns correspond to methods, and rows to individual samples. Green dots indicate correct result; orange dots indicate unexpected result (i.e. method unspecificity or contamination of the sample). As shown, only results related to three method/sample combinations do not correspond to expectation. In those cases confirmation was not possible due to unavailability of certified controls.*

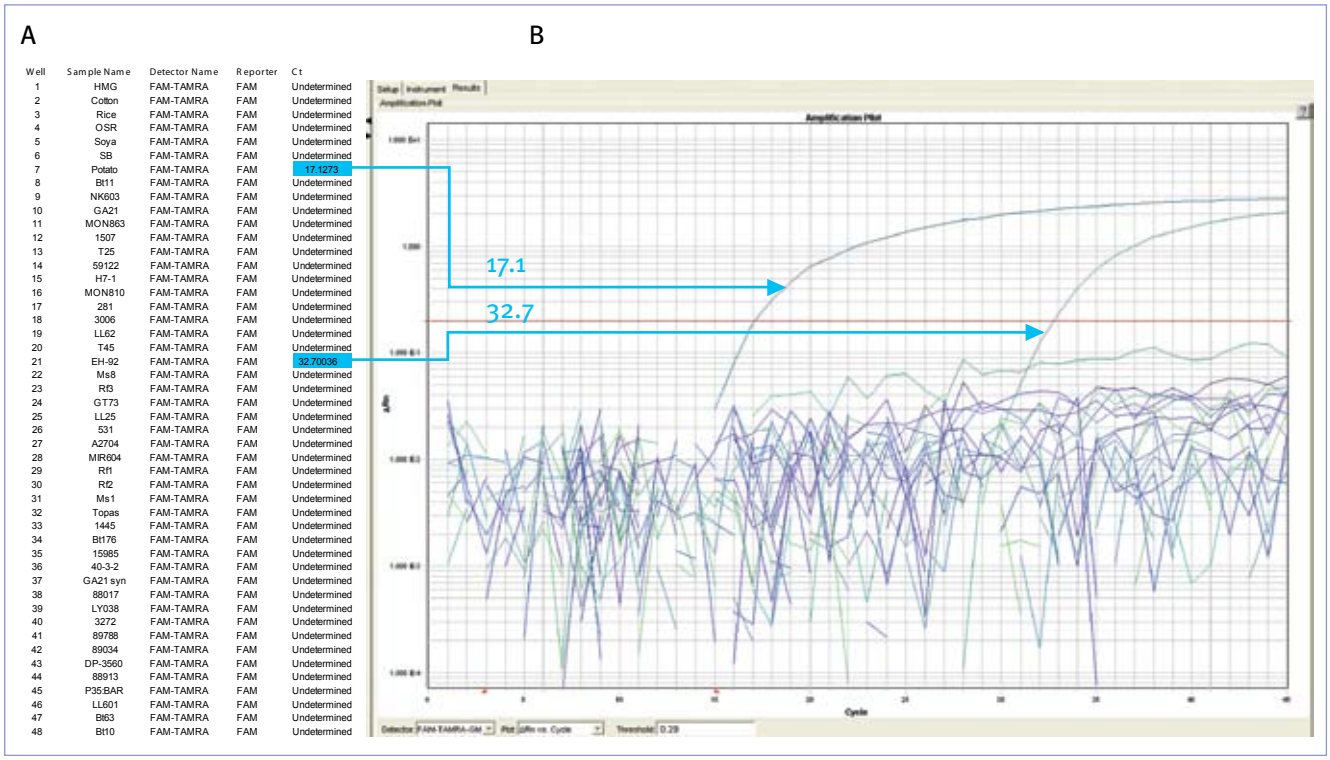

***Figure 4**. Detection of potato event EH92-527-1. A. Interpretation of the results from the table: well A7 corresponds to the potato reference gene method while well B9 corresponds to the EH92-527-1 event-specific method. B. Graphic representation of results: curves above the threshold line (red horizontal line) indicate positive reaction for potato reference gene and for event EH92-527-1.*

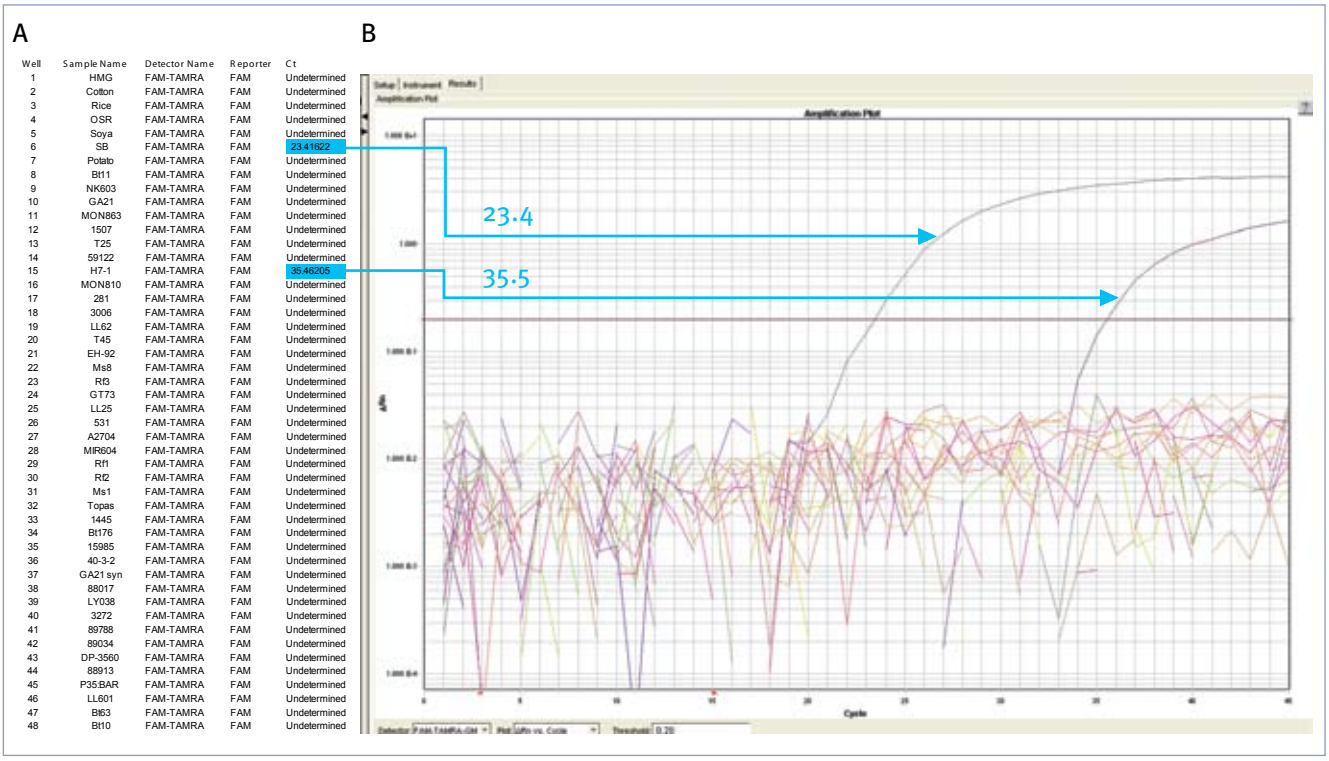

***Figure 5**. Detection of sugar beet event H7-1. A. Interpretation of the results from the table: well E6 corresponds to the sugar beet reference gene method while well F3 corresponds to the H7-1 event-specific method. B. Graphic representation of results: curves above the threshold line (red horizontal line) indicate positive reaction for sugar beet reference gene and for event H7-1.*

A

Well	Sample Name	Detector Name	Reporter	Ct
1	HMG	FAM-TAMRA	FAM	Undetermined
2	Cotton	FAM-TAMRA	FAM	25.41579
3	Rice	FAM-TAMRA	FAM	Undetermined
4	OSR	FAM-TAMRA	FAM	Undetermined
5	Soya	FAM-TAMRA	FAM	Undetermined
6	SB	FAM-TAMRA	FAM	Undetermined
7	Potato	FAM-TAMRA	FAM	Undetermined
8	Bt11	FAM-TAMRA	FAM	Undetermined
9	NK603	FAM-TAMRA	FAM	Undetermined
10	GA21	FAM-TAMRA	FAM	Undetermined
11	MON863	FAM-TAMRA	FAM	Undetermined
12	1507	FAM-TAMRA	FAM	Undetermined
13	T25	FAM-TAMRA	FAM	Undetermined
14	59122	FAM-TAMRA	FAM	Undetermined
15	H7-1	FAM-TAMRA	FAM	Undetermined
16	MON810	FAM-TAMRA	FAM	Undetermined
17	281	FAM-TAMRA	FAM	Undetermined
18	3006	FAM-TAMRA	FAM	Undetermined
19	LL62	FAM-TAMRA	FAM	Undetermined
20	T45	FAM-TAMRA	FAM	Undetermined
21	EH-92	FAM-TAMRA	FAM	Undetermined
22	Ms8	FAM-TAMRA	FAM	Undetermined
23	Rf3	FAM-TAMRA	FAM	Undetermined
24	GT73	FAM-TAMRA	FAM	Undetermined
25	LL25	FAM-TAMRA	FAM	Undetermined
26	531	FAM-TAMRA	FAM	37.62348
27	A2704	FAM-TAMRA	FAM	Undetermined
28	MIR604	FAM-TAMRA	FAM	Undetermined
29	Rf1	FAM-TAMRA	FAM	Undetermined
30	Rf2	FAM-TAMRA	FAM	Undetermined
31	Ms1	FAM-TAMRA	FAM	Undetermined
32	Topas	FAM-TAMRA	FAM	Undetermined
33	1445	FAM-TAMRA	FAM	Undetermined
34	Bt176	FAM-TAMRA	FAM	Undetermined
35	15985	FAM-TAMRA	FAM	35.40573
36	40-3-2	FAM-TAMRA	FAM	Undetermined
37	GA21 syn	FAM-TAMRA	FAM	Undetermined
38	88017	FAM-TAMRA	FAM	Undetermined
39	LY038	FAM-TAMRA	FAM	Undetermined
40	3272	FAM-TAMRA	FAM	Undetermined
41	89788	FAM-TAMRA	FAM	Undetermined
42	89034	FAM-TAMRA	FAM	Undetermined
43	DP-3560	FAM-TAMRA	FAM	Undetermined
44	88913	FAM-TAMRA	FAM	Undetermined
45	P35:BAR	FAM-TAMRA	FAM	Undetermined
46	LL601	FAM-TAMRA	FAM	Undetermined
47	Bt63	FAM-TAMRA	FAM	Undetermined
48	Bt10	FAM-TAMRA	FAM	Undetermined

B

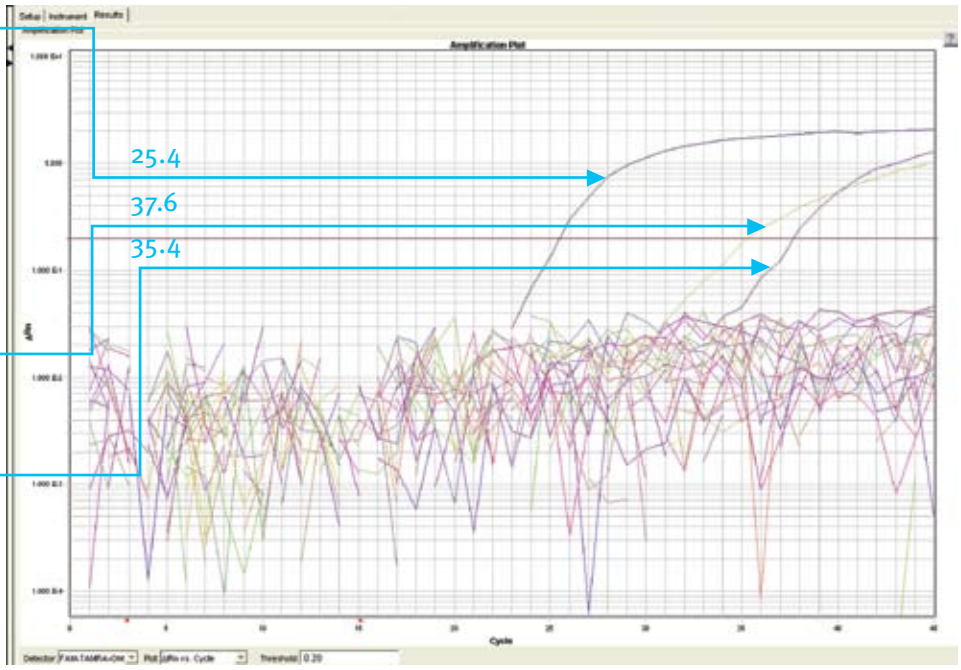

***Figure 6**. Detection of cotton event MON15985. A. Interpretation of the results from the table: well E2 corresponds to the SAH7 cotton reference gene method, wells G2 and G11 correspond to the MON531 and MON15985 event-specific methods, respectively. B. Graphic representation of results: curves above the threshold line (red horizontal line) indicate positive reaction for SAH7 cotton reference gene and for events MON531 and MON15985.*

A

Well	Sample Name	Detector Name	Reporter	Ct
1	HMG	FAM-TAMRA	FAM	24.1
2	Cotton	FAM-TAMRA	FAM	Undetermined
3	Rice	FAM-TAMRA	FAM	Undetermined
4	OSR	FAM-TAMRA	FAM	Undetermined
5	Soya	FAM-TAMRA	FAM	Undetermined
6	SB	FAM-TAMRA	FAM	Undetermined
7	Potato	FAM-TAMRA	FAM	Undetermined
8	Bt11	FAM-TAMRA	FAM	Undetermined
9	NK603	FAM-TAMRA	FAM	Undetermined
10	GA21	FAM-TAMRA	FAM	Undetermined
11	MON863	FAM-TAMRA	FAM	Undetermined
12	1507	FAM-TAMRA	FAM	Undetermined
13	T25	FAM-TAMRA	FAM	Undetermined
14	59122	FAM-TAMRA	FAM	Undetermined
15	H7-1	FAM-TAMRA	FAM	Undetermined
16	MON810	FAM-TAMRA	FAM	Undetermined
17	281	FAM-TAMRA	FAM	Undetermined
18	3006	FAM-TAMRA	FAM	Undetermined
19	LL62	FAM-TAMRA	FAM	Undetermined
20	T45	FAM-TAMRA	FAM	Undetermined
21	EH-92	FAM-TAMRA	FAM	Undetermined
22	Ms8	FAM-TAMRA	FAM	Undetermined
23	Rf3	FAM-TAMRA	FAM	Undetermined
24	GT73	FAM-TAMRA	FAM	Undetermined
25	LL25	FAM-TAMRA	FAM	Undetermined
26	531	FAM-TAMRA	FAM	Undetermined
27	A2704	FAM-TAMRA	FAM	Undetermined
28	MIR604	FAM-TAMRA	FAM	Undetermined
29	Rf1	FAM-TAMRA	FAM	Undetermined
30	Rf2	FAM-TAMRA	FAM	Undetermined
31	Ms1	FAM-TAMRA	FAM	Undetermined
32	Topas	FAM-TAMRA	FAM	Undetermined
33	1445	FAM-TAMRA	FAM	Undetermined
34	Bt176	FAM-TAMRA	FAM	Undetermined
35	15985	FAM-TAMRA	FAM	Undetermined
36	40-3-2	FAM-TAMRA	FAM	Undetermined
37	GA21 syn	FAM-TAMRA	FAM	Undetermined
38	88017	FAM-TAMRA	FAM	Undetermined
39	LY038	FAM-TAMRA	FAM	Undetermined
40	3272	FAM-TAMRA	FAM	Undetermined
41	89788	FAM-TAMRA	FAM	Undetermined
42	89034	FAM-TAMRA	FAM	Undetermined
43	DP-3560	FAM-TAMRA	FAM	Undetermined
44	88913	FAM-TAMRA	FAM	Undetermined
45	P35:BAR	FAM-TAMRA	FAM	Undetermined
46	LL601	FAM-TAMRA	FAM	Undetermined
47	Bt63	FAM-TAMRA	FAM	Undetermined
48	Bt10	FAM-TAMRA	FAM	35.53

B

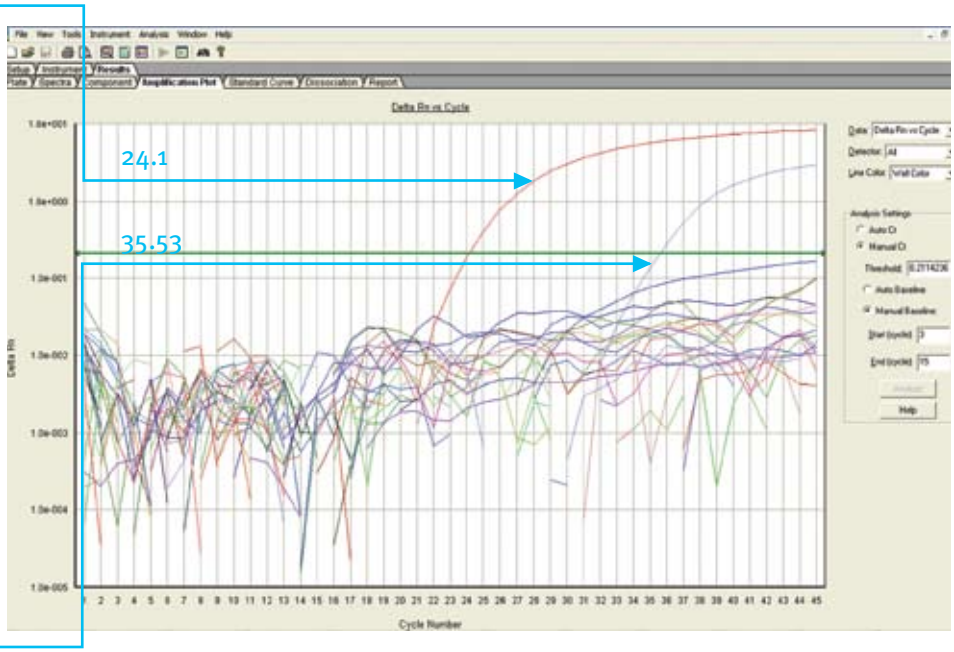

***Figure 7.** Detection of unapproved maize event Bt10. Event-specific detection. A. Interpretation of the results from the table: well A1 corresponds to the maize reference gene method while well D12 corresponds to the Bt10 event-specific method. B. Graphic representation of results: curves above the threshold line (green horizontal line) indicate positive reaction for maize reference gene and for event Bt10.*

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European Commission

EUR 23542 EN – Joint Research Centre – Institute for Health and Consumer Protection

Title: **Scientific and technical contribution to the development of an overall health strategy in the area of GMOs**

Authors: Maddalena Querci, Gijs Kleter, Jean-Paul Malingreau, Hermann Broll, Guy Van den Eede

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Abstract

The present report is the outcome of Project "Scientific and technical contribution to the development of an overall health strategy in the area of GMOs" carried out by the Biotechnology & GMOs Unit of the Institute for Health and Consumer Protection (Joint Research Centre) in the frame of contract CT 30249, requested by the European Parliament Committee on Industry, Research and Energy. The study provides current knowledge on the issue and up-to-date opinions of experts on the assessment of the potential short, medium and long term health impact associated with the consumption of GMOs. In addition, it contains the outcome of a research component that lead to the development of a multi-target analytical tool for the simultaneous identification of 39 GMOs.

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