



CURRENT AWARENESS
OF ISSUES RELATED TO
GENETICALLY MODIFIED FOOD
AND FOOD FROM CLONED ANIMALS

January - June 2008

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1. SUMMARY

This report is one of a series intended to provide NZFSA with information on current and emerging food safety issues related to GM Foods, and foods derived from cloned animals, which contributes to effective food policy, regulatory and risk management activities.

This report covers selected developments in the period January to June 2008, and includes:

1. Evaluation of protein safety in the context of agricultural biotechnology

One of the important components of the safety assessment of foods from GM crops is the evaluation of the safety of newly expressed proteins. During the reporting period the International Life Science Institute (ILSI) International Food Biotechnology Committee published a report (Delaney *et al.*, 2008) outlining a two tiered, weight-of-evidence strategy to assess the safety of novel proteins used in the context of agricultural biotechnology. This strategy incorporates a number of the components currently provided in most risk assessments for GM crops. The report provides useful background to the rationale for these assessments and concludes with some suggestions for areas where further research could strengthen the safety assessment of novel dietary proteins.

2. Multigenerational feeding studies

In the safety assessment of foods from GM plants the requirement for long duration rodent feeding trials is optional. One of the criticisms of the safety assessment of GM food by opponents of GM technology is the lack of information from long-term multigenerational feeding trials. During the reporting period a study was published describing a three generational feeding trial of rats with Bt corn. Overall the conclusion was drawn that, although the results obtained from the study showed minor histopathological and biochemical effects on rats fed with Bt corn, long-term, three generational consumption did not cause any severe health concerns for rats. It is suggested by the authors that this work provide a basis from which to extend multigenerational feeding trials with GM crops to other species as a tool in the suite of analyses undertaken to assess the safety of GM foods.

3. A detection method for dietary DNA from GM foods incorporated into a recipient genome

A food safety issue of particular concern to the public is the possibility that GM material from food could be transferred into the recipient's genome after ingestion (known as horizontal gene transfer, HGT). The concerns are that this could result from the direct ingestion of GM food by humans, or the ingestion of GM feeds by food animals that are then a source of human food; and that if a dietary DNA fragment is internalized in the genome of a recipient organism mutagenic or oncogenic consequences may occur. However, the ability to detect integrated DNA from a potential HGT event has remained problematic. Without the ability to conclusively show the integration of dietary DNA into a recipient's genome the likelihood and potential risks from HGT remain speculative only. During the reporting period researchers published a modified PCR method capable of detecting a single integration in a single dividing cell in a tissue sample from salmon. This method has the potential to be able to detect a specific target DNA, in a high background of genomic DNA, to a level capable of detecting potential HGT events.

4. Unauthorized rice line 'Bt63' in rice food products from China

The detection of the unauthorized rice line 'Bt63' highlights ongoing issues with the monitoring and surveillance of food products for GM material. The issue of unknown GM events entering the international food chain illustrates the need for rapid response with suitable testing methods. The 'Bt63' rice situation illustrated how a Rapid Alert System and the availability of laboratories with expertise in test development can enable an unknown GM event to be detected and monitored. It also highlights the need for robust testing methods that can be rapidly modified to incorporate new testing requirements.

5. Development of a modular system for detecting GM components in foods

Any monitoring for compliance with regulation for labelling of GM material in food products requires robust testing systems able to detect an increasingly large number of GM events. With the increasing number of GM foods reaching the market place there is a major analytical challenge from the need to continuously develop and establish further assays. This requires the availability of flexible testing systems that can be easily expanded. Established PCR based systems for GMO detection and quantification are increasingly unable to cover the range of assays needed in a single, rapidly expandable platform. A recent publication from Germany outlines the development of a modified PCR assay system that could be used

as a modular system for detection of GMOs in foods. In summary, this methodology provides the opportunity to establish a profiling-like platform in which simultaneous testing can be carried out for taxon-specific reference genes, screening elements for GM material and construct/event specific sequences. This would enable the simultaneous detection of authorized, unauthorized and unknown GM events. The modular system should enable easy extension of the targets within an assay to incorporate new sequences of interest.

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2. INTRODUCTION

This project is intended to provide the New Zealand Food Safety Authority with an independent source of current information on genetically modified foods (GMFs) and foods from cloned animals. The principal activity of this project is to survey the current scientific literature to keep abreast of developments in key areas of food safety, selecting five key articles within the subject areas specified and providing comment on the significance to NZFSA for use in its policy, regulatory and risk management activities.

The studies/topics have been chosen from within the following subject areas:

- Novel techniques for developing GM plants/animals and the implications on current detection methods;
- Animal feeding studies – specifically within the area of foods derived from GMFs or foods from cloned animals;
- Food safety and/or composition studies on GMFs and/or foods from cloned animals;
- Adventitious presence issues for GMFs and new GM varieties approved for food use, with particular emphasis on describing how other countries have responded with regard to audits and/or testing regimes and safety assessments, and providing relevant information and discussion of the actual food safety risks.

This is the second report for the 2007/2008 year and covers the period from January to June 2008.

Wider issues concerned with environmental or social effects of genetic modification and genetically modified organisms (GMOs), biodiversity, gene transfer, insect resistance, etc., are not covered in this report. This reflects the division of responsibility for genetically modified material, between the New Zealand Food Safety Authority and Food Standards Australia New Zealand (FSANZ) for GMFs on one hand, and the Environmental Risk Management Authority (ERMA) for GMOs on the other.

For consistency, some alternative terms have been standardised in this report. “Corn” and “maize” are interchangeable; in this document “corn” is used throughout. Canola is a genetic variation of rapeseed (or oilseed rape) developed by traditional plant breeding to be low in both erucic acid and glucosinolates (“double low” variety). In this document “canola” is used for this “double low” variety of rapeseed.

Source material:

Specific studies that are discussed in this report are referenced at the end of the relevant section. Any additional source of background material is also referenced. Where there is no reference to background material it is either taken from the study document or is general scientific knowledge of the report author.

2.1. Abbreviations used throughout this document

EC: European Commission

EU: European Union

FSANZ: Food Standards Australia New Zealand

GMO: genetically modified organism

OECD: Organisation for Economic Co-operation and Development

PCR: polymerase chain reaction

US EPA: United States Environmental Protection Agency

3. FOOD SAFETY AND COMPOSITION STUDIES

3.1. Evaluation of protein safety in the context of agricultural biotechnology

Whilst the generation of transgenic crops involves the insertion of a transgene into the plant's DNA, the result of this modification, in most cases¹, is the expression of novel protein(s) in the plant. In many cases the novel protein will be present in food or feed obtained from these GM plants. Therefore one of the important components of the safety assessment of foods from GM crops is the evaluation of the safety of novel expressed proteins. During the reporting period the International Life Science Institute (ILSI²) International Food Biotechnology Committee published a report (Delaney *et al.*, 2008) outlining a two tiered, weight-of-evidence strategy to assess the safety of novel proteins used in the context of agricultural biotechnology. This strategy incorporates a number of the components currently provided in most risk assessments for GM crops. The report provides useful background to the rationale for these assessments and concludes with some suggestions for areas where further research could strengthen protein safety assessment. Information provided in the report is summarized below. A discussion on the assessment of novel dietary proteins as potential allergens is specifically omitted from the ILSI report as this has been covered extensively by other researchers³.

3.1.1. *Introduction to the biology and chemistry of proteins*

The DNA content of an organism, its genome, can be considered as a 'building blue print' for life. The genetic information in the 'blue print' (i.e. that is encoded in the genes) is translated into proteins which are essential to cellular metabolism and provide a range of functions. These include enzymes to facilitate metabolic reactions, structural molecules, storage molecules, transport molecules, antibodies involved in cellular defence mechanisms and hormones. Proteins are complex macromolecules made up of amino acid units. A group of twenty amino acids is utilized by cells to synthesize proteins. The amino acids are joined together like beads on a string and the

¹ Where the GM crop is developed using RNAi techniques the modification will not result in the direct expression of a novel protein but more likely the reduced expression or complete absence of a protein traditionally found in the plant. See Section 2.2 CA Report FW08007 for a discussion of the use of RNAi techniques in biotechnology.

² ILSI is a non-profit, worldwide foundation established in 1978 to advance the understanding of scientific issues relating to nutrition, food safety, toxicology, risk assessment and the environment.

string can then fold and twist around itself (see Figure 1). The specific amino acid content of the string and the relative position of the amino acids are determined by the DNA sequence of the gene encoding that protein.

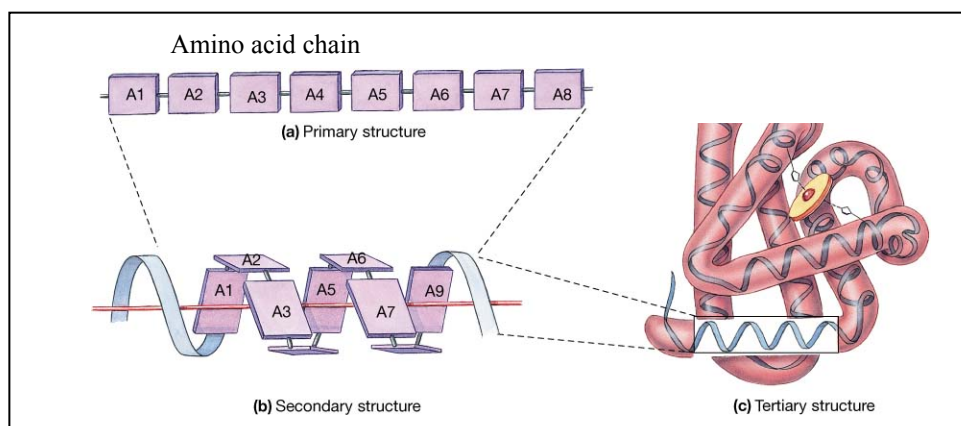


Figure 1 Amino acids joined to give a protein molecule

Taken from: www.stat.rice.edu

The number of theoretically possible combinations of amino acids used to produce proteins is practically limitless. Hence the number of proteins that exists in nature is very large. To date, the amino acid sequences of more than 2.8 million proteins of diverse structure and function have been either experimentally determined or predicted based on experimentally determined DNA sequence. The most fundamental aspect of proteins as functional biological molecules is that the molecular structure and chemical properties of the various amino acids along the string determine the 3-D shape of a protein which determines the molecular stability and, ultimately, the protein's functionality. Most importantly, loss of protein structure leads to loss of biological function. Further background information on amino acids and protein structure can be found in Section 3.1.1 CA Report FW08007.

Despite the theoretically huge number of proteins that could be present in nature, most of the proteins identified to date can be classified into a limited range of families based on their relatedness in structure and function. Most of these protein families are present in both animals and plants and could therefore be consumed in the diet.

³ See Section 2.2.2 CA Report FW07007 for information on the evaluation of human allergenic potential of novel proteins in GM foods.

3.1.2. *Proteins in the diet*

During normal cellular metabolism proteins are constantly being synthesized and degraded in body tissues. Therefore a constant supply of new amino acids is required. Some of these amino acids are supplied directly from breakdown of proteins in the body while others are synthesized by the body from precursors or by modification of other amino acids. For example, breakdown of enzymes and structural proteins from mucosal cells released into the gastrointestinal tract (GI) from the intestinal walls are thought to contribute between 35 and 200g of amino acids daily. Another source of amino acids is the diet. However, humans can only synthesize 11 of the 20 common amino acids and so need to obtain the other 9 (the essential amino acids) from their diet. Several important metabolic conditions can occur if the diet has an imbalance in the range of amino acids, including Kwashiorkor (a form of malnutrition). For example; corn, rice, wheat and potatoes are not good staple diets as they do not contain all of the necessary essential amino acids, while they would be provided by a diet of corn and beans. The remaining amino acids are then synthesized by the body or recycled from protein breakdown.

When proteins are ingested in the diet the structural integrity and functional activity of most of these proteins is denatured and broken down by the physical conditions (temperature, pH and degree of emulsification) in the GI tract, in combination with proteolytic enzymes (e.g. pepsin, trypsin, chymotrypsin and bacterial proteases). The potential for systematic absorption of large, intact dietary macromolecules like proteins is much lower than it is for small molecular weight molecules such as the amino acids. Therefore the likelihood of an intact functional protein being directly absorbed into the body after ingestion is low. All dietary proteins are essentially subject to the same digestive processes regardless of protein source or function. Protein digestion in the human GI tract is an efficient system. Only 6-12% of the daily protein intake from food and recycled enzymes and mucosal cells in the GI tract is lost in faeces.

Despite the efficient system of protein degradation in the GI tract, some dietary protein can survive intact in small quantities. The stable egg protein ovalbumin has been detected in both plasma and lymph fluid after oral administration. There are also examples of larger proteins being broken down into smaller fragments (peptides) that are bioactive and can be absorbed. Certain food matrices and composition can have a semi-protective effect on protein degradation. Factors that

influence the digestive and absorption processes of the GI tract can also influence protein degradation and absorption and may be associated with certain sub-populations. For example; age related factors (e.g. prevalent in infants or the elderly), diseases such as gastroenteritis or Crohn's disease and certain types of medication.

3.1.3. *Known toxic dietary proteins and proteins that may constitute a dietary hazard*

A large number of proteins have been isolated from nature. Proportionally, very few of these have been shown to be toxic to humans or other mammals. Of those that have, even fewer have shown toxicity when administered orally. This is largely due to the lability of proteins in the GI tract. Potentially hazardous dietary proteins fall into several classes:

1. Toxins from pathogenic bacteria

Some bacterial protein toxins are present in food contaminated with a particular organism, e.g. the botulism toxins produced by *Clostridium botulinum*, and are therefore ingested directly with the food. Botulism protein toxins are interesting as they actually utilize the 'degradation barrier' of the GI environment. They are produced in the food as a pro-toxin which is progressively degraded in the GI tract until it forms a biologically active protein toxin that is taken up into the systemic circulatory system. Other bacterial toxins are produced by pathogenic organisms in the small intestine after ingestion. These toxins, often called enterotoxins, therefore by-pass the protein degrading environment of the stomach.

2. Plant and animal protein toxins

The number of known protein toxins produced by plants and animals is considerably smaller than the number known to be produced by bacteria. Protein toxins that have been identified in plants include antifungal proteins and lectins. These proteins are thought to act in the plant against plant pathogens and insects. Some are also toxic to mammals. The most toxic plant lectin identified to date is ricin, from the seeds of the castor bean plant. Another example is the digestion-resistant lectin PHA (*Phaseolus vulgaris* haemagglutinin) which is found in kidney beans. This molecule binds to and damages epithelial cells in the intestines and results in impaired digestion. However, while resistant to digestive degradation, the structure and therefore functional activity of PHA is destroyed by cooking. This allows

kidney beans to be safely eaten when cooked appropriately. Some plants also produce proteins known as antinutrients. These are not toxins *per se* but can interfere with the normal digestion and absorption of nutrients in the GI tract and if ingested in large amounts and/or consistently can have a negative effect on growth of an organism. Antinutrients include enzyme inhibitors; for example trypsin inhibitor and amylase inhibitors. Some antinutrients can become toxic if ingested at high concentrations. Protein toxins are also produced in the venom of some snakes and scorpions, however, exposure to these toxins is unlikely to be via an oral route.

Some of the dietary proteins that are known to cause oral toxicity have been characterised and their sequence is present in general protein sequence databases for scrutiny. In some cases these proteins are known to be resistant to digestive processes that degrade other innocuous dietary proteins.

3.1.4. *Evaluation of the safety of dietary proteins*

As described above, proteins are an important component of the human diet and, while some naturally occurring dietary plant protein toxins are known, the consumption of proteins is not generally considered to be hazardous. However, because proteins introduced into transgenic crops may not have formed part of the historical human diet, the safety of these proteins may not be known. Therefore a safety assessment of novel proteins expressed in transgenic plants should form a part of the overall safety assessment of GM crops as food.

The predominant differences between dietary proteins and most other food additives or chemicals relate to:

- (i) their macromolecular size, which tends to preclude direct absorption from the GI tract, and;
- (ii) their biological activity, which is dependant on 3-D structure and which is largely destroyed by the GI tract environment.

However, the basic principals of safety assessment as applied to other food additives or chemicals provide a starting point for the assessment of dietary proteins. Therefore important aspects to be considered in the safety assessment of dietary proteins can be found in the concepts and approaches

to the risk assessment of food additives or GRAS⁴ (generally recognized as safe) food ingredients and chemicals. Guidelines for these assessments are relatively well established (US FDA, 1983).

Two types of approach can be taken when undergoing a safety assessment:

- ♦ The decision tree approach, which provided distinct yes/no decisions that lead to a specific next step, or;
- ♦ The weight-of-evidence approach, which focuses on the holistic totality of the evidence.

A weight-of-evidence strategy has been proposed for the safety assessment of dietary proteins from GM crops. This strategy is a two tier approach (outlined in Figure 2) and draws on knowledge about the physical and chemical characteristics of proteins as well as the available testing methods for assessing potential food hazards. Components of Tier I of the assessment strategy are able to be applied early in the development of the GM crop, in some cases even before the genetic modification is carried out. When data from the Tier I assessment is incomplete, equivocal or identifies a potential hazard the strategy proposes that Tier II components should then be addressed. This would be done on a case-by-case basis. Tier II assessments would likely require the large scale production, isolation and characterization of the novel protein expressed in the GM crop. Such large scale production usually requires the expression of the novel protein in a bacterial system and a requirement of the assessment would include demonstration that the bacterially-derived protein was equivalent to the protein produced *in planta*.

⁴ Generally Recognized as Safe (GRAS) is a United States of America Food and Drug Administration (FDA) designation that a chemical or substance added to food is considered safe by experts, and so is exempted from the usual Federal Food, Drug, and Cosmetic Act (FFDCA) food additive tolerance requirements. GRAS exemptions are granted for substances that are generally recognized, among experts qualified by scientific training and experience to evaluate their safety, as having been adequately shown through scientific procedures to be safe under the conditions of their intended use.

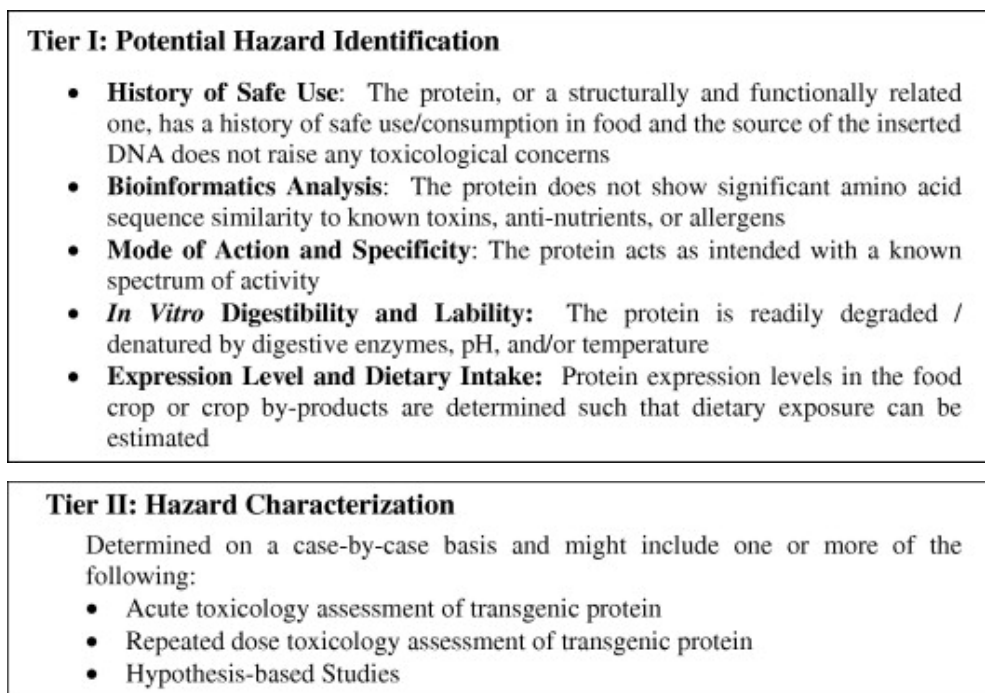


Figure 2 Two tier weight-of-evidence testing strategy proposed for the safety assessment of dietary proteins from GM crops. (Taken from Figure 1 & Figure 2 Delaney *et al.*, 2008)

An extensive background to the various assessments in the two tiers is provided in the report and is summarised below:

Tier I

(i) History of safe use

History of safe use (HOSU) of any dietary chemical or protein requires evidence of dietary consumption by animals or humans over a significant period of time, usually many years, and in a defined population, such that it can be concluded that existing dietary exposure has demonstrated a reasonable certainty of no harm. A complete absence of HOSU does not necessarily mean that a novel protein would constitute a hazard, however it may indicate that Tier I of the assessment is incomplete. This could lead to recommendations for further analysis of Tier I components, or possible additional toxicological assessments from Tier II to be undertaken. The major limitation with the HOSU concept is the lack of a precise scientific definition. This is not problematic when a novel protein is identical to one found otherwise in the diet, but becomes a limitation when ‘similar’ proteins are introduced into a GM crop. A degree of subjectivity then occurs in extrapolation of ‘safe use’ information from one protein to the other. Information about the properties of the organism from which the novel protein is derived, such as known human pathogenicity, ability to produce toxic or allergenic effects in humans, etc. can also contribute to the concept of HOSU. However, it needs to be remembered that a protein from a pathogenic microbe will not be hazardous just because it derives from a pathogenic organism.

(ii) Bioinformatics analysis

In the context of protein safety assessment, bioinformatics describes the comparison of the amino acid sequence of the novel protein with other known protein sequences. The assessment seeks to define the degree of amino acid similarity, phylogenetic relationship

and the orthology⁵ between different proteins. The sequence of the novel protein expressed in the GM crop is compared to publically available databases of known protein sequences. The most commonly used bioinformatics computer tools are the FASTA and BLAST comparison searches available at the US National Institute of Health website (www.ncbi.nlm.nih.gov). The information obtained from bioinformatics analyses can be used to define or refine the mode of action of specific proteins and to determine the phylogenetic relationship between groups of proteins based on sequence and/or function. Specific guidelines have been developed for bioinformatics analyses of allergenicity in terms of what constitutes a similarity match that would suggest a potential allergenic hazard. No such guidelines have yet been determined to define a 'toxicity similarity threshold'. The most common strategy is to demonstrate a lack of similarity between a novel dietary protein and proteins known to be toxic. A general recommendation is that proteins that share less than 20% amino acid identity over 100 or more amino acids should not be considered as homologous. Currently bioinformatics analysis is largely limited to the comparison of linear amino acid sequence. As research into the 3-D structural and functional properties of proteins continues, structure/function comparison database systems should become more readily available and these will contribute substantially to the safety assessment of dietary proteins.

(iii) *Mode of action and specificity*

In terms of the safety assessment of dietary proteins, mode of action is defined as the mechanism by which a protein acts *in vivo*. The uncertainty of safety of a novel protein expressed in a GM crop can be reduced if the mode of action of the protein can be shown to have a low relevance for humans. Specificity is one of the most important factors influencing the potential for a novel protein to be hazardous. The better the specificity of a novel protein is understood, the greater the potential to predict whether it will have an adverse effect on humans. Useful information includes whether the novel protein can perform its function in the body of humans, other mammals or other non-target organisms. This can be influenced by such things as the availability of co-factors, receptor binding sites or substrate molecules. It should be noted that there is no single method available to determine the mode of action and specificity of a protein and assessments need to be done on a case-by-case basis and with reference to the particular protein being assessed. It is however, important that studies be undertaken in a relevant model for the target species. Included in this step of the assessment is a detailed characterisation of the novel protein expressed in the GM plant in terms of size, charge (isoelectrical focusing point), post-translational modifications such as acylation, amino acid sequence and stability to changes in pH and temperature. Any alterations to these parameters in the protein expressed in the GM plant, compared to the original source protein, could suggest a requirement for further safety assessment.

(iv) *in vitro digestibility and lability*

Proteins that are unstable in the GI tract are less likely to be hazardous than those that remain intact. This is related to the structure/function relationship that is intrinsic to protein molecules. A protein that has lost its functionality due to degradation in the GI tract is unlikely to pose a dietary hazard. Assessment of the safety of dietary proteins includes the

⁵ Orthology describes genes in different species that derive from a common ancestor. Orthologous genes may or may not have the same function. See <http://www.ncbi.nlm.nih.gov/Education/BLASTinfo/Orthology.html>

analysis of their stability to thermal and pH effects and to digestive enzymes. Analyses should focus on the sequence integrity of the protein in the presence of digestive enzymes. Simulated mammalian gastric fluids can be used to assess the *in vitro* digestibility of novel proteins from GM crops. These contain a range of digestive enzymes in a solution of the appropriate pH to simulate exposure to the GI tract. The functionality of the protein is the end point of the assessment when the protein is exposed to temperature and pH effects. This assessment can also be extended to treatment of the protein with conditions that the GM crop may be exposed to during processing by the food industry (e.g. dry milling of grain), although this is not usually a requirement of a safety assessment. Where there is a concern about the potential toxicity of a novel protein due to its function *per se*, the loss of functionality resulting from digestibility and/or lability will contribute to the safety assessment of that protein in food or feed.

(v) *Protein expression level and dietary intake*

Expression levels of the novel protein in the GM crop and dietary intake levels should be assessed to help determine the level to which the population is likely to be exposed to the novel protein in their diet. Expression levels of the protein *in planta* should be determined for a range of tissue types and in a range of environmental and agronomic conditions appropriate for the crop. The intake of a protein is typically estimated by considering the level of expression in tissues likely to be consumed along with an evaluation of food consumption practices in the population. Sensitive sub-populations should be included in this evaluation if appropriate. It is also important to consider the effect of processing treatments on the protein during food production. Mathematical models for combining food consumption and food composition data to estimate dietary intake and nutritional status have been in use for many years. The most appropriate model to use should be chosen on a case-by-case basis depending on the specific analysis and the available data.

Tier II

(i) *Acute toxicity assessment*

Acute toxicity testing of transgenic proteins is used to assess the potential for mammalian toxicity following a single exposure to high concentrations of the protein. These tests are done in rodents (mice or rats). Currently the US EPA and OECD guidelines recommend a high dose (limit dose) assessment for acute oral toxicity for protein-based pesticides, including Plant-Incorporated Protectants (PIPs). Generally it is not considered a necessity to undertake acute toxicity testing for non-pesticide transgenic proteins. For example, the EFSA does not currently require acute or repeat dose testing for non-pesticide proteins with a history of safe use. However, some such assessments have been carried out. To date, no tested transgenic protein from a GM crop has demonstrated any adverse effects in acute toxicity tests, even at extremely high doses. For example; no adverse effects were seen in mice for the insecticidal Cry1Ab Bt protein administered orally at 4000 mg/kg body weight, or for the non-pesticide CP4 EPSPS protein (that confers tolerance to the herbicide glyphosate) at 1000 mg/kg body weight. If acute toxicity tests are carried out it is important that routes of exposure reflect *in vivo* exposure. For example; administration of the protein via intravenous injection or intraperitoneal injection would effectively bypass the degradative processes of the GI tract and could result in misleading toxicity results. Acute toxicity testing of transgenic proteins with potential for antinutritional activity may also give misleading results as these proteins may not show acute toxicity even at high levels, while

they may have a long term negative effect on animal health. These proteins may be better assessed using repeated dose toxicity studies.

(ii) *Repeat dose toxicity testing*

In most cases repeat dose toxicity testing studies have not been considered necessary for transgenic proteins from GM crops. It is suggested that if they were to be carried out the study design should be consistent with the OECD guidelines for testing food ingredients. There is no evidence to suggest that protein digestion is altered by repeat exposure. Therefore if a transgenic protein from a GM food is susceptible to degradation upon ingestion this is unlikely to be altered by repeat exposure to the protein. Except perhaps for the case of suspected antinutritional proteins, repeat dosing at high concentrations is therefore unlikely to provide any additional information to an overall assessment of protein toxicity. As with single dose acute toxicity studies, caution needs to be taken that excessive doses and use of inappropriate routes of exposure do not provide evidence for toxicity and mechanisms of action that may not occur in the normal exposure scenario.

(iii) *Hypothesis-based studies*

It is suggested that if one of the Tier I and/or Tier II components of the safety assessment of a transgenic protein suggests a possible hazard, hypothesis-based testing could be considered on a case-by-case basis. For example, if a protein or protein fragment were shown to be relatively stable to digestive enzymes then a determination of the biological fate of the protein could be considered.

The report demonstrates the effectiveness of the two tier weight-of-evidence approach by assessing the safety of six transgenic proteins. A summary of the results from these assessments is shown in Table 1. From these examples the Antifungal Protein (plant defensin, AFP) demonstrates a situation where a Tier I assessment raised some cause for concern leading to further assessment using a Tier II criterion. Bioinformatics analysis of AFP showed similarity between the protein and not only other plant defensins, but also insect defensins (e.g. scorpion protein toxin). Some classes of insect protein toxins are known to be toxic to animals and humans, therefore a hypothesis-based testing strategy was undertaken to assess the mammalian toxicity of AFP. Several *in vitro* studies showed a lack of AFP specificity for mammalian neurons. However, it is suggested that based on this assessment AFP should not be further developed for commercialization until the stability of the protein can be better evaluated and *in vivo* toxicological testing undertaken.

Table 1 Summary of examples of two tier safety assessment of transgenic proteins

Examples	Tier I: Potential hazard identification					Tier II: Hazard characterization		
	History of safe use	Bioinformatics	Gene source	Mechanism of action/function	Digestibility <i>in vitro</i>	Acute toxicity	Sub-chronic toxicity	Hypothesis-based testing
Y coat protein from potato virus Y	Potato virus Y commonly infects as many as 20% of all potato tubers consumed (need exact data for Y coat protein)	Potato virus Y coat protein is not similar to allergens or toxins, and is similar to other plant virus coat proteins commonly consumed	Plant viruses are consumed incidentally	Plant viral coat proteins have no enzyme activity, and the mechanism of 'immunity' is to prevent further infection by Potato virus Y	No data required	No data required	No data required	N/A
Phosphomannose isomerase (PMI)	Found in various plant and animal foods, and is found in many bacteria – also endogenous to humans	PMI shares similarity to other sugar isomerases and no similarity to toxins	<i>E. coli</i>	Well described enzymatic activity with known substrate specificity that poses no concern	Digestible	Non-toxic (NOEL 3030 mg/kg)	No data required	N/A
Phosphinothricin acetyltransferase (PAT)	Found ubiquitously in the environment and on foods	PAT shares similarity to other acetyltransferases and no similarity to toxins	<i>Streptomyces</i>	Well described enzymatic activity with known substrate specificity	Digestible	Practically non-toxic (NOEL 10 mg/kg iv; MOS > 1000)	No data required	N/A
5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS)	EPSPSs are ubiquitous in all plants, microbes and many fungi	CP4 EPSPS shares similarity to other EPSPSs and no similarity to toxins	<i>Agrobacterium</i>	Well described enzymatic activity with known substrate specificity that poses no concern	Digestible	Practically non-toxic (MOS > 1000)	No data required	N/A

Examples	Tier I: Potential hazard identification	Tier II: Hazard characterization
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	History of safe use	Bioinformatic	Gene source	Mechanism of action/function	Digestibility <i>in vitro</i>	Acute toxicity	Sub-chronic toxicity	Hypothesis-based testing
Delta-endotoxin Cry1Ab	Found in many common microbial pesticides used for 40 years (with prior toxicological evaluations)	Cry1Ab shares similarity to other Bacillus crystal proteins and no similarity to mammalian toxins	<i>Bacillus thuringiensis</i>	As an insecticidal protein, the specificity and potency has been characterized and shown to be restricted to Lepidopteran insect pests with no practical toxicity to organisms outside of insects	Digestible	Practically non-toxic (MOS > 1000)	No data required	N/A
Antifungal protein (plant defensins) (AFP)	Found in various plant and animal foods	AFP shares similarity to other plant defensins as well as insect defensins (such as scorpion toxin). Some classes of insect defensins are toxic to animals and humans	Plants	AFP's act by interfering with membrane functionality and growth of fungal hyphae. Insect defensins act directly as neurotoxins via membrane integral channel proteins on the basis of protein-protein interactions	Stable	Not assessed	No data required	Several <i>in vitro</i> studies were completed to show lack of AFP specificity for mammalian neurons – lack of toxicity to mammalian neurons

Taken from Table 4, Delaney *et al.*, 2008.

In summary, this report outlines a robust safety assessment strategy for transgenic proteins that uses a weight-of-evidence approach with two tiers. The first tier involves a hazard identification. If a potential hazard is identified, or if information is missing or equivocal, the second tier assessment is undertaken, involving a hazard characterization. Many of the analyses involved in this strategy are already in use for safety assessment of novel proteins in GM crops and the methodologies well established. This weight-of-evidence strategy puts the assessments into a prioritized order from which the appropriateness of ongoing assessments can be determined. It is suggested that, using this strategy, a number of assessments already undertaken for transgenic proteins in GM crops have included unnecessary testing, particularly with respect to toxicity testing and that in these instances the additional tests should be considered as confirmatory and not as stand-alone safety assessments.

Several areas in the safety assessment of novel dietary proteins are identified that could benefit from ongoing research. These include:

- ♦ Bioinformatics analysis of protein toxicity, which is currently not as well defined as it is for protein allergenicity. There are as yet no guidelines to define the level of similarity between a novel protein, known safe dietary protein and protein toxins. There are also no dedicated databases currently available of sequences for proteins considered to be toxic. Further work in these areas would help to standardize the safety assessment by setting criteria to improve the predictive capacity of the bioinformatics analysis.
- ♦ Development of the concept of threshold of toxicological concern (TCC) for proteins. TTC refers to the establishment of a human intake threshold value for all chemicals, below which there is a very low probability of risk to human health. It relates to a predicted exposure threshold at which toxicological data from structurally similar compounds indicates large margins of safety. This concept is currently not applied to proteins, due in part to insufficient data to define a TCC. However, it is suggested that it might be possible to use this concept to evaluate existing data to determine if TCC values can be defined for specific proteins or classes of proteins.

The report concludes that, as additional proteins are considered as candidates for use in agricultural biotechnology, the importance and weight given to history of safe use and mode or mechanism of action of proteins will be increasingly important. It is also anticipated that Tier II hazard

characterization could become more relevant for future proteins for which this Tier I assessment information is not well defined.

References:

Delaney, B., Astwood, J.D., Cunny, H., Conn, R.E., Herouet-Guicheney, C., MacIntosh, S., Meyer, L.S., Privalle, L., Gao, Y., Mattsson, J. and Levine, M. (2008). Evaluation of protein safety in the context of agricultural biotechnology. *Food and Chemical Toxicology* 46, Supplement 2: S71-S97.

US FDA (1983). Toxicological principles for the safety assessment of direct food additives and color additives used in food. Redbook. US FDA, Bureau of Foods, Washington DC.

3.2. Multigenerational feeding studies

The validity of and potential information that can be obtained from animal feeding studies continues to be an issue associated with the safety assessment of GM foods. This issue has been discussed in previous reports. During this reporting period further study results have been published, in this case on a multigenerational feeding trial. The background to animal feeding studies in food safety assessment, below, is largely taken from CA Report FW08007. This is followed by a discussion of the results presented by Kiliç and Akay (2008) on a three generational feeding trial with rats fed Bt corn.

3.2.1. Background to the use of animal feeding studies in food safety assessment

The need to develop approaches to assess the safety of whole foods was first highlighted in the 1950s when food irradiation was introduced, followed by development of novel proteins from fungi in the 1960s. The first guidelines for safety assessment of whole foods were set up in 1969 and 1970 by the “Protein Advisory Group for Single Cell Protein”, and in 1984 by the UK Department of Health and Social Security. Use of gene technology for the generation of novel foods first appeared in the 1980s and the challenge for assessing the safety of foods derived using this technology was considered by a number of national and international regulatory bodies (e.g. FAO/WHO in 1991).

In 1997 the EC issued its regulation concerning novel foods and food ingredients, including GM foods (EU Regulation 258/97). Recommendations contained in these regulations, and based on the opinion of the Scientific Committee on Food (SCF), suggested a case-by-case approach where chronic or sub-chronic animal feeding studies may be appropriate when preceding data does not enable an acceptable settlement of safety issues. The SCF had expressed its doubts about the usefulness of animal feeding studies, both from a scientific and safety point of view. Issues they raised included:

- Difficulties encountered in the interpretation of data from animal feeding trials when high levels of the novel food are included in the animal’s diet. This may be due to general distortions in the nutritional profile of the diet unrelated to the genetic modification.
- Animal feeding studies do not allow for the use of large uncertainty factors that are generally applied for safety assessment of food additives.

The SCF suggested a decision–tree approach to safety assessment of novel foods, including an optional requirement for animal feeding studies to provide missing information.

While various nutritional analyses and short-term feeding studies have been published to demonstrate the possible effects of GM foods on human and animal health, little information is available on the possible food safety effects of GM crops through multigenerational feeding. These type of studies are often cited by opponents of GM technology as lacking; for example: “Consumers are right to be cautious about GM food as there are no long term, peer reviewed, multigenerational feeding trials to show whether or not it is safe to eat.” (www.madge.org.au). To begin to address this issue, researchers from Turkey published a report in early 2008 on a feeding trial in which three generations of rats were fed a GM Bt corn line as a part of their diet. This study is discussed below.

3.2.2. *A three generational study with genetically modified Bt corn in rats.*

The study was set up using female Wistar albino rats that had been mated in a controlled manner with male rats of the same type. Light microscopy evaluation of vaginal lavages was used to determine that females had mated and to set a Day1 time for the feeding trial. The pregnant female rats were randomly assigned to one of three feed groups (6 rats per group). The feed diets were:

- Group I a diet of 100% standard laboratory rat basal diet
- Group II a diet of 20% reference corn + 80% standard diet
- Group III a diet of 20% transgenic corn + 80% standard diet

In order to avoid any effects from long term feeding of an unbalanced diet, the total level of corn (20%) in Group II and Group III diets was chosen to be below the accepted safety margin of 33% of a single ingredient in a feed. The transgenic corn in Group III feed was a GM Bt corn line, however, the exact GM event was not disclosed in the paper. The reference corn line was described as a line with the same breeding and genetic background as the Bt line, but lacking the Bt transgene. The composition of the two corn lines (Bt and reference corn) was determined to be essentially equivalent in terms of percentage content of water, dry nutrient, crude protein, crude fat, starch and sugar.

The pregnant female rats (F₀) were started on the diet on Day 1 of the trial (assigned by Day 1 of pregnancy) while the male rats used to mate the F₀ females were taken out of the study. The dams and their pups were fed the diets during the periods of gestation, lactation, offspring care and pubescence. The offspring of different dams in each group of each generation were then mated among themselves throughout three generations (F₁, F₂ and F₃). Feeding with the diets was continued through the mating period in these generations, and as above through gestation, lactation, offspring care and pubescence, with both male and female progeny retained in the trial. Progeny numbers were within the range 19–37 progeny per feed group per generation. The F₃ rats were fed with the appropriate diet until they reached 3.5 months of age.

At the end of the treatment all F₃ rats were weighed and sacrificed. A number of analyses were then undertaken to assess potential impacts of the feed diets on rat health. These included:

- ♦ Histopathological assessment of stomach, small intestine, liver and kidney tissue;
- ♦ Assessment of blood samples for urea*, urea nitrogen*, creatinine*, uric acid*, total protein, albumin and globulin content (*reflect kidney function);
- ♦ Measurement of enzyme activities (from blood samples) of aspartate amino transferase[#], alanine aminotransferase[#], alkaline phosphatase[#], gamma-glutamyltransferase, creatine kinase and amylase ([#] reflect liver function).

Details are given in the paper on the statistical methods used to analyse the data, which included an evaluation of homogeneity of variance and normality of distribution.

All generations of rats were observed during the trial and no adverse effects were seen in clinical appearance of newborns in all three generations. Progeny from each generation were fertile, and no significant differences were seen in number of progeny produced in each feed group, suggesting no adverse effect on reproduction from any of the trial diets. There were no significant differences in final body weights of F₃ rats in all feed groups. However, there was a statistically significant decrease in the relative kidney weight of F₃ male rats in Group II, fed a reference corn/standard feed diet, compared to those fed the control Group I diet. Female F₃ rats showed a statistically significant decrease in relative kidney weights for Group II and relative liver weights for Group II and Group III diets compared to those fed the control Group I diet.

The histopathological assessment of tissues from F₃ rats showed:

- (i) No difference in the stomach and small intestine of rats fed the Group III (Bt corn) diet compared to the other feed groups;
- (ii) Some differences in liver tissues between rats fed the Group III Bt corn diet and those fed either the non-transgenic corn or the standard diet. Changes that were seen were granular degeneration, focal infiltration, congestion and nuclear border changes. The type and incidence of statistically significant changes was not consistent between male and female F₃ rats;
- (iii) Changes in the kidney tissue between feed groups. This included a decrease in the diameter of glomeruli and a decrease in glomerular volumes that was statistically different for Group II and Group III diets, compared to the control Group I diet. Some changes were also seen in the thickness of the kidney cortex tissue in the F₃ rats however there was no statistically significant difference between feed groups.

Biochemical analysis of blood samples from F₃ rats also showed some differences between feed groups. Alterations in the amount of creatinine, globulin and total proteins were statistically significant between groups. Creatinine levels were different between the genders. There were increases in the amount of creatinine in Group II females, while a decrease was seen for Group III males compared to the other feed groups. The amount of globulin and total protein was seen to be significantly different in Group II (non-transgenic corn) fed animals compared to the control Group I diet, but was not different in Group III (Bt corn) fed individuals. No statistically significant differences between feed groups were seen for any of the enzyme activities assayed.

Results for both histopathological and biochemical analyses tended to concur with results from other studies in the literature where single generations of rats were fed a diet containing a GM crop component (see references in Kiliç and Akay, 2008).

Conclusions drawn from the study were that:

- ◆ No adverse behavioural or clinical effects were seen between diets, based on clinical observations of all generations, and reproductive fitness was not affected by the feeding of Bt corn in the diet.

- ◆ Final body weights did not differ significantly between F₃ rats from the three feed groups, suggesting the feeds were all nutritionally wholesome.
- ◆ Decreases seen in relative weights of liver and kidney tissues appeared randomly among all feed groups and between sexes, so were considered to be diet independent.
- ◆ Based on histopathological assessment of the stomach and small intestine of F₃ animals, the Bt corn based diet caused no deformations in the gastrointestinal tract.
- ◆ The liver is the main organ involved in biotransformation and detoxification and any changes suggest an alteration in metabolic processes. The specific changes in the liver of animals fed the Bt corn based diet suggest that this diet may cause excess fatty supply for animals. However it was noted that changes to liver tissues were also seen to some extent in rats fed the control diet and those fed the non-transgenic corn based diet and that changes were variable between the genders.
- ◆ Histopathological changes in both liver and kidneys were in accordance with biochemical analyses and suggested changes were minor and not critical to animal health.

Overall the conclusion was drawn that, although the results obtained from the study showed minor histopathological and biochemical effects on rats fed with Bt corn, long-term, three generational consumption did not cause any severe health concerns for rats. It is suggested by the authors that this work provide a basis from which to extend multigenerational feeding trials with GM feed to other species as a tool in the suite of analyses undertaken to assess the safety of GM foods.

One criticism of the publication is that the methods section did not fully explain the feeding schedule of the diets to the extent that another researcher could replicate the trial. It is unclear whether F₁ and F₂ offspring were fed the test diets exclusively after weaning through until they bore and raised progeny or if they underwent a period(s) during which other diets were fed. It is also unclear for how long the F₁ and F₂ generations were monitored by clinical observation, particularly for the males, and if they were fed the test diets exclusively during this period.

This publication does, however, provide important data on the effects of long-term, multigenerational feeding of a GM crop (specifically Bt corn) and includes information on

reproductive fitness of animals in the trial. The study design overcomes one of the issues raised by the SCF in regards to animal feeding trials, specifically that:

- Difficulties may be encountered in the interpretation of data from animal feeding trials when high levels of the novel food are included in the animal's diet. This may be due to general distortions in the nutritional profile of the diet unrelated to the genetic modification.

The use of the GM feed component at below 'critical percentage' in the diet over a long period showed no results that would suggest a serious distortion in the nutritional profile of the diet unrelated to the genetic modification. Ongoing studies of this nature should provide an increasingly robust database of information on the safety and health effects of GM crops.

References:

Kiliç, A. and Akay, M.T. (2008). A three generation study with genetically modified Bt corn in rats: Biochemical and histopathological investigation. *Food and Chemical Toxicology* 46: 1164-1170.

3.3. Detection method for dietary DNA from GM foods incorporated into a recipient genome

A food safety issue of particular concern to the public is the possibility that GM material from food could be transferred into the recipient's genome after ingestion (known as horizontal gene transfer, HGT). The concerns are that this could result from the direct ingestion of GM food by humans, or the ingestion of GM feeds by food animals that are then a source of human food; and that if a dietary DNA fragment is internalized in the genome of a recipient organism, mutagenic or oncogenic consequences may occur.

A number of studies have looked at the issue of ingestion of foreign DNA, however, the ability to detect integrated DNA from a HGT event has remained problematic.

3.3.1. Background to the known fate of ingested DNA

Throughout evolutionary history all organisms have been exposed to foreign DNA. This can be via ingested food or from sources such as infection by viruses or other microorganisms. After ingestion of food, nucleic acids are largely degraded by the conditions in the gastrointestinal (GI) tract. For example; studies were undertaken in 2004 by Netherwood and co-workers on human volunteers (twelve healthy and seven who had undergone ileostomies) who were fed a GM soy food product. The transgene DNA did not survive passage through the intact gastrointestinal tract of healthy volunteers. However, the amount of transgene DNA surviving passage through the small intestine was determined from the volunteers with ileostomies and there was evidence for low frequency recovery of transgene DNA, up to a maximum of 3.7% recovered in one individual. This suggests that, depending on the degree of protection of the DNA afforded by resistant food constituents, a small amount of dietary DNA could remain intact within regions of the GI tract. A number of other studies have reported that dietary DNA may be taken up from the GI tract and transferred to the blood and organs of mice, cows, calves, chickens and pigs (see Section 3.2 CA Report FW0771).

Due to analytical limitations the proof, from sequence information, for insertion of dietary DNA into a recipient's genome by HGT is still lacking. While the development of PCR analysis has enabled the detection of very small amounts of a DNA target in a heterologous DNA background,

the limit of detection of this method may still not be low enough to detect the potentially minute amount of DNA that could be transferred into a large genome background in a HGT event. Similarly, traditional PCR methods rely on detection of a known DNA target. While it is possible to target a potential sequence that may be involved in a HGT (for example a known transgene), the position within the genome into which this may be inserted will not be known, making it difficult to prove the insertion event using traditional PCR. Dietary DNA that could undergo a HGT event is also likely to be very short, increasing the difficulty of detecting it within a large recipient genome. To illustrate: the human haploid genome is approximately 3 billion base pairs in size, making the full genomic DNA complement of a diploid cell approximately six billion base pairs. If as much as 1000 bases of foreign DNA were to insert in a HGT event this insertion would only comprise approximately 0.0001% of the total DNA from one cell. If a HGT event did occur the integration may only be present in one or a few cells within a specific tissue, which would limit the available sample for PCR analysis, and increase the likelihood of false-negative results.

Without the ability to conclusively show the integration of dietary DNA into a recipient's genome the likelihood and potential risks from HGT remain speculative only. During the reporting period researchers from The National Veterinary Institute in Norway (Nielsen *et al.*, 2008) published a modified PCR method capable of detecting a single integration in a single dividing cell in a tissue sample from salmon. This method, described below, has the potential to be able to detect a specific target DNA to a level capable of detecting potential HGT events.

3.3.2. Anchored PCR for possible detection and characterisation of insertion events at near single molecule levels

The potential food safety risks that could be related to HGT events if they should occur are not limited to ingestion of GM foods directly by humans. Concern has also been expressed that a HGT event occurring in a food animal that consumed a GM feed could have flow-on effects up the food chain. To this end Nielsen and co-workers established a model system mimicking a putative HGT event from soy feed meal containing Roundup Ready[®] (RR) soy, fed to farm-bred salmon. DNA extracted from RR soy was mixed into a high background of salmon genomic DNA isolated from liver tissue. The insertion event is mimicked by the well characterised insertion event in RR soy while the genomic background is a chimera of the soy and salmon DNA.

The development of the anchored PCR method describes three slightly modified methods that all gave very low level detection of the model insertion event. Only the method giving the best detection is described in detail here. The other methods followed the same basic steps with slight modifications.

The overall methodology is based on digesting the total DNA with an enzyme that cuts it into variable sized fragments, followed by an initial anchor PCR with a solid-phase purification step that concentrates the target. A nested PCR for the target sequence is then carried out, followed by verification of the product by sequencing. Using this method a product is identified that contains both insertion and genomic sequence, and thus able to verify the insertion of the foreign DNA into the recipient genome. The method is outlined in Figure 3.

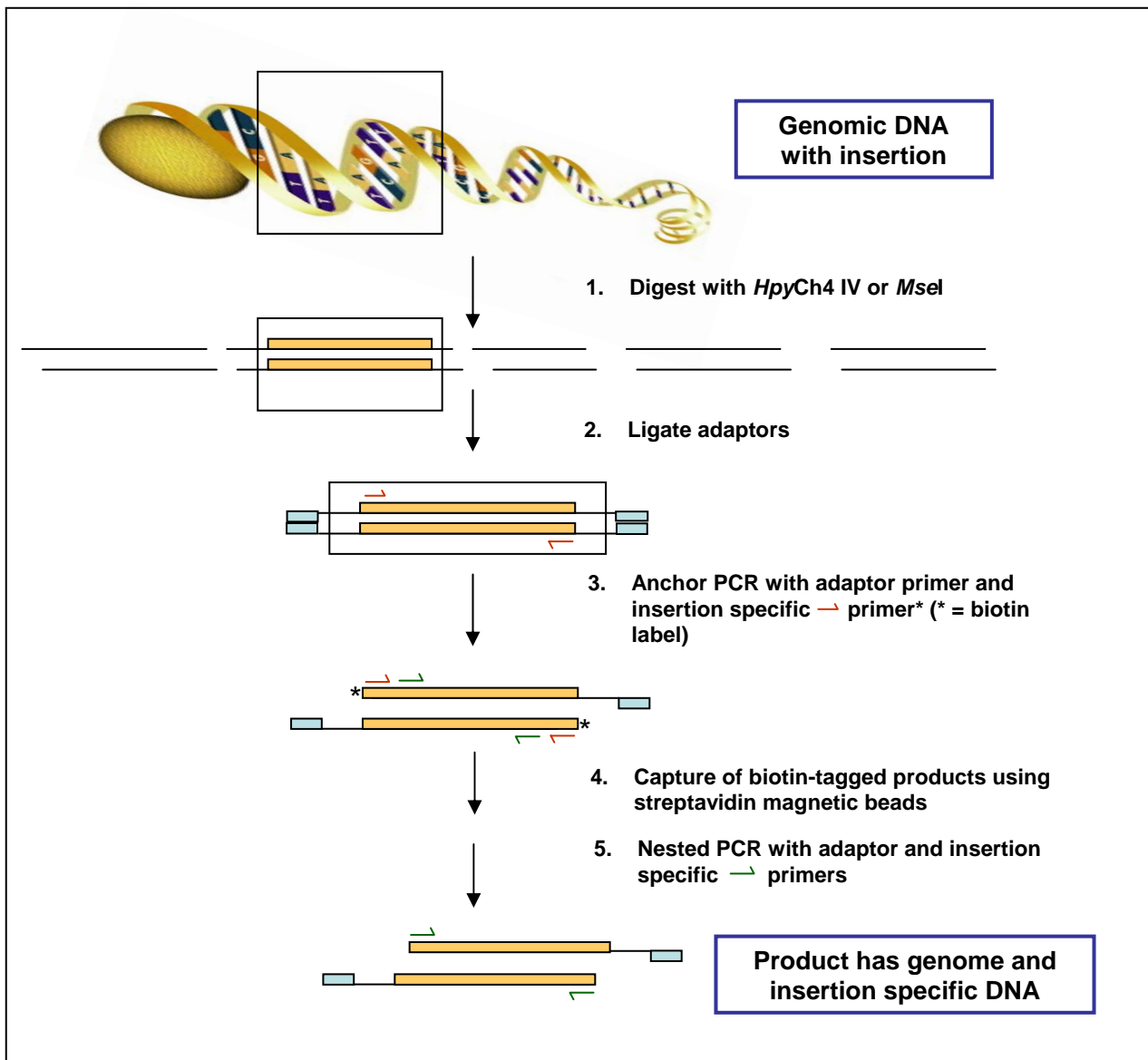


Figure 3 Outline of anchored PCR method for detection of insertions in a large genomic DNA background (Modified from Figure 1. Neilsen *et al.*, 2008).

Step 1: The genomic DNA containing the putative insertion is cut into variable size fragments using a restriction endonuclease enzyme. The enzyme is chosen so as to cut the DNA into a large number of fragments. If the complete sequence of the target insertion is known, the enzyme can also be chosen so as not to cut within this target, but only within flanking genomic DNA sequences. In the study described, the salmon/RR soy DNA was digested with either the enzyme *HpyCh4 IV* or *MseI*, both of which cut the DNA into fragments with an average size of 256 base pairs (bp). This

provides short fragments that should incorporate the size expected from insertion of degraded dietary DNA by a HGT event.

Step 2: Ligation of an adaptor sequence to the restricted DNA. An adaptor is a synthetic oligonucleotide with a known sequence. One end of the adaptor is complimentary to the restriction site generated during the digestion of the DNA and so allows the adaptor to be joined (ligated) to this sequence. Adaptors will ligate to all of the fragments generated by the digestion of the DNA. Assuming that the restriction digest only cuts within the genomic DNA the adaptor will therefore be joined to genomic sequences only. The adaptor also contains a known primer binding sequence for PCR amplification.

Step 3: Anchored PCR amplification of the fragment containing the target insertion sequence. The PCR is called anchored as one primer (the adaptor primer) is anchored to unknown sequence(s) in the genomic DNA. This PCR is performed using the adaptor-specific primer and an insertion-specific primer (in this case specific for the insertion in RR soy). The insertion-specific primer is 'tagged' with a biotin molecule. During this PCR amplification only restriction fragments that contain genomic adaptor-ligated DNA and the target inserted DNA will be amplified. The specificity of the PCR was increased by using a touchdown PCR protocol, which is a modification of conventional PCR that can result in a reduction of nonspecific amplification. It involves the use of an annealing temperature that is higher than the target optimum in early PCR cycles. The annealing temperature is then decreased by 1°C every cycle or every second cycle until a specified or 'touchdown' annealing temperature is reached. The touchdown temperature is then used for the remaining number of cycles. This allows for the enrichment of the correct product over any non-specific product. Even so the researchers reported a range of products generated from this PCR step.

Step 4: The biotin tag on the target specific primer will bind to the protein molecule streptavidin. Magnetic beads coated with streptavidin can therefore be used to separate the amplified PCR product generated in Step 3 away from background DNA. This enables the enrichment for specific targets from within a high background of genomic DNA. Using this step the researchers noted they could detect the same number of starting copies of target DNA (~1.8 copies) in a ten-fold increase in the level of background DNA (25 ng of salmon DNA compared to 2 ng).

Step 5: A ‘nested’ PCR amplification is carried out using the adaptor-specific primer and an insert-specific primer within the amplicon generated in Step 3. This ‘nested’ PCR step allows amplification of the target insertion and its flanking genomic DNA with reduced opportunity for amplification of non-specific background products. The researchers reported that the predominant product of this PCR step was amplicon of the expected size. While there were also some non-specific amplicons generated, sequence analysis confirmed that the product of expected size was the expected target. The presence of some non-specific background would, however, suggest that when using this method to detect an insertion in an unknown genomic region the products should be sequenced to verify their identity.

In summary this method allowed the researchers to detect ~1.8 copies of the target DNA (the insertion from RR soy) in a background of 25 ng of salmon genomic DNA. This corresponds to a single integration site in a single dividing cell in a tissue sample. Based on the estimated genome size of salmon, 25 ng of DNA is equivalent to approximately 7,500 cells. The potential limit of detection (LoD) of the method in terms of HGT events/recipient cell, based on this RR soy/salmon model, was therefore 1 HGT event per 3750 cells. This LoD would, however, be influenced by the tissue from which the DNA is extracted and the method of DNA extraction.

Ideally a method to detect HGT should be independent of any prior knowledge about the specific integrated sequence. In reality, however, the requirement for insert-specific primers in this method would mean that screening for HGT events from GM food in the diet would have to focus on specific candidate genes or regulatory sequences. In the short term, given that HGT from dietary DNA is still only speculative, this method does demonstrate that it may be able to be used to detect and characterise a few copies of a short target sequence in a large background of genomic DNA and could be used to further study and characterise the potential for HGT events.

Note: Section 3.2 of Current Awareness Report FW0771 reviewed current literature reports on the fate of transgenes in stock feed, in which there was no evidence for GM feed affecting meat. Results in these reports showed that while most ingested plant DNA (transgenic or non-transgenic in origin) is degraded in the gastrointestinal tract some DNA fragments have been found in animal tissues. However, in these studies no fragments of transgenic DNA were detected in any tissue or

organ sample, including eggs and milk obtained from animals fed GM plant materials. As some plant DNA seems to be able to be absorbed into the animal's system from ingested feed it can't be ruled out that transgenic fragments may not also be absorbed. Based on results from these studies the potential frequency of this occurring is likely to be extremely low. Due to degradation of DNA in the GI tract, the food safety risk associated with HGT events from dietary DNA is not likely to be related to insertion of full length genes able to be expressed in the recipient's cell. Mutagenic and/or oncogenic effects from insertion of small DNA fragments would be a more probable effect. These could occur from insertion of DNA from any dietary source and would not necessarily be limited to transgenic DNA. Consider, for example, the ingestion of a burger containing a soy patty. While the soy patty may contain some RR soy there could also be dietary DNA from the wheat component of the flour in the bun as well as any milk in the bun, the tomatoes in the ketchup, the onion and lettuce in the filling and the eggs in the mayonnaise. While public concern tends to focus on GM food, the food safety risk of putative HGT events should be considered in the context of all foods and not just GM food, and without evidence to the contrary shouldn't be ruled out as a possible generic risk factor for development of chronic disease. The method described in this report provides a tool by which studies of the potential for HGT could be undertaken.

References:

Netherwood, T., Martín-Orúe, S.M., O'Donnell, A.G., Gockling, S., Graham, J., Mathers, J.C. and Gilbert, H.J. (2004). Assessing the survival of transgenic plant DNA in the human gastrointestinal tract. *Nature Biotechnology* 22: 204 – 209.

Neilsen, C.R., Berdal, K.G. and Holst-Jensen, A. (2008). Anchored PCR for possible detection and characterisation of foreign integrated DNA at near single molecule level. *European Food Research and Technology* 226: 949-956.

4. ADVENTITIOUS PRESENCE ISSUES FOR GMOS IN FOODS

4.1. Unapproved event 'Bt63' in rice

Rice event ‘Bt63’ is an unapproved insect resistant GM rice that has been found in rice products exported from China. A brief summary of the GM event and steps taken by the international community in response to this unauthorized line is provided.

4.1.1. Notification of unauthorized GM rice in products from China

The EU has a Rapid Alert System for Food and Feed (RASFF) in place to enable member countries to notify the presence of unauthorized food and feeds. The description of the system taken from the EU website is as follows:

“The legal basis of the system is Regulation (EC) No 178/2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety (O.J. No L 31 of 1 February 2002). The purpose of the rapid alert system for food and feed (RASFF) is to provide the control authorities with an effective tool for exchange of information on measures taken to ensure food safety.”

(see http://ec.europa.eu/food/food/rapidalert/resources/publications_en.htm).

In September 2006 imported rice products originating from China that contained an unknown GM component were detected by France, Austria and Germany. These imports were notified to the RASFF (see Table 2).

Table 2 Summary of notifications to RASFF, September 2006, for rice products from China containing unauthorised GM material

DATE	NOTIFIED BY	REFERENCE	REASON FOR NOTIFYING	COUNTRY OF ORIGIN
Week 2006/36				
07/09/2006	France	2006.0575	unauthorised genetically modified rice used to manufacture rice sticks	China
Week 2006/38				
21/09/2006	Germany	2006.0616	unauthorised genetically modified rice pasta	China via The Netherlands
Week 2006/39				
27/09/2006	Austria	2006.0646	unauthorised genetically modified rice noodles	China via Germany
27/09/2006	Germany	2006.0647	unauthorised genetically modified rice noodles	China
28/09/2006	Germany	2006.0649	unauthorised genetically modified rice noodles	China
29/09/2006	Germany	2006.0661	unauthorised genetically modified rice noodles	China
29/09/2006	Germany	2006.0662	unauthorised genetically modified rice noodles	China via The Netherlands

Taken from RASFF archive at http://ec.europa.eu/food/food/rapidalert/archive_2006_en.htm

4.1.2. EC response to notification of unauthorised GM rice

Following the RASFF notifications, the EC contacted Chinese authorities requesting information on the unauthorised GM line and its genetic structure, and asked for provision of suitable control samples and an event-specific detection method. Meanwhile the international scientific community undertook to characterize the molecular structure of the insertion region in the GM rice and to develop a detection strategy.

The EC Reference Laboratory for Genetically Modified Food and Feed (CRL-GMFF) is one of the reference laboratories under the auspices of the JRC. The JRC is a Directorate-General of the European Commission under the responsibility of the Commissioner for Research. The role of the

JRC is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of EU policies. The JRC operates six Community Reference Laboratories (CRLs), including the CRL-GMFF. The CRLs are analytical laboratories designated by Community directives and regulations. They are an integral part of the European risk management system. In October 2006 the CRL-GMFF obtained samples from the GMO testing company GeneScan (Germany) that possibly contained Bt rice, as tested by GeneScan with a 'Bt63' construct-specific method. Referred to as 'Bt63', this GM rice contains rice lines Shanyou 63 and Jinyou 63, and was developed in China. The inserted construct in Shanyou 63 is described by Tu *et al.* (2000) as containing a Bt fusion gene construct of *cry1A(b)* and *cry1A(c)* under the control of the promoter sequence from the rice actin gene and the *nos3'* termination sequence from *Agrobacterium tumefaciens*.

Ongoing interception of unauthorized GM rice from China led to the EC issuing a decision in April 2008 requiring mandatory testing of a specified list of rice products for food and feed originating in or consigned from China (EC Decision no 2008/289/EC of 3 April 2008 on emergency measures regarding the unauthorised genetically modified organism 'Bt63' in rice products). Under the directive, such products can only be placed on the EU market if either:

- ♦ They are accompanied by an original analytical report issued by an official or accredited laboratory which demonstrates that the product does not contain, consist of, or is not produced from the genetically modified organism 'Bt63'
- or
- ♦ Satisfactory results of analysis are received by the food authority at the point of entry to the EU, following sampling carried out by or under the supervision of that authority.

The decision also requires that Member States take appropriate measures, including random sampling and analysis, concerning the listed products presented for importation or already on the market.

As a result of the analyses undertaken on the rice line, the JRC suggested that the European Network of GMO laboratories (ENGL) and the EU enforcement laboratories use the event-specific 'Bt63' detection method described by German researchers Mäde, Degner and Grohmann (2006).

This real-time PCR method is construct-specific and targets the transition region between the Bt *cry1A(b)* and *cry1A(c)* fusion genes and the *nos3'* termination sequence.

The European Commission again requested to the Chinese authorities that they supply appropriate control samples for the detection of 'Bt 63' rice. This time samples were forthcoming and genomic DNA of 'Bt63', consisting of genomic DNA extracted from single plants previously tested for the presence of the 'Bt63' construct, was provided. Upon receipt of the genomic DNA reference material, CRL-GMFF experimentally verified the performance of the detection method developed and published by Mäde *et al.* (2006). Details of this method and the validation of the limit of detection and specificity of the method (Savini *et al.*, 2008) can be found at <http://gmo-crl.jrc.it/BT63update.htm>.

GM rice line 'Bt63' continues to be detected in rice products exported from China. For example, since April 2008 the British Food Standards Agency has destroyed four and recalled three rice food products due to the presence of unauthorised rice 'Bt63' (see <http://www.food.gov.uk/news/newsarchive/2008/jul/bt63update>).

The detection of the unauthorized rice line 'Bt63' highlights ongoing issues with the monitoring and surveillance of food products for GM material. This situation illustrated how a Rapid Alert System and the availability of laboratories with expertise in test development can enable an unknown GM event to be detected and monitored. Prior to detection this GM line was not on the EU (or FSANZ) register of authorised GM lines, nor was it listed as an unapproved or withdrawn line. Without access to specific information to this effect, it is assumed that the GM material was detected when products were randomly screened for other known GM rice lines, and that when testing was positive but did not confirm the presence of known rice events further investigation was undertaken. The presence of a Rapid Alert System enabled information on the issue to be rapidly disseminated to the international community. Information published in the scientific literature, from the country of origin of the suspect products, on rice GM events under development presumably enabled a calculated 'guess' to be made as to what the GM event may have been and allowed independent development of a detection strategy to confirm this.

The issue of unknown GM events entering the international food chain highlights the need for rapid response with suitable testing methods. It also reinforces that generic screening methods need to be retained for GM food testing as too heavy a reliance on event-specific detection methods could potentially miss an unknown event. This highlights the need for robust testing methods that can be rapidly modified to incorporate new testing requirements. This issue is discussed in detail in Section 4.2 of this report, where a novel system for establishing a modular testing platform for GM food material is described.

References:

Mäde, D., Degner, C. and Grohmann, L. (2006). Detection of genetically modified rice: a construct-specific real-time PCR method based on DNA sequences from transgenic Bt rice. *European Food Research and Technology*. 224 (2): 271-278.

Savini, C., Querci, M., Ermolli, M., Mazzara, M., Cordeil, S. and Van den Eede, G. (2008). Report on the verification of the performance of a method for the detection of “Bt63” rice using real-time PCR. CRL-EM-02/06, CRL-GMFF: Verification Report Rice Bt63. <http://gmo-crl.jrc.it/BT63update.htm>

Tu, J., Zhang, G., Datta, K., Xu, C., He, Y., Zhang, Q., Singh Khush, G. and Kumar Datta, S. (2000). Field performance of transgenic elite commercial hybrid rice expressing *Bacillus thuringiensis* δ -endotoxin. *Nature Biotechnology* 18: 1101-1104.

4.2. Development of a modular system for detecting GM components in foods

4.2.1. Background to detection methods for GMOs in food

A large number of countries now have legislation that covers the marketing and labelling of GM material in food products. Any monitoring for compliance with these regulations requires robust testing systems able to detect an increasingly large number of GM events. With the increasing number of GM foods reaching the market place there is a major analytical challenge from the need to continuously develop and establish assays for (i) forthcoming authorized GMOs, (ii) to detect non-authorized GMOs, and (iii) to detect unknown GMOs. This requires the availability of flexible testing systems that can be easily expanded.

Testing systems for GM foods fall into two broad categories:

1. Testing for the presence of protein in the food that is the result of expression of the introduced gene. The main disadvantage to this type of testing is that it is very unreliable when used with processed food products. Processing such as heat treatment and mechanical stress can result in proteins being degraded and therefore unable to be detected by this method. This testing also presumes that the genetic insertion has resulted in the expression of a foreign protein.
2. Testing for DNA sequences using PCR (see Figure 4). Qualitative and quantitative testing using PCR can both detect very small amounts of a GM product in a mixed food matrix. While food processing can have an impact on this method it is less so than for detection of proteins and the method will detect the presence of a GM component whether protein is present or not.

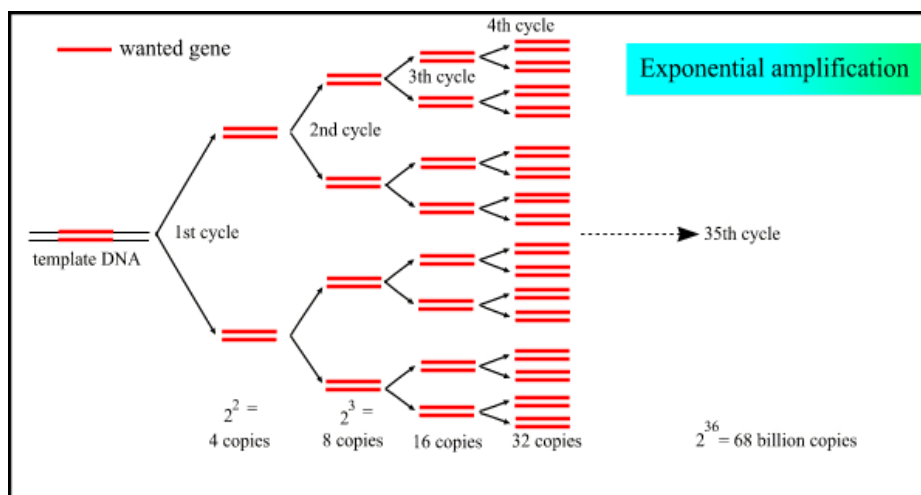


Figure 4 Amplification of a target DNA sequence by PCR

Initial GM testing regimes relied on PCR screening for the presence of generic GM insertions such as the 35S promoter sequence (from cauliflower mosaic virus, CaMV) and the nos3' termination sequence (from *Agrobacterium* sp.) (Anklam *et al.*, 1998). These are GM elements found in all or most, respectively, GM food material. As more GM events came onto the global market there was an increase in the number of construct specific and event specific tests developed. Construct-specific tests target DNA sequences specific for the inserted region in the modification. For example, there has been on-going development in the construct-specific testing for Roundup

Ready[®] soy (soy event MON 40-3-2). This GM food crop was first released by Monsanto in 1996. The genetic modification was the result of insertion into soybean of the plasmid PV-GMGTO4. The inserted DNA contains a 35S promoter region fused to a chloroplast targeting peptide from petunia driving the expression of two bacterial EPSPS genes that confer tolerance to the herbicide glyphosate. Initial construct-specific tests targeted various regions within the inserted DNA in this crop. van Hoef and co-workers (1998) described an event-specific test that targeted the junction between the 35S promoter and the chloroplast targeting peptide. A similar test described by Hurst *et al.* (1999) targeted the junction across the 35S promoter sequence and into the EPSPS gene, incorporating the entire chloroplast targeting peptide. However, as more crops have been engineered to be resistant to the herbicide glyphosate, by using very similar gene cassettes, these construct-specific tests are less able to detect the specific GM crop. To this end, recent event-specific tests have been developed that target the junction between the plant DNA and the insertion sequence. These are highly specific tests for a particular GM event for a specific crop. The EC CRL-GMFF released a validated method of this type for the detection and quantification of Roundup Ready[®] soy in September 2007 (available at <http://gmo-crl.jrc.it/statusofdoss.htm>).

With the increasing number of GM events that require detection, multiplex PCR assays have been developed that allow the simultaneous detection of several GMOs in a single reaction. These assays are, however, limited by difficulties associated with amplification of more than one set of primers in a single PCR, including interaction between different primer pairs and their possible non-competitive amplification. Detection systems can be based on separation by electrophoresis through a gel matrix, however, this requires relatively long amplification products to allow distinction between different targets. This becomes problematic when used for detection of GM material in highly processed and complex food matrices as degradation of DNA during processing can limit the length of DNA target available in an assay. Use of real-time fluorescent detection PCR allows analysis of DNA that is more highly degraded as it relies on detection of smaller amplification products. However, most real-time PCR thermocyclers only allow 4-5 reactions to be detected simultaneously. Therefore, the opportunity to expand and alter multiplex PCRs to incorporate new GM events is limited.

An alternative approach for the simultaneous detection of multiple GM events is the use of microarray analysis. In this method an array of target DNA sequences (for different GM events) is

immobilized on a microchip. An extract from the material to be tested is then labelled with a detection moiety (usually a fluorescent marker) and exposed to the chip. Matching DNA sequences will be detected on the chip giving an indication of whether a GM event is present in the test material (see Figure 5). While effective, this method of testing can be laborious to perform, requires specialized equipment and is currently expensive, making it largely unrealistic as a routine screen for GM material in foods. Similar to multiplex PCR testing, microarray analysis is also difficult to expand readily to accommodate new GM events – a complete new array would need to be developed.

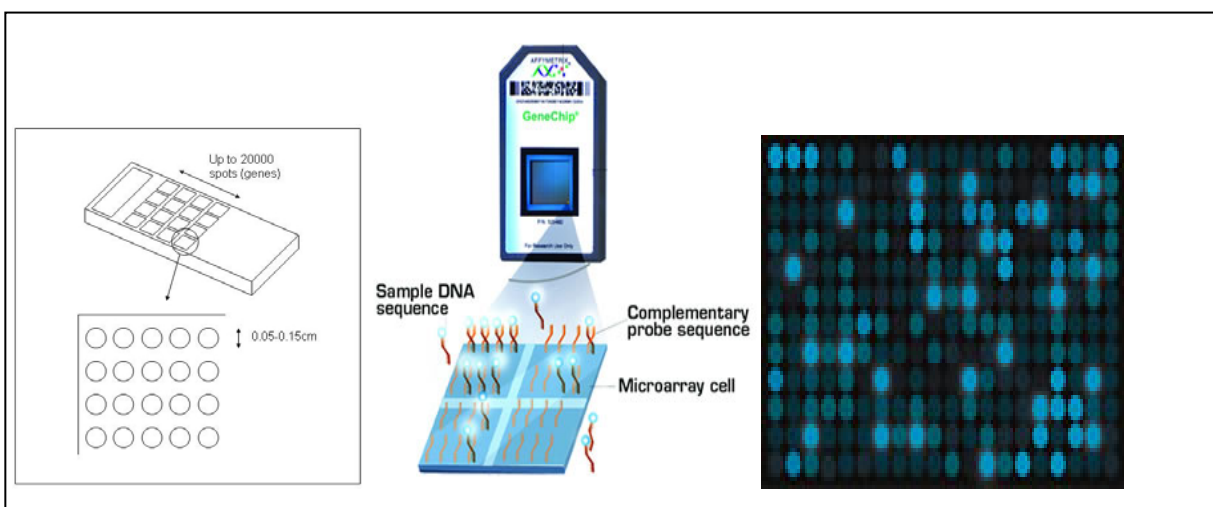


Figure 5 Microarray detection of GMOs. Example of a microarray output. The technology uses a PCR-based amplification of the target DNA with labelled primers and the subsequent microarray-based detection of amplicons with specific DNA capture-probes.

A recent publication from Germany (Ehlert *et al.*, 2008) outlines the development of a modified PCR assay system that could be used as a modular system for detection of GMOs in foods. This method is based on ligation-dependent probe amplification (LPA).

4.2.2. *Background to ligation-dependent probe amplification*

Ligation-dependent probe amplification was originally developed in the field of medical diagnostics. It has particular applications in the determination of gene sequence polymorphisms associated with genetic disease.

The basic method involves:

- (i) binding first and second oligonucleotide ligation probes to adjacent sequences within a target DNA sequence;
- (ii) ligating (joining) together the two oligonucleotide probes at their juxtaposed termini to form a ligated target probe template; and
- (iii) using the target probe template generated in a PCR reaction to amplify the product.

Oligonucleotides are designed with a target specific hybridisation region at one end and a generic primer region at the other end. Multiplex ligation-dependent probe amplification (MLPA) relies on designing oligonucleotide pairs of a unique length for each target. This is done by incorporating a ‘spacer’ sequence between the hybridisation sequence and the primer sequence. The ‘spacer’ sequence may be only a few nucleotides long. A fluorescent tag is attached to one primer sequence and the PCR products separated using an automated sequence analyzer capillary electrophoresis system. The product size is determined by comparison to known size standards. This methodology allows for the simultaneous resolution of a large number of labelled DNA fragments that differ in size by as few as four nucleotides. The method is outlined in Figure 6.

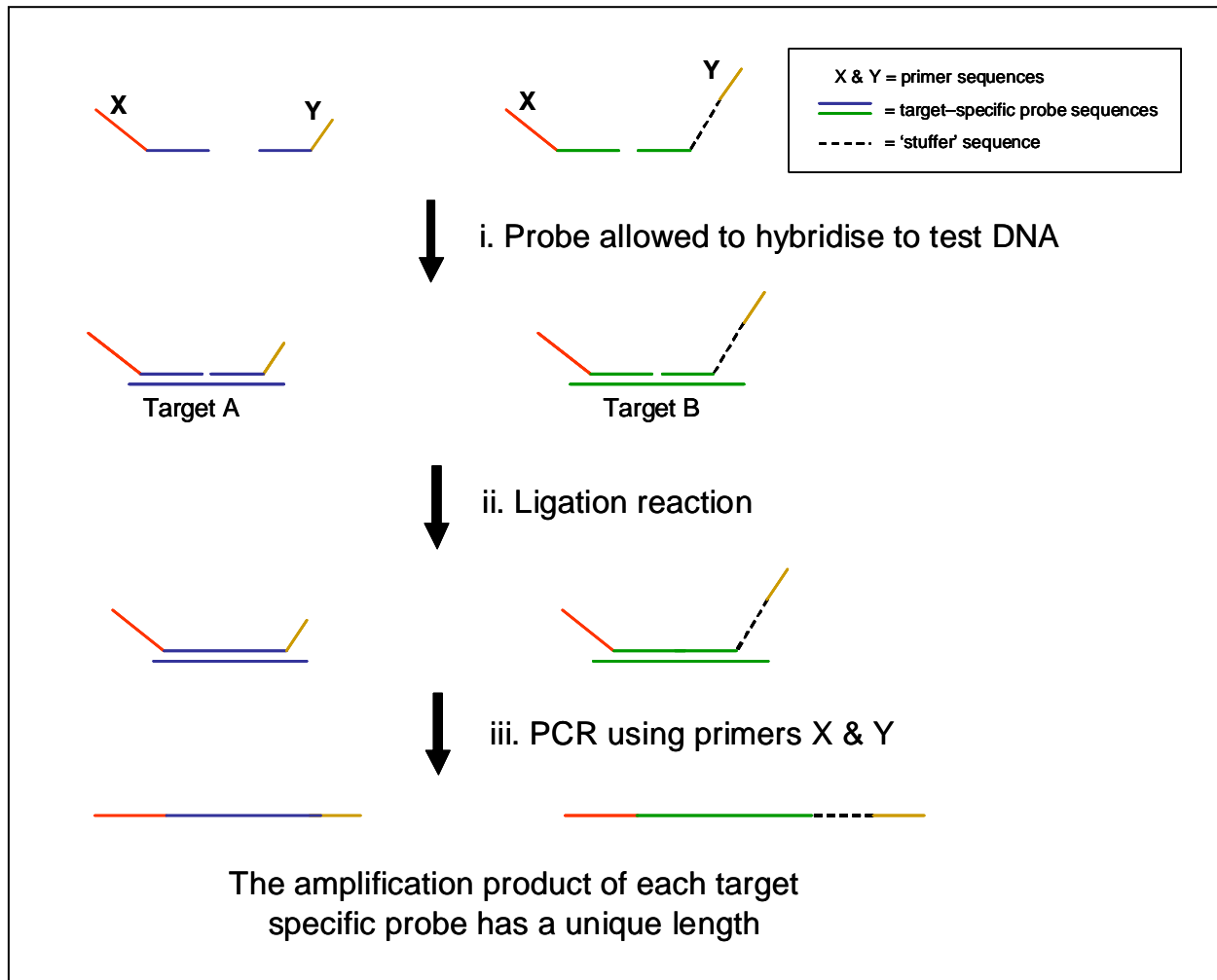


Figure 6 Schematic diagram of MLPA. Modified from Schouten *et al.* (2002).

The advantages of this system are:

- by using common ends to each oligonucleotide pair all probes can be amplified using one primer pair and it is the target probe sequence that is being amplified and not the original target DNA sample. Hybridization of multiple probes to a DNA sample is technically easier than multiplex PCR amplification from a DNA sample. Therefore this method overcomes the limitations of multiplex PCR;
- Target specific binding regions can be significantly less than 100 nucleotides in length, which enables detection in highly degraded DNA samples. Real-time PCR assays optimally detect targets of between 90-200 nucleotides;
- Multiple amplified 'tagged' probe sequences can be easily resolved using fluorescent capillary electrophoresis and the intensity of the measured signal is related to the quantity

of the target amplicon, giving a quantitative output to the analysis. An example of an analysis output is shown in Figure 7.

This method has been used to detect and measure the relative quantity of forty nucleotide sequences, simultaneously, from human DNA (Schouten *et al.*, 2002).

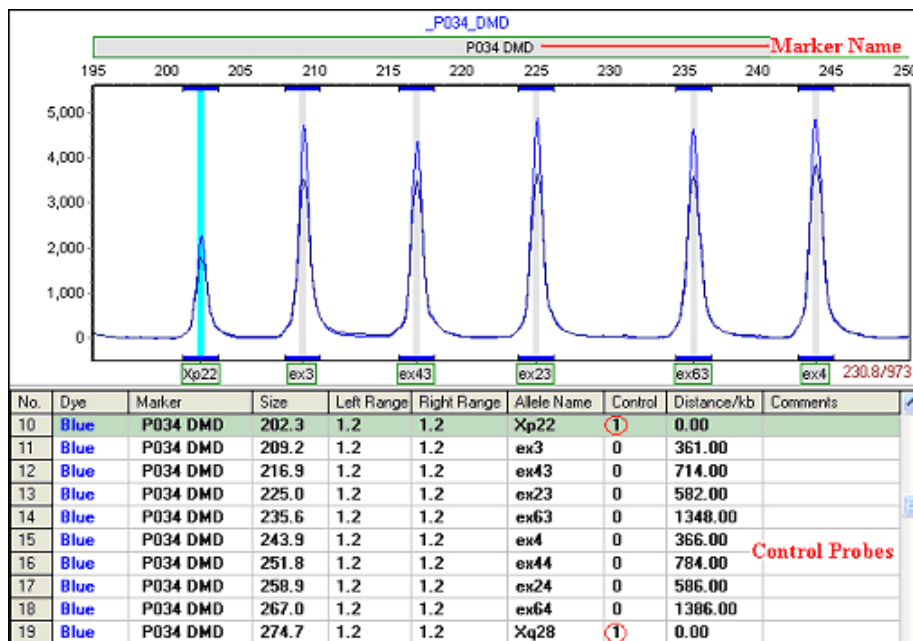


Figure 7 Typical output of a MLPA analysis. Using GeneMarker[®] Luminex-MLPA software. The individual peaks represent different length products. Peak height is a measure of fluorescence signal intensity and is directly related to the amount of starting template. Taken from : SoftGenetics Ltd www.softgenetics.com

4.2.3. Use of MLPA for detection of GMOs in food

The use of MLPA for the detection and relative quantification of GMOs was first described by Moreano *et al.* in 2006. The method was used to simultaneously detect and quantify the DNA from two GMOs using commercially available standards for GM corn and soy. Further development of the method was then reported by the same group early in 2008 (Ehlert *et al.*, 2008). In this work the method was optimised to enable the detection of a greater number of targets, with differing levels of specificity, in one assay.

Target specific probe sets were designed to detect:

- ♦ a taxon-specific soy sequence (*Le1*)
- ♦ a taxon-specific corn sequence (*HMGa*)
- ♦ a taxon-specific rapeseed sequence (*BnACCg8*)
- ♦ the CaMV 35S promoter sequence
- ♦ the construct-specific P-35S-*pat* junction sequence (able to detect LibertyLink™ constructs)
- ♦ an event-specific sequence from corn MON810
- ♦ an event-specific sequence from Roundup Ready™ (RR) soy

The length of the target probe binding sites influences the ability to detect DNA in a food sample. Degradation of the DNA during food processing can negatively affect the detection of long sequences, resulting in false-negative results. For this reason target-specific hybridization regions were selected that were no longer than 71 nucleotides. This length of sequence should be able to be detected in food samples even when the DNA is highly degraded by processing. All probe sets were then designed to contain identical primer binding sequences (PBS). Spacer DNA was introduced into some probe/primer sequences in order to enable size discrimination. Examples of probe sets are shown in Table 3.

Table 3 Examples of LPA probes used for GMO detection

Taken from Ehlert *et al.* (2008), Table 1

Target gene	Left probe	Right probe	Size of ligation product (bp)
Soy <i>Le1</i>	5'- GGGTTCCCTAAGGGTTGGA CCTTGTAGTCAAA CCACACATAAGAGAGGA-3'	5'- TGGATTTAAACCAGTCAGCACCGTAAGTATATAG TGAT TCTAGAATTGGATCTTGCTGGCAC -3'	110
RR soy	5'- GGGTTCCCTAAGGGTTGGA <i>tggtgtgtgt</i> AATGAT GGCATTGTAGGAGCCACCTT-3'	5'- CCTTTCCATTGGGTTCCCTATGTTATTTAACC TGTA TCTAGAATTGGATCTTGCTGGCAC -3'	122

GGGTTCCCTAAGGGTTGGA = forward primer sequence

TCTAGAATTGGATCTTGCTGGCAC = reverse primer sequence

tggtgtgtgt = spacer sequence

UPPERCASE normal = target specific probe sequence

The MLPA system was tested against: (i) DNA extracted from certified reference flour standards from MON810 corn and RR soy in proportions from 0.1%-5%, along with non-transgenic corn and

soy standards; (ii) DNA extracted from non-transgenic rapeseed; and (iii) a synthetic DNA construct containing the rapeseed specific sequence and the LibertyLink™ specific junction region, P-35S/*pat*. Different mixtures of DNA were prepared to simulate composite food products containing different proportions of GM material. For example; a sample mixture containing 5% MON810 corn and 5% RR soy in a conventional non-transgenic rapeseed DNA background.

The system was shown to be able to detect all seven target sequences in a single reaction. No false-positive signals were obtained with any of the test mixtures, or when DNA extracts from other plant and animal species were used. The fragment lengths obtained for each target were highly reproducible (<1 nucleotide shift in absolute length). The sensitivity of the method for the detection of trace amounts of GM material in foods was assessed using certified reference materials with defined GMO proportions. Detection of 0.1% RR soy was shown in a non-transgenic background, with both the CaMV 35S screening target and the event-specific target detected. The presence of 0.1% RR soy could also be detected in a background of other target sequences, for example, in the presence of both 5% MON810 corn and rapeseed LibertyLink™ synthetic construct.

The time taken to analyse samples is indicated to be comparable to real-time PCR analysis and the researchers demonstrated the robustness of the system by showing that there was no difference in sensitivity of the method when performed by different operators. The sequence analyzer capillary electrophoresis equipment required to analyse the assay products is more readily available to most laboratories than the more highly specialized equipment required for analysis of microarrays.

In summary, this methodology provides the opportunity to establish a profiling-like platform in which simultaneous testing can be carried out for taxon-specific reference genes, screening elements for GM material and construct/event-specific sequences. This would enable the simultaneous detection of authorized, unauthorized and unknown GM events. The modular system should enable easy extension of the targets within an assay to incorporate new sequences of interest. It should be noted, however, that this method has been tested using purified DNA from reference materials and from single plant species. Further testing with processed food matrices is needed to determine the practical limits of detection of the method and the overall robustness of the system for testing food products.

References:

Anklam, E., Lipp, M., Brodmann, P. and Pietsch, K. (1998). Screening method for the identification of genetically modified organisms (GMO) in food. DG JRC Environment Institute, Consumer Protection and Food Unit. 9 June 1998.

Ehlert, A., Moreano, F., Busch, U. and Engel, K-H. (2008). Development of a modular system for detection of genetically modified organisms in food based on ligation-dependent probe amplification. *European Food Research and Technology* 227: 805-812.

Hurst, C.D., Knight, A. and Bruce, I.J. (1999). PCR detection of genetically modified soya and maize in foodstuffs. *Molecular Breeding* 5: 579-586.

Moreano, F., Ehlert, A., Busch, U and Engel, K-H. (2006). Ligation-dependent probe amplification for the simultaneous event-specific detection and relative quantification of DNA from two genetically modified organisms. *European Food Research and Technology* 222(5-6): 479-485.

Schouten, J.P., McElgunn, C.J., Waaijer, R., Zwijnenberg, D., Diepvens, F. and Pals, G. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependant probe amplification. *Nucleic Acids Research*, 30 (12): e57.

Van Hoef, A.M.A., Kok, E.J., Bouw, E., Kuiper, H.A. and Keijer, J. (1998). Development and application of a selective detection method for genetically modified soy and soy-derived products. *Food Additives and Contaminants* 7: 767-774.