Review of Technologies for Detecting Genetically Modified Materials in Commodities and Food

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Foreword

The last decade has seen the development and commercialisation of an increasing number of genetically modified (GM) crops. Whilst only two GM crops, cotton and carnation, are approved for commercial production in Australia, almost twenty different GM crops have been approved for import as food. This is less than half of the total number of GM crops grown worldwide, though each country has its own list of approved GM crops.

Labelling legislation and trade requirements differ from one country to another, leading to the rapid development, internationally, of numerous tests to detect GM material. However, GM samples vary from raw commodities to highly processed foods and testing requirements extend from a general GM screen to a method capable of identifying and quantifying a specific GM crop. The method that is most suited for each foodstuff must be determined on a case-by-case basis.

This review responds to an urgent need to provide an understanding of the complexities of GM testing to relevant Australian industry and government bodies. Progress towards international harmonisation of test results and development of the necessary infrastructure in this area of biomeasurement is presented. This document will assist commercial and regulatory decision-making, to meet domestic and international market requirements for commodities and processed foods.

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1 Executive Summary

This review is designed for use by anybody involved with food production, from growers and distributors to regulators or quality control managers in food production companies. It aims to explain how methods for detecting genetically modified organisms (GMOs) work, to explain what information can be produced from each method and to enable people to understand the results and limitations of each method. In this way, the review will provide people with the knowledge to ask the right questions when providing samples for testing.

Production of a GM food involves integration of a novel DNA sequence into the plant's genomic DNA. This is known as a transformation 'event'. The novel DNA 'construct' contains all the information needed to produce the new characteristic or 'trait' and, in most instances, this includes production of a novel protein. For example, in the case of insect resistance traits, the GM plant produces a novel protein that confers resistance to a particular insect or group of insects.

All testing methodologies currently available for GMOs, therefore, detect either the novel DNA or the novel protein. These detection methods can be divided into four categories. *Screening* methods have the broadest application, as they are suitable for detecting multiple GMO traits. *Trait-specific* methods detect a specific novel protein whilst *construct-specific* methods detect a specific DNA construct used to introduce the novel trait. Finally, *event-specific* methods provide unambiguous identification of a specific transformation event. In many situations, a test will be required that not only detects the presence of GMOs in commodities or food but also measures the amount of GMO present in the sample. This additional requirement for quantification will impact on the most appropriate testing method for that application.

The majority of trait-specific or protein detection methods are immunoassays or Enzyme Linked Immunosorbent Assays (ELISAs) and make use of the properties of antibodies. ELISAs are easy to use, robust and cheaper than DNA detection methods. The lateral flow strip formats are ideally suited to on-site testing and require a minimum of sample preparation. Protein detection methods are best suited to raw or partially processed samples, as many food-processing steps, including cooking, will cause the proteins to unfold or 'denature'. To overcome this limitation, some antibodies have been produced that recognise the protein in its denatured state and so are suited for testing cooked food. Protein detection methods are generally less sensitive than DNA detection methods. Protein detection relies on the amount of protein produced or 'expressed' by the novel DNA construct and also on whether that protein is expressed in the part of the plant being tested.

DNA detection methods are very sensitive, although careful sample preparation is required to extract DNA free from assay inhibitors that are naturally present in many food products. The high sensitivity of DNA methods is achieved by amplifying the copy number of specific target DNA sequences. The most commonly used DNA amplification method is the Polymerase Chain Reaction (PCR), though there are other amplification methods to suit specific applications. DNA detection methods are versatile. By varying the DNA sequence being targeted, the method can be changed from a general screening to a construct- or event-specific method, provided the relevant sequence information is available to the laboratory developing the method. DNA detection can also be used to achieve relative quantification, required for compliance

with labelling legislation and export of raw produce. DNA detection is generally laboratory-based and requires a higher skill level and more expensive equipment than protein detection.

The primary question that this review addresses is how to determine the most appropriate method for detecting GMOs in a particular application. Unfortunately, it is not possible to derive a single table listing types of samples and the appropriate testing method. The range of sample types, from raw commodities to highly processed food, is extensive and the reasons for GMO testing are diverse. In addition, the number of different GMOs grown commercially increases each year. Due to these factors, plus others, each sample must be assessed on a case-by-case basis to determine the most appropriate testing method. The relevant factors are identified and discussed in this review, to explain how they impact on the choice of method.

Prior to embarking on a testing program, a series of questions should be asked:

Why is the product being tested for GMOs?
What level of information is being sought by the test?
Is this a raw commodity, an intermediate material or a highly processed product?
Is the product homogeneous?

The answers to these questions will help to identify the most appropriate testing method for a specific application. It is important to ensure that the chosen test method has been validated for the specific application required. This is particularly important with highly processed foods since certain processing steps may damage or destroy the plant protein and/or DNA. A sampling protocol should be used that reflects the type of product being tested. Communication between clients and the staff at testing laboratories is important in gathering the information required to address these questions and this can only be achieved by an increase in the level of understanding on both sides.

With the diversity of testing options for analysis of GM foods, one of the major challenges that remains with GM testing is the development of an infrastructure that will promote harmonisation of test results across laboratories. Whilst there is only a limited availability of GMO reference materials at this stage, programs are in place to expand the range of reference materials commercially available. Proficiency studies have been established to enable international comparison of laboratory performance and are providing the testing community with valuable feedback on the suitability of their test methods for various levels of processed foods. Criteria for method validation are being discussed in international forums and implemented in several laboratories to ensure that methods being utilised are fit-for-purpose. Whilst testing for GM material is still in its infancy compared to more traditional chemistry-based analytical techniques, significant progress has been made in recent years towards international harmonisation of test results.

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2 Background

2.1 What is a Genetically Modified Organism?

Genetically modified organisms (GMOs) are living organisms whose genomes have been modified as a result of gene technology, resulting in the introduction, removal or alteration of a specific characteristic or trait.

Humans have been altering plants for thousands of years, by selecting and crossing plants with desirable attributes. Until the development of recombinant DNA (rDNA) technologies or gene technology, the extent of gene combinations was limited to sexual reproduction and was a relatively slow process. Irradiation has also been used to alter DNA, but this is a rather more random approach. Gene technology enables the exchange of genes not only within species but also across species boundaries, between organisms that are not sexually compatible. This powerful technique can be used to create novel organisms containing desirable traits derived from potentially any other living organism [1]. Gene technology has many attributes that complement conventional breeding. It can be more rapid than conventional breeding, particularly for novel traits, and allows for greater precision in the selection of desired traits [2]. For a full definition of the technical terms, see **Chapter 3** or the **Glossary** in **Appendix 5**.

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– SECTION 10

Genetically modified organism means:

(a) an organism that has been modified by gene technology; or
(b) an organism that has inherited particular traits from an organism (the initial organism), being traits that occurred in the initial organism because of gene technology; or
(c) anything declared by the regulations to be a genetically modified organism, or that belongs to a class of things declared by the regulations to be genetically modified organisms;
but does not include:
(d) a human being, if the human being is covered by paragraph (a) only because the human being has undergone somatic cell gene therapy; or
(e) an organism declared by the regulations not to be a genetically modified organism, or that belongs to a class of organisms declared by the regulations not to be genetically modified organisms.

(W 65[*])

[*] **Where web sites are referenced in this document, a bracketed reference number annotated with a W has been inserted into the text. This number refers to the web site entry in the list of Relevant Web Sites (Appendix 7).**

2.2 Traits Targeted in GMOs

Several traits have been targeted for improvement by gene technology. The first targets included herbicide tolerance, disease and insect resistance, quality improvements [3] and tolerance to abiotic stresses (eg drought). An example of herbicide tolerant GMOs is the Monsanto Roundup Ready® group of products that have been genetically modified by introducing a gene from the plant pathogen *Agrobacterium tumefaciens* strain CP4. This gene increases the plant's tolerance to the herbicide glyphosate. Insect tolerance has been developed in several crops by introducing one of a family of genes from the bacteria *Bacillus thuringiensis*. This results in the production of a protein that is toxic to certain insects, such as the corn borer - a pest of maize[*].

These traits are providing economic benefits to the agrochemical industry, seed producers and farmers due to enhanced productivity. They also potentially benefit the environment due to a reduction in the use of chemicals or a shift to the use of more environmentally friendly chemicals. Furthermore, a reduction in the amount of tilling required can lead to improvements in soil structure and reduced erosion (W 102).

As yet genetically modified traits have provided little direct benefit to the consumer, other than a reduction of pesticides or herbicides potentially found in food. The next generation of genetically modified (GM) products is focusing on foods with improved nutritional or health values, which are of direct benefit to the consumer. Quality improvements include altering the fatty acid composition of oil products, decreasing the level of anti-nutritional compounds in food or changing harvest characteristics of the food product. Golden rice, for example, has a high level of the Vitamin A precursor, beta-carotene, and has been developed by a group in Switzerland to address the Vitamin A deficiency that is common in children from developing countries. Golden rice contains a gene from daffodils that encodes the enzyme phytoene synthase. This enzyme is necessary for synthesis of beta-carotene and is produced in the rice endosperm [4], giving the rice it's golden colour. The beta-carotene is converted to Vitamin A in humans. Incorporation of beta-carotene into the staple diet of these children promises to prevent blindness and other debilitating diseases [1]. Other examples of products in the pipeline include the introduction of novel proteins such as glutenins into wheat to improve the quality of bread, modified coffee plants that inhibit caffeine synthesis and provide 'naturally decaffeinated' coffee [3] and onions that do not make eyes water [4].

Reduced allergenicity of foods such as peanuts could be achieved by altering the properties of some proteins [3]. The production of vaccines in plants such as rice [5], bananas or potatoes [3] is currently under evaluation and has been referred to as 'pharming' [6], for the production of pharmaceutical products in GM plants or animals. A novel approach to the use of GM food is the introduction of a gene that encodes for an antibody to the bacteria associated with dental caries, *Streptococcus mutans*, potentially reducing the incidence of dental carie formation [3]. Other applications for GMOs that are under consideration include production of biodegradable polymers for the plastics industry, plants that can be used for bioremediation of polluted sites such as GM rice that can degrade chlorinated compounds [7] and forest trees with enhanced growth and improved timber qualities [8].

[*] In this review we have used the name maize, though this can be used interchangeably with corn. Likewise, we have used the names soy and canola but soya and oilseed rape, respectively, could also be used.

2.3 The Fundamental Building Blocks of Life and their Role in Gene Technology

Deoxyribonucleic acid (DNA) is present in all living organisms, with the exception of some viruses. DNA provides the genetic blueprint or code for an organism and is identical in each cell of a particular organism. It contains all of the information necessary to synthesise proteins – the molecules that provide the functionality of an organism. However, DNA not only encodes this information but also the instructions to control the **amount** of each protein synthesised and **where** and **when** in the organism a protein will be synthesised.

DNA is composed of four chemical units known as nucleotide bases; namely, adenine (A), guanine (G), cytosine (C) and thymine (T). These nucleotide building blocks are the 'letters' in the DNA code and are arranged in a chain-like molecule or 'DNA strand', linked by a sugar-phosphate backbone (**Figure 2.1 a**). DNA consists of two strands of 'complementary' sequence that are held together by hydrogen bonds. Complementarity refers to the fact that the sequence of one DNA strand is not identical to the other strand but is complementary, since A can only pair with T in the opposite strand, and C with G, due to their chemical structure. The two DNA strands are intertwined forming the DNA double helix.

The complete set of DNA sequences present in each cell of a given species is referred to as that species' genome. In humans, this genome is approximately 3,200,000,000 nucleotide pairs long. The DNA in eukaryotes is carefully packaged around special packaging proteins to form chromosomes in the cell nucleus. Each eukaryote species has a characteristic number of chromosomes. Humans have two sets of 23 different chromosomes, one set inherited from each parent. The potential for variability with respect to the sequential ordering or 'sequence' of nucleotide bases that comprise a DNA strand accounts for the observed and striking differences among living forms.

Within the enormous expanse of a DNA molecule, particular functional regions of nucleotide sequence are defined as genes. The human genome contains at least 30,000 genes. Each gene is composed of a promoter, an open reading frame (ORF) and a terminator. The promoter can be considered as the control switch, part of the cellular machinery that determines whether a gene is active or inactive. The terminator represents the endpoint of a gene or the DNA equivalent to a full-stop. It is the sequence of DNA within the ORF of a gene that ultimately determines the sequence of amino acids in a protein.

The synthesis of protein from DNA occurs in a two-step process. First the DNA sequence of the ORF is copied, using one DNA strand as a template, to produce a single strand of ribonucleic acid (RNA), a molecule very similar chemically to DNA though much more supple. This process is known as RNA transcription and occurs in the cell nucleus, where the DNA is stored (**Figure 2.1 b**). The resultant RNA strand is transported out of the nucleus for the second stage of protein production, namely protein translation. This step occurs at the ribosomes and involves the translation of nucleic acid sequence (RNA) into protein sequence, to form an amino acid strand (**Figure 2.1 c**). Amino acids are the building blocks of all proteins. The newly synthesised amino acid strand is then folded and often modified to produce the functional protein.

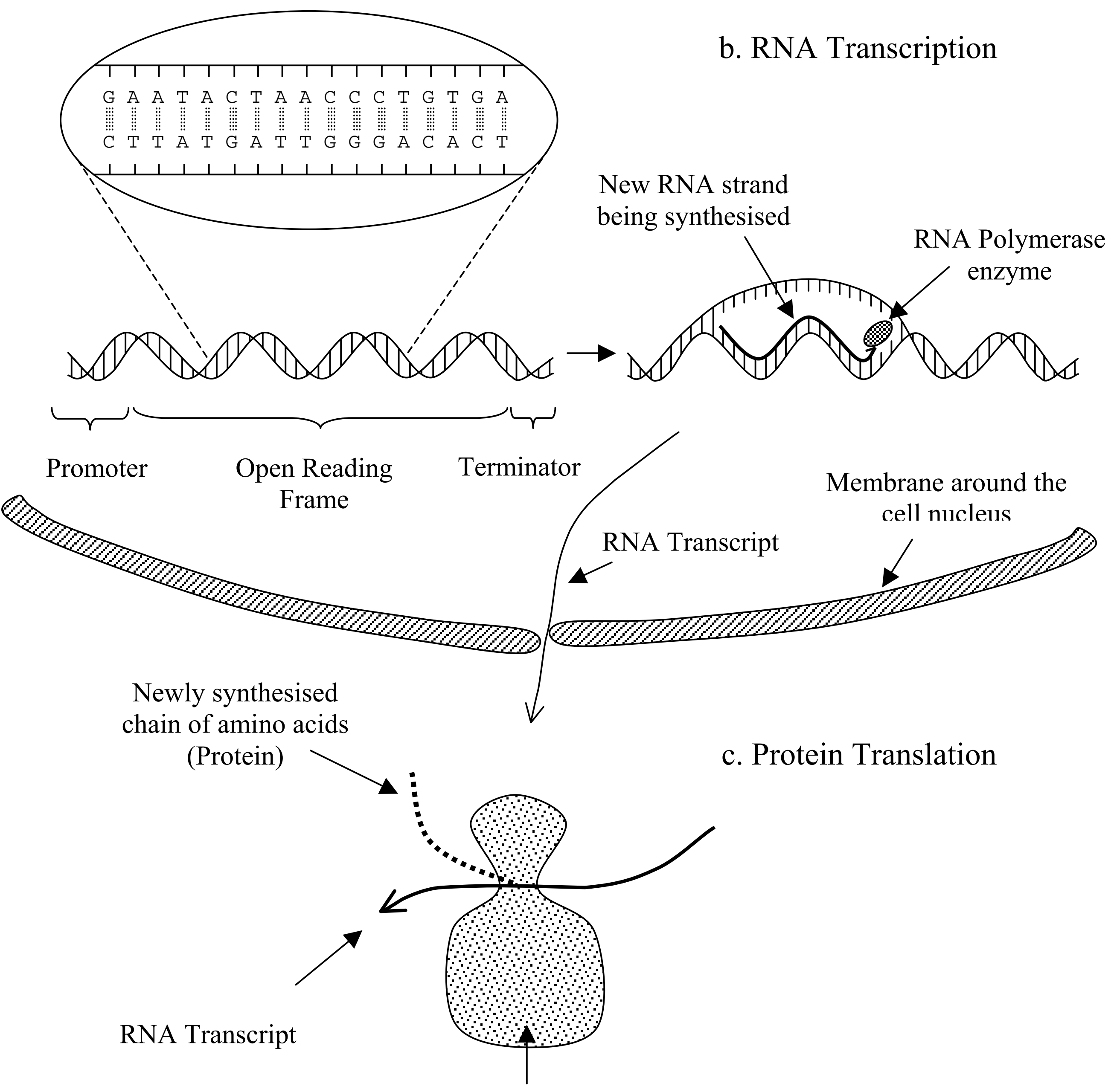


Figure 2.1 Simplified Diagram of Translation from DNA to Protein

Protein production occurs in two stages: (1) transcription of DNA into RNA and (2) the translation of RNA into protein. Following the diagram above, an RNA strand is transcribed from the template DNA strand of the gene's ORF, by the RNA polymerase enzyme (b). The single stranded RNA then leaves the nucleus and moves to the ribosomes. The nucleic acid sequence (RNA) is translated into a protein sequence (c), consisting of a strand of amino acids. The newly synthesised strand of amino acids is then folded and modified to produce a functional protein.

Put simply, specific genes encode the information required for synthesis of specific proteins. The promoters and terminators influence the production of proteins, leading to expression of different amounts of specific proteins in different cell types or at different stages of the cell's life cycle. For example, proteins produced by the liver are, in many cases, different to those required by the heart muscle, although all heart and liver cells of an individual will possess the same DNA.

Whilst the DNA composition is identical in each cell of an organism, the protein composition varies considerably from one cell type to another within the same organism and can also vary at different stages of the cells' life cycle.

2.4 How are GMOs Designed to Achieve the Desired Traits?

The desired trait or characteristic will influence the approach that is taken in designing or 'constructing' the foreign DNA to be inserted into the plant genome. The introduced gene will comprise of the ORF of the gene of interest (eg an insecticidal gene), together with a promoter and terminator to control gene expression at the desired time, level and location. Each of these three components of the introduced gene may be derived from a different source. In the case of an insect tolerance trait, a promoter might be chosen to ensure expression of the protein only in the leaves and stems. It would be of little benefit for the plant to be synthesising large quantities of this particular protein in the root system, which is not attacked by the pest. A different style of promoter may be chosen in the construction of a gene that would impart delayed fruit-ripening characteristics. Thus gene technology, in principle, makes it possible to introduce any gene from any organism into any plant and to express that new product in a specific part of the plant, be it seed, leaf, root or tuber.

In some cases, the desired effect of the genetic modification is to suppress protein production from a pre-existing plant gene rather than produce a new protein. This is usually accomplished by the introduction of a synthetic gene, its DNA sequence being complementary to that of the gene to be suppressed. In this scenario DNA transcription results in two populations of RNA molecules, one from the original gene and one from the synthetic gene. These RNA molecules have complementary sequences and so form RNA duplexes. The formation of double-stranded RNA molecules activates a degradation system that chops up the RNA duplex and thus prevents protein production from the naturally occurring gene. Several varieties of genetically modified tomatoes that exhibit delayed fruit ripening traits are produced by this style of genetic control.

In principle, gene technology makes it possible to introduce any gene from any organism into any plant and to express that new product in any part of the plant, be it seed, leaf, root or tuber.

2.5 How are GMOs Produced?

2.5.1 Transformation Methods for Plants

Transformation is defined as the uptake and integration of DNA in a cell, in which the introduced DNA is intended to change the characteristics of the recipient organism in a predictable manner (W 121). For transformation of plants, segments of the target plant, a culture of callus cells or dedifferentiated seed cells are used. The most successful technique reported uses the plant pathogenic bacterium *Agrobacterium tumefaciens* as a means of introducing new genes into plant genomes. This bacterium contains an extra piece of DNA, present as a large loop or plasmid, called the Ti plasmid and can naturally infect dicotyledonous plants. A part of the Ti plasmid is passed from the bacterium into the plant cell and integrates into a plant chromosome where it remains permanently. This new DNA sequence contains instructions that change the plant's cell division pattern and converts the cell into a crown gall tumour. Researchers have exploited the Ti plasmid for many years for the production of GM plants. Instead of the tumour-forming sequences, novel gene sequences are inserted into the Ti plasmid, which are then stably integrated into the plants genome via this naturally occurring transformation system [9]. More recently, this transformation tool has been modified to allow transformation into important monocotyledonous crop plants such as rice, corn, wheat and sugarcane [10].

Physical techniques of plant transformation involve 'particle gun' or 'gene bombardment', perforating the cell walls with tiny particles of tungsten coated DNA, or high voltage electrical pulses to punch holes in the outer membrane of the cells to allow entry of naked DNA (called electroporation). Another physical method of piercing the cell wall involves vortexing cultured plant cells with needle shaped crystals of silicon carbide, an industrial abrasive.

When a plant cell is transformed, the introduced piece of DNA becomes stably integrated somewhere in the plant genome. Ideally it should be present in a single copy at this stage, though multiple insertions very often occur during the transformation process. The number of copies partly depends on the transformation method used. The *Agrobacterium tumefaciens* method is more commonly used for transforming dicotyledon plants and results in low copy numbers of insertions per genome. The bombardment methods are mainly used for monocotyledons, such as the cereal crops. They usually lead to higher numbers of copies per genome, as the DNA often forms concatemers prior to integration, where individual copies of the DNA construct join up end-to-end. For example the GM maize, Bt-176, has multiple copies of the cry1Ab gene (ANZFA Final Risk Analysis Report. Application A385 Food Derived From Insect-Protected Bt-176 Corn) [11]. In both types of transformation methods the multiple integrations usually occur at one location.

Once the transformant has been selected, the cells are induced to grow into a mature plant (differentiated), using plant growth hormones. Each independently selected transformant contains the same foreign DNA sequence, but with the sequence integrated into a different location in the plant's genome and so each is defined as a different transformation 'event'.

All plants have at least two copies of each chromosome, but the introduced gene is only integrated onto one of these copies. Just as for animals, when plants produce gametes (pollen and egg cells or ovules) cell division occurs and the chromosomes are sorted so

that only one copy of each chromosome pair is transferred to each gamete. In order for every gamete to carry a copy of the novel DNA sequence, the GM plant must be allowed to self-pollinate. Individuals are then selected that contain two copies of the novel DNA, one on each copy of a chromosome pair. These lines are said to be homozygous for the novel DNA sequence and this step ensures that the new trait is stably maintained in subsequent generations.

2.6 What are the Major Considerations when Testing for GMOs?

GMO testing is a challenge for many reasons. These include the number of GM crops being grown worldwide and the even greater variety of foods that potentially contain GMO derived ingredients. It is quite possible that a food product may contain more than one ingredient that is derived from a GM crop. Therefore, the testing process may need to not only detect the presence of GMOs but be able to identify and quantify the amount of each particular GMO that is present. Finally, foods may range from raw commodities to highly processed foods such as cakes and breads. Any of the processing steps can affect the GMO detection. Ideally, a testing regime should be able to detect the presence of GMOs in a range of sample types.

It is not hard to appreciate why there is not a single method that suits all purposes. In this review some of the difficulties involved in testing are highlighted and explanations of why some methods are suited to certain applications and not others are provided. This will help to decide which method best suits the specific application. All testing methodologies currently available for GMOs detect either the novel DNA or the novel protein. The method that is most suited for each foodstuff must be determined on a case-by-case basis.

Prior to embarking on a testing program, a series of questions should be asked.

Why is the product being tested for GMOs?
What level of information is being sought by the test?
Is this a raw commodity, an intermediate material or a highly processed product?
Is the product homogeneous?
(W 108)

2.6.1 Why is the Product being Tested for GMOs?

Testing for GMOs may be undertaken to comply with labelling requirements, to ensure a product meets export market requirements or to determine the presence of approved or unapproved varieties of GMOs. Testing can also be used to complement an identity preservation system that involves ‘chain of custody’ documentation from the farm to the final product. In these circumstances, an audit trail is maintained with confirmation testing at various production stages. (Traceability and Identity Preservation are discussed further in **Chapter 7**.)

2.6.2 What Level of Information is being Sought by the Test?

This is a very important question to consider and may, on its own, determine the type of test that is required to analyse a sample. If the requirement is simply to determine

whether a commodity or food product contains GMOs, a general qualitative screen would be the most appropriate approach initially. Promoter and terminator gene sequences such as the cauliflower mosaic virus (CaMV) 35S promoter and the *Agrobacterium tumefaciens* nopaline synthase terminator (NOS) are used in a number of commercially released GMOs. Tests designed to detect either of these two DNA sequences are suitable as general screens that would detect a large number of currently available GMOs.

It may be necessary to determine if a specific GM event is present in a commodity or food product. In this case, a highly specific test would need to be conducted that unequivocally demonstrates the presence or absence of a particular GM event. Quantitative analysis cannot be undertaken using a screening technique and requires analysis using a more specific protein- or DNA-based method. Appropriate methods for quantification are discussed in **Chapters 4** and **5**.

As a rule of thumb, the more demanding the requirements, the more precise the testing procedure and so the more expensive the cost of providing the answers.

2.6.3 Is this a Raw Commodity, an Intermediate Material or a Highly Processed Product?

Testing protocols can be designed around detection of either the novel DNA sequence or the novel protein present in a GMO. However, the range of tests available for analysis generally decreases with an increase in the level of processing. Raw agricultural products can be analysed using either a DNA- or protein-based detection method, provided that the protein is expressed in the plant tissue being examined. However, various processing steps such as solvent extraction, refining or cooking can remove or damage either the DNA or protein so that it is no longer recognised or detected in a GMO test. A list of the types of processing methods used in food production is given in section **6.2 Processing steps and their effects on protein and DNA**.

In general, protein detection methods are most suitable for raw or partly processed products such as ground flour. Proteins are generally denatured by heat and so usually cannot be recognised in cooked food, though there are some kits on the market designed to detect specific denatured proteins. DNA is much more stable than protein and can be detected in raw ingredients as well as products that have been heated, provided that the test has been designed to detect smaller fragments of DNA. Methods have been developed that can detect DNA in rendered meat used in animal feed. This meat has been cooked at high pressure for extended periods of time.

For highly processed products, such as sugar and oils, the production process removes the proteins and DNA and so it is not possible to determine whether they were derived from GMOs. If the presence of GMOs needs to be determined, sampling and testing would have to be carried out at an earlier stage of the processing.

As a general rule, the more processed the product, the fewer the options for analysis (W 108).

2.6.4 Is the Product Homogeneous?

No matter which method of analysis is used, the sampling protocol is critical and, if not designed carefully, can introduce a significant source of error. For example, when testing for the unintended presence of GMOs in a container full of grain, the GMO contamination would be unlikely to be found evenly distributed and so inadequate sampling can lead to inaccurate results. For raw commodities, the United States Department of Agriculture (USDA) Grain Inspection, Packers and Stockyard Administration (GIPSA) have put out some sampling guidelines, based on statistical calculations of probability (W 106).

The chosen sample size depends on the product being tested, the particle size, the expected level of homogeneity, the required limit of detection, the level of confidence required and other individual requirements. For instance, a test for whole grain that has a required limit of detection of 0.1% (w/w) must be capable of detecting 1 grain in 1000 grains. Thus the absolute minimum sample size is 1000 grains although larger sample sizes would increase the confidence of the test result. For this reason it is often easier to sample partially processed food, such as flour, as there is a far greater degree of homogeneity.

Sampling devices must be free of material before collecting the next sample. Excessive grain dust has been known to cause false positive results. Processing can also be a source of contamination, as grinding produces dust that can drift (W 108).

More details, plus examples of a sampling plan can be found in **Chapter 6**.

The answers to these four questions form the starting point in determining the most appropriate testing method. Staff at the testing laboratory may not know these answers, since they either relate to what has happened to the sample prior to collection or they relate to the customer's specific information needs. This highlights the importance of communication between clients and the staff at testing laboratories. Improved communication can only be achieved by an increase in the level of understanding on both sides.

2.7 Safety Regulation of GMOs in Australia

2.7.1 Office of the Gene Technology Regulator (OGTR)

In December 2000, the Federal Government passed The *Gene Technology Act 2000* and associated Acts, which then came into effect in June 2001 along with the commencement of the ***Office of the Gene Technology Regulator (OGTR).*** *The Act 2000*, plus complementary State and Territory Gene Technology Acts, regulate all dealings (as defined in *the Act 2000*) with GMOs in Australia, including research, field trials, manufacture, production, commercial release and import of genetically modified living things and their offspring. The OGTR carries out extensive risk assessments for GMOs before granting approval for either field trials or commercial release. So far only two GMOs have been given approval for commercial production in Australia: Cotton and Carnations. The details of sites approved for field trials are available on the OGTR web site (W 70).

The Gene Technology Act 2000 requires the OGTR to consult with other Commonwealth Government agencies, the States, the Gene Technology Technical

Advisory Committee, the Commonwealth Environment Minister and appropriate local councils regarding all applications for licenses to undertake dealings involving intentional release of GMOs into the environment. The OGTR consults with these bodies in order to seek advice on the preparation of risk assessments and risk management plans.

2.7.2 Agriculture, Fisheries and Forestry – Australia (AFFA)

The Australian Quarantine and Inspection Service (AQIS) is within AFFA and regulates the importation into Australia of all animal, plant and biological products that may pose a pest or disease risk in accordance with the *Quarantine Act 1908.*

The Quarantine Act requires an importer to give notice of a proposed importation of a GMO. ***Biosecurity Australia (BA)*** via an assessment system, based on the weed risk assessment system, considers the pest and disease ramifications of any GM based products compared to their conventional counterparts.

AQIS certifies exports of agri-food products and commodities from Australia in accordance with the requirements of the *Export Control Act 1982*. Based on identification and inspection of products and commodities, AQIS certifies that products and commodities for export from Australia meet the requirements of importing country governments in accordance with the Sanitary and Phytosanitary (SPS) Agreement.

In providing this certification, AQIS relies upon inputs from a number of sources, mostly third party, State or Commonwealth Government agencies and testing laboratories to provide verification data to underpin certification. When an importing country has a government requirement for certification of the GM status of a product or commodity that is consistent with SPS protocols, AQIS attaches to its export certification a statement from the OGTR that there has been no commercial release of that commodity in Australia.

When commercial GM food crops are introduced in Australia, AQIS will no longer be able to issue export certificates with OGTR statements. In the absence of such OGTR statements, and if certification concerning the GM status of foods or crops is required by importing countries who have a legitimate SPS-based reason for requiring notification of any GM inputs, the following two options would have to be addressed before AQIS could certify the commodity or product for export:

- a traceable and auditable identity preservation system; and/or
- a robust and reliable testing regime.

2.7.3 Food Standards, Australia New Zealand (FSANZ)

The safety of GM-derived food products is the joint responsibility of ***Food Standards, Australia New Zealand (FSANZ***), formerly the Australia New Zealand Food Authority (ANZFA), and the State and Territories' Government, under Standard 1.5.2 of the Food Standards Code. This Standard requires that all GM food or ingredients to be sold in Australia and New Zealand must undergo an extensive, mandatory pre-market safety assessment to ensure that it is safe for human consumption. Based on these assessments, FSANZ will grant approval for specific GMOs to be imported.

FSANZ is primarily responsible for setting food standards, determining the frequency of inspection for particular foods and deciding which test will be performed on these foods. AQIS is responsible for providing operational services to carry out the necessary

inspections and tests. There is currently no identified threat to public health and safety from GM foods.

2.8 Labelling Regulation of GMOs in Australia

In addition to the safety assessments, since December 2001 the Standard requires the mandatory labelling of all GM food sold in Australia and New Zealand when novel DNA or protein is present in the final product or if the food has altered characteristics. This allows consumers to make informed choices on whether to buy GM food. The Compliance Guide to Standard A18 for labelling GM food can be found at:
www.foodstandards.gov.au/whatsinfood/gmfoods/complianceguidea18gm.cfm.

Some highly processed foods contain no traces of DNA and/or protein. In these cases, there is no analytical method available to identify whether these products are derived from GMO so and they do not need to be labelled (**Figure 2.2**). These products must be prepared using an 'approved' method, one that that has been demonstrated to remove protein and DNA from the product to below detectable levels. Some examples of food ingredients that do not normally contain traces of protein and/or DNA include starch derivatives (maltodextrin, glucose), protein hydrolysates, highly heat-treated finished products, refined oils, purified enzymatic preparations, sugar and soy sauce [12].

2.8.1 Australian Competition and Consumer Commission (ACCC)

The ***Australian Competition and Consumer Commission*** has particular interest in ensuring the any GM or GM-free labelling is in compliance with the Trade Practices Act 1974. The ***ACCC*** closely monitors the voluntary claims that businesses make over and above their obligations in relation to the Food Standards Code to ensure that any voluntary claims do not constitute false, misleading or deceptive conduct under section 52 of the Trade Practices Act. In regard to voluntary 'GM-free' or other negative GM claims, the ***ACCC*** looks for documented verification systems underpinned by an effective Trade Practices compliance program. Where a food or ingredient is known to have the unintentional presence of a GMO it cannot truthfully carry a negative claim. For voluntary negative GM claims the *ACCC* advises the claim should be made in full, for example using statements such as *"best endeavours have been made to use non-genetically modified food ingredients"* (FSANZ Compliance Guide to Standard A18 for GM Labelling).

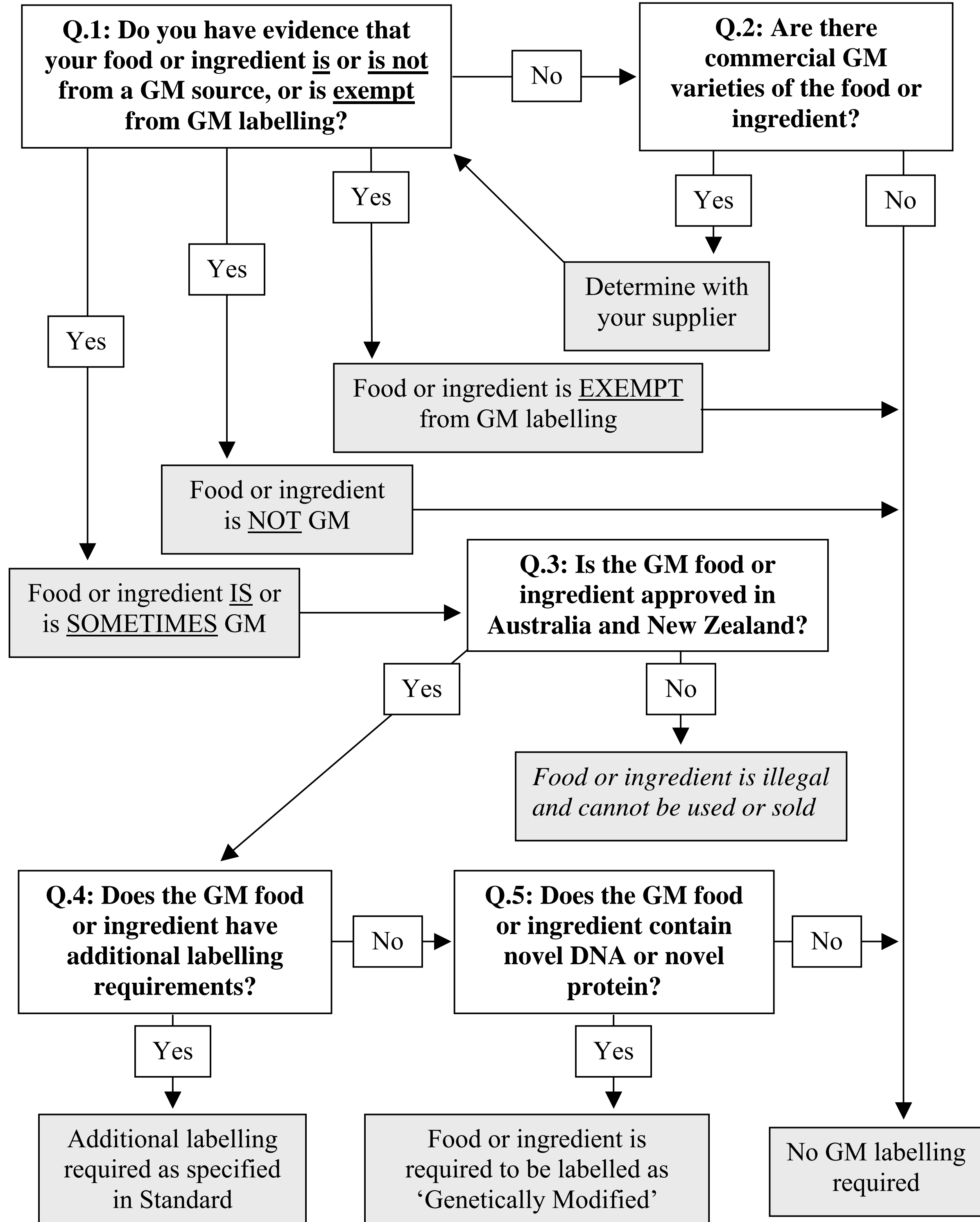


Figure 2.2 Decision Tree on Labelling for GM Food.

This decision tree is taken from the FSANZ 'Compliance Guide to Standard A18, Labelling Genetically Modified Food'. Further information on each question is provided in the Guide. To answer Questions 1, 3 and 5, if verifiable documentation is not available from the supplier, appropriate testing may be required.

2.9 International Regulation of GMOs

Legislation regulating the safety assessments and labelling of GMOs varies considerably between different countries [12]. Most countries require a mandatory pre-market safety assessment, based on Organisation for Economic Co-Operation and Development (OECD) and joint Food and Agriculture Organisation of the United Nations (FAO)/World Health Organisation (WHO) guidelines. The United States of America (USA) use a 60-day notification period for intention to apply for approval. The Codex Alimentarius guidelines for safety assessment of food derived from GM plants are now finalised and are at step 8 of the Codex procedure, and it is likely these guidelines will become accepted as the benchmark for many countries to harmonise with. The Codex Alimentarius Commission (Codex) is the joint FAO/WHO body responsible for compiling the standards, codes of practice, guidelines and recommendations for the international food code (W 112).

The use of a common set of guidelines for the safety assessments used in different countries reduces potential trade barriers. These assessments include scientific data provided by the company producing the GMO, including compositional and nutritional analysis, toxicological and allergenicity analysis, and molecular characterisation of the novel DNA construct. In Australia, New Zealand and Japan, there is also requirement for the full DNA sequence of the novel construct, plus sequence from the adjoining plant genome to be provided. This allows assessment of the feasibility of producing truncated proteins due to integration of the construct into a plant gene.

FSANZ and Health Canada have been running capacity building workshops within the ASEAN (Association of South East Asian Nations) countries to demonstrate how Australian and Canadian safety assessments on GM foods are carried out and to assist these countries in developing their own guidelines and regulatory frameworks. Workshops have been conducted in Singapore and Kuala Lumpur, and more are planned for Thailand and Indonesia next year (Paul Brent, FSANZ, personal communication).

The labelling legislation is not so well harmonised internationally. Whilst Australia has a tolerance level of 1% adventitious GMOs, other countries such as Japan and Korea currently have a higher tolerance level. The requirement for labelling in the USA is voluntary. On the other hand, the European Commission (EC), on behalf of the European Union (EU), have proposed new legislation to drop the threshold for adventitious contamination with GMOs from 1% to 0.5%. The EU are also planning to adopt a process-based rather than the product-based GM food labelling approach, so that any product derived from gene technology would have to be labelled, even if the novel DNA and/or protein were completely removed. The EC also plan to phase out the use of antibiotic resistance genes as markers for the production of GMOs. However, recent indications are that the EC member states are still not unanimous on this new legislation (Paul Brent, FSANZ, personal communication).

GMO legislation is always subject to change and the latest information on individual countries should be obtained from the relevant national authority.

The Cartagena Protocol on Biosafety is the first Protocol to the Convention on Biological Diversity and will provide a framework for addressing environmental impacts of bioengineered products (referred to as living modified organisms or LMOs) that cross international borders. The Protocol establishes an internet-based Biosafety

Clearing-House to help countries exchange scientific, technical, environmental and legal information regarding LMOs. It will require bulk shipments of LMO commodities that are intended to be used as food, feed or for processing, to be accompanied by documentation stating that such shipments "may contain" LMOs and are "not intended for intentional introduction into the environment". This will allow governments to prohibit the import of GM food if they have not been approved in that country. The Protocol will enter into force 90 days after the 50th country has ratified it, which may be in early 2003, due to the accelerated number of countries registered since June 2002 (W 112).

2.10 Where are the Majority of GMOs Produced?

By 1999, more than 40% of the maize, 50% of the cotton and 45% of the soy acres planted in the USA were GM and at least 60% of the food in the USA supermarkets contained GMOs [13]. By the end of 2001, over 52.6 million hectares of GM crops were planted worldwide (W 115). The USA, Canada, Argentina and China currently share 99% of the area worldwide planted with GM crops. Australia accounts for less than 1% of the total global area of GM crops (W 111)

3 Definitions That Apply to Testing Methods for GMOs

3.1 Constructs, Events, Traits and Lines

When creating a GMO, there are a series of sequential steps to go through and there is specific terminology that goes with these steps.

First the novel DNA sequence is constructed (the **construct**), often by taking the separate components from different sources of DNA. A gene is chosen that will confer the desired characteristic or **trait**, such as resistance to a herbicide. The appropriate promoter and terminator sequences are selected so that the protein encoded by the gene will be produced, or **expressed**, in the correct plant tissue (eg leaf and stem). In most cases a marker gene is also used, such as an antibiotic resistance gene, that allows the selection of the plant cells receiving the novel DNA construct. In the case of a herbicide tolerance trait, the herbicide gene itself may serve as both a selectable marker and a whole plant herbicide tolerance trait. In some instances a detectable marker gene such as β-glucuronidase (GUS) is used that will allow transformed cells to be visually identify using a simple colour staining. These selectable marker genes allow the researcher to track the construct during the development and commercialisation of the GMO.

The DNA construct, once assembled, is then transformed into plant cells by one of the transformation methods described in **Chapter 2**. From here the construct can integrate into one of the plant's chromosomes, though this integration step actually occurs at a very low frequency. Once inserted, the new construct will remain stably integrated in this location. This is referred to as an integration **event**. These rare transformants are identified using the marker gene. In the case of a herbicide resistance marker gene, the plant cells are exposed to the herbicide and only those that contain the integrated construct will survive this selection process. Each independently selected transformant will have an insertion at a different location in the plant's genome and will therefore be a different event.

Theoretically, all events resulting from a transformation should contain the same construct and should express the same trait. However, this is not always the case and each transformant must be thoroughly investigated to ensure that it contains the novel DNA and expresses the desired novel trait. Using the Roundup Ready® traits as an example, this would mean checking that the protein that confers glyphosate resistance was produced in suitable quantities in the correct tissue types. During this process, many of the transformation events are eliminated because the construct has either integrated into and inactivated a functional plant gene, or the construct has been rearranged during the transformation process, making the gene sequence no longer functional. Once an event is selected that has all the desired attributes, it will be induced to produce self-fertilised seeds and the next generation of GM plant, to be used in stability tests. Following government approval, this new GM **line** would be grown on a much larger scale, for field trials, safety tests and risk assessments, and possibly bred into a variety of high performing elite varieties of the particular crop plant for commercial use later.

Definitions for GMOs

Construct – An engineered chimeric DNA sequence designed to be transferred into a cell or tissue. Typically, the construct comprises the gene or genes of interest, a marker gene and appropriate control sequences as a single package (W 121).

Event – Transformation of an organism by inserting a piece of DNA (the Construct) into the genome. Events vary in the particular location that the construct is inserted into the host genome and may vary in the precise DNA sequence inserted into the organism [1].

Expression (of a gene or protein) – The process by which a gene's coded information is translated into RNA and/or protein molecules (W 122) (See also **Figure 2.1**).

Trait – Genetic traits are those aspects of an organism controlled by genes, for example, eye colour in humans (W 122), resistance to the herbicide glyphosate in Roundup Ready® soy. Genetic traits are inherited.
A GMO is named by referring to its novel trait, although in every other way it is identical to its conventional counterpart.

Line (Lineage) – A group of individuals related by common descent (W 121), for example, a GM variety derived from a single transformation Event.

3.2 Styles of GM Testing Methods

The initial test carried out on a sample is generally a **screening** test that may detect a range of GMOs. This can be followed by a specific test to **identify** the type of GMO present in the sample and in some cases this test is also designed to **quantify** the amount of a specific GMO.

3.2.1 Methods for Screening for GMOs

Screening methods detect the presence of a range of different GMOs but do not identify the type of GMO present. For example, DNA-based methods could detect a particular DNA sequence that many GMOs have in common. Currently, the most frequently used promoter and terminator sequences are the CaMV 35S promoter and the *Agrobacterium tumefaciens* NOS terminator. A combination of two screening methods to detect these gene sequences would detect 80% of the currently approved GM crops worldwide and would detect all but one of the GMOs currently approved by FSANZ for use in Australia. By extrapolating this concept, microarray methods using multiple probe sequences can be designed to carry out a large number of tests for detecting many different GMOs on a single microchip. Currently microarray methods only indicate the presence of GMOs but not how much is present. However, microchip technology is

improving rapidly in terms of sensitivity and accuracy and may be quantitative for multiple GMOs in the future [2].

In general, protein-based methods are not suitable as a screen for detecting a range of GMOs (see section **4.4.3**).

Definitions used for GM tests

Screening – Tests that detect the presence of multiple GMOs. These methods are not quantitative and cannot confirm the presence of specific GM traits.

Trait-Specific – Protein-based tests that detect the novel trait or characteristic of a GM line.

Construct-Specific – DNA tests that are designed to detect sequences that have been constructed, so are not found in nature. Examples include the junctions between the different components of the construct (eg promoter and ORF junction). Some construct-specific tests will also detect a GM trait.

Event-Specific – DNA tests that are designed to detect the junction sequence between the inserted construct and the genomic DNA. These tests are suitable for Relative Quantification and for identification of a specific GM event.

Quantitative (absolute) – tests that measure the amount of a substance, eg how many milligrams of a specific protein or how many copy numbers of a specific DNA sequence are present. Results are presented as an absolute value. The larger the size of the sample being tested, the higher the absolute quantity.

Quantitative (relative) – tests that measure the amount of a substance relative to another substance, eg how many milligrams of a specific protein are present per gram of total protein or how many copy numbers of a specific DNA sequence are present per genome. Results are presented as a percentage. This percentage does not change with an increase in the size of the sample being tested. For compliance to the GMO labelling legislation, relative quantification is required.

3.2.2 Methods for Identification of GMOs

Identification of GMOs can be considered at two levels. The first is identification of the GM trait or the DNA construct. For example, the identification of GM maize expressing the *Bacillus thuringiensis* δ-endotoxin cry1Ab protein, protecting it from the European corn borer. In this case, protein-based assays detect the presence of the cry1Ab protein

and can be referred to as a trait-specific, whilst DNA-based assays identify the specific DNA construct and so are called construct-specific (see **Figure 5.1** in **Chapter 5**).

The second approach for identification is called an event-specific test and this, as the name suggests, unequivocally identifies a specific GM event by targeting the junction between the plant genomic DNA and the inserted construct DNA. All event-specific tests are DNA-based (see **Figure 5.1** in **Chapter 5**). It is not possible to have an event-specific protein-based method.

3.2.3 Methods for Quantification of GMOs

Quantification of GMOs can be either absolute or relative depending on the type of assay used. However, labelling regulations in Australia require knowledge of the proportion of any commodity or ingredient in a food product that is GM, (ie the relative amount of an ingredient that is GM). An absolute measurement of the level of GM ingredient in a food product cannot be used to determine compliance with labelling requirements.

3.3 Limit of Detection

For each testing method, a Limit of Detection (LOD) is defined to show the sensitivity of the method and is usually expressed as a percentage. For example, a method with an LOD of 1% (w/w) for Roundup Ready® soy would be able to detect Roundup Ready® soy in a batch of soy flour when present as 1% (w/w) of the total soy flour. It is important to note that this LOD value is written in an abbreviated form and actually means the “LOD for Roundup Ready® soy is 1% (w/w) of total soy flour *if the product is comprised of 100% soy*”.

For agricultural commodities, the LOD percentage is a stand-alone value, since samples being tested are comprised of close to 100% of a single crop type (allowing for low levels of adventitious contamination by other crops).

For food products containing several ingredients, estimation of the LOD is complicated by a number of factors. Firstly, a method with an LOD for Roundup Ready® soy of 1% (w/w) of total soy flour *given the product is comprised of 100% soy* may not detect the presence of 1% (w/w) GM soy in total soy if this is only one of several ingredients in the food product. For instance, if the 1% (w/w) GM soy flour were used as a baking ingredient in cake, the soy flour may make up only 0.5% (w/w) of the total cake ingredients. The GM soy would thus be only 0.005% (w/w) of the total sample, which would be well below the LOD for this method. Secondly, whilst the LOD is normally defined on a % (w/w) basis, the actual measurement of GM ingredients is based on either DNA or protein and this DNA- or protein-based measurement is not necessarily directly transferable to a % (w/w) measurement. Some ingredients in a food product may contain no DNA (eg water and sugar) while other ingredients vary widely in their individual weight-to-DNA-content ratios and genome sizes. Even though total DNA can be extracted from a cake sample prior to testing, it is not possible to determine what percentage of the DNA was derived from soy, GM or otherwise. Hence, a method LOD should be defined based on analysis of a product comprising a single ingredient, so should be applied to complex food products with caution.

To deal with the issue of LOD when testing food products containing more than one ingredient, a clause has been introduced into some labelling legislation stating that any ingredient present in less than a defined percentage (w/w) of the total product would fall

outside of the labelling requirements and so would not need to be tested. For example, if the threshold were set at 1% (w/w) of the total product, any ingredient that is present at less than 1% (w/w) of the total product would not need to be tested. This would mean that if the threshold for labelling is 1% GMO in non-GMO ingredient (as it is in Australia) and the ingredient is present at 1% of the total product, the actual LOD would have to be at least 0.01% in order to detect the GMO. This detection limit is achievable using DNA amplification methods following extensive optimisation, provided the DNA is relatively undamaged and free of PCR inhibitors.

This clause would not allow for the differences between genome size and weight-to-DNA-content ratio for each ingredient but would still be an improvement leading to attaining realistic testing and labelling goals. Australian legislation already includes flavourings present at or below 1g/kg (0.1%) in the final food amongst the products that are exempt from labelling (W 64).

In Japan, the threshold for GM labelling is 5%. For products with a single ingredient, if the GM content is 5% (w/w) or more it must be labelled “GM ingredient used”. For food products containing more than one ingredient, if any ingredient is present in less than 5% (w/w) of the total product it does not need to be labelled, even if that ingredient is derived from a 100% GM source. For example, if a cake were made with 4% (w/w) flour from GM soybeans, the whole cake (ie the finished food) would not need to be labelled, as it would be below the 5% threshold. Whereas the flour, if sold as a single ingredient, would be labelled as it is made from GM soybeans (Richard Kerr, AFFA, personal communication). In terms of LOD, if an ingredient were present as 5% (w/w) of the total product and that ingredient were derived from 5% (w/w) GM source, the product would have to be labelled “GM ingredient used”, and the LOD of the testing method used to detect the GMO would need to be at least 0.25%.

4 Protein Detection Methods

4.1 Description of Methods

A summary is presented of the protein detection methods most commonly used for detecting and quantitating GM materials in Australia. For less commonly used methods and those under development or evaluation, refer to **Appendices 2** and **3**.

All cells contain a huge variety of different proteins. The more complex the sample being tested, the more proteins that will be present. To identify a specific protein the mix can be separated in one dimension by size (1-D electrophoresis) or in two dimensions by overall charge and size (2-D electrophoresis), followed by a suitable detection method. These techniques, however, do not lend themselves to routine testing. By far the most common approach to identifying specific proteins involves the use of antibodies, with little sample preparation required.

4.1.1 Methods Based on Immunoassays

4.1.1.1 ELISA

The technique called ELISA (Enzyme Linked Immunosorbent Assay) uses antibodies that recognise specific proteins. Antibodies come in an almost endless array of different shapes. When they find another molecule with a compatible shape, like pieces of a jigsaw puzzle, they can lock together. For ELISA assays, antibodies are used that specifically bind to the protein of interest. Further background information on antibodies and their production is included later in this chapter (see section **4.2**).

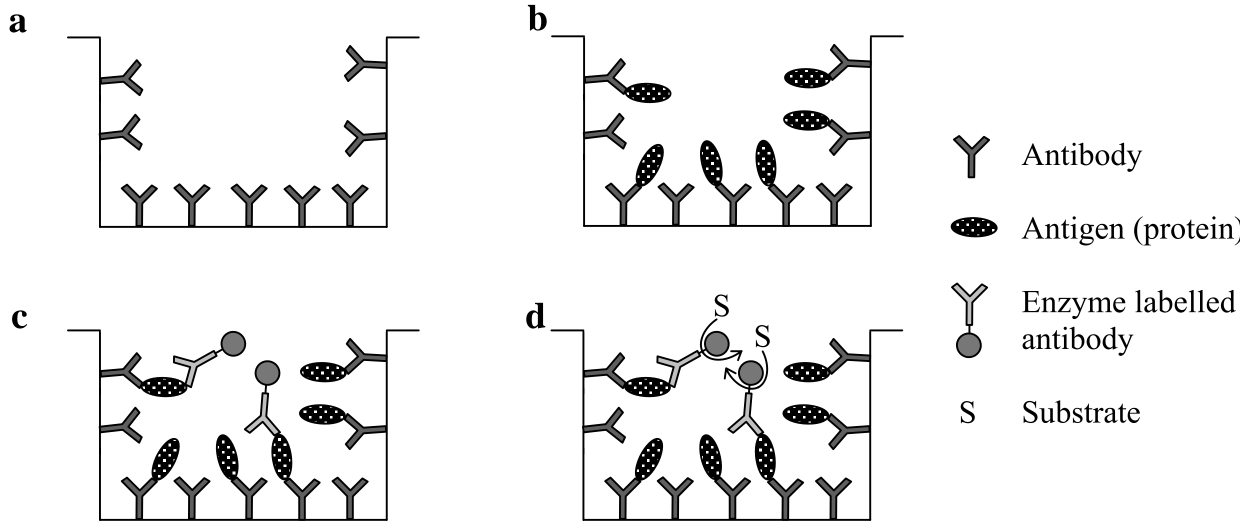


Figure 4.1 Simplified Diagram of an ELISA

For the basic ELISA, antibodies specific to the target protein are bound to the walls in each well of a microtitre plate (**Figure 4.1 a**) and used to ‘capture’ the target protein, if present (**Figure 4.1 b**). A second antibody, that is also specific to the target protein, is

then added (**Figure 4.1 c**). This antibody is labelled with an enzyme that catalyses a colour reaction. If the target protein is present, the second labelled antibody binds to it and any unbound labelled antibody is washed away. Appropriate reagents are then added to allow the colour reaction to proceed (**Figure 4.1 d**). The amount of colour is proportional to the amount of target protein present.

ELISAs have been designed to detect a novel GM protein or trait. Some ELISAs will detect the novel protein such as the CP4 EPSPS protein from *Agrobacterium tumefaciens* expressed in Roundup Ready® plants, whether it be soy, canola or cotton. Other ELISAs are more specific for a trait expressed in one plant species and do not cross-react with the same trait expressed in another plant species. ELISAs range from very sophisticated assays that provide semi-quantitative results to relatively cheap systems that are suitable for on-site use and simply provide a qualitative result that is interpreted visually. Some of these inexpensive kits are designed as a screening tool to test for several proteins such as the Cry1Ab, Cry1Ac and Cry9C proteins, although the test will not distinguish between these three proteins.

All ELISAs detect a novel GM trait but not all traits can be detected or differentiated by an ELISA.

Table 4.1 Characteristics of ELISA Methods

Purpose	To identify and semi-quantify a specific protein related to a GM trait
Advantages	
	Moderate sample preparation
	Relatively fast assay (2-4 hours, including sample preparation)
	Qualitative or semi-quantitative [14]
	Low-medium relative cost (AUS$10-50)
	Robust and simple assay formats
	Suitable and cost-effective for batch analysis of samples [14]
	Economical compared to DNA detection methods
	Less skill required than for DNA detection methods
	Equipment cheaper than for DNA detection methods
Disadvantages	
	Less sensitive than DNA detection methods (see below) [13]
	ELISA Kit must be stored at 4°C [15]
	Moderate equipment costs include an ELISA plate reader required for some kits [15]
	Lack of availability of relevant antibodies
	Quantification may be questionable since the amount of GM protein produced can be influenced by external factors such as climate, soil conditions and nutrient availability
	Development of appropriate antibodies can take months to years

Table 4.1 Characteristics of ELISA Methods

Limitations	Generally, a laboratory analysis False positives can occur due to cross-reactions with other components in the sample analysed [15] ELISA tests are not event-specific Sensitivity is ~0.5 to 1% GMO Some GMOs do not express a detectable level of the target protein [13], whereas others are designed that do not produce a novel protein at all There may be limited or no expression of the novel protein in the plant tissue that is used for food production. Commercial kits are only available for a limited number of GMOs [16] Antibody production is slow and difficult The majority of ELISA kits detect only one protein each [16] Suitable for raw, whole or ground, uncooked commodities but not always suitable for cooked products due to denaturation of proteins by heat [16] Suitable for detecting gene flow into conventional crops of the same species, but the protein may be expressed in a modified form or not be expressed at all if the novel construct were to end up in a different plant species

4.1.1.2 Lateral Flow Strips

Lateral flow strip technology is a variation of ELISA, but the antibodies are immobilised onto a test strip in specific zones (**Figure 4.2 a**). The test is provided in kit form and does not require any major equipment. Lateral flow strips are suitable for field or on-site use, with minimal training required. Similar types of kits are sold in pharmacies for pregnancy testing. Sample preparation simply involves crushing the sample and mixing it with the protein extraction solution provided in the kit.

The lateral flow test strip is dipped into the prepared sample in extraction solution and the sample migrates up the strip by capillary action. As it moves up the strip, the sample passes through a zone of reagent that contains antibodies, usually labelled with colloidal gold. This labelled antibody binds to the GM protein, if present in the sample (**Figure 4.2 b**). The antibody-protein complex then continues to move up the strip until it reaches a second zone of antibodies, which in this case are immobilised onto the test strip. The complex concentrates into this immobilised antibody zone where the gold becomes visible as a red band (**Figure 4.2 c**). The test strip also contains an immobilised control zone that binds a control complex that is present in the extraction solution and also produces a visible line. If there is no target GM protein present only a single line will form at the control zone. A result is called positive when both the control line and the line indicating presence of target GM protein change colour.

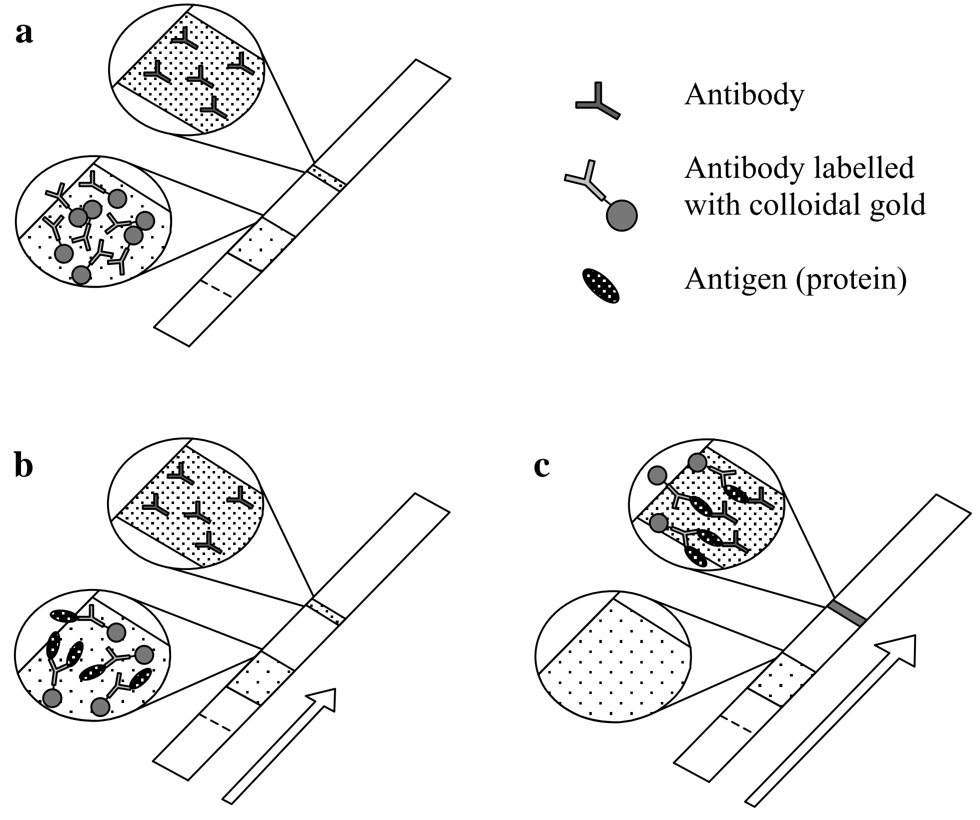


Figure 4.2 Simplified Diagram of a Lateral Flow Strip

Table 4.2 Characteristics of Lateral Flow Strips	
Purpose	To provide a rapid test for the detection of a GM trait
Advantages	
	Minimal sample preparation Rapid results (5-10 minutes) Qualitative Relatively cheap, (AUS$5-20) Disposable [17] Strips can be stored at room temperature prior to use [15] Simple to perform with minimal training No expensive equipment required [15] Suitable for on-site or field testing [17]
Disadvantages	
	Not very sensitive (~1% (w/w) GM protein) Lack of availability of relevant antibodies Development of appropriate antibodies can take months or years

Table 4.2 Characteristics of Lateral Flow Strips

Limitations	
	Limited to 1 or a small number of traits per test
	Lateral Flow Strips are not event-specific
	Some GMOs do not express a detectable level of the target protein [13]
	Only available for a limited number of GM products [16]
	Most suited as a rapid test for raw, whole or ground, uncooked commodities

4.1.1.3 Other Immunoassay Formats

Another format for immunoassays uses magnetic particles as the solid support surfaces. Magnetic particles are coated with the capture antibody and reaction is carried out in a test tube. The particles with bound proteins are separated from unbound proteins in solution using a magnet. The advantages include superior kinetics, as the particles are free to move in the reaction solutions, and increased precision owing to uniformity of the particles [13].

4.1.2 Future Developments in Immunoassay Technology

Several approaches are being taken to extend the application of immunoassays for GM testing. These include lateral flow strips that simultaneously detect multiple proteins [13] and development of antibody-based methods for testing processed foods. Such methods use antibodies that will bind to linear fragments of proteins and will therefore recognise proteins after processing steps such as heating [14]. Improvements in antibody specificity and binding strength will result in higher sensitivity and reduced sample preparation [17]. Recombinant antibody technology can be used to address problems associated with antibody production and generation of antibodies capable of reacting normally at extremes of pH or in high concentrations of salt or solvent [14]. Finally, there is the potential to incorporate ELISA methods for detecting GMOs into fully automated instruments capable of running hundreds of samples an hour [17] and to use combinations of methods such as immunoassay/mass spectrometry/biosensors based on real time antibody binding to target molecules [14].

4.2 Background on Antibodies

Antibodies are proteins that are naturally produced in humans and higher animals as part of a defence mechanism, to protect against infections and foreign molecules. Antibodies will bind to foreign organisms or molecules found in the body and this binding leads to the identification of the molecule as being unwanted and finally to its destruction and/or removal, as part of an immune response. This binding also triggers the production of more antibodies with the same shape as the first, to allow the rapid removal of any other foreign organisms or molecules of the same type. Antibodies used in immunoassays are produced in laboratory animals, as part of an immune response triggered by exposure to a purified, target protein or antigen. An antigen is simply any molecule that generates an immune response. Scientists have been exploiting antibodies for many years as an analytical tool in immunoassays.

Antibodies from laboratory animals may be used as a mixture (polyclonal antibodies) or as single, very specific antibodies (monoclonal antibodies). Polyclonal antibodies are

generated following the initial injection of an animal with the target protein or antigen. Monoclonal antibodies are then produced by isolating the cells that are expressing this mixture of antibodies and fusing them with an immortal cell line. Specific fused cell lines are then selected that express a large amount of a single type of antibody that binds strongly to the target protein. Monoclonal antibodies have the advantage over polyclonals in that they can be produced indefinitely in any quantity just by growing up more of the fused cell line, an important consideration for the commercial production of antibody test kits, but are more difficult and expansive to produce.

When an antibody finds another molecule with a compatible shape, they can lock together. Antibodies are produced in a vast number of different random shapes, so that there will be at least some antibodies compatible with virtually any foreign particle that invades the body. The more compatible the shapes, the stronger the binding. The challenge for laboratory-based antibody production is to induce, isolate and purify an antibody that is compatible with the protein of interest, in this case the novel protein expressed by a GMO. High **sensitivity** from the antibody is achieved when the antibody binds strongly to the protein antigen. High **specificity** is achieved when the antibody binds only to that protein. This also means that only minimal sample preparation would be required. When new antibodies are isolated, they must be carefully screened for these desirable properties.

Immunoassays have the capacity to be widely implemented on a commercial scale for the detection of novel or modified proteins in raw commodities and foods. Ideally, an antibody must be selected that will specifically bind to the shape of the protein of interest, even when there are thousands of other proteins present, each with their unique shapes. Once a specific antibody has been developed, it can be incorporated into an immunoassay by labelling with fluorescent or coloured dyes, or attachment to a surface where it is used to 'capture' the protein of interest.

The design of immunoassays for detection of proteins also requires knowledge of protein expression levels and stability during food production and processing. Matrix effects are an important aspect of immunoassay method validation, particularly when assessing antibody specificity, due to the large diversity of other proteins and components in food products, any one of which could cross-react with the antibodies.

The majority of protein detection methods are based on immunoassays. Protein detection methods have the potential to identify the presence of a specific GM trait and to provide absolute quantification of the level of GMO present. However, these methods are not event-specific.

Protein methods cannot be event-specific.

4.3 General Advantages of Protein Detection Methods

Protein detection methods for GMO testing vary from those that are relatively cheap and easy to perform to more sophisticated assays requiring moderately expensive instrumentation. In some formats, protein detection can be carried out by unskilled persons on site, producing an answer in minutes. Sample preparation prior to testing is

often minimal and may simply involve crushing a certain number of seeds in a solution provided in a detection kit. Protein detection methods are highly suitable for monitoring specific GM traits during handling of raw products, provided the protein is expressed in the part of the plant being tested. Protein detection methods can be used to detect GM traits in some processed foods, such as flour, but are less suited to cooked products where the protein may be denatured and, therefore, unrecognised by the antibody. In the future, it is likely that tests will be developed that detect multiple novel proteins using a single lateral flow strip.

4.4 General Disadvantages and Limitations of Protein Detection Methods

4.4.1 Production of Suitable Antibodies

Laboratory production of antibodies is a slow and difficult task that requires a great deal of skill and experience. It sometimes takes up to two years to develop a single specific antibody. A highly purified sample of the novel protein that had been isolated from the GM plant must be available to inoculate into a mouse, rat or rabbit to induce an immune response. Alternatively synthetic peptides, or small fragments of the protein, can be used for inoculation. Animal ethics clearance is needed and method validation must include testing the antibody with many different matrices to check for cross-reactivity.

4.4.2 Recognition of the Foreign Protein by the Antibody

The main limitation of antibody-based detection methods is that antibody binding relies on shape, so the target protein must be correctly folded in order to be recognised by the antibody. If a protein is heated, such as in a food product that has been cooked, it will denature or unfold. Exposure to strong acids or alkalis will also denature proteins. If the antibody has been developed to recognise the protein in its native folded state, in most cases, it will not recognise the protein once unfolded. A few antibodies have been selected to recognise a protein in its unfolded shape and thus are more suited to detection of GMOs in highly processed foods. Some forms of processing, such as grinding, do not affect the protein shape and so will not affect reactivity with the antibody.

An important part of selecting a suitable antibody for use in a detection method is the binding capacity. How strong the antibody binds will affect the sensitivity of the test, the stronger the binding, the more sensitive the test and this reduces the risk of false negative results.

4.4.3 Limited Application as a General Screening Method

Protein detection methods are less suited to general GM screening, as a single antibody will only recognise one particular protein. There are no structures common to all GM proteins or groups of GM proteins that would allow one antibody to be used to detect a number of GMOs. It is feasible to mix antibodies in order to develop a general screening. This would not allow quantification, though, as the binding capacity of each antibody affects the sensitivity of its detection.

4.4.4 Protein Levels Are Not the Same in All Cells

As mentioned in the **Chapter 2**, protein composition varies considerably from one cell type to another within the same organism and can also vary at different stages of the cells life cycle. Some GMOs have been specifically designed to express the foreign protein in a particular tissue and this may not necessarily be the part of the plant that is

harvested. Therefore, a protein-based detection method for GMOs will only be feasible if the foreign protein is expressed in the tissue undergoing analysis. If this is not the case, a DNA-based detection method is the only option.

In some cases, different GM events have been developed that express the same foreign protein but in different parts of the plant. For example, the foreign protein produced in both the GM maize events Bt-11 and Bt-176 is the *Bacillus thuringiensis* δ-endotoxin cry1Ab, which protects the plant against lepidopteran attack from insects such as European corn borer. In the case of Bt-11, the CaMV 35S promoter has been used and this results in expression of cry1Ab in all plant tissues, although the amount of GM protein per milligram of plant protein still varies considerably from one tissue type to another. On the other hand, Bt-176 uses a combination of two maize-derived, tissue-specific promoters that result in gene expression only in plant green tissues and pollen. The novel protein in Bt-176 is not expressed in the kernels and therefore will not be detected when the kernels are tested using a protein-based assay [12], (FSANZ Draft Final Risk Analysis Report, Application A386 - Food derived from insect-protected, herbicide-tolerant Bt-11 corn).

4.4.5 Protein Assays Produce an Absolute Rather than Relative Quantification

Protein assays used for quantification generally produce an absolute value, such as the total amount of a novel protein present. To comply with labelling legislation, the relative quantity of the GM trait needs to be determined, or in other words, the **proportion** of a particular commodity or ingredient that is derived from a genetically modified plant. If the product being tested has only one ingredient, for example raw soybeans, then relative quantification to meet labelling requirements could be achieved by determining the percentage of the GM protein in total protein using an immunoassay, provided that a suitable reference material were available. There is a risk involved in this type of quantification though, as the amount of protein present is dependent on expression levels from the gene. This level varies between plant tissue types, as discussed above, and can also vary with growth conditions and seasons [18].

If the product being tested has several ingredients so that soy comprises less than 100% of the product, then quantitative testing to conform to labelling legislation would not be possible, as the total protein would be derived from more than one source. The legislation requires that the percentage of GM soy out of total soy is determined, not the percentage out of the total product.

Protein-based detection methods are generally not suitable for determining the relative amount of an ingredient that is genetically modified unless it is the sole ingredient in the sample and a suitable reference material is available.

4.4.6 Protein Assays are Not Always Appropriate for Monitoring Gene Flow

Protein detection methods could be used to detect gene flow from GM to conventional crops of the same variety. These methods could also potentially be used to monitor gene flow to related wild plant species, provided that the novel protein is expressed in exactly the same form in the new plant so that it is still recognised by the antibody. The

majority of proteins undergo some form of modification after being produced. These modifications may include trimming of the protein, the addition of carbohydrate molecules (glycosylation) or a change to the overall protein conformation. If any modification occurs to the novel protein in the new plant host that affects antibody recognition, it is possible that the protein will not be detected. The more distantly related the new plant host, the less likely it is that a protein detection method will work.

5 DNA Detection Methods

5.1 Description of Methods

A summary is presented of the DNA methods most commonly used for detecting and quantifying GM materials in Australia. For more detailed descriptions of these, of the less commonly used methods and those methods under development or evaluation, refer to **Appendices 1-3**.

The accuracy of a method, particularly a quantitative one, is dependent on the type of GMO, the food matrix with its complement of assay inhibitors, the DNA extraction method and the degree of processing of the food sample. The lower the concentration of GMOs and/or DNA in a sample, the greater the potential error (W 111). For these reasons, there are three key factors that determine the success of DNA detection methods. These are the **quantity** or amount of DNA extracted; the **quality**, which relates to the amount of damage inflicted on the DNA during food processing steps prior to extraction, and the **purity** which reflects the amount of contaminants co-purified with the DNA.

As almost all living organisms contain DNA, measuring the total amount of DNA will provide very little information when testing for the presence of GMOs. The assay method must differentiate and identify the DNA sequence that is specific for the GMO amongst a massive excess of 'background' sequences. This is equivalent to searching for a particular sentence in an instruction manual. All DNA-based detection methods rely on the inherent stability of the double stranded DNA structure and the ability of a single strand of DNA to 'find' and bind to its complementary sequence.

For many applications, there may be very little DNA present, such as in highly processed food. In other applications there may be plenty of DNA but very little of the sequence of interest, for example, detecting low levels of GM grain in a sample of conventional grain. In order to detect these low amounts of target sequence, several methods have been developed to increase or **'amplify'** the number of copies of the specific DNA sequence so that they can be more easily detected. This amplification step forms the basis for the majority of DNA detection techniques currently available and is the reason why DNA amplification methods are very sensitive. The most frequently used DNA amplification method is called the polymerase chain reaction or PCR.

5.1.1 DNA Extraction Methods

DNA extraction is often the most time consuming step of a DNA-based detection method and can form the bottleneck for high-throughput testing. However, the efficiency of the DNA extraction step can be critical for successful amplification since there are many compounds that inhibit DNA amplification that can be co-purified with the DNA. These compounds include proteins, fats, polysaccharides, lipids and polyphenols [19]. Chemicals used in the DNA extraction itself, such as phenol, SDS and EDTA, can also inhibit amplification, as the DNA polymerase enzyme, frequently used for DNA amplification, is very sensitive to their presence [20].

Procedures for isolating DNA from plant tissues for the purpose of GMO detection involves a number of steps. First, chemical agents, most commonly anionic detergents, are used to disrupt cells in order to release DNA into a solution. Second, proteins and other cellular components are largely removed by a protein precipitation step during and

after which the DNA is maintained in solution. Finally, the DNA is selectively isolated from most of the remaining contaminants by precipitation in alcohol. Optionally, the DNA may be further purified using DNA binding resins or chelating agents that remove non-DNA components that co-precipitate with DNA in alcohol. Generally, with the inclusion of additional DNA purification steps, a marked decrease in DNA recovery or 'yield' from a given sample will be observed. This is an important trade-off to consider when analysing samples that contain low amounts of DNA. For a full list of extraction procedures, see Table 1 in the review by E. Anklam *et al*, 2002 [20].

5.1.2 PCR Methods

PCR is a technique for amplifying the number of copies of a specific DNA sequence by many thousands of times. These methods must be very **sensitive** – to <u>always</u> amplify the sequence of interest if it is present, so as to prevent false negative results, and be highly **selective** – to <u>only</u> amplify the intended target sequence and no other, so as to prevent false positive results.

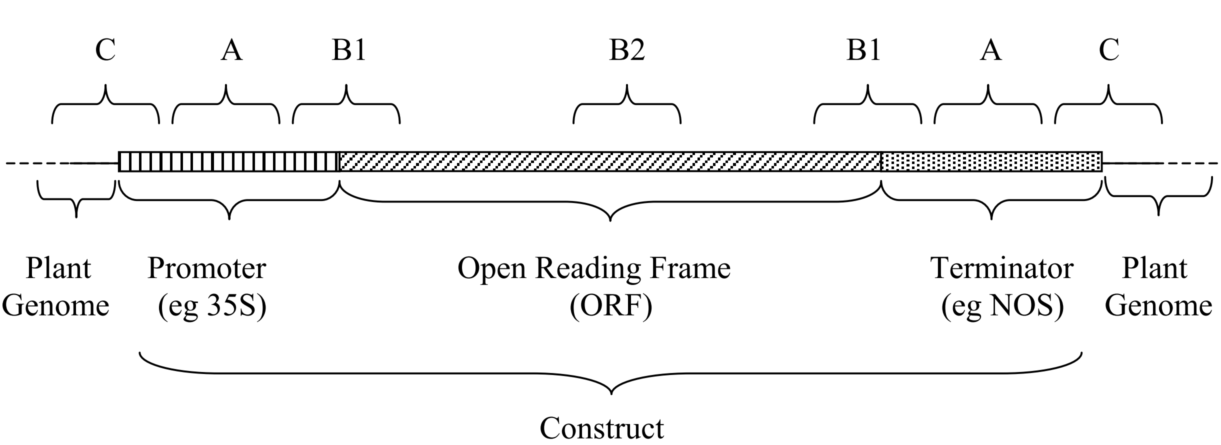


Figure 5.1 Simplified diagram showing a novel gene construct integrated into a plant genome and the PCR amplification styles that are possible

The regions designated 'A' are found in many different GMOs and thus are suitable for amplification in a screening method, though these sequences can also occur in nature. Both 35S promoter and NOS terminator sequences are frequently used in GM constructs.

The regions designated 'B1' can be amplified in construct-specific, qualitative methods. These junction regions do not occur in nature, eg between the promoter and ORF. If the DNA sequence for the ORF has been altered from the original, these sequences can also be targeted in qualitative, construct-specific methods (B2). The regions designated 'B' cannot be used for event-specific testing, as there are examples where the same construct has been used more than once to produce different events.

The regions designated 'C' can be used for event-specific amplification methods as they cover the junction regions between the construct and the plant genomic DNA, so are unique to each event. These regions can be used for both qualitative and quantitative detection.

The sequence to be amplified can be targeted very specifically by the design of two DNA primers. These are short, single-stranded lengths of DNA that are commercially synthesised. One primer is complementary to one end of the target sequence, in the 'Forward' direction and the other is complementary to the other end of the target, in the 'Reverse' direction (see **Figure A1.1** in **Appendix 1** for more details). In order to design these primers, the analyst must have knowledge about the relevant DNA sequences. Further details on the types of PCR detection methods are given later in this chapter and a detailed technical description of PCR methodology can be found in **Appendix 1**.

An extensive list of specific PCR methods for GMOs and their target sequences is presented elsewhere (see Anklam *et al* review, 2002: Table 3 PCR methods in GMO detection for maize; Table 4 PCR methods in GMO detection for Roundup Ready® soy and Table 5 PCR methods in GMO detection for tomato, potato, sugarbeet, cotton, papaya, alfalfa and tobacco [20]).

Table 5.1 General Characteristics of PCR Methods

Advantages	
	Very Sensitive
	Any tissue type can be analysed since all plant cells carry the same DNA
	Suitable for a range of applications from screening to event-specific methods
	Provide relative quantification suitable for monitoring compliance with labelling legislation
	Methods can be designed by laboratories in-house, provided DNA sequence information is available
Disadvantages	
	Time consuming sample preparation
	Total assay time 1-2 days
	Generally more expensive than protein-based methods (AUS$100-500 per test)
	PCR reagents must be stored at 4°C [15]
	Moderate-high equipment costs include a thermocycler and electrophoresis apparatus
	Susceptible to inhibitors that may be present in food ingredients
	Strict quality assurance required to minimise risk of cross-contamination
	Generally, a laboratory analysis
Limitations	
	Suitable for a range of raw to processed products provided that DNA can be extracted in sufficient quantity and quality
	Moderate technical skills required
	There is no single PCR method that will detect all GMOs

5.1.2.1 PCR Methods for Screening

Most of the currently approved GMOs worldwide contain any of three genetic elements that can be targeted for GMO screening. These elements are the CaMV 35S promoter, the NOS terminator from the soil bacterium *Agrobacterium tumefaciens* and the kanamycin resistance marker gene (NPTII) [13, 19]. These sequences also occur naturally in plants and soil micro-organisms, therefore a positive result will not necessary confirm the presence of GMO, but will suggest that it is probable [19, 20]. If both the 35S and NOS results were positive, for example, the probability of this being due to the presence of a GMO is far greater than if only one were positive. GMOs lacking either the 35S promoter or the NOS terminator will not be detected by such as screen and an alternative screening test will be required.

To confirm definitively the presence of a GMO, a sample with a positive signal in 35S and/or NOS screening should be further analysed using a construct-specific or event-specific method. Alternatively, the sample could be analysed for the presence of either naturally-occurring CaMV or *A. tumefaciens* infection, respectively. However it should be considered that the host range of the CaMV is restricted to cruciferous plants such as canola, and that the NOS terminator sequence is found only in certain strains of *A. tumefaciens*, which are only pathogenic to certain crop species. In addition, the *A. tumefaciens* frequently found in soil is generally not virulent and does not carry the Ti plasmid (see Transformation Methods in **Chapter 2**), so the NOS gene and its control elements are not present in these naturally occurring strains [20].

Table 5.2 Specific Characteristics of PCR Screening Methods

Purpose	To screen for the possible presence of GMOs
Advantages	
	Qualitative
	Sensitivity is ~0.1% GMO
	Several commercial tests available
	Multiple different GMOs can be detected with a single test
Disadvantages	
	Cannot be used for quantification
	Screening tests target naturally occurring DNA sequences and cannot be used to unambiguously confirm the presence of GMOs
Limitations	
	There is no single screen that will detect all GMOs
	Since screening tests target naturally occurring DNA sequences, they are poorly suited for monitoring gene-flow from GMOs into related plants due to the ambiguous nature of the result

5.1.2.2 Construct-Specific, Qualitative PCR Methods

Construct-specific methods are designed to confirm the presence or absence of a specific sequence that is not found in nature. Amplification of a trait-specific sequence such as a modified gene sequence, or the junction between a promoter and gene sequence provides far more information than a screening method. These constructed sequences do not occur in nature (**Figure 5.1**, style B).

Table 5.3 Specific Characteristics of Construct-Specific Qualitative PCR Methods

Purpose	To detect and identify the presence of a specific DNA construct
Advantages	
	Qualitative
	Sensitivity is ~0.1% GMO
	Specific DNA construct can be identified
	Commercial tests available for some constructs
	Could be used to detect the construct in other related plants resulting from gene flow
Disadvantages	
	In general, not suitable for quantification
Limitations	
	Detects a specific construct but not an event
	Due to its specificity, not suitable for detecting adventitious presence of GMOs, unless this trait is the only likely source of contamination

5.1.2.3 Event-Specific, Qualitative PCR Methods

For unequivocal identification of a specific GM event, the PCR product to be amplified must span the junction between the construct and the plant genomic DNA (**Figure 5.1**, style C). This last style of PCR, known as event-specific PCR, is the most informative, as the junctions at each end of the construct are always present in two copies per genome (one copy on each chromosome) in approved GMOs (see section **2.5.1**) and are absolutely specific for that event. For example, when a GMO trait is developed, many transformants are produced initially but maybe only one will be chosen to be put forward for approval. The different GM transformants contain the same DNA construct but the construct has integrated into different locations in the plant genome so are different ‘events’. The simplest way to distinguish between these events is to amplify the genome/construct junction from each. These different events would be indistinguishable using style A or B PCRs (**Figure 5.1**, above) or by protein detection assays.

The choice of PCR style depends on the requirements for the test. For example, several countries have approved the commercial release of Ingard® cotton event 531 but not Ingard® 757 (W 94). An event-specific method is the only technique that can distinguish between the two Ingard® events and would be required to ensure that a shipment of cotton was Ingard® 531. On the other hand, if cotton is being exported to a

country that will not admit any GM seed, then a less discriminating method that would detect both events would be more suitable.

5.1.2.4 Quantitative PCR Methods

With the introduction of mandatory labelling legislation for food products containing GMOs, there has been increasing demand on testing laboratories to develop or adopt quantitative methods to assure compliance. Although the GMOs approved for human consumption have been through stringent scientific testing and have been deemed safe, labelling allows consumers to make a choice in selecting the food that they feel comfortable with [13].

PCR is most commonly used to provide a relative rather than absolute quantification for the level of GMO in a sample. Relative quantification is achieved by amplifying two DNA sequences and calculating the ratio between the amounts of amplified products obtained. A sequence of DNA from the inserted construct and a sequence of DNA from an endogenous gene from the host plant genome are amplified. For example, the lectin gene is usually chosen as the endogenous gene for soy since it is present in all soy varieties. An amplification method, by definition is an indirect way of measuring the starting amount of a DNA sequence. An understanding of the amplification efficiency is necessary in order to extrapolate back to the starting amount, using comparisons with controls and reference materials. Reference materials are critical as they reflect the ratio of lectin gene to construct in the GMO trait and also the relative amplification efficiencies (see **Chapter 7** for more details on Reference Materials). Relative quantification can be achieved using either End-Point or Real-Time PCR. These methods are described in more detail in **Appendix 1**.

Table 5.4 Characteristics of Event-Specific Qualitative and Quantitative PCR Methods

Purpose	To identify and quantify the amount of a specific GM event
Advantages	
	Qualitative or Quantitative
	Very Sensitive
	Specific GM event can be identified
Disadvantages	
	Commercial tests available for limited number of events
	Real-time PCR thermocycler preferred, but this is a more expensive piece of equipment than a conventional thermocycler as fluorescence detection is incorporated
	Requires highly skilled analyst with experience in data analysis
Limitations	
	DNA sequence information for the integrated construct and adjacent plant genomic DNA is required to design event-specific methods
	Due to its specificity, not suitable for detecting adventitious presence of GMOs, unless this GM event is the only likely source of contamination, though event-specific methods could be used to quantify any adventitious contamination, once identified.

5.1.2.5 Is Event-Specific Amplification Necessary for Quantification?

It is strongly recommended that event-specific methods be used for quantification for several reasons. One reason emerges from the transformation process, when the DNA construct is integrated into the plant genome. In the ideal situation, this DNA construct would be integrated into the plant genome as a single, linear copy. Unfortunately, sometimes the DNA gets rearranged and duplicated during the transformation process, so that the final construct in the genome ends up with extra copies of some sequences (see Transformation Methods in **Chapter 2**). As long as there is one uninterrupted copy of the promoter-ORF-terminator unit, the transformation would be successful. In such a construct, quantification using a PCR method that targets sequences present in more than one copy would result in an overestimation of the percentage of GMOs present. If there were two copies per insertion site, the quantification would be twice as high as the correct value. If there were three copies then the quantification would be three times higher than the correct value, etc. Event-specific PCR overcomes this problem because this amplifies the junction of the inserted DNA and plant genome.

If raw ingredients are being analysed, such as a batch of maize flour, amplification styles other than event-specific ones cannot be used for accurate quantification. This is particularly true for screening PCRs, such as amplification of the 35S promoter, where the same sequence can be found in multiple GMOs. For example, there could be different types of GM maize in a batch, some with one copy of the 35S promoter sequence per insertion, others with more copies per insertion. The problem is further exacerbated by the latest style of GM crops where new traits are 'stacked', so that one GM plant contains more than one construct. When the variation caused by non-homogeneity is added, one can imagine the range of answers possible from this mix.

When screening a food product with multiple GM ingredients, quantification of specific traits becomes even more complicated. For example, a food product might contain both GM soy and GM maize. If both of these GM crops contained the 35S sequence, the method would not be able to distinguish whether this sequence came from the soy or maize so quantification using a screening method would clearly not be possible in this case.

Although event-specific methods are most suitable for quantification, the DNA sequence information (ie the DNA sequence at the junction between the inserted DNA and plant genome) required to design this type of method is not always available to testing laboratories.

5.2 General Advantages of DNA Detection Methods

5.2.1 Suitable in a Range of Applications from Screening Methods to Event-Specific Methods

Because there have been a limited number of promoters and terminators used in the GMOs currently available, DNA-based tests that are designed to detect these DNA sequences can be used as general screening methods. These methods would even detect unapproved varieties, if these varieties use a common promoter or terminator. At the other extreme, highly specific tests can be designed that will detect a single GM event. For instance, it is possible to design 'event-specific' tests that will distinguish between the two Ingard® cotton events, 531 and 757, that contain the same gene but inserted

into a different location in the plant genome. A protein-based test would not be able to make this distinction.

5.2.2 DNA Composition is the Same in All Cells of an Organism

One of the major advantages that DNA-based detection methods have over protein-based methods is that the DNA composition is the same in all cells and tissue types of an organism. In this respect, in principle, any part of a plant can be used to detect for the presence of GMOs provided that the DNA can be extracted efficiently from that tissue.

5.2.3 DNA-Based Assays Provide Relative Quantification

DNA-based quantitative methods are designed to measure not only the amount of the foreign DNA, but also the amount of a gene that is naturally occurring in the plant (see Section 5.2.1.4). The relative level of these two sequences provides a means of determining the amount of GM ingredient relative to the total amount of that ingredient in the product. This relative quantification provides a percentage measurement, as required by the labelling legislation.

5.3.4 DNA-Based Methods are Versatile and Sensitive

The principles behind DNA-based testing methods are very generic, since the same basic approach can be used to detect a completely different GMO or be changed from a screening to an event-specific method simply by designing the necessary DNA primers for the relevant target sequence (**Figure 5.1**). A DNA method to detect a new GM trait, an unapproved variety or even to monitor the possibility of gene flow into wild strains can be developed relatively quickly, provided the relevant sequence information is available. In contrast, protein methods require the prolonged procedure of antibody development.

Since DNA-based methods involve amplification of the target DNA they are extremely sensitive.

5.3 General Disadvantages and Limitations of DNA Detection Methods

5.3.1 Labour Requirements

Methods are generally expensive and require moderate to high levels of skill from the analyst. Currently, most DNA tests are unsuitable for on-site testing.

5.3.2 Some Processing Procedures Can Damage or Remove DNA

Although DNA is very stable relative to proteins, it can be degraded by excessive heat, ultraviolet light, acidic conditions and nuclease activity (enzymes that specifically destroy DNA). Even if a large quantity of DNA is extracted from a highly processed food, a DNA-detection method may not work if the DNA is ‘cut up’ into short lengths during the processing. DNA-based methods used to test highly processed food should be designed to detect very short fragments of DNA (< 200 base pairs in length) to increase the probability of detecting the DNA. The critical minimum average length of DNA fragments for successful analysis by PCR is estimated to be 400 base pairs [1]. DNA fragments with an average length of 100-400 base pairs have been extracted from soybean protein products and processed tomato products (W 111).

In general, no DNA is detectable in highly heat-treated food products, hydrolysed plant proteins, purified starch derivatives and refined oils derived from GMOs [19]. Failures in extracting detectable DNA levels have also been reported for soybean sauce, refined sugar and distilled ethanol produced from GM potatoes [20]. Although highly refined oils do not need to be labelled when produced from GM plants, an exception is cold-pressed oil as this still contains DNA [4].

5.3.3 Some Food Ingredients will Inhibit DNA Amplification and thus Prevent Detection of DNA

There are many compounds in food or plants that can be co-extracted with the DNA that will inhibit DNA amplification in techniques such as PCR. These inhibitory factors include some proteins, fats, polysaccharides, cocoa extracts and caramelised sugar [13, 19]. In fact, chocolate is one of the more difficult matrices to test, due to the inhibitory factors. In one study on dark, milk and white chocolate containing soy lecithin, two DNA extraction kits and a non-commercial extraction method were compared. DNA could be extracted using the kits but it was very poor quality and the yields were low. This DNA could only be amplified by PCR following extensive optimisation of the amplification method [21].

When analysing processed food, the DNA extraction protocols need to be assessed on a case-by-case basis to allow for the extremely varied compositions and degrees of processing [21].

5.3.4 Detection Methods that Rely on DNA Amplification are Susceptible to Cross-Contamination

As PCR is an amplification method, it is prone to accidental contamination of samples and reagents by cross-contamination or carry-over from previous PCRs, so testing laboratories must be scrupulous with their technique and laboratory layout. Due to this high risk, individual PCR steps should be separated in terms of physical space and equipment used, to prevent these problems [22]. With every set of samples, a series of positive and negative controls are run to ensure the accuracy of the results.

5.3.5 Sequence Information is Required to Design a DNA-Based Method

One of the essential requirements before designing a DNA-based method is information on the foreign DNA sequence as well as the DNA sequence of the plant genome at the site of insertion of the foreign DNA. This information is not always available to the laboratories involved in method development. You cannot make assumptions on the DNA sequence based on knowledge of the trait (**Figure 5.2**).

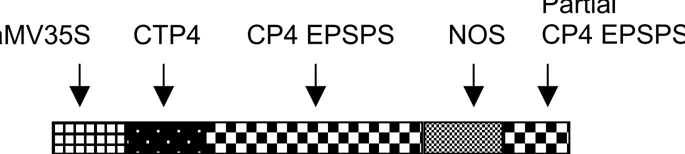
Whilst some of the gene sequences are available on publicly available databases, small changes are often made to the novel DNA sequence in the construct. These changes do not always affect the amino acid sequence of the novel protein but are designed to increase expression of the protein in the plant because the altered DNA is more suitable for that particular plant host. For example, the cry1Ab gene in Bt-176 shows only 65% homology with the naturally occurring gene even though the resultant protein product is identical in amino acid sequence to the native toxin (ANZFA Draft Risk Analysis Report Application A385 - Food derived from insect-protected Bt-176 corn). In addition, during the transformation process it is possible that rearrangements in the DNA sequence may have occurred. For instance, Roundup Ready® soy has an additional segment of the CP4 EPSPS gene inserted immediately adjacent to the NOS terminator (**Figure 5.2**).

5.3.6 An Illustration of the Features and Differences between Construct- and Trait-Specific Tests

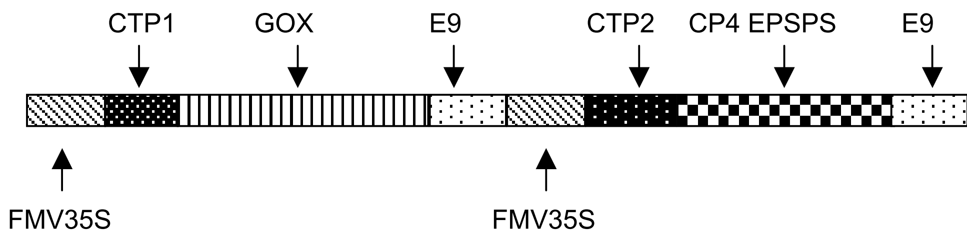
Roundup Ready® canola, soy and cotton all contain the gene for expression of the CP4 EPSPS protein that confers tolerance to the herbicide, glyphosate (**Figure 5.2**). ELISA-based protein assays, such as the Strategic Diagnostics Inc. (W 25) RUR NK603 lateral flow strip kit detects the CP4 EPSPS protein but cannot distinguish whether the protein was derived from a canola, soy or cotton-based ingredient. However, such an assay would be useful as a general screen for detecting foods containing the Roundup Ready® trait or the CP4 EPSPS protein, though if a mixed food contained, for example canola and soy ingredients, the ELISA assay would not be able to determine which one of these ingredients was genetically modified.

A construct-specific PCR assay could be designed to amplify a region of the Roundup Ready® canola construct between the CTP2 sequence and the CP4 EPSPS gene (**Figure 5.2**). This same assay will most probably also detect the Roundup Ready® cotton construct provided that there are no subtle changes to the novel DNA sequence in this area for the cotton construct. However, it will not detect the Roundup Ready® soy construct as it has a CTP4 sequence in place of a CTP2 sequence. Even PCR assays that are designed to amplify a region entirely within the CP4 EPSPS gene of one of these three constructs may not amplify the gene in the other two constructs (AGAL unpublished observations). This is presumably due to subtle changes in the novel DNA sequence that have been included to optimise expression of the foreign protein in each plant type.

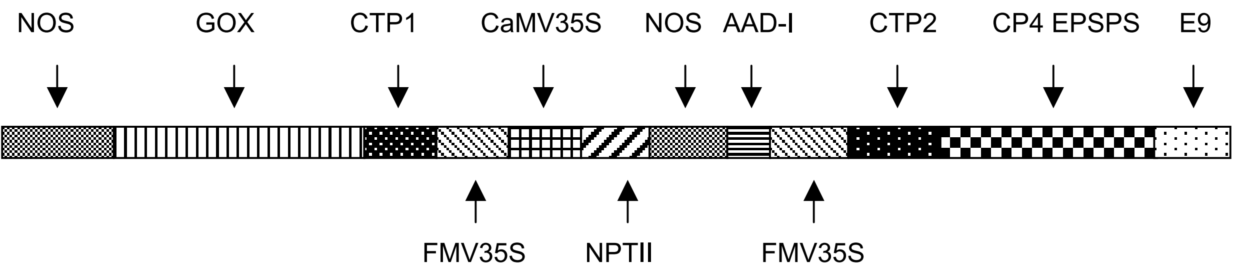
Roundup Ready® Soy (40-3-2)



Roundup Ready® Canola (RT73)



Roundup Ready® Cotton (1445)



Key (see **Table A4.7** in **Appendix 4** for definitions of these abbreviations)

Figure 5.2 Comparison of Roundup Ready® Constructs

Adapted from ANZFA Draft Risk Analysis Reports, Application A355 - Food produced from glyphosate-tolerant cotton line 1445, ANZFA Draft Risk Analysis Reports, Application A363 - Food produced from glyphosate-tolerant canola line GT73 [23].

Constructs not drawn to scale.

6 Testing – From Raw Commodities to Processed Foods

6.1 Sampling Protocols

One of the major considerations in analytical testing of almost any product is the sampling procedure. The sample analysed must be representative of the material from which it is taken otherwise the testing regime is flawed.

The majority of this sampling section focuses on raw commodities. Sampling errors have a more significant effect on testing accuracy for raw commodities, compared to processed food, due to increased particle size and reduced sample homogeneity. For example, if a sample contained one hundred soybeans, the largest possible number of plants represented in this sample is one hundred. If the sample consisted of the same weight of soy flour, theoretically each grain of flour may be derived from a different plant and so the sample is far more representative.

Sampling Considerations

Sample plan
Sample size
Sample representivity
Sample preparation
Sample analysis
Reporting of results

6.1.1 The Sample Plan

It is critical to design a sampling plan that takes into account the type of sample being analysed and the final outcome of results expected in terms of sensitivity of the detection method. If a sampling strategy is not employed and the product is tested in a random manner, then the final test results will only apply to the sample analysed. If, however, an appropriate sampling plan has been developed and implemented then the final test result can be applied to the whole batch. The sampling plan should benefit from the same care and investment as the final analysis since the best analytical procedures will only be as good as the sampling procedure used to provide the sample(s) to be analysed [14, 24].

Sampling plans are likely to be negotiated between buyers and sellers who should agree to use a sampling plan that is statistically acceptable and affordable and that provides acceptable risk management to both parties. The optimum sampling strategy will always be a balance between sensitivity, cost and degree of confidence required [13].

The sample plan should define the sample size, minimum number of samples taken, sample preparation and final method of analysis. The samples should be random and an upper limit should be placed on sample size. The analytical methods must be acceptable or 'fit-for-purpose' since the sensitivity of the analytical method will determine, in part, the appropriate sample size [25].

A sampling plan must ensure that the field sample is taken in a statistically representative manner with respect to the larger lot of material and, especially, to its state of homogeneity. It also needs to take into account the intrinsic variability associated with each step in the sampling process, as these errors are additive [25]. Cross contamination with sampling equipment, sample dust, transportation and conveying equipment can also affect the integrity of the sample. The degree of heterogeneity or uneven distribution of a given sample and the actual threshold limit, which is set for acceptance of the presence of GM material, will define both the number of samples to be taken and the appropriate sample size [19, 20, 24]. The more heterogenous the material, the more difficult the sampling procedure as the degree of distribution of ingredients will be less uniform. Whilst raw materials may have a high degree of heterogeneity, a more consistent distribution is normally found for processed foods. However, processed foods may have more than one potential source of GMO and this should be considered in a sampling plan. The lower the threshold limit for GM contamination, the greater the demands will be upon the sampling plan.

Factors Affecting Development of a Sampling Plan

What is being sampled?
(grain, seed, food ingredient, final food product)

Why are the samples collected?
(eg acceptance by a food producer, for regulatory or enforcement purposes or for surveillance)

Where in the food supply chain will the sampling occur?
(early at the commodity level or later to confirm the integrity of the material)

ILSI workshop 2000 [25]

The USDA GIPSA have produced an informative paper on sampling errors associated with detecting the presence of GM varieties in grain lots entitled "Sampling for the Detection of Biotech Grains" (W 106). It includes discussion on random and systematic sampling theory, grain sampling devices, cross-contamination issues, sample size and risk management, qualitative and quantitative analysis, sample plans containing multiple independent samples and sample preparation including grinding, homogenising and final particle size.

GIPSA also provide a statistical formula and sample size calculator that can be used to design a sampling plan for single and multiple testing of raw commodities (W 46). The GIPSA Sample Size calculator will determine the sample size required, the probability of accepting/rejecting a lot or the threshold limit depending on knowledge of the other two parameters.

The sampling procedure or plan for food products, agricultural commodities and bulk materials are covered in several ISO standards (eg ISO 2859, 3951, 8422, 8423, 8550, 10725) [26]. The EC is working towards development of harmonised sampling plans

and recommend that these plans be coordinated on a worldwide basis by a multinational organisation, such as the International Life Science Institute (ILSI). The ILSI is a worldwide, public, non-profit scientific foundation, affiliated with WHO and the FAO [27].

Statistical Formula for Determining the Probability of Accepting or Rejecting a Lot

$$1-(r/100) = (1-(p/100))^n$$

where

n is the sample size given in the number of kernels,
p is the percent biotech kernels in the lot and
r is the probability of rejecting the lot, given as a percent (the probability of accepting a lot is 100-r).

(W 46)

Use of the GIPSA Sample Size Calculator

Example 1: Changing the probability of rejection and the threshold limit for GMO. For example, a buyer wants to be able to detect GM contamination of a lot at the level of 1% GM with a confidence level of 95%. In this case, the size of the sample would need to be 299 kernels or beans and the risk that the buyer accepts a lot with more than 1% GMO content is 5%. If the buyer changes the criteria so that he would like to detect GM contamination of a lot at the level of 0.5% GM with a confidence level of 95%, then the sample size would need to be increased to 598 kernels. If the sample size is maintained at 299 kernels, the risk that the buyer accepts a lot with more than 0.5% GMO contamination increases to 22%.

Example 2: Changing the threshold limit for GMO. If the desired GMO concentration for a lot is less than 5%, then a sample size of 60 kernels may be acceptable. On the other hand, if the buyer requires a maximum GMO concentration of 0.1% then a sample size between 3000 and 5000 kernels would be recommended. Thus testing for the lower concentrations requires larger sample sizes.

Example 3: Compromising between precision and cost of analysis. If a buyer requires detection of 0.01% GMO in a lot with a 99% probability then the required sample size would be 46,050 kernels. Thus sample size often involves a compromise between precision and cost of analysis.

(W 106)

6.1.2 Sample Size

Sample size is a major factor that determines the sensitivity or limit of detection of a given analytical method [20] and must be sufficiently large to allow reliable detection at the desired sensitivity. If the sample size is too small the test will not be sensitive enough to detect GMO material that is not uniformly distributed throughout the lot. Increasing sample size reduces the risk to both buyer and seller. However, both the lower and upper limits for sample size selection must be balanced with the number of samples, cost and detection sensitivity [20, 24].

Other factors to consider when determining sample size for analysis include the expected proportion of target ingredient in the final product [26], the extraction efficiency of the analyte (either DNA or protein), the coefficient of variation of the sample extraction procedure and the amount of analyte remaining in the food product. For instance, lecithin products will only have very low levels of DNA and the sample size required to obtain sufficient DNA for analysis may be impractical.

6.1.3 Sample Representivity

The sampling procedure determines the 'representivity' of a result, therefore a sampling plan must not only consider sampling of a lot but also the process required to subsequently reduce the field sample to laboratory and test samples [20]. This is important as it ensures that the sample analysed in the laboratory is representative of the sample submitted for analysis by the customer. Also when sampling and testing small sub-lots of a bulk commodity, these results may be non-representative of the bulk, if stratification is present in the batch [26].

Physical sampling should take place according to ISO standards [26]. This essentially involves combining increment samples to form a bulk sample that is reduced to a laboratory size sample via grinding and the extraction process [26]. The laboratory sample size should correspond in proportions of GMO/non GMO to the original bulk sample. Ideally this is achieved through a perfect random selection process [26].

General Principles for Sampling of Grain

"A sample is obtained from the seed lot by taking small portions at random from different positions in the lot and combining them. From this sample, smaller samples are obtained by one or more stages. At each stage, thorough mixing is followed either by progressive sub-division or by the abstraction and combination of smaller portions at random." [28]

6.1.4 Sample Preparation

Significant errors can be introduced during the sample preparation stage unless adequate care is taken. In order to minimise sample preparation errors a representative composite sample must be ground to an appropriate particle size. The sample must then be thoroughly mixed prior to analysis. Sample carryover during grinding is also another

potential source of error that can be introduced into the final result unless adequate care is taken.

The quality and quantity of the analyte may also vary depending on the sample preparation. The efficiency of the extraction method can adversely affect the final results, especially if the level of GM in the sample is present at close to the Limit of Detection. Generally for each type of sample matrix the extraction method may require some modification. This can complicate the overall testing regime, especially with processed or finished products, due to the sheer number and variety of samples potentially containing GMOs. Sample preparation is thus a crucial step in the process of GMO detection [20].

6.1.5 Sample Analysis

The method used to analyse samples can vary depending on the sample type and even the country where the sample is analysed. In the USA protein immunoassays are favoured for analysing large numbers of samples, whereas in Europe PCR methods are generally preferred. Different methods are also used depending on the type of sample (grains, flour, processed), the GM trait, or the type of information required (qualitative or quantitative). If a threshold limit is to be enforced however, a relative quantitative detection system is required. Further details on methods for sample analysis are found in **Chapters 4** and **5**.

6.1.6 Reporting of Results

The reporting of results should include relevant details on the sampling procedure [26]. If the test is negative then the report should state that “the lot contains less than a specific level of GM material, at a certain probability level”. If a qualitative test is positive then the report can only state a qualitative conclusion.

Example: Reporting a Negative Result

Laboratory sample size:	800 kernels
Test result:	Negative
Test conclusion:	The lot contains <0.37% GM* material, 95% probability level

Example: Reporting a Positive Result

Laboratory size:	800 kernels
Test result:	Positive
Test conclusion:	The lot contains GM material

[26]

* This probability was calculated using the GIPSA sample size calculator (W 46).

6.2 Processing Steps and Their Effects on Protein and DNA

The term 'processed' can be used to define a very broad range of different procedures, so should be used with caution when choosing an appropriate testing method. Processing steps may or may not affect the protein and DNA in a sample, so it is critical to select the appropriate testing method, one that has been fully validated using samples processed in the same way.

Processing steps in food production include but are not limited to:

Grinding
Heating (cooking)
Retorting (canned food)
Rendering (animal feed)
Separation/Fractionation (phases/oils)
Acid/Alkali treatments
Fermentation/Enzymic digestion
Crystallisation

Many of these procedures can affect protein structure. Heating, strong acids and strong alkalis can denature proteins, so if a protein detection method can only detect the intact novel protein, it would be inappropriate for testing foods exposed to these conditions. Yet some protein detection methods can also detect denatured proteins, so would still be suitable. On the other hand, some processes such as separation/fractionation steps can remove proteins completely, in which case no protein detection methods would be suitable.

DNA is more stable than proteins, so can withstand heating and alkaline conditions. Both treatments cause the two strands of the DNA to separate, though the strands can come back together once the sample is cooled or neutralised. Single stranded DNA is less stable than double stranded DNA, so more vulnerable to degradation. Strong acids can cause DNA fragmentation, leading to degradation. Acidic conditions are found in many common food products, such as those containing tomatoes or vinegar. Retorting and rendering combine heat and high pressure to sterilise and cook food and animal feed respectively. The higher the temperature and/or pressure, and the longer the treatment, the greater the damage to the DNA. Although large amounts of DNA may be extractable following each of these processes, the level of degradation is the critical factor here in determining whether specific sequences can be detected.

There are some approved refining processes that remove all of the protein and DNA, such as those used to produce crystalline sugar and sugar syrups, refined oil and purified starches (see below). For this reason, no GM labelling is required in Australia for these products. Cold pressed oils can still contain detectable amounts of protein and DNA and so would need to carry a 'genetically modified' label if appropriate (FSANZ Compliance Guide to Standard A18 for Labelling GM Food).

Processing Steps

There are many different processing steps used in the production of food and animal feed. Some of these steps affect the protein and/or DNA and other steps have no affect. When choosing an appropriate method to meet the testing requirements, it is important to consider the processing that has been carried out and whether this will affect the protein or DNA.

Some methods state whether or not they are suitable for testing processed food without defining what is meant by 'processed'. For example, flour has been processed but is still suitable for protein testing methods. In fact, the first step of protein ELISA methods is to grind the sample.

6.2.1 Detecting DNA from Highly Refined Products

There have been very few systematic investigations to determine how various food processing steps affect the level and quality of either DNA or protein [29]. Some processing steps are known to remove or destroy the DNA and protein entirely, if the final products do not have altered characteristics they do not require labelling as they do not fall under the definition of GM food.

A close analysis of the refining process for soybean oil has been made, with DNA extraction carried out after each step [8]. Large quantities of degraded DNA could be extracted from crude oil produced from crushed soybeans. This DNA could still be amplified by PCR. Following the degumming stage, no amplifiable DNA could be detected in the oil. This stage involves heating the oil, mixing it with water and then neutralising with sodium hydroxide before centrifuging to separate the two phases. As DNA is water soluble, it is concentrated into the water phase, along with many other components of the crude oil. Some components concentrate to the interphase, including the food additive lecithin, and are also removed from the oil at this step [30]. Although the DNA could not be detected in the refined oil, more sensitive detection methods may be developed in the future. Currently, if there is a specific requirement to test for the presence of oil derived from GM soy, the DNA could be extracted from the crude oil or crushed beans.

Although there is no GM sugar commercially grown in Australia, analysis of sugar crystals and syrup derived from GM sugarcane has been carried out at a research level [31]. A sugarcane plant line was produced that was resistant to the sugarcane mosaic virus. PCR methods were developed to detect both the novel DNA construct and a naturally occurring sugarcane gene. The leaves, fibre and extracted juice tested positive for the DNA sequences, but no DNA could be detected from the filtrate, syrup and crystals. To check that these results were not due to PCR inhibition, the filtrate, syrup and crystal solutions were 'spiked' with DNA which could then be amplified [31].

Table 6.1. Detection Methods Suitable for Canola, Cotton, Maize and Soy Products

Suitable Detection Methods	Protein-Based Techniques: Lateral Flow ELISA	Protein-Based Techniques: ELISA	DNA-Based Techniques: General Screen for Multiple GM Traits	DNA-Based Techniques: Construct-Specific	DNA-Based Techniques: Event-Specific	DNA-Based Techniques: Event-Specific Quantification
Canola Products						
Whole Canola Products						
Raw whole canola						
Milled Products						
Canola meal						
Oil Products						
Cold Pressed Oil	–	–				
Refined Oil/Margarines	–	–	?	?	?	?
Cotton Products						
Whole Cotton Products						
Cottonseed						
Milled Products						
Cottonseed meal						
Oil Products						
Refined Oil	–	–	?	?	?	?
Maize Products						
Whole Maize Products						
Raw whole maize and ground maize flour						
Canned maize						
Milled Products						
Grits						
Maize meal						
Cooked Products						
Doughs, tacos, chips	–					
Cereals	–					
Other Products						
Refined Oils	–	–	?	?	?	?
Starch	–	–	?	?	?	?
Nutritive Sweeteners	–	–	–	–	–	–
Bioproducts/ Amino acids and Antibiotics	–	–	–	–	–	–
Soy Products						
Raw whole seed and ground soy flour						
Soy sprouts						
Cooked full fat soy flour	–					
Roasted soybeans	–					
Traditional soyfoods (eg. tofu, soymilk)	–					
Soybean Protein Products						
Soy flour concentrates	–					
Soybean meal	–					
Refined Soyoil						
Oils/ Margarines	–	–	?	?	?	?
Soybean Lecithin						
Bakery products/ Candy/chocolate coatings	–	–	?	?	?	?

Both DNA and protein-based methods are suitable for this product.

This processing step may alter, damage or remove protein and/or DNA. The method must be validated to ensure that GM product can still be detected after this processing step.

? This processing step removes both protein and DNA, though some products may still have sufficient DNA to enable detection of GM content.

6.3 Traceability and Identity Preservation

6.3.1 Traceability

Traceability is a mechanism or system for tracking GMOs and GMO products. It covers production stages from cultivation to distribution and can be used as part of a quality assurance procedure. Product traceability is based on the ability to identify products uniquely, by physical markings and/or packaging and aims to facilitate accurate labelling of GMOs. Traceability may involve product testing at critical control points to monitor compliance.

In July 2001, the EC proposed a new regulation (Directive 2001/18/EC) called the Traceability and Labelling (T&L) regulation. The T&L regulation provides a framework for the traceability of food and feed products produced from GMOs at all stages in the production chain (GMO regulations: traceability and labelling GM food and feed approvals, (W 78). Under the T&L regulation, GMOs and GMO derived products require specification of the identity of imported bulk shipments in terms of GMO content. If a product contains GMOs then it must be labelled "this product contains genetically modified organisms". Eventually a relevant code will be assigned to the particular GMO. Development of unique codes is fundamental to the proposed traceability scheme for products containing GMOs. If the information on whether or not a shipment contains GMOs is not available by the exporter, then the product is sampled and tested. The information is then supplied to the operator who must pass on this information to all other operators receiving the product and must also retain the information for at least 5 years (W 78).

6.3.2 Identity Preservation (IdP)

Identity preservation (IdP) is a more active process than traceability. It involves any system of crop or raw material management that preserves the identity or source of a crop or product and confirms that this has happened by using documented evidence [32]. IdP is thus "a tool for providing consumers with the opportunity to exercise choice in the selection of food products based on the presence or absence of specified characteristics" [25]. It enables purchase of crops anywhere in the world with the assurance of desired characteristics.

IdP systems tend to be market or demand driven rather than based on safety issues. However, currently consumer demand based on public perception of the 'proven' safety is creating a demand for choice between organic, conventional and GMO products. There is also increasing demand from processors and suppliers for specific quality traits, the differentiation between GM and non-GM crops and products is part of this trend. Labelling requirements for GM foods in several countries are also driving the market towards IdP systems even though this may mean significant extra cost.

This IdP system allows for the segregation of GMOs from non-GMOs by careful documentation during the various stages of the production chain [33]. Information can be retrieved from any point in the food supply chain. IdP extends beyond traceability as stringent specifications based on agreement between suppliers and their customers must be met. A company making a 'GMO free' claim must verify that the product is free of all GMOs in the market. This would require 'chain of custody' documentation to ensure identity preservation (W 115).

Moving from a traceability system to an IdP system incurs higher costs. Empirical evidence relating to soy suggests that the additional costs range from 5% to 15% of the farm-gate price of soy (W 109). These costs are related to the level of difficulty and number of actions required to provide physical segregation, the cost of testing, the tolerance level for unintentional presence of GM and the relevant information that certifies integrity has been maintained at all stages of the supply chain (W 115).

In the EU, the aim is to develop an internationally agreed-upon general IdP procedure guideline for biotechnology derived products. A general guideline is required to develop an IdP system as there is too much variation in products to develop one specific program to suit all biotechnology-derived products. The standard may also develop quality analysis critical control points (QACCP) to ensure the integrity of the IdP protocol [25].

As confirmation of product integrity is required for each step in the production and supply process, the appropriate test must be chosen for each particular application. For instance, to certify or guarantee the integrity of a GM product requires exact identification or event-specific testing. Screening tests and construct–specific tests have limited applications, as they do not identify the specific GM event involved. On the other hand, an IdP system for a GMO free product may involve use of an initial screening method to monitor for the presence of generic GM elements which, if positive, would be followed up by more specific construct- or event-specific PCR testing. Quantitative testing is necessary to determine whether the level of GM contaminant is below the acceptable limit for adventitious presence of a GM ingredient.

7 Quality Assurance and Progress towards Harmonisation

One of the major challenges still facing GM testing is the harmonisation of test results. This does not necessarily mean that GM methods must be standardised but that 'in-house' analytical methods are thoroughly validated and shown to be 'fit-for-purpose'. The major requirements to achieve harmonisation are Reference Materials (RM), target DNA sequence information, method validation procedures and performance criteria, proficiency studies and inter-laboratory studies or ring trials.

RMs are a cornerstone of any analytical method validation. They enable performance-based assessment of 'in-house' analytical methods and can also be utilised in inter-laboratory method validation procedures. Method validation is a process designed to evaluate the accuracy, specificity, reproducibility and ruggedness of an analytical procedure over the specified range of analysis.

7.1 Reference Materials

A Reference Material is defined as:

Material or substance one or more of whose property values are sufficiently homogenous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials.

A Certified Reference Material has an additional specific definition:

A material or substance accompanied by a certificate, one or more of whose property values are certified by a procedure which establishes its traceability to an accurate realisation of the unit in which the property values are expressed, and for which each certified value is accompanied by an uncertainty statement at a stated level of confidence.

[34]

With respect to food analysis, the issues relating to the production of RMs suitable for DNA-based or protein-based analyses are considerable. For example, an RM based on soy flour may be suitable for evaluating method performance using raw soy ingredients but may not be sufficient to demonstrate the performance of a method for analysing highly processed soy products such as crude soy bean oil, soy milk or soy lecithin. Furthermore, the potential for assay inhibition by various ingredients in complex food matrices needs to be assessed.

The production of RMs is very labour- and cost-intensive. Certified Reference Materials (CRMs) should be produced under International Organisation for Standardisation (ISO) guidelines and Good Manufacturing Practice to ensure batch to batch continuity, consistency and quality. Therefore, consideration as to the purpose of the RM should be

addressed prior to manufacture [25]. Clearly, it is not feasible to develop standardised GM RMs for every different food matrix. One possibility is to develop RMs for key commodities such as raw materials and basic ingredients [17, 19] and to test the ingredients for GM content at specific critical control points rather than analyse the end product. Final GM content could be determined from the earlier processing events and verified through documentation chain of custody [17]. Kuiper has suggested incorporating RMs containing known concentrations of GMO in the same matrix as the test material, to evaluate method performance [19]. However, whilst this approach may be useful for monitoring assay inhibitors, it does not address the problem of damage to sample DNA and protein during processing steps prior to testing and the effect that this may have on method performance.

A joint workshop between the International Life Sciences Institute (ILSI) Europe Novel Food Task Force, the European Commission's Joint Research Centre (JRC) and ILSI International Food Biotechnology Committee was held in 2000 to discuss "Method development in relation to regulatory requirements for the detection of GMOs in the food chain". The aim of this workshop was to investigate progress made in the development of analytical methods over the previous two years [25]. A selection of the presentations at this workshop have been published in the special guest editor section of the May/June 2002 issue of Journal of AOAC International, volume 85, number 3. Due to the delay in publishing, some of the published information has been updated since the workshop.

This ILSI Europe Workshop identified a number of priority issues with regard to development of GM RMs. They recommended the development of an international agreement (principally between the United States and Europe) on recognising common and mutually acceptable CRMs for biotechnology-derived products. They also recommended that developers of biotechnology derived products supply protein and DNA CRMs, together with parental material such as seed or grain, to a central repository as part of the product authorisation process.

7.1.1 GM Reference Materials Currently Available or Under Development

Currently, the availability of GM RMs is very limited. There have been two main approaches to the development of GM RMs. The first of these is based on preparation of grain flour of defined GMO content, whilst the second approach involves development of DNA-based RMs. Flour-based RMs have application in both DNA- and protein- based detection methods. However, they are not suitable for protein-based methods where the foreign protein is only expressed in specific part of the plant, such as leaf tissue.

Factors that are critical in the development of suitable flour-based RMs include homogeneity of the preparation, long-term stability of the analyte in the sample and a particle size that optimises the efficiency of extraction of DNA and protein from the sample matrix. Another critical factor that has been recognised is the need to prevent DNA degradation during production of the RM [25].

The Institute for Reference Materials and Measurements (IRMM) in Belgium was the first group to develop and certify RMs for GMOs. IRMM has produced CRMs for Roundup Ready® soy, Bt-176, Bt-11 and MON 810 maize. These CRMs consist of dried flour containing different mass fractions of powder from GM and non-GM crops. Different techniques have been developed to mix the mass fractions of powder.

Unfortunately, a wet-mixing technique that was used for the production of Bt-176 and Bt-11 CRMs resulted in instability of the DNA and these CRMs were recently withdrawn. The original wet-mixing process is being replaced by a dry-mixing process. However, this does highlight the issue of developing RMs with long term stability.

In the data certificate for the IRMM MON810 maize CRM, quantification has been carried out using a real-time PCR kit (GMO Quant MON810 Yield Guard Corn DNA, BioInside) with calibrants based on plasmid DNA and an ELISA kit (Bt Maize Test kit, Stratagene Diagnostics Inc) with calibrants supplied in the kit. Although both methods produced linear results for the CRMs, both were consistently biased, the ELISA method over estimating the GM concentration and the PCR method under estimating the GM concentration, by 30-40% (Certified Reference Material IRMM-413 certificate, W 91). This highlights the importance of harmonising RMs, particularly for quantification.

DNA reference materials have been produced in the form of 'plasmids' or loops of DNA. Since this DNA has no ends it is less vulnerable to nuclease enzymes and therefore more stable than linear DNA. Standard plasmid sets for GMO maize and soy are available for both qualitative and quantitative analysis from Nippon Gene. They comprise a series of dilutions ranging from 20 copies to 250,000 copies of the plasmid, which can be used to produce standard curves. The maize plasmid set detects the endogenous maize gene, SSIIb (starch synthase gene – internal standard), construct DNA sequences from the events: Bt-11, GA21, Bt-176, Mon810 and T25, plus the CaMV 35S promoter and the NOS terminator sequences. The soy plasmid contains sequences from the endogenous lectin gene, construct DNA sequence from Roundup Ready® Soy, plus CaMV 35S promoter and the NOS terminator sequences. Nippon Gene GMO plasmids are distributed in Australia by Novachem Pty Ltd (W 93) (For details on how these RMs are used for DNA quantification, see **Appendix 1**).

There are currently no reference materials specifically designed to be used in conjunction with GM canola testing. Some GM canola varieties, including the Bayer CropScience T45 canola, contain the CaMV 35S promoter sequence, so the Nippon Gene GMO plasmids may be suitable when testing these traits. The plasmid RMs would have to be fully validated for canola testing prior to use with commercial samples.

The National Institute of Standards and Technology (NIST), USA, is currently developing DNA RMs to standardise equipment and protocols used in detecting and quantifying the presence of DNA. These RMs are designed to be generic and although they will not contain DNA sequences specific to GMOs, they will have application in monitoring method performance during detection of GMOs (W 92).

Future requirements for GM RMs include developing RMs for all biotechnology-derived products and developing RMs to address the changes that protein and DNA undergo during food processing. It will also be important to develop negative control standards [25].

7.2 Method Development

Although the majority of readers will never be involved in method development and validation, it is important to have an understanding of these processes in order to develop an appreciation of what is involved and to improve communication between clients and those carrying out the testing.

Successful monitoring of GM products can only be achieved with the development of appropriate methods of detection. These methods are generally based on the detection of the novel proteins or DNA and include ELISA and PCR. Provided that sequence data is available, DNA-based methods for detecting GMOs can be designed and developed in-house by individual laboratories. It is more difficult to develop in-house protein-based methods because of the need to access suitable antibodies. Methods for the detection of GMOs have been developed by companies producing GMOs to track and identify their products; by primary producers to define their products; by Government organisations to monitor and control use of GM products and by commercial companies to provide assay kits.

Preliminary efforts are underway in the EU through the JRC to establish a molecular register, consisting of a database of information and accompanying bioinformatic tools regarding GMOs, especially the DNA sequence of introduced genetic elements and their integration sites (W 125). This database will have restricted access for participating laboratories only and has been designed for monitoring purposes as required under EC Directive 2001/18/EEC. The monitoring is envisaged not only for GM products approved for market release but also for any other GMO released for field trials worldwide [20]. A publicly available database of methods used to test GMOs has also been set up. This includes whether the method is suitable for screening, identification or quantification; whether protein or DNA is detected; method validation data and whether the method is a commercially available test or kit (W 125).

A number of large projects have also been established to develop testing methodologies for GM food. A European Research Project, “Development of Methods to Identify Foods Produced by Means of Genetic Engineering” DMIF-GEN (Project no. SMT4-CT96-2072) was conducted from 1996-1999 and involved twenty-four laboratories across twelve European countries. The aims of the project were to develop and standardise detection methods for identification of GM foods, to establish DNA extraction methods for raw, processed and complex foods, to perform inter-laboratory validation of several methods and finally to study possible means of enhancing efficiency of analysis and sample throughput. A database containing information on GM food worldwide was also developed [35].

The PCR-based Qpcrgmofood project was set up by ENTRANSFOOD (European network - safety assessment of genetically modified food crop) to develop sufficiently reliable and validated methods for the testing of GM foods. This project runs from February 2000 - January 2003. One of the major objectives of this project is to develop reliable, event-specific individual and multiplex tests for qualitative and quantitative detection of GM ingredients in food. The project is coordinated by the National Veterinary Institute in Norway and has thirteen partners from seven European countries. It will address issues such as matrix-limitations for a standard DNA extraction protocol, sequence characterisation of transformation events (junction between the construct and insertion site in the host genome), development and testing of event-specific primer-probe sets and validation of techniques in inter-laboratory studies (W 9).

The Codex *Ad Hoc* Inter-governmental Task Force on Foods Derived from Biotechnology established a Working Group on Analytical Methods in 2000. This Working Group is compiling a list of appropriate methods that includes those for the identification of foods or food ingredients derived from biotechnology as well as extraction methods for DNA and sampling. Information relating to method performance

criteria and specificity is also included. These groups and projects are all leading the way towards international method harmonisation.

7.3 Method Validation

Method validation is formally defined as "Confirmation through the provision of objective evidence that the requirements for a specific intended end use or application have been fulfilled" [36]. In the case of GMOs, it is the "process of demonstrating that the combined procedures of sample preparation (extraction, cleanup etc) and analysis will yield acceptably accurate, precise and reproducible results for a given analyte in a specified matrix" [37]. In other words, it is a process of establishing that a method is 'fit-for-purpose'. An integral part of method validation also involves documentation of all of the related information needed to perform the entire analytical procedure, including background information and method validation data [37].

Method validation data should include evaluation of performance parameters such as limit of detection, sensitivity, specificity, repeatability (closeness of repeated measurements under the same conditions) and reproducibility (closeness of repeated measurements under different conditions such as different days, different instruments etc). The robustness or ruggedness of the method relates to the effect of small changes in analytical conditions on the result and may also need to be considered. Performance criteria should also be developed for each analytical method.

Case Study – The need for method validation to address suitability of a method for processed foods

The Food Analysis Performance Assessment Scheme (FAPAS), Central Science Laboratories, UK, run a series of proficiency studies for detection of GMOs in many different matrices. In Round 09, completed by April 2002, cans of meat test material were prepared which contained soy flour, including an undisclosed amount of Roundup Ready® soy. The cans were vacuum-sealed and 'retorted' or heated to 121°C under pressure to cook and sterilise the contents. These canned samples were sent out to 68 GMO testing laboratories worldwide for analysis. These laboratories could use any DNA extraction method and qualitative or quantitative PCR methods or they could use a protein-based method.

When the results were collated, one third of the laboratories were unable to detect any GM soy. Many of the laboratories that failed to detect the GM soy were using accredited methods. The discrepancy between the laboratories most probably reflects the differential ability of laboratory methods to detect GMOs in the retorted sample.

There is some evidence to suggest that certain regions of DNA are less susceptible to heat damage (Heinz Schimmel, IRMM, personal communication). This could be a cause for the high level of variation seen in this study, as detection of GMOs in the

retorted sample may depend on the precise section of the DNA molecule that was targeted in the assay.

This proficiency study highlights the importance of ensuring that a method has been validated for the specific matrix and processing conditions that the GM component has been exposed to. It also highlights the difficulties with method harmonisation that still need to be resolved.

The European Committee for Standardization (CEN) (W 74) Technical Committee 275 'Food analysis-Horizontal methods', Working Group 11 'Detection of genetically modified organisms in the food chain' is currently working on European Standards for sampling, DNA extraction, qualitative and quantitative DNA methods and protein based detection methods. These standards aim to support harmonisation of results across laboratories by ensuring compatibility of methods and general principles. The objective of the draft standards is to ensure that methods are compatible and that general principles used to perform analyses are the same in all laboratories [35].

7.4 Inter-Laboratory Studies

One approach to establishing the reproducibility of a specific method is to conduct an inter-laboratory study as part of the validation process. This is not always practical and is not an essential component of method validation. However, a number of such studies have been conducted for specific GM test methods and they allow a formalised assessment of both method and laboratory proficiency.

The American Association of Cereal Chemists (AACC) and IRMM selected Strategic Diagnostics (SDI) Cry1Ab (MON810) ELISA kit for their first inter-laboratory study of a specific method. Forty laboratories from twenty countries participated in the study. The test targets the Cry1Ab insecticidal protein from *Bacillus thuringiensis* (Bt) in Yieldguard corn. Results from the study indicated that the concentration of cry1Ab protein could be accurately and precisely determined in ground maize and cornflour. The performance of the laboratories undertaking the study were evaluated and 85% of laboratories successfully analysed the samples in the first attempt and 100% determined concentrations within accepted limits in two attempts [38].

An inter-laboratory study has also been conducted on an ELISA kit (SDI) for the detection and quantification of CP4 EPSPS protein in Roundup Ready® soy. This European ring trial coordinated by the JRC involved thirty-eight laboratories. For samples containing less than 2% (w/w) GMO, the rate of false positive and false negative results was 1% and 6%, respectively [39]. The relative standard deviation under repeatability (RSD_r) and reproducibility (RSD_R) conditions were 7% and 10%, respectively, which is acceptable. The detection limit was approximately 0.35% GMO [20]. However, comparability in quantitative tests was poor and Erickson concluded that this was possibly due to a lack of internationally recognised RMs [39].

The performance of an ELISA kit (Envirologix) for the detection of Cry9C protein in Starlink maize was determined in an inter-laboratory study involving seven laboratories in the United States. All laboratories used the same methods and the study was conducted in accordance with the Association of Official Analytical Chemists (AOAC)

International guidelines. The final results indicate that the method is applicable for the determination of Cry9C protein in eight maize products (tortillas, muffins, bread, flakes, puffs, chips, oil and starch) at levels greater than or equal to 2 ng/g of sample [40].

7.5 Proficiency Testing

Laboratory proficiency testing is an essential part of a quality assurance program, particularly with an increasing demand for independent proof of competence from regulatory bodies and customers. This external quality check has allowed laboratories to compare their results with other laboratories, to gauge their standard on an international level [41]. These programs may involve the laboratory using a specified method or their own in-house method for the analysis of a defined sample. A number of proficiency testing schemes for testing GM foods exist.

FAPAS at the Central Science Laboratories, UK, first introduced proficiency testing for GM food in 2000 (FAPAS series 23 round 01) and this has subsequently been named the GeMMA (Genetically Modified Material Analysis) scheme (W 90). The individual GeMMA scheme proficiency tests are referred to as rounds. For each round, the samples are sent to participating laboratories for analysis, the results are submitted, statistically analysed and a report produced. Test sample matrices have included soy and maize flours, soy in biscuit crumbs and in canned meat. **Table 7.1** shows results for the first twelve rounds of GM testing. Test materials are often available for sale after the study is completed. Note that in the First round, 91% of participating laboratories produced a false positive result from qualitative analysis of non-GMO soy protein isolate (Sample C). This may have been due to higher than expected levels of sensitivity of the methods, as subsequent investigation by the GeMMA team confirmed that the 'non-GM' soy in this initial round did, in fact, contain trace amounts (~0.01% w/w) of GM soy. This is a good example of how proficiency trials assist in method assessment and improving method accuracy. Although at first glance, the results in **Table 7.1** may look alarming, they actually represent a general trend of improvement, as each Round involves testing progressively more difficult samples, either due to increased processing of the sample or lower percentages of GM ingredients present in the samples.

The USDA GIPSA conducted a Proficiency Study to assess the capability and reliability of DNA-based testing for USA commercialised biotechnology events in maize. Private and government laboratories in the USA and Europe participated in the study. The study showed that the capability of laboratories to analyse for biotechnology events varied significantly. To help improve the reliability of testing, on February 7 2002, GIPSA began offering a Proficiency Program for organisations testing for biotechnology-derived grains and oilseeds. Participants receive a set of maize and soy test samples each quarter, to allow them to validate their own methods. GIPSA provide each participant with their specific results and post a summary report of the quarterly results on the GIPSA web site (W 105).

The AACC organise a check sample series for GM grain analysis. Bimonthly samples can be analysed qualitatively and/or quantitatively. Ground maize samples provided include eight samples per bimonthly round that may include Yieldguard® (Cry1Ab protein), Starlink® (Cry9C protein) and/or Libertylink® (PAT protein) at various concentrations, plus conventional seed. Soy samples (per bimonthly round) are provided for analysis of glyphosate tolerance-Roundup Ready® at various concentrations, plus conventional seed (W 88). Whilst the AACC approved methods are recommended, any method can be used providing the method is described (W 89).

Table 7.1 Genetically Modified Material Analysis Scheme (GeMMA): Summary of results from series on testing for GMOs

Round	Number	Test Material	Ingredient	GM Ingredient[a]			Laboratory results		
				GM Trait	% of GM Ingredient	% in Test Product	No. Results	% of results present	% of results absent
1	2301A	Soy flour	Soy flour	Roundup Ready	1	1	85	94	6
	2301 B	Soy flour	Soy flour	Roundup Ready	0.2	0.2	81	89	11
	2301 C	Soy protein isolate	Soy protein isolate	-	0	0	91	91	9
	2301 D	Soy protein isolate	Soy protein isolate	Roundup Ready	1	1	94	99	1
	2301 E	Soy protein isolate	Soy protein isolate	Roundup Ready	0.5	0.5	92	98	2
2	2302 A	Maize flour	Maize flour	Bt176	0.75	0.75	74	97	3
	2302 B	Maize flour	Maize flour	-	0	0	74	26	74
	2302 C	Maize flour	Maize flour	Bt176	1.5	1.5	74	99	1
3	2303 A	Soy flour	Soy flour	-	0	0	63	16	84
	2303 B	Soy flour	Soy flour	Roundup Ready	2	2	63	92	8
	2303 C	Wheat flour/soy flour	Soy flour	Roundup Ready	10	0.5	63	97	3
4	2304 A	Maize flour	Maize flour	Bt176	0.5	0.5	98	99	1
5	GMO-05A	Soy milk powder with soy protein isolate	Soy protein isolate	Roundup Ready	1	0.15	67	100	0
6	GMO-06A	Snack Food Crumbs	Soy flour	Roundup Ready	0	0	57	14	86
	GMO-06B	Snack Food Crumbs	Soy flour	Roundup Ready	2	Unknown	58	95	5
7	GMO-07	Maize/soy flour mix	Soy flour	Roundup Ready	1	0.5	78	96	4
			Maize flour	Bt176	0.25	0.125	67	91	9
			Maize flour	Bt11	0	0	43	53	47
8	GMO-08A	Soy-based baked biscuit crumbs	Soy flour	Roundup Ready	2.5	0.05	91	98	2
9	GMO-09A	Soy in canned meat	Soy flour	Roundup Ready	5	0.1	81	67	33
10[b]	GMO-10A	Maize flour/wheat flour	Soy flour	Roundup Ready	0	0	92	4	96
			Maize flour	Bt176	0.5	0.025	97	86	14
			Maize flour	Bt11	0	0	65	6	94
	GMO-10B	Soya flour/maize flour	Soy flour	Roundup Ready	1	0.25	107	97	3
			Maize flour	Bt176	1	0.025	91	84	16
			Maize flour	Bt11	0	0	65	38	62
11[b]	GM11A	Soy flour/ wheat flour	Soy flour	Roundup Ready	1.5	0.075		90	10
			Maize flour	Bt176	0	0		8	92
			Maize flour	Bt11	0	0		0	100
	GM11B	Soy flour/ wheat flour	Soy flour	Roundup Ready	0.5	0.025		82	18
			Maize flour	Bt176	0	0		10	90
			Maize flour	Bt11	0	0		0	100
12	GM12	Powdered bread	Soy flour	Roundup Ready	3	0.01		81	19

[a] '% of GM Ingredient' is calculated as % (w/w) GM ingredient in total ingredient. '% in Test Product' is calculated as % (w/w) GM ingredient in total Test Material.
[b] Results for information only as IRMM Maize Reference Materials withdrawn due to degradation

8 Scenarios

8.1 Scenario 1. Testing Raw Canola

Question 1. Why is the sample being tested for GMOs?

To check for the adventitious presence of GM canola in a sample of conventional canola

Question 2. What level of information is being sought?

Qualitative screen

Question 3. Is this a raw commodity, an intermediate material or a highly processed product?

Raw commodity

Question 4. Is the product homogeneous?

No

Answer: DNA screening methods to detect the CaMV 35S promoter, FMV 35S promoter and NOS terminator DNA sequences would be most suitable, as all of the canola GMOs grown worldwide contain at least one of these three sequences. A negative result would indicate that no GM canola was present in that sample, within the limit of detection of the method used.

If a positive result were obtained, follow up testing would be recommended to confirm that the amplified target sequence was derived from a GMO and not the naturally occurring gene sequence. The CaMV naturally infects the *Brassica* family, which includes canola (*Brassica napus*). The specific GMO may then need to be identified, to confirm that it was a trait approved for use in Australia. This could be done using a construct-specific, or preferably an event-specific DNA method. The traceability documentation may indicate the possible source of contamination and so help to narrow down the choice of target GMOs to test for.

Protein detection methods would be less suitable for the initial screen as there are several GM canola varieties grown worldwide, each expressing a different novel protein, plus one trait with modified fatty acid content that cannot be identified using a protein detection method (see **Appendix 4 Table A4.1**). Each different trait would have to be detected using a different ELISA, assuming all these ELISA kits were available. If the initial DNA screen produced a positive result and documentation indicated the potential contamination with a specific GM canola, a trait-specific ELISA could then be used to positively identify the trait.

There are currently two GM canola applications under consideration by OGTR, for approval for growth in Australia: Monsanto Roundup Ready® canola and Bayer InVigor® canola (W 70). The Strategic Diagnostics (SDI) TraitChek Roundup Ready® lateral flow strip kit (W 25) is designed to detect the CP4 EPSPS protein produced in the range of Roundup Ready® GM traits and so is suitable for detecting raw Roundup Ready® canola seeds. The SDI TraitChek LibertyLink® Grain lateral flow strip kit will detect the PAT protein expressed by several GMOs, including InVigor® canola (W 25). This latter kit is currently unavailable in Australia but is expected to become available shortly.

Since this canola is not homogeneous, an appropriate sampling plan is required in order to collect representative samples for testing.

In some instances, quantification may also be needed, for example, to see if the percentage of GM canola is below the threshold for adventitious presence prescribed for a particular market. Due to risks associated with producing a false positive or a false negative result, the sampling technique is highly critical in collecting representative samples for testing in this scenario. As this is a raw product, if the sample came from a much larger batch of seed there is a high probability that very little mixing occurred prior to collection. GMO contamination is unlikely to be found evenly distributed, so an appropriate sampling plan would have to be adopted, taking into consideration the level of confidence required for detecting unintended GMOs, the threshold of acceptable levels of contamination, if present, and the cost of testing. The larger the sample size and number of samples, the higher the testing cost will be, but the smaller the risk of failing to detect any adventitious GMOs that may be present. A testing method with a suitably sensitive Limit of Detection (LOD) is also necessary.

To minimise the risk of false positive and false negative results, the method must be fully validated to test performance parameters such as sensitivity, specificity, repeatability, reproducibility and ruggedness prior to use on commercial samples (see **Chapter 7** section **7.3 Method Validation**).

8.2 Scenario 2. Testing Soy Flour

Question 1. Why is the sample being tested for GMOs?

Confirm sample is Roundup Ready® soy as part of a traceability audit

Question 2. What level of information is being sought?

Qualitative identification

Question 3. Is this a raw commodity, an intermediate material or a highly processed product?

Intermediate

Question 4. Is the product homogeneous?

Partially mixed

Answer: Either an event-specific DNA detection method or a trait-specific protein detection method would be suitable.

The soy flour has been ground, so is partially processed. This processing will not significantly affect the protein or DNA in the sample so both can be targeted for detection, though these molecules may degrade over time if the flour is not stored appropriately. The grinding step also results in mixing of the particles, so that the sampling plays a slightly less significant role in achieving the correct answer, as this sample would be more homogeneous than a sample of unground soybeans.

The Roundup Ready® EPSPS protein is expressed from the constitutive CaMV 35S promoter (see **Appendix 4 Table A4.6**) so the protein can be detected in all parts of the GM soy plant, including the soybeans. This means that a protein detection method can be used to confirm the identity of Roundup Ready® soy flour, so an ELISA that detects the EPSPS protein would be suitable for this application. The ELISA lateral flow strip kits (see **Chapter 4**) are cheap and simple to use, suited to on-site testing. ELISA kits for the detection of the EPSPS protein are available from SDI (W25).

A trait-specific method could also detect the same novel protein expressed by different crops. For example, the EPSPS protein is expressed by all of the Roundup Ready® GM

crops: canola, maize, soy, cotton and sugar beet (**Appendix 4**). Since this sample contains only soy flour, a positive result with an ELISA designed to detect the EPSPS protein will most likely confirm the presence of Roundup Ready® soy, but does not eliminate the possibility of detecting adventitious contamination with a different Roundup Ready® crop. On the other hand, an event-specific DNA detection method will identify conclusively the presence of a particular GMO. Event-specific methods target either of the two junction regions between the novel integrated construct and the plant genome (see **Chapter 5 Figure 5.2**). There are several published event-specific detection methods for Roundup Ready® soy available for use by testing laboratories. Irrespective of the detection method used, appropriate method validation for testing ground produce will be required by the kit producers or testing laboratories.

If a promoter had been used that restricted expression of the EPSPS protein to the green parts of the plant, for example, then a protein detection method would not have been suitable when testing soybean-derived flour. The DNA is the same in all parts of the plant, so this is not a necessary consideration for DNA detection methods.

8.3 Scenario 3. Testing Bread Containing Soy Flour

Question 1. Why is the sample being tested for GMOs?
Compliance with labelling legislation

Question 2. What level of information is being sought?
Quantitative

Question 3. Is this a raw commodity, an intermediate material or a highly processed product?
Highly processed

Question 4. Is the product homogeneous?
Yes

Answer: Event-specific quantitative PCR would be suitable, validated for cooked produce and with an appropriate LOD.

For this sample, the most important considerations are (i) whether the method has been validated for testing cooked products and (ii) whether the LOD of the method is suitably sensitive to meet the needs of the client.

Both GM soys approved for use in Australia use a constitutive promoter so that the novel protein will be present in the flour. However, the cooking process denatures most proteins so the novel protein in the bread is not likely to be recognisable using an ELISA, unless the ELISA has been specifically designed for use on cooked products.

DNA is generally more stable than proteins, so DNA detection methods would be more appropriate. The success will depend on the amount of soy present in the bread and whether the method is sensitive enough to detect it (see section **3.3 Limit of Detection**). Since there are two GM soy varieties approved for use in Australia, an event-specific method for each would be required. Although both GM varieties contain the 35S promoter and NOS terminator sequences, these sequences may be present in multiple copies, so are not suitable target sequences for quantification (see section **5.1.2.5**).

Appendix 1. Technical Description of DNA Amplification and Detection Methods

A1.1 Polymerase Chain Reaction

PCR reactions are used to amplify a target DNA sequence so that it can be detected above the background of other DNA sequences. The PCR reaction allows the amplification of the specific target DNA from as low as 1 copy to over a million copies. This is achieved by cycling the target DNA, mixed with other specific reagents, through various temperatures (**Figure A1.1**). The PCR reaction consists of the target DNA of interest, primers that anneal to the target DNA, nucleotide triphosphate (dNTP) bases and a thermostable DNA polymerase. The two primers are designed to bind to the DNA at either end of the sequence of interest, to opposite strands of the DNA in a 'forward' and 'reverse' direction (**Figure A1.1 c**). Primers can also have tags attached such as fluorescent compounds which can be used for detection, or biotin which can be used to separate the primers from a mixture of DNA.

The first step of PCR is a 'denaturation' step, where the sample DNA is heated to around 94°C. This heating causes the two strands of the DNA to separate (**Figure A1.1 b**). The second step is an 'annealing' step that allows the primers to bind to the specific target sequence. The annealing temperature can vary from 45-72°C, depending on the length and sequence of the primers. Generally, the longer the primers the higher the annealing temperature (**Figure A1.1 c**). The third step is the 'elongation' step which allows the DNA polymerase enzyme to attach to the free end of the primers, and using the DNA as a template, synthesise the second strand of DNA by incorporating the dNTP bases (**Figure A1.1 d**). The elongation temperature is usually about 72°C, as the enzyme works most efficiently at this temperature. DNA Polymerases can incorporate around 1000 bases per minute.

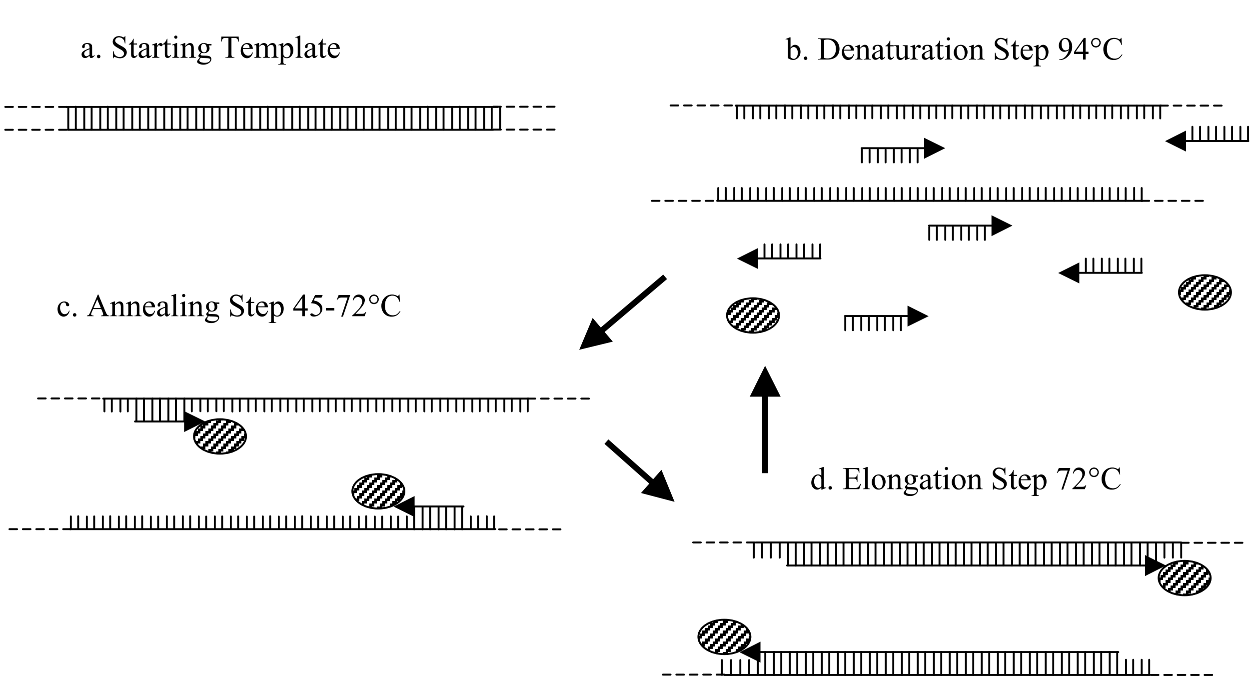


Figure A1.1 Polymerase Chain Reaction

The DNA polymerase enzymes used in PCR reactions were originally isolated from bacteria or archea growing at high temperatures, such as hot pools, so the enzymes can tolerate exposure to the elevated temperatures used in a PCR.

This cycling of temperatures is repeated many times, sometimes up to 50 cycles, though 30-40 cycles is more common. In each cycle, the copy number of the sequence of interest doubles, so that the total copy number increases logarithmically. Theoretically, if you started with a single copy of the sequence of interest, after 30 cycles you would have a billion copies. For each PCR method, the temperatures, length of time for each step and number of cycles have to be carefully optimised.

A confirmation/verification step is required to ensure that the right DNA sequence has been amplified.

A1.1.1 PCR Techniques

When DNA is amplified, the product can be detected and quantified either during the amplification (**Real-Time PCR**) by monitoring an increase in DNA associated fluorescence or at the end of a defined number of cycles (**End-Point PCR**). Methods that are used to detect a product from end-point PCR are referred to as **heterogeneous** since the sample must be taken out of the tube for detection. In contrast, real-time PCR makes use of **homogeneous** detection methods, since detection is carried out in the same tube as the PCR. In general, heterogenous detection methods are more susceptible to cross-contamination than homogeneous detection methods, since the PCR product must be transferred from the tube for the detection step.

End-point PCR requires a standard thermocycler. These are made by many different manufacturers and are relatively cheap. For real-time detection, the PCR product is monitored throughout the amplification process by measuring the increase in fluorescence with each cycle. For this type of analysis, a highly specialised thermocycler is required that also allows emission and detection of specific wavelengths of light. The first two real-time PCR machines on the market were the Lightcycler (Roche) and the ABI PRISM 7700 (Applied Biosystems). These are expensive pieces of equipment, though there are others on the market now, such as the Australian-made Rotor-Gene from Corbett Research (W 30) and the portable Smart Cycler (W 31). These newer models reduce the up-front cost of setting up real-time PCR analysis and increase testing flexibility. The Smart Cycler is light and robust and can be programmed using a lap top computer.

A1.1.2 Homogeneous Detection of Product Following Real-Time PCR

For real-time PCR, the level of fluorescence is monitored during each cycle. The intensity of fluorescence is directly related to the amount of amplified product. A fluorescence threshold is defined, that is above the background 'noise' but within the early logarithmic phase of amplification. The threshold cycle, also referred to as the C_T value, is the cycle number for each reaction at which the level of fluorescence intersects the fluorescence threshold (**Figure A1.2**). The more copies of target sequence present at the start of the amplification, the sooner the amplification signal will pass the threshold and the lower the C_T value, so that the C_T value is inversely proportional to the log of the initial amount of target DNA.

A range of fluorescent chemistries is available for detection of amplified products during real-time PCR. These detection systems lend themselves to high-throughput screening and automation [42].

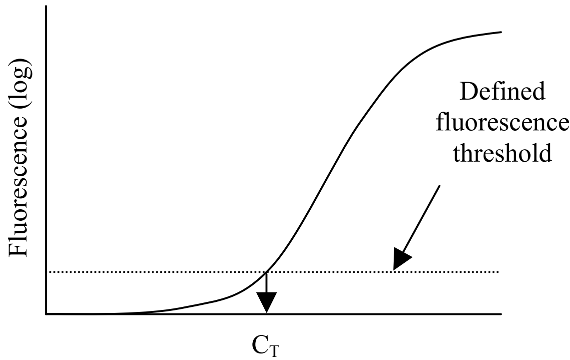


Figure A1.2 Schematic Diagram of a Fluorescence Plot from Real-Time PCR
This figure depicts a typical amplification plot for real-time PCR, monitored by an increase in fluorescence with an increase in target DNA after each PCR cycle. The C_T value is the cycle number at which the level of fluorescence intersects the fluorescence threshold, a level set above the background signal but within the early linear-log phase of amplification.

A1.1.2.1 SYBR Green

SYBR Green is a dye that fluoresces strongly when bound to double stranded DNA [43, 44]. Detection using SYBR Green is not suitable for carrying out more that one amplification in the same tube (multiplexing) as it binds to all double-stranded DNA in a non-specific fashion.

A1.1.2.2 Melting Curve Analysis

Every PCR product has a particular temperature at which the two DNA strands will separate or ‘melt’. This temperature depends on the length of the PCR product and the sequence, as it is dictated by the number of hydrogen bonds between the two DNA strands. There are three hydrogen bonds between each Guanine:Cytosine pair and two between each Adenine:Thymine pair. The sample is slowly heated up to 95°C and the temperature at which the PCR product melts is determined. Melting curve analysis can be used in conjunction with SYBR Green detection to confirm that the melting curve characteristics correspond to those of the expected PCR product.

A1.1.2.3 Probes

There are a number of styles of probes now available that can be used for detection of product in real-time PCR. Taqman probes have a fluorophore attached at one end and a quencher attached at the other end. Hydrolysis of these probes by the DNA polymerase enzyme leads to release of the fluorophore and an increase in a fluorescent signal [45-47]. FRET Probes involve hybridisation of two labelled probes to adjacent DNA sequences within the target. This hybridisation leads to Fluorescence Resonance Energy Transfer (FRET) between the two fluorophores that emit light at different wavelengths. Molecular Beacons Probes involve fluorogenic conformational changes [44, 48, 49]. Self-probing or Scorpion primers are really primer/beacon hybrids, as a single oligonucleotide is used as both primer and probe [42].

Currently up to five different PCR products can be amplified simultaneously in a single reaction by using a different fluorophore to monitor each PCR product. This is referred

to as multiplexing [47]. Detection methods using probes are much more specific than those using SYBR Green, leading to improved sensitivity of detection.

A1.1.3 Heterogenous Detection of Product Following End-point PCR

For end-point PCR, a variety of methods can be used to detect and verify the PCR product following amplification.

A1.1.3.1 Gel Electrophoresis

DNA can be separated by size using gel electrophoresis and then visualised using a stain such as Ethidium bromide [50, 51]. However there is a risk that an artefact of the same size as the target sequence has been amplified, so the product can be additionally digested with restriction enzymes prior to separation to confirm the expected sizes of the fragments [52]. These PCR products can be quantitated by scanning the gel or by CCD photography followed by densitometry [50]. Multiple products can be analysed simultaneously provided they differ in size.

A1.1.3.2 Gel Electrophoresis Followed by Blotting onto a Membrane

PCR products can be labelled during synthesis, such as incorporation of digoxigenin (DIG) or ^{32}P [53]. The products are first separated on a gel by size, then blotted onto a membrane to allow detection. The DIG label can be visualised by a colour change or fluorescence using an anti-DIG antibody labelled with either horseradish peroxidase or alkaline phosphatase (colour), or fluorescein (fluorescence) [53-55]. The ^{32}P labelled products can be visualised by exposing the membrane to X-ray film. Alternatively, the unlabelled PCR product can be blotted and incubated with a specific DNA probe to allow hybridisation (see Southern blotting in **Appendix 3**) [52].

A1.1.3.3 Selective Immobilisation and PCR-ELISA

One of the PCR primers can be biotinylated prior to use. Biotin has a very strong binding affinity to streptavidin, so by attaching this molecule to magnetic beads [56] or to microtitre wells [54, 55], the biotinylated PCR products can be ‘captured’ and separated from the reaction mix. The PCR product is then denatured leaving a single strand of the PCR product still captured onto the microtitre well or magnetic bead. A DNA probe, labelled with an enzyme-coupled antibody, is hybridised to the trapped sequence. Detection is achieved via a chemiluminescence or a colour reaction. Alternatively, a specific probe that is complementary to the PCR product can be biotinylated and immobilised, so that target sequences can be captured by hybridisation to these probes [53].

The advantage of PCR-ELISA over endpoint PCR followed by gel electrophoresis is the increase in specificity offered by the ELISA detection system.

A1.1.3.4 Nested PCR

A second round of PCR is performed where the PCR product is re-amplified with a new primer pair that binds to sequences within the first PCR product. This enables increased specificity over a single PCR, plus higher sensitivity.

A1.1.3.5 Sequencing

The PCR product can be purified and sequenced. **DNA sequencing is the most reliable method to confirm the authenticity and identity of the PCR product.**

A1.1.3.6 Mass Spectrometry

Historically, the application of mass spectrometry to nucleic acids (DNA and RNA) has been impeded because of the high polarity of these molecules due to the phosphodiester backbone, which is completely ionised at pH<1 [57]. Recent advances in ionization processes, especially matrix-assisted laser desorption/ionization combined with time-of-flight (MALDI-TOF) mass spectrometry (MS) and electrospray ionisation MS (ESI), have allowed for mass spectrometric analysis of large biomolecules including DNA primers and PCR products. The main issues regarding the analysis of PCR products by MS concern the size of the PCR products and the clean-up procedure prior to analysis.

Although PCR products larger than 100 base pairs have been measured, currently MALDI-TOF is best suited to the analysis of DNA molecules that contain less than 100 bases, due to sensitivity and resolution. The optimum size for PCR products tailored for MALDI analysis is presently in the 50-100 base pair range. Some examples of the applications of this technology include identification of PCR products (56 and 99 base pairs) from bacteria used in bioremediation processes [58] and use of delayed extraction (DE) MALDI-TOF MS for analysis of short tandem repeats (STRs) of up to 92 base pairs [59]. For PCR products between 88 and 114 base pairs, the mass accuracy of ESI-MS can distinguish single or multiple nucleotide insertions/deletions [60]. The use of DE improves mass resolution and measurement precision of PCR products and continues to extend the accessible mass range [59]. Electrospray ionisation fourier transform cyclotron resonance (ESI-FTICR) MS is the only technique currently used for accurate molecular weight analysis of PCR products above 100 base pairs [60].

Another important issue in the application of MALDI to the analysis of DNA is the necessity of a rapid and efficient sample preparation procedure. PCR products contain relatively high concentrations of salts, buffers, dNTPs, primers and other components that interfere with either desorption or ionisation of the analyte in MALDI. Low molecular weight species in the PCR mix such as dNTPs and primers ionise preferentially over the higher molecular weight PCR products, dramatically dampening the signal on MALDI-TOF analysis. Alkali metal cations (Na^+) form adducts with the negatively charged phosphodiester backbone to give a mixture of DNA salt forms, each with a different mass to charge ratio. These salt forms appear as either resolved or broad unresolved peaks depending on the salt concentrations and increase the complexity of the spectra. Various methods have been described for removing these interfering components from PCR products prior to mass spectrometric analysis. These methods include sample cleanup through a reverse-phase cartridge and addition of ammonium-form cation-exchange beads to the final sample [58], reducing cation adducts by ammonium acetate precipitation together with chelating agents to reduce metal ions in solution [57] and affinity capture and magnetic separation yielding single stranded DNA (ssDNA) [59].

The application of mass spectrometry to GMO analysis has not been thoroughly investigated to date. The main advantage of applying MS to GMO analysis would be in situations where high throughput is required and MS is used in tandem with microchip technology (see **Appendix 2**). To be compatible with MALDI-TOF MS, the PCR product would need to be less than 100 base pairs. This is not considered an issue, as short PCR products for GMO analysis are preferred in order to overcome any problems due to degradation of DNA during sample processing prior to testing.

Table A1.1 Characteristics of Mass Spectrometry

Purpose	To provide accurate mass determination of PCR products
Advantages	
	Accuracy and precision in molecular weight determinations
	Speed of measurement
	Robust, high-throughput tool
	Compatible with the next generation of high performance genetic analysis technology ie biochips
Disadvantages	
	Ionisation processes extremely intolerant to the presence of salts, and other PCR components. PCR products must be carefully purified to avoid affecting detection limit and mass accuracy
	Equipment very expensive
	High level technical skill required
Limitations	
	Accurate measurement of PCR products restricted to less than 100 base pair when using MALDI-TOF [58]
	ESI-FTICR MS can measure larger PCR products than MALDI-TOF. However, ESI-FTICR MS instrumentation is more expensive and less user-friendly, therefore it is unlikely to be used for high-throughput analysis [60]

A1.1.3.7 Surface Plasmon Resonance (SPR) and Biosensors

Surface plasmon resonance (SPR) is a micro-technology that has been applied to both protein and DNA analysis. The recent development of SPR based biosensors enables one to perform biospecific interaction analysis (BIA) for monitoring a variety of molecular reactions in real time. This optical technique detects and quantifies changes in refractive index in the vicinity of the sensor chip surface due to interaction of biomolecules (analytes) with ligands that have been immobilised to the surface of the chip. For DNA analysis the system relies on hybridisation of the target single stranded PCR product to an immobilised DNA probe.

Mariotto *et al* have used SPR for screening purposes in GMO analysis [61]. Probe sequences that are internal to the 35S promoter and the NOS terminator were developed. The system was optimised using synthetic oligonucleotides, then applied to real sample analysis. Samples containing 35S and NOS were amplified and then detected with the SPR biosensor [61]. SPR based formats have also been assessed to determine their usefulness in detecting Roundup Ready® soy gene sequences. Both oligonucleotide and PCR generated probes were tested against target single stranded PCR products. Results suggested that these procedures are useful for real-time monitoring [62].

A DNA-based biosensor has been developed for GM organisms where oligonucleotide probes are immobilised onto carbon screen-printed electrodes. Probes were designed to target the 35S promoter and the NOS terminator. Following incubation of amplified DNA from samples with the DNA-biosensor, double-stranded hybrids were detected via the intercalation of an electrochemical marker, daunomycin. The daunomycin anodic

peak was used to detect whether hybridisation took place. This technique was used successfully to analyse dilutions of 2% GM soy flour [63].

A1.1.3.8 Capillary Electrophoresis

Capillary electrophoresis (CE) is a separation technique that can be used to accurately determine DNA fragment size. Applications include size measurement of PCR products, PCR-Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP) or RFLP fragment sizes. CE instrumentation consists of two electrolyte chambers linked by a thin capillary, typically of 50-100 μm internal diameter. The high electric fields allow fast, reproducible separation (separation of fragments 80 to 1000 base pairs can be achieved in 30 minutes) with high-resolution [64]. The DNA may be detected using UV absorption, however laser induced fluorescence (LIF) provides extremely high sensitivity (~100 times higher than UV absorption) [65]. CE has been evaluated in conjunction with both UV and LIF for the detection of GM maize amplified products. Sensitivity using UV detection was 1% (w/w) GM maize in maize flour [66] whilst LIF detected levels of 0.01% GM maize in maize flour [64]. Levels less than 0.01% were not tested but theoretical calculations indicate that the sensitivity using LIF is significantly below 0.01% [64].

Capillary array electrophoresis (CAE) offers all the advantages of conventional CE, but also allows high throughput, with up to one hundred samples analysed simultaneously in parallel capillaries [65]. The development of microchip technology will also increase the capacity, automation and speed of analysis. Small CAE chips have the ability to rapidly (2-3 minutes) size DNA fragments in parallel in multiple different samples. Analysis is 10-fold faster than conventional CE and uses less reagent and sample (submicroliter level). Using a glass-wafer microchip with a 96-capillary array, PCR fragments have been analysed in less than 8 minutes. Rapid separation of DNA restriction fragments ranging from 75 to 1,632 base pairs were also achieved in 50 seconds using a microfabricated chip formed by 2 glass layers. The miniaturisation and coupling of PCR instrumentation with CE on a microchip will also facilitate rapid analysis. PCR-CE can offer an alternative to real-time PCR and can analyse several DNA target sequences simultaneously. These instruments will also be applicable for field use [65].

Table A1.2 Characteristics of Capillary Electrophoresis

Purpose	To rapidly separate and detect DNA fragments of varying sizes
Advantages	
	High throughput by using large numbers of capillaries in an array
	High resolution
	Suitable for automation
	Samples easily loaded in parallel through standard electrokinetic injection techniques
	High sensitivity achieved by fluorescently labelling DNA fragments and detecting with LIF
	Fast analysis
	Can be integrated with other instrumentation, in particular PCR
	Suitable for miniaturisation ie microchip
	Can be developed for use on-site

Disadvantages	
	Significant level of technical skills required
	Currently not widely used for GM analysis
Limitations	
	Essentially a research tool at present

A1.1.4 Quantitative PCR

Following the introduction of labelling thresholds for GMOs in food, quantitative detection methods have been required to allow enforcement of the legislation [67, 68]. PCR can be used to achieve absolute or relative quantification, but for this application a relative value is required.

Measuring the amount of the target DNA sequence after amplification is an indirect approach, since this amount depends on the amplification efficiency of the reaction. The amplification efficiency can vary between different reactions and also within the same reaction, particularly in the later cycles of the PCR when reagents become rate limiting (W 110). Conventional PCR relies on end-point measurement, when the reaction has often gone beyond the exponential phase. The amount of product therefore does not reflect the starting amount of target DNA. To address the problem of establishing the relationship between the amount of target DNA at the start of the reaction and amount of amplified product at the end, two alternative techniques have been developed: real-time PCR and end-point quantitative competitive (QC) PCR (W 110). These methods reduce the effects of variable amplification efficiencies by (i) measuring the amplified product at an early stage of the reaction while the amplification efficiency is constant and exponential (real-time PCR) or (ii) co-amplifying a DNA standard added to the reaction, to correct for the decrease in amplification efficiency in the later stages of the reaction (QC-PCR).

A1.1.4.1 Relative Quantification using Real-Time PCR Analysis

Real-time PCR analysis measures the kinetics of PCR amplification, cycle by cycle, rather than measuring the amount of amplified DNA at the end of the reaction. This amplification is monitored by an increase in fluorescent signal. There are a variety of different chemistries available for this homologous detection, as discussed above (**A1.1.2**). These detection methods eliminate the need for post-PCR analysis, such as gel electrophoresis, and so speed up the testing procedure.

Relative quantification is achieved by carrying out two PCR reactions and calculating the ratio of the two amplified sequences, using the C_T value for each reaction (see section **A1.1.2** and **Figure A1.2**). One reaction would be designed to amplify a plant-specific DNA sequence (an endogenous gene sequence), such as the soy lectin gene sequence found in all soy genomes, whether GM or conventional, while the other would amplify part of the GM construct. Ideally, an event-specific amplification should be used for the GM construct PCR [69, 70]. The C_T value for the amplified plant-specific PCR product is subtracted from the C_T value for the construct PCR product to calculate the difference in C_T value (ΔC_T).

Reference Materials with different percentages of GM to non-GM derived DNA are used to construct a calibration curve of the %GM against ΔC_T. This curve can then be used to determine the %GMO in the sample [70, 71]. There is a limited range of commercial reference materials available for production of calibration curves (see

Chapter 7), so some researchers have produced their own calibration standards using purified genomic DNA [72] or target DNA sequences cloned into plasmids [69].

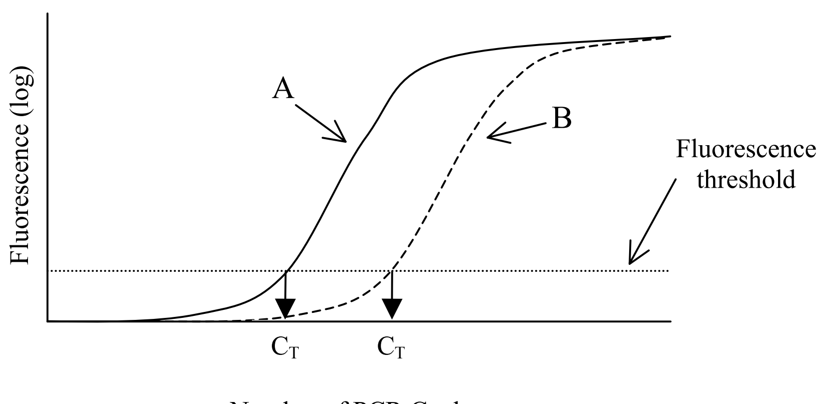


Figure A1.3 Real-Time PCR can be Used to Measure Relative Quantification

This figure depicts two amplification plots, for the endogenous gene sequence (A) and for the construct sequence (B). The ΔC_T value is calculated by subtracting the C_T value derived from curve A from C_T value derived from curve B.

The ratio of the two target sequences must also take into account the copy number of each per plant genome. The ratio would give a percentage value, eg the percentage of soy DNA derived from GM soy, expressed as a % (genome/genome). Ideally, the amplified DNA sequences are kept as small as possible. This allows the method to be used to test processed samples, as the smaller the target size, the better the detection of DNA degraded during food processing [68].

When fluorescent probes are used for detection, these two reactions can be multiplexed, or carried out simultaneously in the same reaction tube, provided different fluorophores are used for each sequence-specific probe [47, 73]. Multiplexing reduces the cost of testing and eliminates minor variations between reactions due to experimental factors such as pipetting errors, therefore improving accuracy.

Real-Time quantification offers several advantages over End-Point quantification. It is well suited to automation and high-throughput screening. Probe systems that are sequence specific can be used for detection, thus giving added confidence of the identity of the PCR product. Currently, real-time PCR is considered to be the most powerful tool for detection and quantification of GMOs in commodities and food (W 110).

A1.1.4.2 Relative Quantification using End-Point PCR Analysis

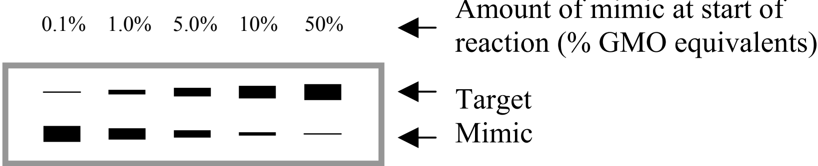
The most common approach to quantitative analysis using end-point PCR is QC-PCR. Generally, a DNA standard or ‘mimic’ is designed that can be amplified using the same pair of primers that are used to amplify the target DNA. However, the DNA sequence for the mimic differs slightly from that of the target, so that the two amplification products can be differentiated. This is achieved by either inserting [51, 74] or deleting part of the sequence of the mimic [51] or even by making a single nucleotide change [50].

When this mimic is spiked into a sample of test DNA, the original target and mimic compete for primers, hence competitive PCR. The quantities of PCR products amplified from both the target and mimic are measured at the end of the reaction. When the target sequence is in excess at the start of the reaction, there will be far more of this product at the end of the amplification. The reverse is true if the mimic sequence is in excess at the start.

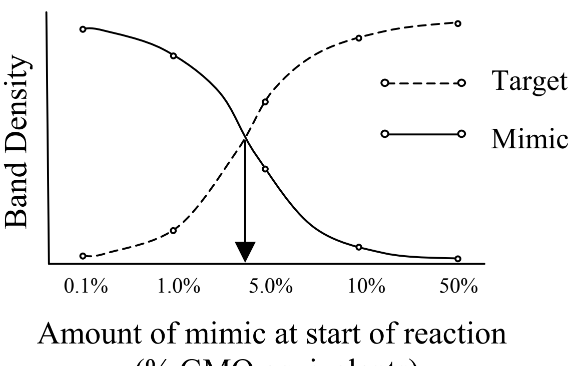
The DNA mimic is first calibrated against a reference material. Using Roundup Ready® Soy as an example, DNA is prepared from 100% conventional soy and from 100% Roundup Ready® soy and these DNA samples are mixed in different proportions. A constant amount of the mimic is spiked into aliquots of each of these mixed DNA samples and then amplified by PCR. The concentration of the mimic can then be adjusted so that the copy number is equivalent to a defined percentage (eg 1%) of GM soy DNA [50].

This calibrated mimic can then be used for semi-quantification by amplification of aliquots of sample DNA containing the mimic spiked at equivalent to 1% GMO. The comparative amounts of PCR product will indicate whether the sample contains more than, less than or equal to 1% GM DNA. For more accurate quantification, a dilution series of the mimic is prepared, containing copy numbers up to the equivalent of, say, 50% GM DNA. A series of identical PCRs are set up, each containing the same amount of sample DNA but spiked with different amounts of the mimic from the dilution series (**Figure A1.4**).

a. PCR products separated on a gel and stained



b. Density of bands are plotted on a graph



Amount of mimic at start of reaction
(% GMO equivalents)

Figure A1.4 Quantitative Competitive PCR using End-point Analysis

Following amplification, the amounts of both target and mimic PCR product are plotted against the %GM equivalent of the spiked mimic. The point at which these two lines intersect represents the equivalence point, where the copy numbers of the target and mimic were the same at the start of the reaction [50]. This method was shown to have good linearity between 1% and 10% and was tested using lecithin, flour, protein and grist samples, using either Roundup Ready® soy or Maximizer® Maize flour RM mixtures for calibration [74]. One advantage of using such an internal control is that PCR inhibitors will be evident as they affect both mimic and target DNA amplification [74].

The above example is suitable for analysis of products comprising a single ingredient, such as soy flour, since it is calculating amount of GM DNA in the total DNA. However, if the soy flour has been used in a product with multiple ingredients, such as cake or bread, the total DNA will be derived from a number of different ingredients. The GMO analysis must determine the percentage of GM soy in total soy, not in the total product. To determine this percentage, two amplifications are required using different primer pairs. One amplifies a region of the novel DNA construct found in GM soy and the other amplifies an endogenous soy gene such as the lectin gene. The difference in amount of amplified product for the two reactions is calculated and compared against a series of standards that have been analysed simultaneously, as described in the previous section.

This approach assumes that the amplification efficiency of both PCRs is the same and that any damage to the DNA that has occurred during processing is the same for both PCR target sequences [46]. If the amplification efficiencies differ, the tests would over or under-estimate the percentage of GMO present. A 5% difference in amplification efficiency can lead to a 50% difference in copy number after 14 cycles. The alternative is to carry out two independent QC-PCRs, referred to as Double-Competitive PCR [46]. For Double-Competitive PCR two mimics are prepared. One of these is the same as that described in the example for QC-PCR whilst the other is constructed so that it is almost identical to the endogenous gene (eg lectin) and is amplified with the lectin-specific primers.

A limited range of internal standards is commercially available for GMO sequences that can be used for this type of quantitative analysis (produced by Nippon Gene and IRMM and distributed through Novachem and Fluka chemicals, respectively; see also Reference Materials section in **Chapter 7**).

A1.2 Other DNA Amplification Methods

PCR has proved to be one of the most useful molecular tools since being introduced in the mid-1980s. However, despite its high sensitivity and selectivity, it does have some limitations, including the need for a thermal cycler, plus the potential variation in amplification efficiency from different targets and in the presence of various inhibitors. Several novel methods have been developed to avoid these problems.

A1.2.1 Rolling Circle Amplification

The rolling circle amplification (RCA) technique uses small single stranded DNA circles as templates for DNA polymerases to generate many concatemerised copies of the circle under isothermal conditions. When a single primer complementary to the circle is employed, product accumulation generally proceeds in a linear mode producing

approximately a 10^5- fold increase in signal (**Figure A1.5**). The introduction of a second primer with a sequence identical to a part of the DNA circle allows geometric amplification to occur resulting in excess of 10^9-fold signal increase. As few as 150 molecules can be detected using RCA (W 36).

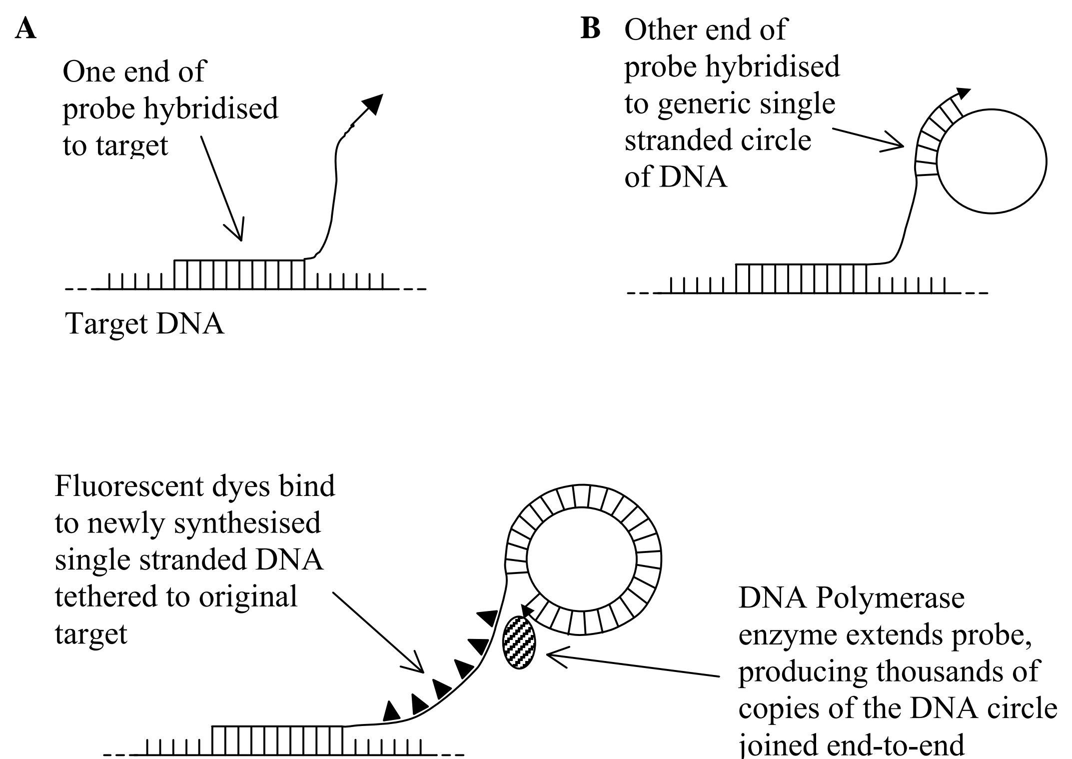


Figure A1.5 Rolling Circle Amplification

The most important advantage of RCA is that the amplified product remains linked to the DNA target. Therefore signals can be generated at specific locations that allow many assays to be performed simultaneously. Thus RCA is well suited to solid phase formats such as microarrays. To date, it has been used successfully on three DNA templates with different topological constraints [75] and has also been used to generate high quality templates for DNA sequencing reactions [76]. RCA can also be used to increase the sensitivity of immunoassays. Signal amplification via immuno-RCA results in an easily detectable high molecular weight nucleic acid molecule at the site of antibody binding. As the product remains attached to the immune complex it is compatible with localisation of the product on a microarray [77].

Table A1.3 Characteristics of Rolling Circle Amplification

Purpose	To amplify DNA *in situ* thus is well suited to microarray technology
Advantages	
	Produces a single amplified product that remains linked to the DNA primer, allowing the analysis of many targets simultaneously
	Very sensitive

	Adaptable to an on-chip signal amplification format
	Isothermal process, which overcomes the need for a thermocycler and its associated costs
	Reduced risk of false positives compared to conventional PCR
	Compatible with other detection/imaging techniques
	Targets can be analysed in solution or solid phase
Limitations	
	Essentially a research tool at present

A1.2.2 Isothermal Ramification Amplification (IRA)

Isothermal Ramification Amplification (IRA) uses a specially designed circular probe (C-probe) in which the two ends are brought together by juxtaposition by hybridisation to a target. A closed circular molecule is generated by incubating the probe with a DNA ligase enzyme. In the presence of excess primers the polymerase extends the forward bound primer along the C-probe and displaces the downstream strand, generating a multimeric ssDNA, analogous to the rolling circle replication in bacteriophages *in vivo*. This multimeric ssDNA then serves as a template for multiple reverse primers to hybridise, extend and displace downstream DNA, generating a large ramified (branching) DNA complex. This ramification process continues until all ssDNA is made double stranded, resulting in an exponential amplification that distinguishes itself from the previously described non-exponential rolling circle amplification [78].

As for RCA, the main advantage of IRA over PCR is that reactions can be localised allowing simultaneous analyses to be performed in a microarray format. In terms of GMO detection, this technology has the potential to allow the identification of many DNA target sequences in a quick and economical manner.

Table A1.4 Characteristics of Isothermal Ramification Amplification

Purpose	To amplify DNA *in situ* thus is well suited to microarray technology
Advantages	
	Ideal for *in situ* amplification as it provides good signal localisation
	Very sensitive
	Adaptable to an on-chip signal amplification format
	Isothermal process, which overcomes the need for a thermocycler and its associated costs
	Reduced risk of false positives compared to conventional PCR
	Generic primers amplify all probes with equal efficiency, resulting in better multiplex capability than conventional PCR
	Probe ends can be ligated regardless of the nature of target (DNA or RNA) thus eliminating the need for reverse transcription for detecting RNA and creating a uniform assay format for both RNA and DNA detection
	Ligation requires that both of the probe ends hybridise with perfect matching, thus allowing detection of a single nucleotide

	polymorphism
	Carryover amplified products eliminated by using exonucleases
Limitations	
	Essentially a research tool at present

A1.2.3 Hybridisation Signal Amplification Method (HSAM)

Hybridisation Signal Amplification Method (HSAM) is similar to *in situ* hybridisation, except the single-stranded probe is circular (o-probe). The probe contains a target complementary sequence and also has a section which is covalently linked to multiple ligands (eg. biotin) (**Figure A1.6**). The ligands bind to a conjugate (eg. streptavidin), which, in turn, binds to a second ligand that is linked to a generic signal nucleic acid complex (gsna) (W 35) for detection.

Whilst HSAM is still a research tool, it has the potential to be a simple and rapid method that is suitable for routine testing. However, the method is susceptible to false positive results as it is essential that any unbound probe is removed in the washing steps. Whilst a thermocycler is not required, the gsna must be purchased commercially. Further validation of this method is required to determine the binding efficiencies of the probe to the target DNA and the conjugate to both the ligand and the gsna. The sensitivity, specificity, reproducibility, efficiency and robustness of the method for specific analyses also need to be evaluated.

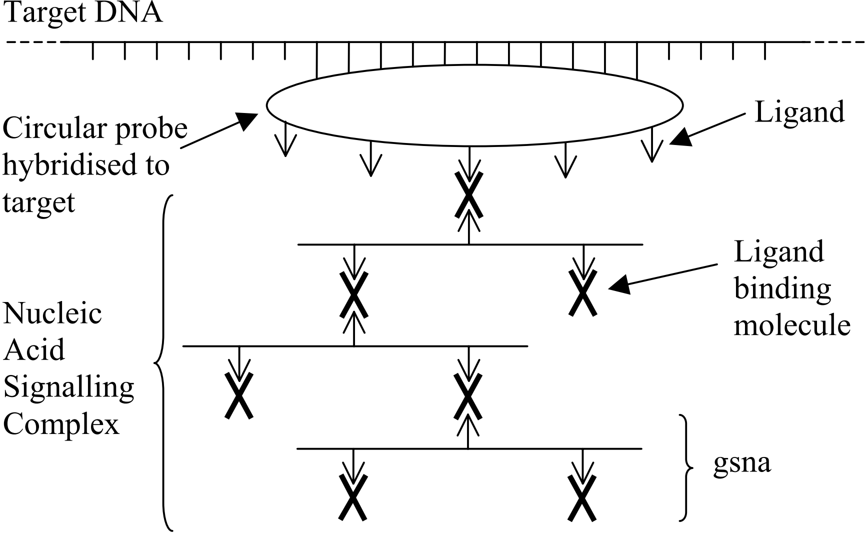


Figure A1.6 Hybridisation Signal Amplification

A1.2.4 Tyramide Signal Amplification (TSA)

Like other molecular detection methods, in Tyramide Signal Amplification (TSA) the target sequence is recognised by a probe [79, 80]. In this case, the probe is linked to multiple biotin molecules (**Figure A1.7**). Horseradish peroxidase (HRP) linked streptavidin binds to the biotin molecules. The signal is amplified when the HRP

activates multiple tyramide molecules that are deposited at the site of hybridisation of the probe. The method is fast. However, stringent washing steps are required to ensure that the background signal is minimised.

Besides HSAM and TSA, other novel methods may also have the potential to be employed for GMO testing on-site. Nevertheless, more studies must be done to determine their robustness and to improve their efficiency before applying in routine testing.

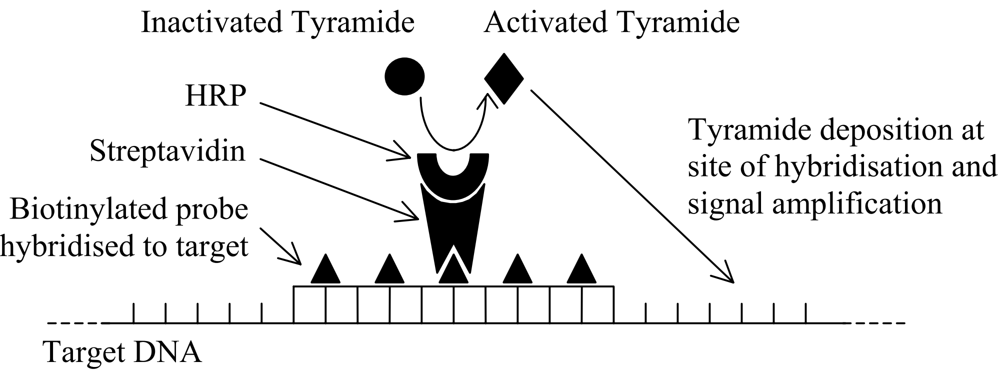


Figure A1.7 Tyramide Signal Amplification Method

Appendix 2. High-Throughput Technologies for Measuring GMOs

As the variety of genes incorporated into GM products increases, this will result in more PCR tests and an increase in cost and turnaround times. New instrumentation will be required for high-throughput screening. Technology based on gene-chips look promising for this type of analysis. Gene-chips, also known as arrays, micro-arrays, biochips or multi-arrays, are small surfaces (about 1cm x 1cm) onto which different sequences are immobilised and analysed in parallel. Hundreds of tests could be run simultaneously and in a cost-effective manner. Quantification could be made on those specific positive reactions where a threshold concentration applied.

A2.1 DNA Detection using Gene-Chip Technology

This is a relatively new technology that has enormous potential for high throughput screening of multiple GMOs simultaneously, provided the relevant DNA sequence information is available. Microarray analysis in theory can combine detection, identification and quantification of a large number of GMOs in one single assay. This technology relies on the complementarity between the two strands of double stranded DNA for its specificity. Short sequences of single stranded DNA are immobilised in tiny spots onto a glass slide. These sequences are complementary to the target sequence, and act as probes.

When the slide is exposed to extracted DNA from a test sample, any target sequences present, can bind to the immobilised probe and be detected. These slides are prepared on a microscopic scale, with many thousands of different probe sequences spotted onto each slide, allowing the potential for detection of many thousands of target sequences. If the DNA sequences that span the junction between the plant genome and construct (event-specific sequences) are used as immobilised probes, then potentially every different GMO grown or approved worldwide could be screened, with unequivocal identification of each trait.

To increase the sensitivity of this method, the target sequence can be amplified using PCR prior to binding to the slide, or targets can be amplified whilst bound to the slide, referred to as *in-situ* amplification. Amplification techniques such as Rolling Circle amplification, solid phase amplification, strand displacement amplification and ramification amplification have been developed for this purpose [79]. These methods allow multiple amplifications to occur simultaneously, with each amplified product remaining localised to its point of attachment. Detection is achieved using specialised gene-chip readers. Currently this technology is not used for quantification of GM traits as there is difficulty getting consistent amounts of probe DNA in each spot, though this may be possible in the future.

Table A2.1 Characteristics of Gene Chip Technology

Purpose	To screen for the presence of a large number of GM constructs or events
Advantages	
	Qualitative screen for a large number of GMOs simultaneously in a single sample
	Sensitive if an initial amplification step is used
	Suitable for high throughput screening
	Potential for reduced costs as multiple GMO traits can be screened simultaneously
	Reduction in reagents consumed (nanoliter quantities)
	Shortened incubation times (minutes to seconds)
	Potential for automation
Disadvantages	
	Time consuming sample preparation
	Total assay time can be long
	Reagents must be stored at 4°C
	Special equipment required
	Requires highly skilled analyst with experience in data analysis
	Strict quality assurance required to minimise risk of cross-contamination
	In many cases both sensitivity and limit of detection need to be determined (ie still at research stage)
Limitations	
	DNA chip systems may not yet be robust enough to deliver reliable and reproducible results with complex food matrices
	Costs of screening many targets simultaneously may be prohibitive at this point in time
	Mainly research tool
	Restricted to laboratory analysis currently

A2.2 Micro-Fluidic Systems

A microfluidic device can be identified by the fact that it has one or more channels with at least one dimension less than 1 mm. The volume of fluid in the channels is typically in nanoliter quantities which increases the speed of analysis and reduces the amount of reagents used. Microfluidic devices have been developed that consist of an integrated thermocycler for PCR, an electrophoretic system and an 'on-chip' DNA concentration technique enabling the detection of PCR products after 10 cycles and a total analysis time of <20 minutes [81].

An integrated PCR-CE microfluidic device was developed by Lagally *et al* in 2001 [82]. This device consisted of a 280 nL PCR chamber etched into a glass substrate and coupled with a gel-filled capillary. The PCR cycle was 30 seconds and the system was capable of detecting a single molecule of target DNA.

An electrophoretic microfluidic device used to analyse STRs achieved baseline resolution separation of a single locus STR in 30 seconds and of 4 loci in less than 2 minutes. The high-speed analysis relies on very short and precisely controlled injection plug widths (100 µm or less). These narrow injections permitted short separation distances and consequently shorter analysis times, which resulted in reduced diffusion. This new format outperformed current slab gel technology in all areas including speed, resolution and cost savings due to large reductions in reagent usage [83].

Protein Detection using Micromosaic Immunoassays

In the past, protein-based assays have generally been more difficult than DNA-based assays to miniaturise and integrate into highly sensitive, practical formats. A recent technique called soft lithography, that can handle the fragile proteins and pattern them on a surface, has enabled development of miniature protein-based assay formats. The technique used is comparable with traditional immunoassays in terms of sensitivity and reliability. Dilute proteins flow through small channels and coat the substrate immobilised on the device. The diffusion path is small therefore the reaction time is reduced.

The device consists of flat poly (dimethylsiloxane) substrate, onto which narrow stripes of antigen are deposited using a network of microfluidic channels fabricated in silicon. A second network of microchannels, perpendicular to the first, delivers the solutions to be analysed (simple capillary forces are used to induce flow in the channels). Binding the analytes results in a mosaic pattern of tiny squares similar to the grids of dots seen with DNA microarrays that can be analysed with a fluorescence microscope [84].

Table A2.2 Characteristics of Protein Micro-chip Technology

Purpose	High throughput protein detection
Advantages	
	Qualitative screen for a large number of proteins simultaneously in a single sample
	Suitable for high throughput, qualitative screening
	Reduction in reagents consumed (nanoliter quantities)
	Shortened incubation times (minutes to seconds)
	Multiple assays can be performed together
	Potential for automation
	Can be interfaced with other instruments
Disadvantages	
	Requires highly skilled analyst with experience in data analysis
	Equipment expensive
	In many cases both sensitivity and limit of detection need to be determined (ie still at research stage)
Limitations	
	Currently a research tool
	Micro-chip systems may not yet be robust enough to deliver reliable and reproducible results with complex food matrices

Appendix 3. Techniques with Limited or Specific Application for Measuring GMOs

A3.1 Near Infrared (NIR) Spectroscopy

At grain buying stations, near-infrared (NIR) spectroscopy is commonly used to assess whole grain composition rapidly and non-destructively [85]. The technique is used to measure moisture, protein, fibre, starch, oil and saturated fats in various grains including wheat, corn and soy. Analysis is quick and is completed in 60 seconds per sample [86].

A preliminary study in 1998 demonstrated a spectral difference between Roundup Ready® and non-GM soy. A calibration study predicted Roundup Ready® soy with 85% accuracy and non-GM soy with 95% accuracy. These results implied that the two types of grain must have a structural or biochemical distinction that would affect NIR spectral properties [86]. However, when this was repeated the following harvest the accuracy was greatly reduced [85] even though the direction of spectral differences between Roundup Ready® soy and non-GM soy was consistently positive across crop years [85].

The source of the Roundup Ready® spectral effect is unknown since the average nutritional value and the content of protein, oil, fibre and saturated fats are not significantly different between Roundup Ready® and non-GM samples (W 33). However, soy composition has long been known to be dependent on environment, and this may also be a factor responsible for the difference in NIR spectral properties [85].

Table A3.1 Characteristics of Near Infrared Spectroscopy

Purpose	To provide a rapid on-site screening method of raw materials for grain handlers [85]
Advantages	
	Extremely Fast [13]
	Potential for automation [86]
	Minimal expertise required [86]
	Suitable for high throughput screening [86]
	Cheap to implement as equipment already available [13]
	No sample preparation [13]
Disadvantages	
	Currently no validated methods
	Currently too much variation in values, therefore making the technique not very accurate compared with other techniques.
	Analysis and data interpretation requires complicated statistics [85]
	A calibration must be developed for each separate GMO [13]
	Generation of statistical models requires large data sets (~ 8000) [13]

Table A3.1 Characteristics of Near Infrared Spectroscopy

Limitations	
	Still at the research stage
	Technique requires clarification as cause of spectral shift unknown

A3.2 Methods based on Fatty Acid Determination

In some cases, a food or commodity being the product of genetic modification might contain no residual DNA or protein, but the modification itself was intended to produce altered characteristics. Soy lines G94-1, G94-19 and G168 (Optimum Quality Grains) have been genetically engineered to contain high levels of oleic acid. Comparative compositional analysis of high oleic soy and the parental soy by Gas Chromatography reveals significant changes in their fatty acid profiles. The oleic acid content increased to 83.8% in the genetically modified varieties compared to 23.1% in the parental soy. Linoleic acid content decreased from 55.4% to 2.2% in the GM varieties (FSANZ Draft Risk Analysis Report, Application A387 - Food derived from high oleic acid soybean lines G94-1, G94-19 and G168).

Investigation of triglyceride patterns using High performance liquid chromatography (HPLC) coupled with atmospheric pressure chemical ionisation mass spectroscopy has also been used to identify GM canola. It was observed that the oils of the GM canola varieties had an increased content of triacylglycerols, showing more oxidative stability for high stearic acid canola oil as well as for high lauric acid canola oil. In addition, fatty acid compositions have been measured by using gas chromatography coupled with Flame Ionisation Detection to support HPLC results [20].

A3.3 Amplified Fragment Length Polymorphism (AFLP)

Amplified Fragment Length Polymorphism (AFLP) is a DNA fingerprinting method that has been used with success to discriminate between and identify plant varieties. This technique has also been investigated for its potential in the combined identification of variety genotypes and the monitoring of very low levels of GM materials. AFLP analysis has limited application when applied to GMO identification as it provides too much information. AFLP is useful however for the development of PCR detection methods, in particular event-specific PCR.

An event specific PCR for the detection of RoundUp Ready® soy was developed using this method [87]. In this approach, target DNA was subjected to restriction digestion and subsequent ligation of adaptor oligonucleotides to the end of each restriction fragment. GMO-specific anchor–primers were designed according to the 35S promoter and NOS terminator sequences and the selected fragments were amplified using a labelled anchor primer and an adaptor primer specific for the adaptor ligated at the end of each restriction fragment. This combination of primers enabled amplification of cross-border (or junction) fragments between insert and plant DNA. The junction region was then characterised by sequencing the PCR product and this then allowed design of primers specific for the identified plant DNA sequences [87].

A similar approach could also be used for identification of unauthorised GMOs. If samples are positive in a screening test but negative for event specific tests, this may suggest the presence of an unknown GMO. Following restriction enzyme digestion of

DNA and ligation of specific adaptors, selected fragments are amplified with primers targeting the adaptor sequence and a selective genetic element. The fingerprint or profile can be compared with the authorised variety. Bands of differing length will be present if the sample is different [33].

Table A3.2 Characteristics of Amplified Fragment Length Polymorphism

Purpose	Development of event-specific PCRs and detection of unauthorised GMOs
Advantages	
	Enables development of event-specific test methods
Disadvantages	
	High technical skills required
	Moderate to high equipment costs requiring Thermocycler and DNA Sequencer
Limitations	
	Essentially a tool for method development

A3.4 Southern Blot

The Southern blot method was developed by Southern in 1975 [88]. This method has been used for many years as a research tool. Although there have been many advances in associated detection methods, the whole process still involves many steps and so is not suitable for high-throughput screening or quantification. DNA is extracted from the sample and then cut into shorter lengths, or digested, using DNA restriction enzymes. The DNA fragments are separated by size on an agarose or acrylamide gel by electrophoresis. The separated DNA is then denatured to form single strands and transferred, or blotted, onto a membrane (nylon or nitrocellulose).

Detection of the sequence of interest is achieved using a DNA probe. This is a synthesised, single stranded length of DNA that is complementary to the GMO target sequence. The probe is labelled to allow detection. Earlier probes were labelled with ^{32}P and detected using X-ray film. However, non-radioactive detection is now available which is much safer to use and allows faster results. The membrane with the immobilised DNA is immersed in heated hybridisation buffer containing the probe, allowing the probe to bind to its complementary sequence, if present. Unbound probe is then washed off. The presence of the target sequence is visualised by exposing the immobilised DNA and bound probe to an X-ray film. A fluorescent or chemiluminescent signal can be detected in under an hour, compared to the 24 hours exposure with ^{32}P labelled probes.

Table A3.3 Characteristics of Southern Blot Techniques

Purpose	Detection of specific DNA fragments
Advantages	
	Provides information about target size

Table A3.3 Characteristics of Southern Blot Techniques

Disadvantages	
	Large quantities of good quality DNA are required
	DNA is not amplified so less sensitive than DNA amplification methods (see below)
	Labour intensive with many steps taking 2-3 days
	Moderate technical skills required
	Not suitable for accurate quantification or automation
Limitations	
	Principally a research tool
	Only limited number of probes can be used simultaneously so not suited to multiple analyte detection [13]

A3.5 Western Blot

Western Blot analysis is frequently used for research purposes but is not suitable for high-throughput testing. The protein is extracted from the food, separated in an acrylamide gel using electrophoresis and then immobilised onto a membrane (eg nitrocellulose). The membrane is then immersed in a solution containing a conjugated antibody that specifically recognises the target protein. Unbound antibody is washed free and the appropriate reagents are then applied to the membrane to allow a colour reaction to proceed. Genetically modified soy and soy products were tested using monoclonal antibodies against CP4 EPSPS in a Western blot format. Sensitivities between 0.5 % and 1 % for raw products were achieved. Soy protein, however could not be detected in highly processed soy products [19].

Table A3.4 Characteristics of Western Blot Techniques

Purpose	Mainly as a research tool for detecting GM proteins
Advantages	
	Useful for insoluble proteins [13]
Disadvantages	
	High technical skills
	Labour intensive with analysis time at least one day [89]
Limitations	
	Qualitative test [13]
	More suited to research than routine [13]

Appendix 4. Genetically Modified Plants Approved Worldwide

Tables A4.1 to A4.6 provide relevant information on the current status of GM crops approved worldwide. Table A4.7 provides a list of abbreviations used in these Tables.

Table A4.1 Approved GMO Plants - Canola

Species	Event	Company	Approval Status by Year[Country]			Intended Use	Introduced Trait	Introduced DNA
			Environment	Food	Animal Feed			
Canola (*Brassica napus*)	GT200 (Roundup Ready®)	Monsanto	1996[4]	1997[4]	-	Not commercial-ised	Glyphosate Herbicide tolerance	CP4 EPSPS, CTP1, CTP2, FMV35S, Goxv247,
	GT73/RT73 (Roundup Ready®)	Monsanto	1995[4] 1996[9] 1999[16]	1994[4] 1995[16] 1996[9] **2000[2]**	1995[4,16] 1996[9]	Food and feed production	Glyphosate Herbicide tolerance	CP4 EPSPS, CTP1, CTP2, FMV35S, Goxv247
	23-18-17, 23-198	Calgene	1994[16] 1996[4]	1994[16] 1996[4]	1994[16] 1996[4]	Industrial applications, food and feed production	Modified seed fatty acid content	Bay TE, NPTII, Oct-T, P-NOS
	OXY-235	Bayer CropScience	1997[4] 1998[9]	1997[4] 1999[9,16] **2002[2]**	1997[4] 1999[9]	Food and feed production	Oxynil herbicide tolerance, including bromoxynil & ioxynil	CaMV 35S, BXN, NOS
	HCN10 (LibertyLink®)	Bayer CropScience	1995[4,16] 1997[9]	1995[4,16] 1997[9]	1995[4,16] 1998[9]	Food and feed production	PPT herbicide tolerance	CaMV 35S, PAT
	HCN92 (LibertyLink®)	Bayer CropScience	1995[4] 1996[9]	1995[4] 1996[9]	1995[4,16] 1996[9]	Food and feed production	PPT herbicide tolerance	CaMV 35S, CaMV 35ST, NPTII, OctT, PAT, P-NOS,
	T45/HCN28	Bayer CropScience	1996[4] 1997[9] 1998[16]	1997[4,9] 1998[16] **2002[2]**	1995[4] 1997[9] 1998[16] **2002[2]**	Food and feed production	PPT herbicide tolerance	CaMV 35S, PAT

Species	Event	Company	Approval Status by Year[Country]			Intended Use	Introduced Trait	Introduced DNA
			Environment	Food	Animal Feed			
	MS8xRF3	Bayer CropScience	1996[4] 1998[9] 1999[16]	1996[16] 1997[4,9]	1996[4,16] 1998[9]	Food and feed production	Pollination control / PPT herbicide tolerance, pollination control system, male sterility, fertility restoration	Bar, barnase, barstar, OctT, P-NOS, PSsuAra, P-TA29
	PHY36	Bayer CropScience	1997[9]	1997[9]	1997[9]	Food and feed production	Pollination control / PPT herbicide tolerance	Bar, barnase, barstar, OctT, P-NOS, PSsuAra, P-TA29
	PHY14, PHY35	Bayer CropScience	1997[9]	1997[9]	1998[9]	Food and feed production	Pollination control / PPT herbicide tolerance	Bar, barnase, barstar, MPT, OctT, P-NOS, PSsuAra, P-TA29
	(i) PGS1 (ii) PGS2	Bayer CropScience	i & ii 1995[4] i 1996[9] ii 1997[9]	i & ii 1995[4] i 1996[9,16] ii 1997[9]	i & ii 1995[4] i 1996[9] ii 1997[9]	Food and feed production	Pollination control / PPT herbicide tolerance	Bar, barnase, barstar, MPT, OctT, P-NOS, PSsuAra, P-TA29

Country Key: Argentina[1]; Australia[2]; Brazil[3]; Canada[4]; China[5]; European Union[6]' France[7]; India[8]; Japan[9]; Korea[10]; Mexico[11]; Netherlands[12]; Russia[13]; South Africa[14]; Switzerland[15]; United States[16]; Uruguay[17]

Table A4.2 Approved GMO Plants - Cotton and Flax

Species	Event	**Company**	**Approval Status by Year[Country]**			**Intended Use**	**Introduced Trait**	**Introduced DNA**
			Environment	**Food**	**Animal Feed**			
Cotton (*Gossypium-hirsutum*)	**Mon1445/1698 (Roundup Ready®)**	Monsanto	1995[16] 1997[9] 1999[1] **2000[2]**	1995[16] 1996[4] 1997[9] **2000[2]** 2001[1]	1995[16] 1998[9] 2001[1]	Food and feed production, cotton fibre	Glyphosate Herbicide tolerance	AAD-1, CaMV 35S, CP4 EPSPS, FMV35S, NOS, NPTII
	BXN	Calgene	1994[16] 1997[9]	1994[16] 1996[4] 1997[9] **2002[2]**	1994[16] 1996[4] 1998[9] **2002[2]**	Food and feed production, cotton fibre	Oxynil herbicide tolerance, including bromoxynil and ioxynil	BXN, NOS, NPTII
	31807/31808	Calgene	1997[16] 1998[9]	1998[16] 1999[9]	1998[16] 1999[9]	Food and feed production, cotton fibre	Resistance to lepidopteran insects; oxynil herbicide tolerance, including bromoxynil	35S-Man, BXN, CaMV 35S, Cry1Ac, NPTII
	Mon531/757/ 1076	Monsanto	*1995[16] **1996[2]** 1997[5,9,11,14] 1998[1] 2002[8]	1995[16] **1996[2,4]** 1997[5,9,11,14] 1998[1]	1995[16] **1996[2,4]** 1997[5,9,11,14] 1998[1]	Food and feed production, cotton fibre	Resistance to lepidoptera	AAD-1, CaMV 35S, Cry1Ac, NOS, NPTII
	19-51A	Dupont	1996[16]	1996[16]	1996[16]	Food and feed production	Sulfonylurea herbicide tolerance	ALS
Flax (*Linum usitatissimum*)	FP967	University of Saskatchewan	1996[4] 1999[16]	1998[4,16]	1996[4] 1998[16]	Food and feed production	Sulfonylurea herbicide tolerance	ALS, BLA, NOS, NPTII, P-NOS,

***Approval in countries [1,2,5,11,14] for event 531 only**

Country Key: Argentina[1]; Australia[2]; Brazil[3]; Canada[4]; China[5]; European Union[6]' France[7]; India[8]; Japan[9]; Korea[10]; Mexico[11]; Netherlands[12]; Russia[13]; South Africa[14]; Switzerland[15]; United States[16]; Uruguay[17]

Table A4.3 Approved GMO Plants - Maize

Species	Event	Company	Approval Status by Year[Country]			Intended Use	Introduced Trait	Introduced DNA
			Environment	**Food**	**Animal Feed**			
Maize (*Zea mays*)	**GA21 (Roundup Ready®)**	Monsanto	1997[16] 1998[1,4,9]	1996[16] 1999[4,9] **2000[2]**	1996[16] 1998[4] 1999[9]	Food and feed production	Glyphosate herbicide tolerance	Act, CP4 EPSPS4, NOS, RuBisCo CTP
	Mon832	Monsanto	-	1997[4]	-	Food and feed production	Glyphosate herbicide tolerance	CaMV 35S, CP4 EPSPS4, Goxv247, NOS, NPTII
	NK603 (Roundup Ready®)	Monsanto	2000[16] 2001[4]	2000[16] 2001[4,9] **2002[2]**	2000[16] 2001[4]	Food and feed production	Glyphosate herbicide tolerance	CaMV 35S, CP4 EPSPS4, CTP2, NOS, P-ract1
	676, 678, 680	Pioneer	1998[16]	1998[16]	1998[16]	Food and feed production	phosphinothricin (PPT) herbicide tolerance	CaMV 35S, Dam, PAT, PinII
	MS3 (InVigor®)	Bayer CropScience	1996[4,16]	1996[16] 1997[4,]	1996[16] 1998[4]	Food and feed production	Male sterility; phosphinothricin (PPT) herbicide tolerance	Bar, Barnase, BLA, CaMV 35S, NOS, p-TA29
	MS6 (InVigor®)	Bayer CropScience	1999[16]	2000[16]	2000[16]	Food and feed production	Male sterility; phosphinothricin (PPT) herbicide tolerance	CaMV 35S, CaMV 35ST, NPTII, Oct-T, PAT, P-NOS,
	B16/DLL25	Dekalb	1995[16] 1996[4] 1999[9]	1996[4,16] 1999[9]	1996[4,16] **2000[9]**	Food and feed production	PPT herbicide tolerance	Bar, BLA, CaMV 35S, Tr7 3’
	Mon863	Monsanto	-	2001[16]	-	Food and feed production	Resistance to corn root worm	CaMV 35S, Cry3Bb1, NOS, NPTII
	Mon80100	Monsanto	1995[16]	1996[16]	1996[16]	Food and feed production	Resistance to European corn borer	CaMV 35S, Cry1Ab, CP4 EPSPS4, CTP2, Goxv247, NOS, NPTII

Species	Event	Company	Approval Status by Year[Country]			Intended Use	Introduced Trait	Introduced DNA
			Environment	Food	Animal Feed			
	Mon810 (Yieldgard®)	Monsanto	1995[16] 1996[9] 1997[4,14] 1998[1,6]	1996[16] 1997[4,9,14] 1998[1,6] **2000**[2,15]	1996[16] 1997[4,9,14] 1998[1,6] 2000[15]	Food and feed production	Resistance to European corn borer	CaMV 35S, Cry1Ab
	Mon802 (Yieldgard®)	Monsanto	1997[4,9,16]	1996[16] 1997[4]	1996[16] 1997[4]	Food and feed production	Resistance to European corn borer, Glyphosate herbicide tolerance	CaMV 35S, CP4 EPSPS, Cry1Ab,CTP1, Goxv247, NPTII, P-NOS
	Mon809 (Yieldgard®)	Monsanto	1996[4,16] 1997[9]	1996[16,4]	1996[4,16] 1998[9]	Food and feed production	Resistance to European corn borer, Glyphosate herbicide tolerance	CaMV 35S, CP4 EPSPS, Cry1Ab, CTP2,Goxv247, NPTII, P-NOS
	Bt176 (NatureGard®)	Syngenta Seeds Inc.	1995[16] 1996[1,4,9] 1997[6]	1995[4,16] 1996[9], 1997[6,12,15,18] 1998[1] **2001[2]**	1995[16] 1996[4,9] 1997[6,12,15] 1998[1] **2001[2]**	Food and feed production	Resistance to European corn borer (*Ostrinia nubilalis*); phosphinothricin (PPT) herbicide tolerance	CaMV 35S, Cry1Ab, PAT
	Bt11	Syngenta Seeds Inc.	1996[4,9,16] 2001[1]	1996[4,9,16] 1998[6,15,18] **2001**[1,2]	1996[4,9,16] 1998[6,15,18] **2001**[1,2]	Food and feed production	Resistance to European corn borer (*Ostrinia nubilalis*); phosphinothricin (PPT) herbicide tolerance	CaMV 35S, Cry1Ab, NOS, PAT
	CBH-351 (StarLink®)	Aventis	1998[16]	-	1998[16]	Feed production	Resistance to European corn borer (*Ostrinia nubilalis*); phosphinothricin (PPT) herbicide tolerance	Bar, BLA, cab22L, CaMV 35S, CaMV 35ST, Cry9C, NOS

Species	Event	Company	Approval Status by Year[Country]			Intended Use	Introduced Trait	Introduced DNA
			Environment	**Food**	**Animal Feed**			
	DBT418 (Bt Xtra®)	Dekalb	1997[4,16] 1998[1] 1999[9]	1997[4,16] 1999[9] **2002[2]**	1997[4,16]	Food and feed	Resistance to European corn borer (*Ostrinia nubilalis*); phosphinothricin (PPT) herbicide tolerance	Bar, BLA, CaMV 35S, Cry1Ac, Oct-T, PinII, Tr7 3'
	TC1507 (Herculex®I)	Dow AgroSciences-Dupont	2001[16] 2002[9]	2001[16] 2002[9]	2001[16] 2002[9]	Food and feed	Resistance to European corn borer (*Ostrinia nubilalis*); phosphinothricin (PPT) herbicide tolerance	CaMV 35S, Cry1Fa2, PAT, Ubi
	T14, T25	Bayer CropScience	1995[16] 1996[4] 1997[9] 1998[1]	1995[16] 1997[4,9] 1998[1,6] **2002[2]**	1995[16] 1996[4] 1997[9] 1998[1,6] **2002[2]**	Grain and silage		CaMV 35S, BLA, PAT
(*Brassica rapa*) Inter-specific cross with GM Brassica napus canola line GT73	ZSR500/502	Monsanto	1997[4]	-	1997[4]	Food and feed	See GT73	See GT73

Country Key: Argentina[1]; Australia[2]; Brazil[3]; Canada[4]; China[5]; European Union[6]' France[7]; India[8]; Japan[9]; Korea[10]; Mexico[11]; Netherlands[12]; Russia[13]; South Africa[14]; Switzerland[15]; United States[16]; Uruguay[17]; United Kingdom[18]

Table A4.4 Approved GMO Plants - Chicory, Melon, Papaya, Rice, Squash, Sugar Beet and Tomatoes

Species	Event	Company	Approval Status by Year[Country]			Intended Use	Introduced Trait	Introduced DNA
			Environment	Food	Animal Feed			
Chicory (Chichorium intybus)	RM3-3, RM3-4, RM3-6	Bejo Zaden BV	1996[6] 1997[16]	1997[16]	1997[16]	Human consumption	Phosphinothricin (PPT) herbicide tolerance	Bar, Barnase, NOS, NPTII, oct-T, PSsuAra, P-NOS, p-TA29
Melon *(Cucumis mel)*	A,B	Agritope Inc.	-	-	-	Human consumption	Delayed ripening	NOS, NPTII, sam-k
Papaya *(Carica papaya)*	55-1/63-1	Cornell University	1996[16]	1997[16]	1997[16]	Human consumption	Resistance to viral infection	CaMV 35S, CaMV 35ST, Gus, NPTII, P-NOS,
Rice *(Oryza sativa)*	LLRICE06, LLRICE62 (LibertyLink®)	Bayer CropScience	1999[16]	2000[16]	2000[16]	Food and feed production	Phosphinothricin (PPT) herbicide tolerance	Bar, CaMV 35S
Squash *(Curcubits pepo)*	CZW-3	Asgrow	1996[16]	1994[16] 1998[4]	1994[16]	Human consumption	Resistance to viral infection	CaMV 35S, CP, NPTII, P-NOS
	ZW-20	Upjohn	1994[16]	1997[16] 1998[4]	1997[16]	Human consumption	Resistance to viral infection	CaMV 35S, CP
Sugar Beet (*Beta vulgaris*)	T120-7	Bayer CropScience	1998[16] 2001[4]	1998[16] 1999[9] 2000[4]	1998[16] 1999[9] 2001[4]	Human consumption	Phosphinothricin (PPT) herbicide tolerance	CaMV 35S, CaMV 35ST, NPTII, PAT, P-NOS,
	GTSB77	Monsanto	1998[16]	1998[16] **2002[2]**	1998[16] **2002[2]**	Human consumption	Glyphosate herbicide tolerance.	CaMV 35S, CP4 EPSPS, CTP1, CTP2, E9 3', *goxv247*, gus, FMV35S,
Tomato (*Lycopersicon esculentum*)	1345-4	DNA Plant Technology Corporation	1995[16]	1994[16] 1995[4]	1994[16]	Human consumption	Delayed ripening	ACC, CaMV 35S, NOS, NPTII, oct-T P-NOS

Species	Event	Company	Approval Status by Year[Country]			Intended Use	Introduced Trait	Introduced DNA
			Environment	**Food**	**Animal Feed**			
	35 1 N	Agritope Inc.	1996[16]	1996[16]	1996[16]	Human consumption	Delayed ripening	Sam-k, NPTII, oct-T, P-NOS
	8338	Monsanto	1995[16]	1994[16]	1994[16]	Human consumption	Delayed ripening	ACCd, CaMV 35S, E9 3', FMV35S, NPTII, oct-T
	FLVR SAVR	Calgene	1992[16] 1995[11] 1996[9]	1994[16] 1995[4,11] 1997[9]	1994[16] 1995[11]	Human consumption	Delayed ripening	CaMV 35S, NPTII, oct-T, PG
	5345	Monsanto	1998[16]	1998[16] 2000[4]	1998[16]	Human consumption	Resistance to lepidopteran pests	AAD-1, CaMV 35S, cong, Cry1Ac, NOS, NPTII

Country Key: Argentina[1]; Australia[2]; Brazil[3]; Canada[4]; China[5]; European Union[6]' France[7]; India[8]; Japan[9]; Korea[10]; Mexico[11]; Netherlands[12]; Russia[13]; South Africa[14]; Switzerland[15]; United States[16]; Uruguay[17]

Table A4.5 Approved GMO Plants - Potato

Species	Event	Company	Approval Status by Year[Country]			Intended Use	Introduced Trait	Introduced DNA
			Environment	**Food**	**Animal Feed**			
Potato *(Solanum tuberosum)*	**ATBT04-6,27,30,31,36/SPB T02-5,7 (NewLeaf®)**	Monsanto	1996[16] 1997[4]	1996[4,16] 1997[9] **2001[2]**	1996[16] 1997[4] **2001[2]**	Production of potatoes for human food supply	Resistance to Colorado potato beetle	ats1a, CaMV 35S, Cry3A, E9 3', NOS NPTII
	BT6,10,12,16,17, 18, 23 (NewLeaf®)	Monsanto	1995[4,16]	1994[16] 1995[4] 1996[9]	1994[16] 1995[4]	Production of potatoes for human food supply	Resistance to Colorado potato beetle	Cry3A, E9 3', NOS, NPTII
	RBMT15-101, SEMT15-02, SEMT15-15 (NewLeaf® Y)	Monsanto	1999[4,16]	1998[16] 1999[4] **2001[2]**	1998[16] 1999[4] **2001[2]**	Production of potatoes for human food supply	Resistance to Colorado potato beetle; resistance to potato virus Y	AAD-1, CP, Cry3A, E9 3', FMV35S, NOS, NPTII, P-NOS
	RBM21-129, 21-350, 22-082 (NewLeaf® Plus)	Monsanto	1998[16]	1998[16] 1999[4] **2001[2]**	1998[16] 1999[4] **2001[2]**	Production of potatoes for human food supply	Resistance to Colorado potato beetle resistance to potato leafroll luteovirus	CP4 EPSPS, Cry3A, E9 3', FMV35S, Hel, NOS, NPTII, P-NOS, Rep

Country Key: Argentina[1]; Australia[2]; Brazil[3]; Canada[4]; China[5]; European Union[6]' France[7]; India[8]; Japan[9]; Korea[10]; Mexico[11]; Netherlands[12]; Russia[13]; South Africa[14]; Switzerland[15]; United States[16]; Uruguay[17]

Table A4.6 Approved GMO Plants - Soy

Species	Event	Company	Approval Status by Year[Country]			Intended Use	Introduced Trait	Introduced DNA
			Environment	**Food**	**Animal Feed**			
Soy *(Glycine max)*	**GTS 40-3-2 (Roundup Ready®)**	Monsanto	1994[16] 1995[4] 1996[1,9] 1997[17] 1998[3,11] 2001[14]	1994[16] 1996[1,4,9,12,15] 1997[17] 1998[3,11] 1999[13] **2000**[2,10] 2001[14]	1994[16] 1995[4] 1996[1,9,12,15] 1997[17] 1998[3,11] 2001[14]	Food and feed production	Glyphosate herbicide tolerance	CaMV 35S, CP4 EPSPS, CTP4, NOS
	DD-Ø26ØØ5-3 (G94-1, G94-19, G168)	DuPont Canada Agricultural Products	1997[16] 1999[9] 2000[4]	1997[16] **2000**[2,4] 2001[9]	1997[16] 2000[4,9]	Production of soy for human consumption	Modified seed fatty acid content, specifically high oleic acid expression	BLA, CaMV 35S, Cong-P,Gm Fad2-1, gus, NOS, Phas
	A2704-12, A2704-21, A5547-35	Bayer CropScience	1996[16] 1999[4,9]	1998[16]	1998[16] 2000[4]	Production of soy for human consumption	PPT herbicide tolerance	CaMV 35S, PAT
	A5547-127	Bayer CropScience	1998[16]	1998[16]	1998[16]	Production of soy for human consumption	PPT herbicide tolerance	BLA, CaMV 35S, PAT
	GU262	Bayer CropScience	1998[16]	1998[16]	1998[16]	Production of soy for human consumption	PPT herbicide tolerance	BLA, CaMV 35S, PAT
	W62, W98	Bayer CropScience	1996[16]	1998[16]	1998[16]	Production of soy for human consumption	PPT herbicide tolerance	Bar, CaMV 35S, gus

Country Key: Argentina[1]; Australia[2]; Brazil[3]; Canada[4]; China[5]; European Union[6]' France[7]; India[8]; Japan[9]; Korea[10]; Mexico[11]; Netherlands[12]; Russia[13]; South Africa[14]; Switzerland[15]; United States[16]; Uruguay[17]

Disclaimer

The information contained within these tables is based on data obtained from second parties including Food Standards Australia New Zealand (FSANZ) and Agricultural and Biotechnologies Strategies (Canada) Inc (AGBIOS). AGAL accepts no responsibility and cannot be held liable for errors or omissions. Environmental approval does not necessarily indicate that approval has been given for environmental release of all events. In Australia, environmental approval refers to approval for commercial release.

GM events in Bold indicate that the crop has been approved in Australia for at least one of environment, food and feed use. GM events that are not in Bold indicate crops that have not been approved in Australia.

Updates

Since the information above represents a snapshot at the time of writing, with new additions likely on an ongoing basis, it is suggested that the interested reader consult the following online resources for updated information on genetically modified plant approvals:

- Australia (food) http://www.foodstandards.gov.au
- Australia (environment) http://www.ogtr.gov.au
- Global (food, feed and environment) http://agbios.com

Table A4.7 Abbreviations used in Tables A4.1 to A4.6

Abbreviation	Definition
35S-man	Chimera of the 35S promoter and the promoter from a mannopine synthase gene from *A. tumefaciens*
AAD-1	3"(9)-O-aminoglycoside adenylyltransferase; conveys (bacterial) resistance to streptomycin and spectinomycin
ACC	Aminocyclopropane-1-carboxyllic acid synthase from tomatoes
ACCd	1-amino-cyclopropane-1-carboxylic acid deaminase derived from *Pseudomonas chlororaphis*
Act	Promoter from rice actin I gene
ALS	Gene encoding Acetolactate synthase which confers tolerance to sulfonylurea herbicide (sulfonylurea tolerance)
atsla	Promoter from *Arabidopsis thaliana* ribulose-1,5-bisphosphate carboxylase gene
Bar	Gene encoding phosphinothricin acetyltransferase from *Streptomyces hygroscopicus*
barnase	Ribonuclease from *Bacillus amyloliquefaciens*
barstar	Specific inhibitor of barnase from *Bacillus amyloliquefaciens*
Bay TE	Gene encoding the enzyme 12:0 ACP thioesterase (bay TE) from the California bay tree (*Umbellularia californica*)
BLA	Beta-lactamase gene from *Escherichia coli* confers resistance to beta-lactam antibiotics
BXN	Bromoxinyl-resistance gene from *Klebsiella pneumoniae*
Cab22L	Leader sequence of the cab22L gene of *Petunia hybridia*
Cab22R 5' utr	5' Untranslated region from the Cab22R gene from *Petunia hybrida*
CaMV 35S	35S promoter from Cauliflower mosaic virus
CaMV 35ST	Terminator from the 35S gene of Caulifower mosaic virus
Cong-P	Promoter from soy alpha subunit of beta-conglycinin gene
Cong-T	Terminator from soy alpha subunit of beta-conglycinin gene
CP	Viral coat protein from *papaya ringspot potyvirus (PRSV)*
CP4 EPSPS	5-Enolpyruvylshikimate-3-phosphate synthase, from *Agrobacterium* sp. (strain CP4)
Cry1Ab	One of several proteins from the crystals in spores of *Bacillus thuringiensis*
Cry1Ac	One of several proteins from the crystals in spores of *Bacillus thuringiensis*
Cry1Fa2	Gene, isolated from the common soil bacterium *Bacillus thuringiensis* (*Bt*) var. *aizawai*, encoding the delta-endotoxin, Cry1F
Cry2Ab	One of several proteins from the crystals in spores of *Bacillus thuringiensis*
Cry3A	One of several proteins from the crystals in spores of *Bacillus thuringiensis*
Cry3Bb1	Gene, from *Bacillus thuringiensis* (subsp. *kumamotoensis*), encoding the insect control protein Cry3Bb1, a delta-endotoxin
Cry9c	One of several proteins from the crystals in spores of *Bacillus thuringiensis*
CTP1	Chloroplast transit peptide from *Arabidopsis thaliana* SSU1A
CTP2	Chloroplast transit peptide from *Arabidopsis thaliana* EPSPS
CTP4	Chloroplast transit peptide from *Petunia hybrida*
Dam	Gene from *Escherichia coli* that encodes the enzyme DNA adenine methylase
E9 3'	Terminator from small subunit of rbcS (ribulose-1,5-bisphosphate carboxylase) E9 gene from *Pisum sativum*
FMV 35S	35S Promoter from Figwort mosaic virus
Gm Fad2-1	Gene for delta-12 desaturase in *Glycine max*

Abbreviation	Definition
Goxv247	Glyphosate oxidoreductase from *Ochromobactrum anthropii*
gus	Beta-glucuronidase, marker gene
Hel	Gene from potato leafroll luteovirus (PLRV) encoding helicase
NOS	Terminator of Nopaline synthase gene from *Agrobacterium tumefaciens* strain T37
NPTII	Neomycin-3'-phosphotransferase from transposon TN5
Oct-T	Octopine synthase gene terminator from *Agrobacterium tumefaciens*
P-NOS	Promoter of nopaline synthase gene from *Agrobacterium tumefaciens*
P-ract1	P-ract1/ract1 intron containing rice actin 1 promoter
P-TA29	Promoter from anther-specific TA29 gene from *Nicotiana tabacum*
PAT	Gene coding for phosphinothricin acetyltransferase from *Streptomyces viridochromogenes*
PG	Gene encoding polygalacturonase from tomato
Phas	Terminator from phaseolin gene from *Phaseolus vulgaris*
PinII	Terminator from gene encoding protease inhibitor from *Solanum tuberosum*
PPT	Phosphinothricin (glufosinate ammonium) herbicide
PRSV	*papaya ringspot potyvirus*
PSsuAra	Promoter from *Arabidopsis thaliana*
RuBisCo CTP	Chloroplast transit peptide sequences from ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCo)
Rep	Gene from potato leafroll luteovirus (PLRV) encoding replicase (RNA dependent RNA polymerase)
sam-k	S-adenosylmethionine hydrolase derived from E. coli bacteriophage T3
Tr7 3'	3' Regulatory region from *Agrobacterium tumefaciens* of the T-DNA transcript 7
Ubi	Promoter from ubiquitin gene of *Zea mays*

Appendix 5. Glossary

Adventitious
The unintended, incidental presence of a product impurity (W 48).

Amino acid
The basic building block of a protein. There are 20 different amino acids. Long chains of amino acids are folded to make up proteins (W122).

Analyte
A molecule or material that is being analysed.

Antibiotic resistance
The ability of bacteria to tolerate antibiotics and remain unaffected by them. Resistance may evolve naturally in bacteria after years of exposure to antibiotics. It is controlled by genes and can be spread between bacteria. Many medically important bacteria have become resistant to one or more antibiotic drugs. Bacteria that have resistance to many different antibiotics are a major medical problem as they may result in infections that are untreatable (W122).

Antibody
A protein, produced by the immune system, that attaches to an antigen. (The antigen is usually a complex biochemical compound that is from outside the organism. Usually antigens are present on infectious pathogens, although they may also be on non-infectious substances such as pollen.) When an antigen from outside is present in the body, it stimulates the production of a specific antibody that will combine with it, usually enabling it to be eliminated. There are many thousands of different types of antibodies (W122).

Base
The basic building block of DNA. There are 4 bases (or nucleotides) - Adenine, Cytosine, Thymine and Guanine. A DNA strand is made up of thousands or millions of these 4 building blocks. The same building blocks are present in all forms of life (W122).

Biochip
Surfaces bearing microscopic spots, each one being formed by specific biological probes such as oligonucleotide and protein probes.

Bioinformatics
The application of information technologies and sciences to the organisation, management, mining and use of life-science information [90].

Biotechnology
(see Genetic engineering)

Chimeric gene
A constructed gene, where a coding sequence is fused to promoter and/or other sequences derived from a different gene. Most genes used in transformation are chimeric (W121).

Chromosome
A structure made of DNA and protein. Most living things more complex than bacteria package their DNA in the form of chromosomes. Each eukaryotic species has a characteristic number of chromosomes. Bacterial and viral cells contain only one chromosome, which consists of a single or double strand of DNA or, in some viruses, RNA, without the packaging proteins.

Closed-tube detection
(see Homologous detection)

Concatemer
A DNA segment made up of repeated sequences linked head to tail (W 121)

Constitutive (promoter)

An unregulated promoter that allows for continual transcription of its associated gene (W121).

Construct

An engineered chimeric DNA sequence designed to be transferred into a cell or tissue. Typically, the construct comprises the gene or genes of interest, a marker gene and appropriate control sequences as a single package (W121).

Construct-specific method

DNA tests that are designed to detect sequences that have been constructed, so are not found in nature. Examples include the junctions between the different components of the construct (eg promoter and ORF junction). Some construct-specific tests will also detect the presence of a GM trait.

Depurination

The breakdown of DNA at the *N*-glycosidic bond in the purine nucleotides, adenine (A) and Guanine (G). This occurs due to exposure to acidic conditions of pH4 and lower [9].

Dicotyledon

A plant with two cotyledons. One of the two major classes of flowering plants (along with the monocotyledons). Examples include many crop plants (potato, pea, beans), ornamentals (rose, ivy) and timber trees (oak, beech, lime) (W121).

Differentiation

The process of change from an unspecialised cell into a specialised one. The cell's structure and function may change (W122).

DNA (deoxyribonucleic acid)

The chemical of which genes are made (except for the genes of some viruses). DNA is a long molecule that looks like a coiled thread. Along its length occur chemical groups called nucleotide bases (see Base). The sequence of bases in the DNA molecule represents the instructions for making proteins. These proteins are essential for all biochemical processes within the body. In nature, DNA is copied every time new cells are made. DNA is usually contained within the nucleus of the cell.

Endogenous gene

Derived from within; from the same cell type or organism (W121).

Endogenous control

A control that is already present in the sample, eg an endogenous gene in a sample of genomic DNA.

Eukaryote

One of the two major evolutionary groups, characterised by having the nucleus enclosed by a membrane, and possessing chromosomes that undergo mitosis and meiosis. Eukaryotic organisms include animals, plants, fungi and some algae (W121).

Event

Transformation of an organism by inserting a piece of DNA (the Construct) into the genome. Events vary in the particular location that the construct is inserted into the host genome and may vary in the precise DNA sequence inserted into the organism [1].

Event-specific method

DNA tests that are designed to detect the junction sequence between the inserted construct and the genomic DNA. These tests are suitable for Relative Quantification and for identification of a specific GM event.

Exogenous control

A control that does not occur naturally in the sample and has to be added at the start of the reaction.

Expression (of a gene or protein)
The process by which a gene's coded information is translated into RNA and/or protein molecules (W122).

External control
A control that is analysed in parallel with the sample.

Gene
The basic unit of inheritance. A section of DNA carrying instructions that code for a specific product, eg a protein molecule. Some are the blueprint for the formation of other molecules. Genes are said to be active or 'expressed' when they are being 'read' and used for the production of RNA and/or protein.

Gene Technology
(see Genetic engineering)

Genechip
(see microarray)

Gene-flow
The spread of genes from one breeding population to another (usually) related population [91].

Genetic code
The code in which the instructions of life are written. The genetic code refers to the sequence of bases in a DNA molecule. There are four possible bases, and their sequence spells out how to build proteins. In turn, the proteins are responsible for constructing and operating the features of the organism (W122).

Genetic engineering
The science of genetically modifying an organism's DNA, such that the transformed organism can express new traits or modified characteristics. Also, DNA technology, Gene technology or Biotechnology.

Genetically modified organism (GMO)
An organism in which the genetic material (DNA) has been altered in a way that does not occur naturally by mating or natural recombination, ie by being genetically modified (GM) or by recombinant DNA (gene) technology.

Genetic modification
The deliberate changing of the genetic material in an organism. Scientists can determine whether or not the change will be passed onto offspring. For most GMOs, the modification is passed on. Genetic modification is a general term that can cover many processes (W122).

Genome
The total genetic material of an individual or species (W122).

Glycosylation
The covalent addition of sugar or sugar-related molecules to other classes of molecule, including proteins or nucleic acids (W121).

Heterogeneous (detection)
The samples have to be taken out of the reaction container in order to be analysed.

Heterologous
From a different source (W121).

Homogeneous (detection)
The samples are analysed whilst still in the reaction container. Also known as Closed-tube detection.

Homologous
1. General definition: from the same source, or having the same evolutionary function or structure. 2. For chromosomes: identical with respect to gene content and linear

ordering. Homologous chromosomes pair and recombine with one another at meiosis. 3. Of DNA/proteins: identical or nearly identical nucleotide/amino acid sequence (W121).

Hybridisation

1. The process of forming a hybrid by cross-pollination of plants or by mating animals of different types. 2. The pairing of two DNA strands, often from different sources, by hydrogen bonding between complementary nucleotides (W121).

Immortal cell line

A cell line which can proliferate indefinitely (W121).

Internal control

The control is analysed in the same tube as the sample.

Line (Lineage)

A group of individuals related by common descent (W121).

Marker gene

A gene, whose presence is easily detectable, which is inserted into a GMO along with the desired gene. The presence of the marker gene allows scientists to know that the insertion of the genes has been successful (W122).

Matrix

The external material in which the analyte is found.

Meiosis

The two-stage process in sexual reproduction by which the chromosome number is reduced from the somatic to the haploid number. The first division, in which homologous chromosomes pair and exchange genetic material, is followed by a mitotic division. The nucleus divides twice, but the chromosomes only once, generating haploid nuclei which develop into the gametes (eggs and sperm in animals; ovules and pollen in plants) (W121).

Microarray

A large set of DNA molecules or proteins immobilised as an orderly grid pattern of microscopic spots onto a solid matrix (typically a glass slide). The major advantage of microarrays is the extent to which screening can be automated, thereby enabling large numbers of samples to be analysed simultaneously (W121). Also called biochips, DNA chips or gene chips though the latter two refer only to DNA screening rather than protein screening.

Molecule

The stable union of two or more atoms; some organic molecules contain very large numbers of atoms (W121).

Monocotyledon

A flowering plant whose embryo has one cotyledon. Examples are cereals (corn, wheat, rice etc.), banana, and lily (W121).

Multiplex

The simultaneous amplification of a number of products in a single DNA amplification reaction, achieved by including more than one set of primers in the reaction mix (W121).

Nucleotide

(see Base)

Oligomer

A molecule formed by the covalent joining of a small (undefined) number of monomers (W121).

Oligonucleotide

A DNA nucleotide oligomer (W121). Often synthesised for use as primers for DNA amplification or probes for DNA detection.

Open Reading Frame (ORF)
A sequence of nucleotides in a DNA molecule that has the potential to encode a peptide or protein (W121).

Pharming
The use of genetically transformed crop plants and livestock animals to produce valuable compounds, especially pharmaceuticals (W121).

Plasmid
A circular self-replicating non-chromosomal DNA molecule found in many bacteria, capable of transfer between bacterial cells of the same species and occasionally of different species. Plasmids are particularly important as vectors for genetic engineering (W121).

Primer
Short length of single stranded DNA (oligonucleotide) used to initiate DNA amplification (used in pairs) or for DNA sequencing (used singly). These can be synthesised by many commercial companies.

Probe
A strand of DNA that is complementary to a portion of the DNA in the target sequence being sought. The probe DNA can be labelled for easy detection, for example with radioactive atoms or with a fluorescent dye. The target DNA is first separated into two strands, then the probe DNA is added. It will bind to a strand at the point where it recognises a sequence of bases. It can then be detected either by its emitted radioactivity or by its fluorescence (W122).

Promoter
A DNA sequence at the start of a gene that controls when and where that gene is expressed and at what expression level [1].

Protein
A type of molecule occurring in all living things. Proteins are made from about 20 basic units (See Amino acid). There is a huge variation in protein size and function depending on how the units are put together. The instructions for how to assemble proteins are usually contained within DNA molecules. Within cells, proteins carry out most of the chemical functions necessary for life – for example, building other proteins, carrying out chemical reactions, controlling what enters and leaves the cell, making structures, controlling the expression of genes (W122).

Quantification (absolute)
Tests that measure the amount of a substance, eg how many milligrams of a specific protein or how many copy numbers of a specific DNA sequence are present. Results are presented as an absolute value.

Quantification (relative)
Tests that measure the amount of a substance relative to another substance, eg how many milligrams of a specific protein are present per gram of total protein or how many copy numbers of a specific DNA sequence are present per genome. Results are presented as a percentage. For compliance to the GMO labelling legislation, relative quantification is required.

Recombinant DNA
The hybrid DNA produced by joining DNA that has originated from different organisms – the DNA is 'recombined' (W122).

Recombinant DNA technology
The techniques and tools employed to produce recombinant DNA (W122).

Reference materials

Material or substance one or more of whose property values are sufficiently homogenous and well established to be used for the calibration of an apparatus, the assessment of a measurement method or for assigning values to materials [34].

Repeatability

Precision under repeatability conditions. Tests performed under conditions that are as constant as possible, with the tests being performed during a short interval of time in one laboratory by one operator using the same equipment.

Reproducibility

Precision under reproducibility conditions. Tests performed under conditions that are not constant, such as over a period of time, in different laboratories, by different operators or using different equipment.

Resistance

The ability of an organism to survive exposure to a toxin [1].

RNA (ribonucleic acid)

A messenger molecule which is a copy of the information from DNA (which is housed in the nucleus), and transports the code outside the nucleus to cellular machinery which reads the code and puts together a long chain of amino acids (protein). The RNA molecule is very similar to DNA (W122).

Sampling

The selection of a certain subset of constitutive elements making up the lot [24].

Screening method

Test that will detect the presence of multiple analytes.

Selectivity

In diagnostic tests, the ability of an assay to selectivity detect the target molecule.

Sensitivity

In diagnostic tests, the smallest amount of the target molecule that the assay can detect. (W121).

Stacking

The introduction of a new DNA sequence into a GMO, so that it confers more than one novel trait.

Terminator

The sequence found at the end of the gene, the DNA version of a full stop.

Trait

Genetic traits are those aspects of an organism controlled by genes, for example, eye colour in humans. Genetic traits are inherited (W122).

Trait-specific method

Protein-based tests that detect the novel trait or characteristic of a GM line.

Transformant

A cell or organism that has been genetically altered through the integration of a transgene(s). Primary: the first generation following the transformation event. Secondary: progeny of the primary transformant (W 121).

Transformation

The uptake and integration of DNA in a cell, in which the introduced DNA is intended to change the characteristics of the recipient organism in a predictable manner (W121).

Transgenic

An organism containing one or more deliberately inserted genes from another species. Examples are bacteria containing the gene for human insulin and plants that contain the gene for a naturally occurring insecticide (Bt toxin) (W122).

Appendix 6. Abbreviations

For abbreviations used only in the Appendix 4 tables, see **Table A4.7**.

Abbreviation	Definition
35S	35S promoter
A	Adenine
AACC	American Association of Cereal Chemists
AAD-1	3'(9)-O-aminoglycoside adenylyltransferase; conveys (bacterial) resistance to streptomycin and spectinomycin
ACCC	Australian Competition and Consumer Commission
AFFA	Agriculture, Fisheries and Forestry – Australia
AFLP	Amplified Fragment Length Polymorphism
ANZFA	Australia New Zealand Food Authority (now FSANZ)
AOAC	Association of Official Analytical Chemists
AQIS	Australian Quarantine and Inspection Service
BA	Biosecurity Australia
BIA	Biospecific Interaction Analysis
bla	Beta-lactamase gene; conveys resistance to beta-lactam antibiotics (e.g. penicillin, ampicillin); from Tn3 (see also ampR)
Bt	*Bacillus thuringiensis*, a bacterium commonly found in soil. It produces a protein (Bt toxin) which is naturally toxic to some insects. Different Bt toxins (from different strains) affect different insect types
BXN	Bromoxinyl-resistance gene (nitrilase) from *Klebsiella*
C	Cytosine
CAE	Capillary Array Electrophoresis
CaMV	Cauliflower Mosaic Virus
CCP	Charged Couple Device
CE	Capillary Electrophoresis
CP4 EPSPS	5-enolpyruvylshikimate-3-phosphate synthase, from *Agrobacterium* sp. (strain CP4)
C-probe	Circular probe
CRM	Certified Reference Material
Cry protein	Any of several proteins that comprise the crystal found in spores from *Bacillus thuringiensis*, providing resistance to certain specific insects; the active form of the toxin binds to cells in the insect gut, ultimately leading to cell lysis and insect death
C_T value	Cycle number in real-time PCR when the fluorescent level is significantly higher than the background 'noise'
CTAB	Cetyl-trimethylammonium bromide
CTP1	Sequence coding for chloroplast transit peptide, CTP1
CTP2	Sequence coding for chloroplast transit peptide, CTP2
CTP4	Sequence coding for chloroplast transit peptide, CTP3
DE	Delayed extraction
DIG	Digoxigenin
dNTPs	Nucleoside triphosphates
DMIF-GEN	EC project for the development of methods to identify foods produced by means of genetic engineering
DNA	Deoxyribonucleic Acid
E9 3'	3' sequence of small subunit of rbcS (ribulose-1,5-bisphosphate carboxylase) E9 gene (from pea)
EC	European Commission

Abbreviation	Definition
EDTA	Ethylene Diamine Tetra-acetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ESI	Electrospray Ionisation
ESI-FTICR MS	Electrospray Ionisation Fourier Transform Cyclotron Resonance Mass Spectrometry
EU	European Union
FAO	Food And Agriculture Organisation Of The United Nations
FDA	Food and Drug Administration (USA)
FMV	Figwort Mosaic Virus
FRET	Fluorescence Resonance Energy Transfer
FSANZ	Food Standards, Australia New Zealand
G	Guanine
GeMMA	Genetically Modified Material Analysis
GIPSA	Grain Inspection, Packers and Stockyard Administration
GM	Genetically Modified
GMO	Genetically Modified Organism
GOX	Glyphosate Oxidoreductase
gsna	Generic signal nucleic acid complex
Gus	β-Glucuronidase
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidase
HSAM	Hybridisation Signal Amplification Method
IdP	Identity Preservation
ILSI	International Life Sciences Institute
IRA	Isothermal Ramification Amplification
JRC	Joint Research Centre
LCR	Ligase Chain Reaction
LIF	Laser Induced Fluorescence
LMO	Living Modified Organism
LOD	Limit of Detection
MALDI-TOF	Matrix-assisted laser desorption/ionization combined with time-of-flight
MS	Mass Spectrometry
NIR	Near-Infrared
NIST	National Institute of Standards and Technology
NPTII	Neomycin-3'-phosphotransferase (aminoglycoside-3'-phosphotransferase gene) from TN5
NOS	Nopaline Synthase gene (from *Agrobacterium tumefaciens*)
OECD	Organisation for Economic Co-Operation and Development
OGTR	Office of the Gene Technology Regulator
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
QACCP	Quality Analysis Critical Control Points
QC-PCR	Quantitative Competitive PCR
RCA	Rolling Circle Amplification
rDNA	Recombinant DNA
RFLP	Restriction Fragment Length Polymorphism
RM	Reference Material
RNA	Ribonucleic acid
RSD_r	Relative Standard Deviation under repeatability conditions

Abbreviation	Definition
RSD_R	Relative Standard Deviation under reproducibility conditions
SPS	Sanitary and Phytosanitary (Agreement)
SDI	Strategic Diagnostics
SDS	Sodium Dodecyl Sulphate
SPR	Surface Plasmon Resonance
ssDNA	Single stranded DNA
STR	Short Tandem Repeat
T	Thymine
T&L	Traceability and Labelling
TSA	Tyramide Signal Amplification
USA	United States of America
USDA	United States Department of Agriculture
Western	Western analysis, immunoblotting
WHO	World Health Organisation
w/w	Weight for weight ratio

Appendix 7. Web sites relevant to GMO Testing

The information contained within this table may be incomplete. We apologise for any unintended omissions, they do not reflect on the companies, organisations and web sites that have not been included.

Methods for Detecting GMOs

w1 Commonly used methods for detecting GMOs in grain crops
Ohio State University extension fact sheet, horticulture and crop science
http://ohioline.osu.edu/agf-fact/0149.html

w2 Database on methods for detection, identification and quantification of GMOs
EU commissioned study - GMOs in food and environment
http://139.191.1.19/gmomethods.htm

w3 Development of methods to identify foods produced by means of genetic engineering
Europa, European Union
http://europa.eu.int/comm/research/quality-of-life/gmo/04-food/04-03-project.html

w4 GMO Testing
Food Service Focus, provides general information on GMO testing
http://foodproductdesign.com/archive/2001/0201fo.html

w5 GMOs: How can you tell that they're there?
Overview information on GMO testing
http://www.ifis.org/hottopics/GMOdetection.html

w6 Jakob Lindenmeyer's home page
Detection methods to identify foods produced by means of genetic engineering
http://www.weboffice.ethz.ch/jl/research.html

w7 Methods for Detection of Transgenic Plants
Lists and links for GMO kits, manufacturers, testing labs, news and organisations
http://sbc.ucdavis.edu/Outreach/resource/detect_biotech.htm

w8 Methods to test food produced by recombinant DNA
Ministry of Health, Labour and Welfare, Japan
http://www.mhlw.go.jp/english/topics/food/sec05-1a.html

w9 Qpcrgmofood project
Concerns validation of quantitative PCR methods, coordinated by Norway Veterinary Institute (JRC)
http://www.vetinst.no/Qpcrgmofood/Qpcrgmofood.htm

Testing Laboratories

w10 AGAL - Australian Government Analytical Laboratories
Provide GMO testing services in Australia
http://www.agal.gov.au

w11 ALTECA Ltd
Provide GMO testing
http://www.alteca.com/index.htm

w12 Food Analysis and Management Centre
Japanese food analysis company, carry out genetic tests on food and sell food test reagents
http://www.fasmac.co.jp/index-e.html

w13 Genescan
Worldwide provider of GMO testing
http://www.genescan.com

w14 HKDNA chips Ltd
Provide GMO testing
http://www.dnachip.com.hk/

w15 IdentiGen
Ireland-based genetic testing company
http://www.identigen.com/

w16 Investigen – DNA Biotechnologies
Provide GMO testing and IdP services
http://www.investigen.com/

w17 Strategic Diagnostics Inc.
Innovative diagnostic testing for food ingredient and food safety, make lateral flow strip tests for GMOs
http://www.sdix.com

w18 US GM grain testing labs
http://www.extension.iastate.edu/Pages/grain/publications/testing/testingcos.html

Testing Kit Producers and Information Regarding Kits

w19 Agdia
Easy-to-use test kits, reagents and laboratory testing services for agricultural diagnostics
http://www.agdia.com/

w20 AOAC rapid test kits for GMO analysis
AOAC International
http://aoac.org/testkits/gmo.html

w21 EnviroLogix Inc.
Company that makes test strips/quick kits for GMOs and other applications
http://www.envirologix.com/

w22 Kit performance results
http://www.usda.gov/gipsa/tech-servsup/metheqp/testkit.htm

w23 Neogen
Produce GMO screening kits
http://www.neogen.com/gmo.htm

w24 SCIL Diagnostics
http://www.scildiagnostics.com/c.php/scil_diagnostics/about_us/about_us.php?

w25 SDI - Strategic Diagnostics Inc.
Company that makes test strips/quick kits for GMOs and other applications
http://www.sdix.com/ProductSpecs.asp?nProductID=19

Testing Equipment

w26 ABI Prism 7700
Real-time PCR machine produced by Applied Biosystems
http://events-na.appliedbiosystems.com/mk/get/7000_LANDING?_A=13077&_D=11862&_V=0

w27 eSensor
A hand held DNA detection system developed through a collaboration between Genescan and Motorola
http://www.motorola.com/lifesciences/esensor/

w28 Lightcycler
Real-time PCR machine produced by Roche
http://www.roche-applied-science.com/lightcycler-online/

w29 Qualicon
Automated PCR machine produced by DuPont
http://www.qualicon.com/abx.htm

w30 Rotor-Gene
Real-time PCR machine produced by Corbett Research
http://www.corbettresearch.com/index2.php

w31 SmartCycler
Portable PCR cycler
http://www.smartcycler.com/

Biochips and Novel Technology

w32 Biochips: A powerful tool for multiple and fast analysis of genes and DNA sequences
http://www.bats.ch/blickpunkt/archiv/juli01.htm

w33 Detection of genetically modified grains by Near Infrared Spectroscopy
Abstract from Pittcon 2000–Hurburgh
http://pittcon.org.technical_program/detailed/fri_am_tech_prog.html

w34 GMO Chips
European commissioned research project in genechips for GMO
http://www.gmochips.org/index.html

w35 HSAM – Hybridization Signal Amplification Method
Novel DNA detection method
www.hamiltonthorne.com/biosciences/reference/pdf/HSAM-Narrative.pdf

W36 RCAT – Rolling Circle Amplification Technology
Novel DNA amplification method
http://www.molecularstaging.com/Pages/RCAToverview_.html

Information on Seed Producers and Testers

w37 AOSA - Association of official seed analysts
Involved in seed testing
http://www.aosaseed.com

w39 AOSCA - Association of Official Seed Certifying Agencies
http://www.aosca.org/

w39 ASTA - American Seed Trade Association
http://www.amseed.com/

w40 ATSC - Australian Tree Seed Centre
Maintains national collection of tree seeds, part of the CSIRO division of forestry and forest products
www.ffp.csiro.au/tigr/atscmain/

w41 Aventis
Producers of agricultural products
http://www.aventis.com

w42 Bayer CropScience
http://www.bayercropscience.com/Bayer/CropScience/CSCMS.nsf/ID/Home_EN

w43 Calgene Inc
Producers of agricultural products, Monsanto is parent company
http://www.monsanto.com/monsanto/layout/default.asp

w44 CSD - Cotton Seed Distributors
http://www.csd.net.au

w45 GIPSA - Grain Inspection, Packers and Stockyards Administration
United States Department of Agriculture
http://www.usda.gov/gipsa/

w46 GIPSA Sample Size Calculator
Excel spreadsheet for designing sample plan
http://www.usda.gov/gipsa/biotech/samplingplan1.xls

w47 ISTA - International Seed Testing Association
Develop, adopt and publish standard procedures for sampling and testing seeds
http://www.seedtest.org/

w48 Monsanto
Producers of agricultural products
http://www.monsanto.com/monsanto/layout/default.asp

w49 SCST - The Society of Commercial Seed Technologists
Seed testing organisation
http://www.seedtechnology.net/

w50 Syngenta
Producer of agricultural products
http://www.syngenta.com/en/index.asp

Government and Related Industries - Australia

w51 ACCC – Australian Competition and Consumer Commission
http://www.accc.gov.au/

w52 AFFA - Australian Commonwealth Department of Agriculture, Fisheries and Forestry
http://www.affa.gov.au

w53 AFMA - Australian Fisheries Management Authority
Manages Commonwealth fishery resources
http://www.afma.gov.au

w54 AGAL - Australian Government Analytical Laboratories
http://www.agal.gov.au

w55 Avcare - Australian National Association for Crop Production and Animal Health
http://www.avcare.org.au/

w56 Biotechnology Australia
http://www.biotechnology.gov.au/

w57 CSIRO - Commonwealth Scientific and Industrial Research Organisation
http://www.csiro.au/

w58 CSIRO - Forestry and Forest products
http://www.ffp.csiro.au/

w59 CSIRO - Marine research
aquaculture and biotechnology
http://www.marine.csiro.au/

w60 CSIRO - Plant industry
http://www.pi.csiro.au/

w61 CSIRO – Stored Grain Research Laboratory
http://www.sgrl.csiro.au

w62 FRDC
Fisheries Research and Development Corporation
http://frdc.com.au

w63 FSANZ - Food Standards - Australia & New Zealand
previously Australia New Zealand Food Authority, ANZFA
http://www.foodstandards.gov.au/

W64 FSANZ
Labelling Genetically Modified Food, Compliance Guide to Standard A18
http://www.foodstandards.gov.au/whatsinfood/gmfoods/complianceguidea18gm.cfm

w65 Gene Technology Act 2000 – Sect 10 Definitions
Legal information retrieval system owned by the Australian Attorney-General's Department
http://scaletext.law.gov.au/html/pasteact/3/3428/0/PA000170.htm

w66 GRDC - Grains Research and Development Corporation
Australian Government Locater Service
http://www.grdc.com.au/

w67 GTGC - Gene Technology Grains Committee
Agrifood Awareness Australia
http://www.afaa.com.au/gtgc.asp

w68 NSW Agriculture
http://www.agric.nsw.gov.au/

w69 OGTR - Office of the Gene Technology Regulator
Australian Commonwealth Department of Health and Ageing
http://www.ogtr.gov.au/

w70 OGTR field trial sites
http://www.ogtr.gov.au/gmorecord/maps.htm
Risk assessment and risk management plan for Bayer CropScience InVigor® canola
http://www.ogtr.gov.au/applications/pdf/dir010finalrarmp.pdf
Risk assessment and risk management plan for Monsanto Roundup Ready® canola
http://www.ogtr.gov.au/applications/pdf/dir011finalrarmp.pdf

Governments and Related Industries - International

w71 AACC - American Association of Cereal Chemists
http://www.aaccnet.org/about/default.asp

w72 ACNFP - Food Standards Agency (UK) Committee on Novel Foods and Processes
http://www.foodstandards.gov.uk

w73 APHIS - Animal and Plant Health Inspection Service
US Department of Agriculture, Marketing and Regulatory Programs
http://www.aphis.usda.gov/index.shtml

w74 CEN - European Committee for Standardization
http://www.cenorm.be/

w75 Codex Alimentarius
Created by FAO and WHO to develop food standards and guidelines under the Joint FAO/WHO Food Standards Programme
http://www.codexalimentarius.net/

w76 Department for Environment, Food and Rural Affairs (UK)
http://www.defra.gov.uk/

w77 DTI - Department of Trade and Industry – Biotechnology (UK)
Overview of biotechnology and pharmaceutical industries and sectors
http://www.dti.gov.uk/sectors_biotechnology.html

w78 EPR - Environment, Planning and Regulatory Publications
Links to GM regulations
http://www.freshfields.com/practice/environment/publications/en.asp

w79 FAO - Food and Agriculture Organisation of the United Nations
http://www.fao.org/

w80 FDA - US Food and Drug Administration
US Department of Health and Human Services
http://www.fda.gov/

w81 GIPSA - Grain Inspection, Packers and Stockyards Administration
US Department of Agriculture
http://www.usda.gov/gipsa/

w82 ILSI - International Life Science Institute
Non-profit foundation, improves the well-being of the general public, studies scientific issues: nutrition, food safety, toxicology, risk assessment and the environment
http://www.ilsi.org/

w83 Japanese Ministry of Health, Labour and Welfare
http://www.mhlw.go.jp/english/index.html

w84 JRC - European Commission Joint Research Centre
http://www.jrc.it/

w85 OECD - Organisation for Economic Cooperation and Development
Main reference for the certification and standardisation of certain agricultural commodities
http://www.oecd.org

w86 US Department of Agriculture
Economic Research Service
http://www.ers.usda.gov/briefing/biotechnology/

w87 WHO - World Health Organisation
http://www.who.int/en/

Proficiency Testing and Reference Materials

w88 AACC Check Sample
Contains information on Check sample series, proficiency testing, reference materials, cost, description of services, food safety series, biotechnology series and standard reference samples
http://www.aaccnet.org/checksample/aboutchecksample.asp

w89 Genetically Modified Food
Containing issues on genetically modified material testing accuracy
http://www.aaccnet.org/checksample/geneticallymodifiedgrains.asp

w90 GeMMA scheme
Proficiency studies for the analysis of GMO materials. CSL (Central Science Laboratory) UK
http://ptg.csl.gov.uk/gmopts.cfm

w91 IRMM - Institute for Reference Materials and Measurements
European Commission Joint Research Centre
http://www.irmm.jrc.be

w92 NIST - National Institute of Standards and Technology (USA)
Producers of Quantitative PCR Reference Materials
http://www.cstl.nist.gov/biotech/bioprocess/quantitativePCR.htm

w93 Novachem Pty Ltd
Australian supplier of chemical, physical and biological material for research, specialising in stable isotopes and standards for environment analysis. Australian distributors of GMO plasmids from the Japanese company Nippon Gene to be used as reference materials and quantification
http://www.novachem.com.au

Safety Issues

w94 Agbios - Agriculture and biotechnology strategies, Canada
Information about the environment, livestock feed and human food safety of GM crops
http://www.agbios.com/default.asp

w95 Essential Biosafety Electronic Library
Electronic library for environmental, livestock feed and human food safety of GM crop plants
http://www.essentialbiosafety.info/biblio.php

w96 European network safety assessment of genetically modified food crops
http://www.entransfood.com/

w97 Genetically engineered food safety problems
Physicians & scientists for responsible application of science and technology
http://www.psrast.org/

w98 International centre for genetic engineering and biotechnology
http://www.icgeb.trieste.it/~bsafesrv/

w99 Safety Aspects of Genetically Modified Foods of Plant Origin
Report of a Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology
http://www.who.int/fsf/GMfood/FAO-WHO_Consultation_report_2000.pdf

Reports and Publications

w100 BATS – Centre for Biosafety and Sustainability, Switzerland
Science reports published by the Swiss National Science Foundation
http://www.bats.ch/ordering.htm

w101 Biogene
Research Institute of Organic Agriculture, Switzerland
Production without genetic modification (GM), focusing on organic farming, GM-free foods and feedstuffs, biotechnology and genetic engineering
http://www.biogene.org

w102 Comparative environmental impacts of biotechnology-derived and traditional soybean, corn and cotton crops
CAST – The science source for food, agricultural and environmental issues
http://www.cast-science.org/biotechnology/index.html#biotechcropsbenefit

w103 Food and Agriculture Organisation (FAO) report
FAO/WHO consultation on Biotechnology and Food Safety
http://www.fao.org/es/esn/biotech/tabconts.htm

W104 Food derived from GMO and detection methods
http://www.bats.ch/abstr/297intro.htm

w105 GIPSA
Marketing information on grains
http://www.usda.gov/gipsa/newsroom/backgrounders/biobackgrounder.htm

w106 GIPSA
Sampling for the Detection of Biotech Grains
http://www.usda.gov/gipsa/biotech/sample2.htm

w107 GM Fact sheet
Food Standards Agency – UK
http://www.food.gov.uk/science/sciencetopics/gmfoods/gmfactsheet

w108 GMO testing
Food Product Design, B. Floyd
http://www.foodproductdesign.com/archive/2001/0201fo.html

w109 Preserving the Identity of non-GM Crops in South Australia
Genetically Modified Food Unit, Environmental Health Branch, Department of Human Services South Australia. Discussion Paper.
http://www.dhs.sa.gov.au/pehs/Food/id-non-gm-crops.pdf

w110 Review of GMO Detection and Quantification Techniques
European Commission, Joint Research Centre
http://biotech.jrc.it/doc/EUR20384Review.pdf

w111 Scenarios for co-existence of genetically modified, conventional and organic crops in European agriculture
Institute for Prospective Technological Studies, European Commission, Joint Research Centre
http://europa.eu.int/comm/agriculture/publi/reports/coexistence/index_en.htm

w112 WHO – 20 questions on genetically modified (GM) foods
http://www.who.int/fsf/GMfood/q&a.pdf

General Information

w113 Ag Biotech Infonet
http://www.biotech-info.net/

w114 AgNIC - Agriculture Network Information Centre
A guide to quality agriculture information on the Internet as selected by the National Agricultural Library, Land-Grant Universities and other institutions
http://www.agnic.org/

w115 AgriFood Awareness Australia
Follow the links to find more information on the use of gene technology in agriculture
http://www.afaa.com.au/

w116 BINAS - Biosafety Information Network and Advisory Service
On-line message board, part of the UN industrial development organisation, monitoring global developments in regulatory issues in biotechnology.
http://binas.unido.org/binas/

w117 Biotech Infonet
http://www.biotech-info.net/

w118 Biotech Life Science Dictionary - glossary
http://biotech.icmb.utexas.edu/search/dict-search.html

w119 Biotechnology Communications
CAST – The science source for food, agricultural and environmental issues
http://www.cast-science.org/biotechnology/index.html

w120 Danish Forest and Nature Agency
Lists of companies carrying out GMO testing or selling kits
http://www.sns.dk/erhvogadm/biotek/images/appendix%204.pdf
http://www.sns.dk/erhvogadm/biotek/images/appendix%201.pdf

w121 FAO Glossary of Biotechnology for Food and Agriculture
http://www.fao.org/biotech/find-form-n.asp

w122 Glossary in layman terms
http://genetech.csiro.au/glossary.htm

w123 Information on GMOs and food around the world
Includes links to full text articles
http://www.checkbiotech.org/root/index.cfm

w124 Molecular Genetic Characterisation Data
Canada-US Bilateral on Agricultural Biotechnology
http://www.inspection.gc.ca/english/plaveg/pbo/usda/usda03e.shtml

w125 Molecular Register of DNA sequences of authorised GMOs
European Commission's Joint Research Centre (JRC)
http://biotech.jrc.it

w126 Motorola Press Release
GeneScan Europe AG & Motorola establish technical collaboration to detect genetically modified crops.
http://www.gmotesting.com/motorola.html

w127 News items on the GM issue
http://www.bioexchange.com/news/gmfood.cfm?start=31

w128 OECD BioTrack Online Product Database
http://www.olis.oecd.org/bioprod.nsf

w129 Qpcrgmofood
European commissioned research project on quantitative PCR for GMO
http://www.vetinst.no/Qpcrgmofood/About.htm

w130 Seed Quest
Links relating to seed manufacturers, dealers, and other information associate with seeds
http://www.seedquest.com/

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