Chapter 33
Monitoring GMOs Released into the Environment and the Food Production System

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GENETIC ID

Introduction

In order to systematically assess the impact of any genetically modified organism (GMO) on health or the environment, one must be able to answer the questions, 'Is the GMO present in the material of interest?' and 'How much of it is present?' This is the first step in assessing whether the presence of a given GMO is correlated with specific effects either on the environment or on health. The ability to track GMOs in the environment and food chain is, therefore, an essential capacity required for biosafety assessment.

The vast majority of countries that have implemented, or are in the process of implementing a biosafety framework recognize the need to track GMOs released into the environment or the food production system. The only notable exceptions are Canada and the US. The latter's system of authorization for environmental release for food purposes is permissive in many ways. The environmental assessments by the US Department of Agriculture and the Environmental Protection Agency are weak at best, and further, the US Food and Drug Administration does not impose mandatory food safety assessment of GMOs before release.

Biosafety frameworks generally identify the following purposes for establishing systems for post-release tracking of GMOs:

- To enable the efficient and timely withdrawal of products, where unforeseen adverse effects on human or animal health or the environment are established
- To facilitate the targeting of monitoring programs to examine potential harmful effects on health or the environment
- To support the implementation of risk management measures in accordance with the Precautionary Principle
- To facilitate accurate labeling of genetically modified (GM) products:
- To ensure that accurate information is available to the food industry and consumers to enable them to exercise freedom of choice
- To enable control and verification of labeling claims
- To verify that GMOs, and the mode of their release into the environment, are in compliance with international accords, such as the Cartagena Protocol on Biosafety to the Convention on Biological Diversity
- To verify that GMOs, and the mode of their release into the environment, are in compliance with national regulations

More broadly, to monitor the movement of released GMOs in the environment and food chain. Analytical methods, aimed at the identification and quantification of specific GMOs, can be integrated with document-based traceability and labeling systems to efficiently, economically, and reliably track the movement of GMOs in the environment and the food chain. This integrated approach is of great benefit, especially to operators within the food chain and to regulators, since it both reduces the need for time-consuming and costly testing, and actually increases the effectiveness of monitoring efforts.

This chapter will begin with an overview that considers document-based traceability and labeling systems, as well as testing, in the context of biosafety assessment of GMOs released into the environment and food chain. The chapter will then discuss GMO testing methods more deeply.

Tools for Tracking GMOs Released into the Environment

Testing—Positive identification, based on empirical evidence, such as test results, is the foundation for the traceability chain of every product. In principle, the traceability data for a given lot of product should document a chain of custody that traces the product and its precursors all the way back to the initial transformation event that generated the specific GMO contained in the product. However, in most cases, the starting point is a test result that identifies and/or quantifies the specific GMO present in the product or in a precursor of that product.
Once the genetic status of a specific lot or consignment of food has been established through testing, documentation systems and labeling can be used to track the movement of that product through the food chain.

Testing continues to play an important role at later stages in the chain, however. Testing and representative sampling are a necessary part of the quality control systems, used by industry to verify that traceability and labeling procedures are operating effectively in the transport, storage, and processing chain. Sampling and testing are also of importance to government regulators charged with operating surveillance programs designed to confirm that suitable traceability or labeling is being maintained for approved GMOs, and to verify that only approved GMOs are being introduced by importers and domestic operators into the environment and the food production system of the nation.

Document-based Traceability Systems—Two different models are used for traceability systems. The most rigorous approach is where a centralized documentation system tracks, handler-by-handler, the chain of custody of a specific lot of product through each step in the journey from the farmer's field to the consumer's dinner plate. At any point in time, the whole chain of custody is fully and immediately available. This is the traceability model that is used in organic certification and a few other applications.

The second, more common, traceability system is the 'one-forward, one-back' system. In this system, each participant in the chain is required to maintain the following four pieces of information, for each specific lot of product that they handle: (a) from whom they received that lot of product, (b) the date on which they received it, (c) to whom they released that lot of product, or lots of product derived therefrom, and (d) the date of release. This system does not provide a chain of custody document for a given lot of product, but imbeds in the supply chain sufficient information to assure that it should be possible to trace any given lot of product back to its source ingredients, if needed.

This second form of traceability has been required since 2005 for every food product and food ingredient sold in the European Union under regulation EC 178/2002. This system is also used in a modified form for traceability of GMOs in the European Union, as outlined in regulation EC 1830/2003 (European Commission 2003). This regulation requires that, in addition to retaining information on the immediate supplier and immediate buyer, the operator must retain, and supply to the buyer, information on the specific GMO contained in the product, if it is a GM product, or, if it is a product derived from GMOs, an explicit declaration that the product 'contains GMOs'. A system similar to that specified in EC 178/202, the 'trace-back system', is under development in the US.

One-forward, one-back traceability is designed to enable regulators, who identify a health hazard associated with a specific package of product, to trace that product back to each of its component ingredients, and thereby locate the source of the contamination. Although this approach to traceability is more economical, insufficient evidence has been gathered to date to demonstrate its consistent effectiveness in practical application.

Labeling–Traceability systems can also make use of labeling, bar-codes, radio frequency (RF) tags, and a diversity of other physical devices. These are useful in maintaining traceability of packaged goods or other strictly defined units, the integrity of which is not compromised as the product changes hands in the chain. Examples would be a package of breakfast cereal, a sealed tank of lecithin, and a living farm animal, such as a cow. Traceability and the validity of the labeling are destroyed as soon as the seal on the lecithin tank is broken, or as soon as the animal is rendered into separate meat products, unless documentation is created that traces the next steps of the production process.

Segregation–Segregation measures are distinct from traceability. Segregation maintains the physical integrity of a given lot of product as it passes through the chain. For instance, a consignment of grain can be traced from the farm, to a centralized storage facility, to a barge, to an export terminal storage bin, to the hold of a boat, and finally to an import storage bin owned by a manufacturer who converts the grain into consumer products. Records can be created accurately documenting each of these steps—this is traceability. However, this documentation does not assure the integrity and purity of the product that the final buyer incorporates into the consumer product. At each step in the transport, storage and processing of the product, contamination can occur; a storage bin may not have been cleaned out properly and may contain residual grain from a previous use. A ship's hold may be loaded with multiple products, creating significant risk of cross-contamination. The manufacturing facility may be operating multiple production lines simultaneously, and inputs or work in progress may spill from one line to another, contaminating the product of that line. Even at the farmer level, contamination can occur due to cross-pollination from a neighboring field.

Segregation measures are procedures designed to preserve the integrity of the product by preventing cross-contamination of the kinds described. The stringency of segregation measures determines the purity and degree of physical integrity of the final product. For instance, non-GMO soy is sold in multiple grades. The highest grade is guaranteed to contain less than 0.1% GM soy, and is used in many countries by operators who want to make claims that their products are 'non-GMO'. The next grade is guaranteed to contain less than 0.9% GM soy, and is often used in the EU by operators who wish to produce products that are exempt, according to Regulation EC 1830/2003, from being labeled as 'genetically modified'.

Identity Preservation—Segregation together with traceability documentation comprise identity preservation. To credibly preserve the identity of a lot of product, it is necessary to both segregate that lot from other lots, and to maintain adequate traceability documentation for that lot.
When used properly, the aforementioned components—testing, traceability, labeling, segregation, and identity preservation—function together to assure that a specific lot of product, whose genetic status is known, can be tracked efficiently, economically, and reliably through the food production chain.

Technically speaking, the most challenging of these components is testing, and this is also the most critical component for assuring the initial identity of the GMO and verifying the accuracy of the traceability system at intermediate points in the chain of custody. The following sections of

this chapter discuss the technical aspects of GMO testing, and how to maximize accuracy and reliability of this critical element of traceability systems.

Basic Rationale of GMO Testing

Gene modification (also called recombinant DNA methods or gene splicing techniques) introduces new genetic information, new DNA sequences, into the genome of an organism. Once introduced into the genome, the transgenic (also called genetically modified) DNA reprograms the cells of the recipient organism to produce new mRNA species and new proteins. The transgenic proteins confer new characteristics or functions upon the organism. GMO detection methods could, in principle, measure transgenic DNA, mRNA, or proteins, or even the novel biosynthetic products or biological functions conferred by the new genes. However, in practice, analytical methods have focused almost exclusively on detection of transgenic DNA and protein. I will consider both of these analytical approaches in some detail.

Immunological Analysis of GMOs

Immunological tests for GMOs detect the transgenic proteins encoded by recombinant genes. These tests employ both the ELISA (enzyme-linked immuno-sorbent assay) and the lateral flow test formats (Lipton et al. 2000, Lipp et al. 2000, Stave 1999).

Although there are many different configurations for ELISA tests, the basic design is illustrated in Figure 33.1. First, antibodies specific for the analyte of interest are immobilized to the wells of the ELISA assay plate. When exposed to a solution containing the analyte of interest, the immobilized antibodies capture the analyte. This immobilized complex is then exposed to a solution containing a second antibody that also recognizes the analyte, and which is also linked to an enzyme. This second antibody becomes immobilized to the complex, as well, where the enzyme catalyzes the conversion of a compound present in the reaction vessel into a second compound that can be quantified colorimetrically or fluorimetrically. Thus, ELISA technology is in essence a method for linking the antibody-analyte recognition reaction to a reaction that generates a colored material that can be detected and quantified.

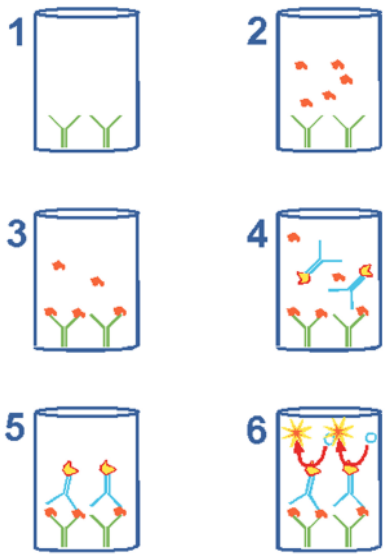

Figure 33.1. ELISA immuno-detection process
The figure shows the basic principles of enzyme-linked immuno-sorbent analysis (ELISA), which is used to detect transgenic proteins for GMO analysis. Step 1, antibodies are bound to the surface of the reaction well. Step 2, analyte (antigen) solution is added to well. Step 3, analyte binds to antibodies. Step 4, a second antibody, with conjugated enzyme is added to the well. Step 5, second antibody binds to the complex between the analyte and first antibody, which is bound to the surface of the well, thereby immobilizing the second antibody to that surface. Step 6, the enzyme conjugated to the second antibody converts colourless substrate (blue circle) to bright coloured or fluorescent reaction product, which can be quantified colorimetrically or fluorimetrically.

The lateral flow test makes use of the same basic immunochemistry but is configured to allow convenient field analysis with visual assessment of results. On the biochemical level, the main difference between ELISA and lateral flow strip tests is that the enzyme-linked second antibody, used in ELISA assays, is replaced in strip tests with antibodies conjugated with colloidal gold. Because immuno-tests require minimal processing of the sample, they can be completed quite quickly (Lipp et al. 2000, Stave 1999). Moreover, in the lateral flow format, immuno-tests are very convenient and easy to carry out, do not require sophisticated equipment, and are inexpensive on a test-by-test basis. This format is particularly useful for field GMO tests, where they can be used to rapidly screen truckloads of soy or maize at the grain handling facility for a single GM trait.

The speed and convenience of immunological tests offer substantial utility. However, the limitations of this method should be recognized in order to assure appropriate application. One crucial limitation of immunology-based tests is in the area of quantification (Stave 2002, Fagan 2001). Although ELISA tests can be configured to function quantitatively, in the context of GMO testing, the capacity for quantification cannot be used advantageously. This is because it is difficult, if not impossible, to translate mass of transgenic protein, measured in the sample extract, into percent GMO.

Percent GMO is the quantitative basis for most national regulations on genetically modified foods, such as in the EU regulation EC 1830/2003 (European Commission 2003). Percent GMO refers to the weight percentage of food derived from genetically modified materials. For example, a truckload of 20% genetically modified maize might contain 5 metric tons of transgenic maize and 20 metric tons of conventional maize.

If one were to conduct a quantitative ELISA analysis of a representative sample of that maize, the analysis would provide information, with reasonably good accuracy and reproducibility, on the mass (nanograms) of a specific transgenic protein, such as Cry1Ab, extracted from a given number of grams of maize. The difficulty arises in accurately extrapolating from this value to percent GMO. This is due to the fact that there is no constant relationship between these two parameters (mass of transgenic protein extracted and mass of maize grain or grain derivatives). Several factors contribute to this.

First, the level of expression of the transgenic protein is not constant, i.e., the ng of transgenic protein expressed per gram of transgenic maize is not constant. If it were, then one could compare the result of this analysis to a series of standards containing known amounts of transgenic maize, to estimate percent GMO. However, expression is not constant. It is influenced by weather, soil, and other cultivation conditions. For example, Roundup Ready soy has been found to express transgenic EPSPS (5-enolpyruvyl shikimate 3-phosphate synthase) at levels ranging from 0.179 to 0.395 ng/mg (Monsanto 1994). This is more than a two-fold range in variation.

In virtually every instance, the standards used for calibrating the analysis will be derived from a different lot of soy cultivated under conditions different from those under which the soy present in the sample were cultivated. Therefore the level of expression in the sample will differ from the reference materials, and it will not be valid to estimate the GMO content of the sample by comparison with those reference materials. A sample judged to contain 1% GM soy based on such a comparison could contain as little as 0.5% or as much as 2%.

A second contributor to variability in expression of transgenic proteins is the fact that different transgenic events are engineered to express the same recombinant proteins at widely varying levels. For example, Bt176, Bt11, and Mon 810 all express transgenic Cry1Ab proteins, but at very different levels. Cry1Ab is present at 0.09 μg/mg, in E176 maize, while the levels in Mon 810 and Bt 11 maize are 0.31 and 4.767 μg/mg, respectively (Ciba Geigy 1995, Monsanto 1996, Northrup King 1995). Thus, if an ELISA test indicated that the Cry1Ab content of a truckload of maize was 0.09 μg/mg, this could indicate that the truck contained 100% E 176 maize, 29% Mon 810 maize, or 1.9% Bt 11 maize, or any combination of the three.

In the real world, the analyst will not know whether a sample is comprised of a single event or of a mixture, nor will the relative proportions of the events that may be present be known. Thus, it is virtually impossible, in practice, to determine percent GMO for maize using ELISA. This problem does not arise at this time for soy, because there is only one transgenic soy event, Roundup Ready, in open, commercial production.

Another factor that influences quantification by ELISA is efficiency of extraction. If the sample and standard reference materials are not ground to the same mesh size and extracted for the same length of time, the transgenic proteins will be extracted with different efficiencies from the reference materials and the sample, making it impossible to make a valid comparison of the two. In summary, due to several confounding factors, the amount of a transgenic protein present in a grain or food is variable and cannot be used as a measure of the proportion of that food which is transgenic. Thus, percent GMO cannot be determined accurately by immunological methods, such as ELISA or lateral flow strip tests.

A similar limitation is apparent in considering processed foods. Proteins, including transgenic marker proteins, are easily denatured during food processing. This either destroys the ability to recognize these proteins with immunological reagents or reduces sensitivity to detection (Lipp et al. 2000, Hubner et al. 1999). Thus, detectability is variable and is process dependent, again compromising the utility of immunological quantification methods. As stated by others (Lipp et al. 2000, Stave 2002), matrix-matched reference materials would be required for valid quantification. Not only would it be necessary to process the standard reference material under conditions identical to those of the sample, but also the proportions of different genetically modified events comprising the standard would have to necessarily match that of the sample. These are conditions that can be fulfilled in only a small fraction of the circumstances where it is necessary to quantify GMO content.

A third limitation of immuno-assays is that the transgenic proteins expressed in some GM crops are not detectable by immuno-analysis. For example, the glyphosate-resistant maize variety GA21 expresses a transgenic EPSPS protein that differs from the native maize EPSPS by only two or three amino acids (Monsanto 1997). The structures of the transgenic and native EPSPS proteins are so similar that all attempts to develop antibodies capable of differentiating the two have been unsuccessful. Thus, to date, no immuno-test exists that is capable of detecting this transgenic event.

Despite limitations, immunological tests serve a useful role. Their application at early stages of the chain is well accepted at this time, especially at points where rapid field tests are needed. For instance, they are often used in checking trucks before they unload their cargoes at grain-handling facilities. The initial results from these tests prevent the introduction of truckloads of maize or soybeans that contain high levels of GM material into silos designated for non-GM products. ELISA is also being used for quantification in situations where economy and convenience are considered more critical than accuracy or where it can be known with confidence that only one event exists that can produce the transgenic marker protein of interest.

Genetic Analysis of GMOs by Using the Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is widely used in genetics-based analysis of GMOs. PCR uses biochemical processes to scan through a sample of DNA and to locate one or more specific DNA sequences, called target sequences. This target sequence is then amplified billions of times, making it possible to detect that target sequence with high sensitivity and also to quantify the proportion of DNA molecules in the sample that contain that target. See Fagan (2003) for a full description of the PCR mechanism.

Because of the powerful amplification that occurs during PCR, this method is highly sensitive. Because the interactions between the primer and target DNA molecules are highly selective, the PCR process is highly specific. A third advantage is that PCR is capable of detecting all GMOs. This is because, even if the transgenic protein is not expressed in the food part of the plant or even if the transgenic protein is indistinguishable from the native protein by immuno-analysis, the transgenic DNA will still be present and can be detected by PCR. A final advantage is that DNA is less subject to denaturation and degradation during food processing than are most transgenic proteins. Thus, even when transgenic proteins have been degraded to the point where immuno-tests are ineffective, PCR analysis can, in most cases, still successfully detect the presence of GM material (Hubner et al. 1999, Jankiewicz et al. 1999).

The robust and versatile nature of this method makes it possible to use PCR to test for the presence of GM material at almost all points in the food chain, from the farmer's field to the consumer's dinner plate. PCR can also be used to quantify GMO content in most food products, including many highly processed foods. The only exceptions are the most highly modified food ingredients, such as certain chemically modified starches, the most highly refined grades of vegetable oil, and highly fermented products, such as soy sauce.

One of the most significant advantages of PCR-based GMO analysis lies in the area of quantification (Hubner et al. 1999, Vaitilingom et al. 1999). The DNA extracted from a sample contains not only the transgene, but also all of the other genes naturally present in the organism. The copy number of each transgene should be invariant in any GMO. Also, the vast majority of endogenous genes of all organisms will be invariant in copy number. The PCR signal derived from a transgene can be used as a measure of the number of GM genomes in the sample. Similarly, the PCR signal derived from a selected endogenous gene (a species-specific reference gene) can be used as a measure of the number of total genomes present in the sample for the species of interest. The ratio of these two signals can be used to accurately calculate the proportion of transgenic genomes—the percent GMO—present in the sample as shown in the following formula:

$$GMO\% = \left(\frac{\text{(Concentration of GMO target sequence)}}{\text{(Concentration of species specific reference gene)}} \right) \times 100$$

This provides a quantitative determination of the percent of GM material present in the sample. In essence, the naturally occurring gene serves as an internal reference point that allows consistent quantification. Immuno-analysis does not make use of such an internal reference and thus fails to provide definitive quantification. Thus, although both immuno-methods and PCR methods can be used effectively to screen for GMOs, PCR is the preferred method when quantification is required.

Because of these advantages, PCR is recognized as the gold standard for GMO testing in Europe and Asia.

Overview of PCR Analysis of GMOs

PCR analysis of GMOs involves five steps: sample preparation, DNA purification, target amplification, detection of reaction products, and interpretation of results.
Sample preparation—For an analytical result to provide meaningful information regarding the original consignment of food, the field sample, drawn from that consignment, must be representative of the consignment as a whole, and the analytical sample, derived from the field sample, must be representative of the field sample.

The first key step is that the field sample must be obtained in a manner that ensures representation from all parts of the lot. Statistical methods are used to define a sampling plan that yields a representative sample. The field sample also must contain a sufficient number of units to ensure that the analysis will be statistically robust at the limits of detection and quantification relevant to the assay. If the sample size is too small, the full power of PCR cannot be exploited.

More specifically, the limit of detection (LOD) for PCR is typically 0.01% or lower. To gain full advantage of an LOD of 0.01%, or 1 part in 10,000 requires that the sample be quite large. For instance, if the true GMO content of a consignment of rice is 0.01%, one must take a sample of 30,000 seeds in order to have 95% confidence that the sample will contain at least one GM rice grain. The probability of picking up at least one GM rice kernel in a sample of, for instance, 1000 seeds would only be 9.5% and the probability for picking up one GM kernel in a sample of 10,000 seeds would only be 63%. For rice, a small seed grain, a sample of 30,000 kernels is not prohibitive, consisting of only 900 g. However, for soy beans, 30,000 seeds would weight *c.*10 kg, and for maize, *c.*12 kg. Thus, sample sizes in this range are on the far outer limit of practicality for most routine applications, except for small grains and for powdered or ground materials, such as soy meal or maize flour.

These examples make it clear that in many cases, the factor limiting the overall sensitivity of GMO detection is not the PCR method, but practical limitations of field sample size.
Sample processing, and the size of the sample taken from the processed and homogenized field sample for DNA extraction and purification (the analytical sample) are also very important in determining whether final analytical results are representative of the original consignment of food. The sample should be finely ground and homogenized to assure that any suitably-sized sub-sample taken from the analytical sample for DNA extraction will be representative of the whole. It is a common error to take sub-samples that are too small to be representative. Typically, samples of 50 mg to 150 mg are used, because this makes it possible to conveniently carry out the

whole DNA extraction procedure in micro-centrifuge tubes. However, empirical studies have demonstrated that samples in this size range fail to yield representative and reproducible results. Only when sample size exceeds 0.5 g to 1.0 g do replicates begin to show acceptable consistency. For routine purposes, samples of at least 2.0 g should be used for DNA extraction of most materials.

DNA Extraction and Purification—To gain reliable and informative results, purification procedures must produce DNA that is free from PCR inhibitors, minimize DNA degradation, and also achieve good yields. Because food products vary tremendously in their physical and chemical compositions, it is essential to customize DNA extraction methods to function optimally for each food matrix. DNA extraction kits purchased from a scientific supply house are unlikely to perform adequately for all sample types. Figure 33.2 compares the performance of a customized system of DNA purification methods, Fast ID, with four kits available in the marketplace today, and with a public domain method, the CTAB method (Murray & Thompson 1980, Scott & Benedich 1988). In this study, DNA was extracted from soybeans and from three soy products. In each case, the kits and methods were used exactly as recommended by their developers. The quality of the DNA prepared using these six methods was then assessed by quantitative real-time PCR. The matrix-specific Fast ID system performed better with all food matrices, but the greatest difference in performance was observed with complex, multi-ingredient products. These were virtually un-analyzable using many of the other methods, while with Fast ID reasonable results were obtained. Similar findings were obtained, as shown in Figures 33.3 and 33.4, where the effectiveness of Fast ID and one commercially available kit were compared for the analysis of other food matrices.

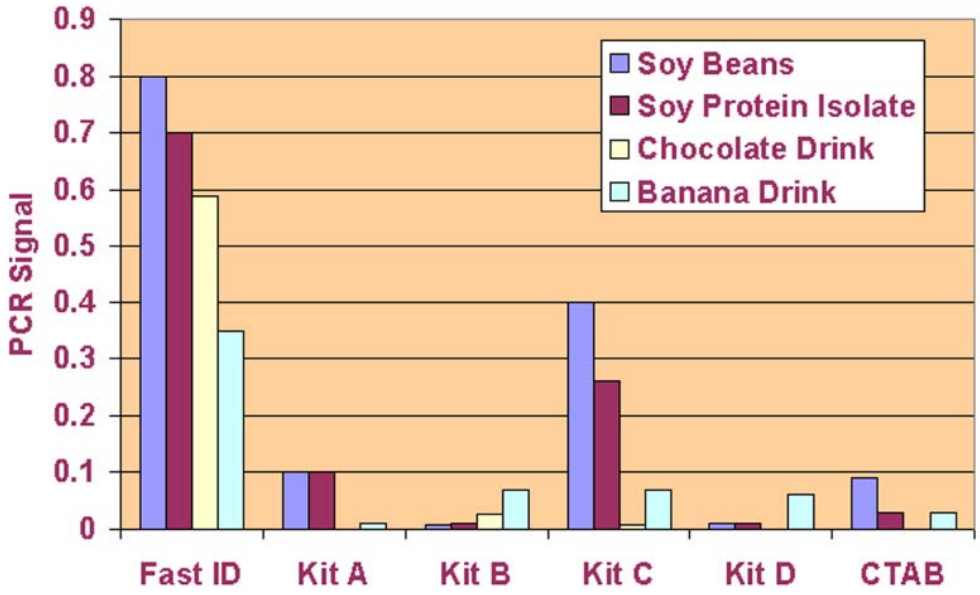

Figure 33.2. Comparison of fast ID DNA extraction with commercial kits and CTAB – real-time quantitative PCR analysis

DNA was prepared from four food samples using the Fast ID method, four DNA extraction kits that are commonly used for analysis of genetically modified foods and agricultural products, and the public domain CTAB method. The quality of the DNA was assessed by real-time PCR. A standard amount of DNA (50 ng, quantified by absorbance at 260 nm) was introduced into each PCR reaction. PCR signals are reported relative to signals obtained with a standard of highly purified soy DNA. Somewhat reduced signals for chocolate and banana drinks for Fast ID are not due to the presence of inhibitors, but to the presence of DNA from other species, derived from other ingredients in these samples.

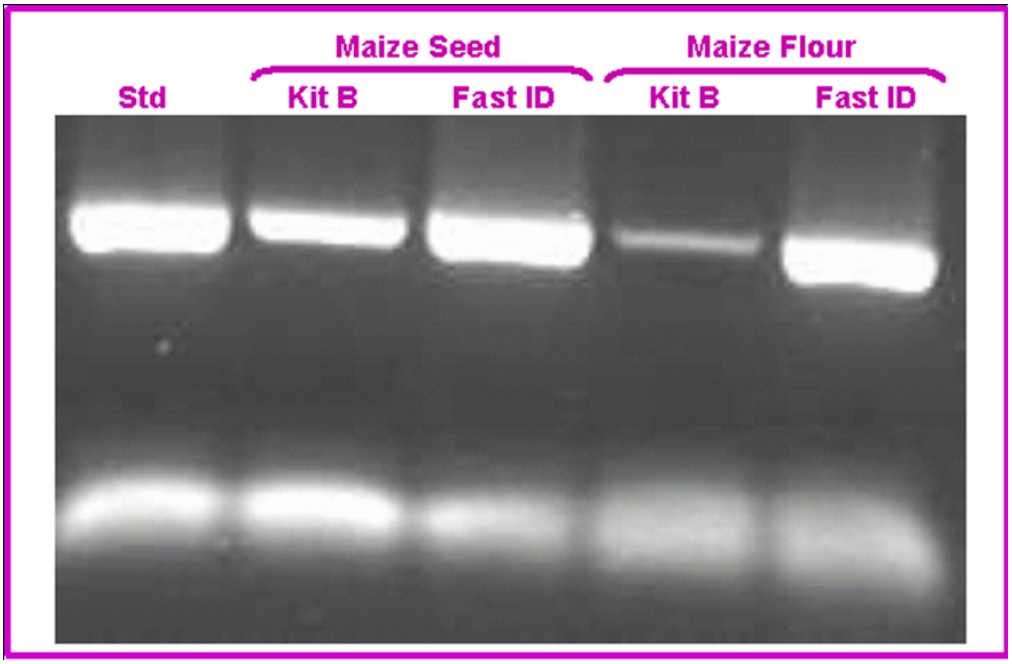

Figure 33.3. Comparison of Fast ID DNA extraction with a commercial kit – analysis by conventional PCR

DNA was prepared from food samples using the Fast ID method and another commonly used DNA extraction kit. The quality of the DNA was assessed by conventional PCR. A standard amount of DNA (50 ng, quantified by absorbance at 260 nm) was introduced into each PCR reaction.

Figure 33.4. Comparison of Fast ID DNA extraction with a commercial kit – analysis by conventional PCR DNA was prepared from food samples using the Fast ID method and another commonly used DNA extraction kit. The quality of the DNA was assessed by conventional PCR. A standard amount of DNA (50 ng, quantified by absorbance at 260 nm) was introduced into each PCR reaction.

PCR Amplification—The ability to amplify a target sequence billions of times is the basis of the sensitivity of the PCR method. PCR is an exponential amplification process. Each cycle doubles the number of target molecules. If one starts with one target molecule, completion of one PCR amplification cycle yields two molecules. In two cycles, 4 molecules, and in 3 cycles, 8 molecules are generated. Ten cycles will generate more than 1,000 copies, and 20 cycles, more than 1,000,000 copies, and so on. If the quality of the DNA preparation is good, between thirty and forty cycles of PCR amplification are more than adequate to yield signals that are easily observed even if the original target sequence is present in only one or a few copies. With this method it is possible to routinely detect the presence of GM material at concentrations well below 0.01%.

Sample size is equally important when taking samples of the DNA extract for PCR analysis. For instance, if the true GMO content of a lot of maize is 0.01%, and proper sampling had been done at both the field sample and analytical sample levels, one should have a DNA preparation whose GMO content is very close to 0.01%. When one takes a sample of 200 ng from this DNA preparation, it will contain approximately 77,000 copies of the haploid maize genome. Using the normal approximation to the binomial distribution, the probability is only 87% that such a sample will contain ± 50% of the true value. That is, the probability is only 87% that the actual GMO content of the sample will be between 0.005% and 0.015%. The limit of detection (LOD) is defined as that concentration of analyte that can be detected with 95% confidence. In the present case, the probability of detection is only 87%, thus, 0.01% is below the LOD. We can calculate that the actual LOD for this sample size would be 0.02%. Thus, despite the fact that the PCR amplification process is fully capable of detecting 0.01%, the limited number of genome copies in the DNA sample subjected to PCR analysis significantly reduces the LOD of the over-all analytical process. In order to achieve a LOD of 0.01%, a sample of 400 ng maize DNA would be required, which is very high, in fact, inappropriately high, except for the most pure DNA preparations.

It is clear from the literature, that most methods employ DNA sample sizes in the range of 50 to 150 ng of maize DNA. For samples in this size range, sample size, not the inherent properties of

PCR amplification, is the limiting factor in determining the LOD of the over-all analytical method. Fortunately, the maize genome is exceptionally large. For a grain such as rice, which has a genome only 17% the size of the maize genome, a sample of 200 ng is quite adequate to achieve detection at the 0.01% level.

Detection of PCR Reaction Products—The products of the PCR amplification process can be detected by several different methods. One of the most common is electrophoretic analysis, where the amplified DNA molecules are resolved into, and appear visually as, distinct bands on an agarose or acrylamide gel, when stained with a fluorescent dye. The other common method is fluorimetric analysis, where the PCR process is modified to generate fluorescent products which are detected in proportion to the number of amplification events that take place. This method is the basis of real-time quantitative PCR technology. Figure 33.2 illustrates the kind of quantitative data that are obtained when fluorimetric analysis is used as part of real-time quantitative PCR. Figures 33.3 and 33.4 illustrate the results of electrophoretic analyses of conventional PCR products.

Applying PCR to the Analysis of Food Samples for GMO Content

For GMO detection, PCR can operate either (a) qualitatively, or (b) quantitatively; and can either (c) target many different varieties (events) of GMOs, or (d) selectively target a single transgenic event. Table 33.1 summarizes the specific analytical questions addressed by each of these four categories of methods.

Table 33.1. Classification of GMO testing methods.

	Qualitative Method	**Quantitative Method**
Broad Spectrum Primers	Broad-spectrum Screening: Is any genetically modified material present in the sample?	Rough Quantification: Approximately how much genetically modified material is present in the sample?
Event-Specific Primers	Event-/Variety-Specific Detection: Specifically which GMO(s) is (are) present in the sample?	Precise Quantification: How much of a particular GMO is present? (one primer set) How much total GMO is present? (total measurements for all GMOs in the sample)

Regardless of whether a GMO analytic system is qualitative, quantitative, broad-spectrum, or event-specific, the basis of all such systems is a core set of PCR reactions that employ primer sets specific for the GMO(s) of interest. There are several additional design elements, controls, and reference reactions that are common to all analytical systems and are designed to achieve the following objectives:

Detection of inhibitors that reduce or block the PCR process.

- Assessment of the degree to which the sample DNA is degraded.
- Verification that the PCR reagents and equipment are functioning properly and that PCR amplification actually occurs.

- Determination of the limit of detection and/or limit of quantification of the PCR method, and confirmation that the PCR process is operating at a consistent level of sensitivity from PCR run to PCR run.
- Confirmation of positive and negative results.

In addition, a practical analytical method that includes all of these design elements must be an integral part of a comprehensive quality assurance/quality control (QA/QC) system in order to achieve the degree of reliability and consistency that is necessary for the analytical system to be of practical utility for industry or government regulatory bodies.
The following sections discuss the four classes of PCR systems used in GMO analysis, and the required assay design features for each, as well as how they can be used within a QA/QC system to assure reliable GMO analysis.

Qualitative PCR and Illustration of Basic Controls Required for All PCR Analyses

A diagrammatic example of electrophoretic analysis of PCR reaction products generated from two food samples is presented in Figure 33.5. Six separate PCR reactions were run for each sample, duplicate reactions with each of three distinct primer sets. These are presented in lanes 1 through 12 of the figure. Lanes 13 through 26 present PCR reactions run with reference DNA samples (lanes 13, 14, and 19 through 26) and no DNA (lanes 15 through 18).

The first primer set recognizes an internal control DNA preparation. These reactions are used as an indicator as to whether or not PCR inhibitors may be present in the sample DNA preparation. If inhibitors are not present the intensity of the electrophoresis bands from the control reactions (lanes 1 & 2 and 7 & 8) will match the intensity of the internal standard control (lanes 13 &14). The intensity of the bands in lanes 1 & 2, and 7 & 8 indicate that no inhibitors are present in either DNA sample #1 or #2.

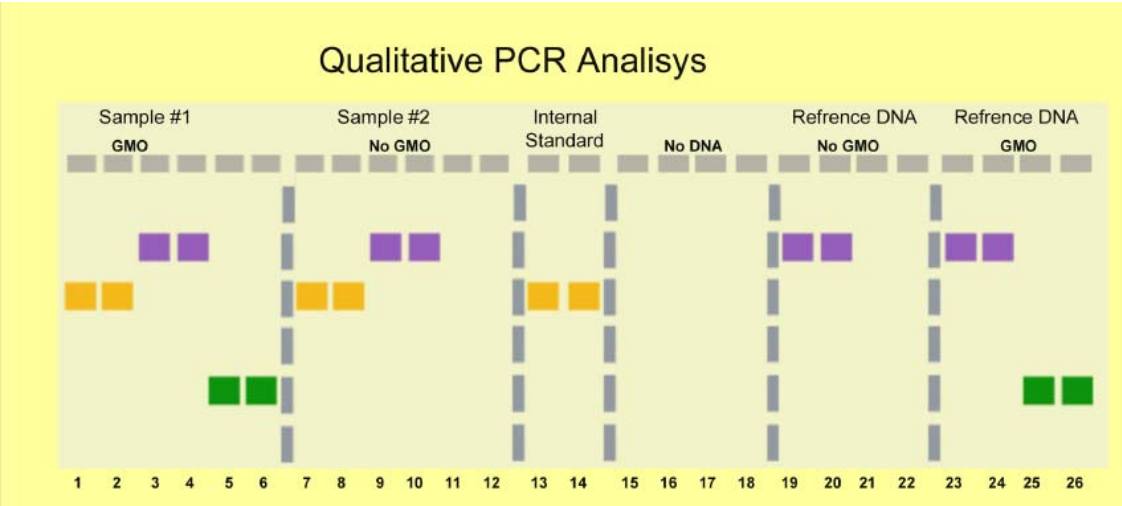

Figure 33.5. Typical configuration for GMO analysis by PCR
Reactions 1 & 2 and 7 & 8 contain sample DNA, internal standard DNA, and primers for internal standard DNA. The products of these reactions are compared to reactions containing internal standard DNA and primers for internal standard DNA only (lanes 13 & 14). If the intensity of bands is comparable, then it implies that the sample DNA does not contain compounds that inhibit the PCR process.
Reactions 3 & 4 and 9 & 10 contain sample DNA and primers for a gene common to all varieties of the genetically modified crop of interest (e.g. soy). The intensity of the bands produced in these reactions is compared to the corresponding standards (lanes 19 & 20 and 23 & 24). Weak or absent bands would imply

poor recovery of sample DNA or degradation of that DNA. Absence of bands could also imply that the PCR system was not functioning properly due to inhibitors or faulty reagents or equipment. These alternatives can be sorted out by comparison of these results with those for Reactions 1 & 2 and 7 & 8.
Reactions 5 & 6 and 11 & 12 contain sample DNA and primers specific for a genetically modified sequence present in the GMO(s) of interest. The intensity of the bands for this primer set is compared to reactions containing non-GMO and GMO DNA, run with the same primer set (lanes 21 & 22 and 25 & 26). Absence of signal in lanes 21 & 22 is expected, implying that the primer set does not interact with sequences in the non-GM DNA, and is, therefore, specific for the GM sequence of interest. Presence of signal in lanes 25 & 26 is expected, implying that the primer set is effectively detecting the GM sequence of interest in the GMO DNA. The results indicate that Sample #1 contains the GMO of interest, while Sample #2 does not.
Duplicates – All analyses are carried out in duplicate, beginning with duplicate sub-samples of the ground and homogenized food. Two independent DNA preparations are made from these food samples and are carried through PCR independently. It is not sufficient to run duplicate PCR reactions from a single DNA preparation.

The principle behind the internal standard is illustrated in more detail in Figure 33.6. In Figure 33.6, no inhibitor is present in the first reaction, but is present in the second (indicated in red). The intensities of the bands corresponding to the first reaction are equal to those obtained when the internal standard is run alone (far right), while the intensities of the bands corresponding to the second reaction are much less than those of the internal standard when run alone. This is due to the effects of the inhibitory molecules (red) present in the second reaction.

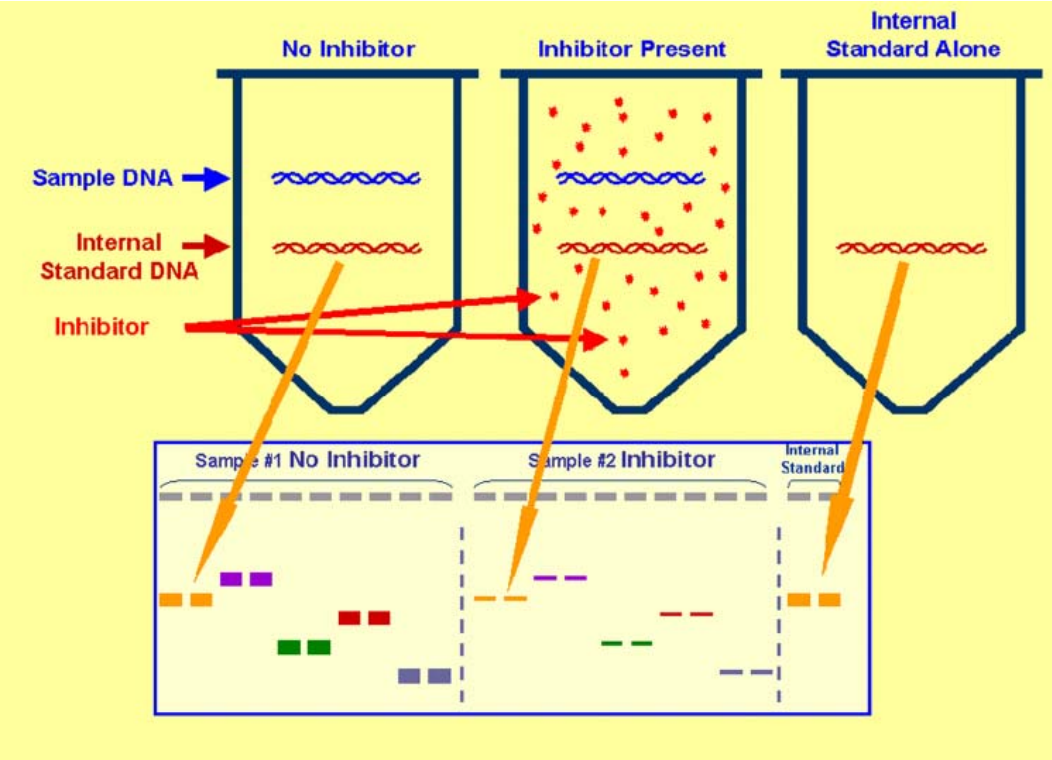

Figure 33.6. The internal standard – A control for the presence of PCR inhibitors
A defined concentration of a known target DNA molecule (internal standard template) is added to all three reaction tubes (red DNA molecule). When this template is amplified in the presence of a DNA sample that is free from inhibitors (left-hand tube) the resultant signal is equal to that obtained when the internal standard template is amplified alone (right-hand tube). In contrast, amplification of this template in the presence of a sample that contains inhibitors (centre tube) results in reduced signal intensity.

The second primer set used in the analysis presented in Figure 33.5 is designed to determine whether the DNA sample is intact and free from degradation. However, results with this primer set must be considered in light of the results obtained with the first primer set, since inhibitors will influence the reactions with the second primer set, as well as those with the first. This primer set is specific for a reference gene relevant to the species of interest. This is termed the species-specific reference gene primer set. For example, to test for GM soy, a primer set would be selected that targets a gene known to be present in all soy, whether transgenic or conventional. Many laboratories use a primer set specific for the soy lectin gene for this purpose. The reaction products generated with samples #1 and #2 using this primer set (lanes 3 & 4, and 9 & 10) are compared to those in lanes 19 & 20, and 23 & 24. If the sample DNA is free from degradation, then the intensity of the bands generated from those DNA preparations (lanes 3 & 4, and 9 & 10) will correspond to the intensity of the bands generated in reactions containing reference DNA preparations (lanes 19 & 20, and 23 & 24).

The principle behind the species-specific reference gene control is illustrated in more detail in Figure 33.7, where it is shown that partly fragmented DNA (right-hand reaction tube) results in weaker PCR bands than intact DNA (left-hand reaction tube).

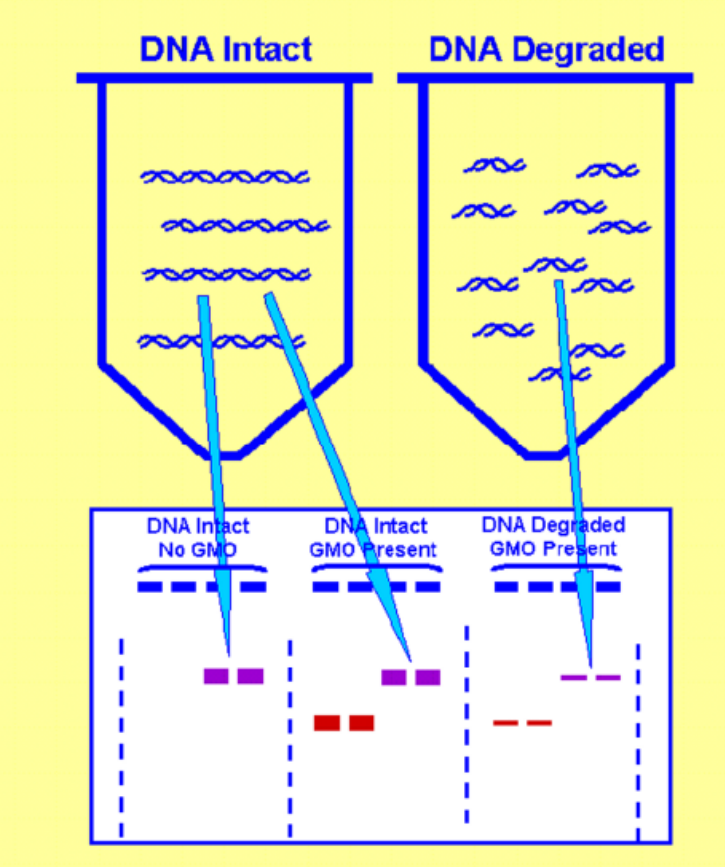

Figure 33.7. Positive control using primers specific for a species-specific reference gene – A control for DNA degradation and reduced DNA recovery
A primer set targeting a gene common to all varieties (conventional or transgenic) of the crop species of interest can be used to assess the integrity of the sample DNA. Both DNA degradation and presence of PCR inhibitors will reduce the signal generated from this primer set. By using the test described in Figure 33.5 to detect inhibitors, the contribution of DNA degradation can be understood.

The final primer set used in Figure 33.5 detects a specific transgenic sequence. The intensities of the signals generated for samples #1 and #2, using this primer set (lanes 5 & 6, and 11 & 12), can be compared to the respective PCR reactions with external standard DNA (lanes 21 & 22, and 25 & 26). This comparison indicates that sample #1 contains GM material, while sample #2 does not.

A successful PCR analysis requires that all duplicates agree, that the no-DNA controls display no bands (lanes 15 through 18), that the controls with both GM and non-GM reference DNA preparations be consistent with the expected characteristics of the primers (lanes 21 & 22 no signal, all other lanes positive), and the internal control reactions (lanes 1 & 2, 5 & 6, and 13 & 14) must all be positive and of roughly equal intensity.

This example illustrates that 20 data points must be considered in determining the GMO content of any given sample: a total of 6 PCR reactions are carried out with DNA derived from the sample, and 14 additional reactions are carried out with reference DNA preparations.

Safeguards to Ensure Reliable PCR Results

Incorporated into the procedure illustrated in Figure 33.5, are eight elements designed to assure the accuracy and consistency of results. These include the following:

1. An internal standard primer set and template are incorporated into the assay to test for the presence of PCR inhibitors in the DNA preparation.
2. A positive control primer set that recognizes a species-specific reference gene is used to assess whether the DNA is intact and free from degradation (also influenced by inhibitors).
3. Each sample is analyzed in duplicate from start to finish. These duplicates do not originate at the PCR stage of the analysis. Instead, duplicate analytical samples are taken from the homogenized field sample and processed in parallel throughout the whole analytical procedure.
4. A set of external reference DNA preparations is employed to verify the sensitivity of the method and to provide evidence that the PCR system is operating properly.
   A number of other measures that are not apparent from this example are also essential to reliable PCR analysis:
5. The PCR reaction conditions and the primer sets used must be optimized for sensitivity and specificity.
6. DNA purification procedures must be optimized for each food matrix to ensure freedom from inhibitors and to minimize degradation.
7. To reduce the risk of cross-contamination, the laboratory must be organized such that the steps of analysis are physically and operationally isolated.
8. Stringent quality control measures must be implemented for all analytical procedures, all reagent preparation, and for data analysis and reporting of results.

When these or equivalent measures are employed, highly accurate and consistent results can be obtained. For instance, Table 33.2 summarizes the results from a ring trial conducted by the Joint Research Centre of the European Commission in 1998 (Scott & Benedich 1988). In this part of the study, the frequency of false positives was 2.1%. The frequency of false negatives was 5.1% for samples that contained 0.1% GM material, but no incorrect results were reported for samples containing 0.5% or 2% GM material. For both false positives and false negatives, incorrect results were reported by only 2 of the 25 laboratories. Thus, the vast majority of laboratories performed perfectly on all analyses.

Table 33.2. Reliability of GMO analysis by PCR.

Actual GMO Content	0.00%	0.10%	0.50%	2.00%
Samples Reported Negative	94	5	0	0
Samples Reported Positive	2	93	105	101
% Samples Reported Correctly	97.9	94.9	100	100
% False Positives	2.1			
% False Negatives		5.1	0	0
Labs Making Errors	2	2	0	0
Labs Performing Without Error	23	23	25	25

1998 Ring Trial Conducted by Joint Research Centre, European Commission

Assay Design Features to Confirm Results

When putatively positive results are obtained in PCR analysis of GMOs, it is necessary to carry out confirmatory analysis before regulatory or other action is taken. The following two approaches are recommended by Swiss and German law (Schweizerisches Lebensmittelbuch 1998, German-Federal-Foodstuffs Act 1998), (a) Southern hybridization of PCR products to a probe known to be homologous to the bona fide amplicon of interest, or (b) cleavage of the PCR products into fragments of expected size using restriction endonucleases.

Real-time PCR analysis, using TaqMan, minor groove-binding, Molecular Beacon, and FRET (fluorescence resonance energy transfer) probes, has built into it a third option for confirmation that is equivalent to Southern hybridization. The probes used in these real-time methods hybridize to the sequences within the amplicon, and a signal will not be generated unless both the primers and the probe are homologous to the target. Thus, hybridization of the probe confirms that the amplicon amplified possesses the sequence of the bona fide target. The requirement that the probe must hybridize to the target sequence provides an additional level of confirmation equal in specificity and stringency to the requirement that amplicons hybridize to a Southern blot or be cleaved by a restriction enzyme into fragments of predicted size. Thus, generation of a real-time signal inherently and automatically confirms the identity of the amplicon.

Although these methods verify the identity of amplicons, they do not differentiate between (a) amplification of the bona fide target sequence present in the genome of the sample and (b) amplification of amplicons from another PCR reaction that might have contaminated the sample. This possibility is very real, because such amplicon contamination is the most common form of contamination in the PCR laboratory. To gain additional confidence that the putative positive result is not due to contamination of the sample with amplicons, multiple primer sets are advantageous as a routine part of analysis. With this approach, results obtained with one primer set are confirmed when amplification is also observed with a second primer set that targets a second, independent site that will be present if and only if the site targeted by the first PCR amplification is actually present. This is a stronger method of confirmation than simply confirming the sequence of amplicons. It constitutes a true, independent confirmation that the sequence of interest is actually present in the DNA of the sample.

Simultaneously running PCR analyses with two primer sets, both of which independently recognize separate domains within the sequence of interest, can be used as a strategy for accelerating delivery of final analytical results. In this case, confirmation is achieved simultaneously with the initial observation of positive results, instead of carrying out amplification with one primer set, and then carrying out a second series of reactions with a

second confirmatory primer set. In addition, the use of multiple primer sets provides greater certainty in avoiding false negatives as well as false positives.

Real Time Quantitative PCR

The existence of thresholds for the GMO content of products, whether mandated by government regulation or by contractual agreement between buyer and seller, necessitates methods for quantifying GMO content of foods and agricultural products. This need has triggered a move to methods that offer increasingly more robust quantification. Real-time quantitative PCR methods are currently the methods of choice (Hubner et al. 1999, Vaitilingom et al. 2000).
While conventional PCR measures the products of the PCR reaction at a single point in the reaction profile, real-time PCR methods generate fluorescent reaction products that can be monitored continuously to follow the time course of several PCR reactions simultaneously (See Fagan (2003) for fully a referenced discussion of real-time PCR methodology). The basis of this approach is the linkage of PCR amplification to the generation of one fluorescent reporter molecule for every amplicon that is generated during PCR. For Taqman technology, this occurs through the use of a fluorescently labeled probe that anneals between primer recognition sites. Taq polymerase has an exonuclease function in addition to its DNA polymerase activity, and during strand elongation, the fluorescence-labeled probe is cleaved from the oligonucleotide allowing it to produce a fluorescent signal that is proportional to the number of amplicons generated during the reaction. With this method, the complete reaction profiles for as many as 96 samples can be determined simultaneously.

A typical series of real-time PCR reaction profiles is presented in Figure 33.8. By comparing the profiles of a sample of unknown GMO content with those of a series of standards of known GMO content, it is possible to quantify with reasonable accuracy the GMO content of the unknown. This is illustrated in Figure 33.9, which shows that, when the log of GMO content of a series of standards of known GMO concentration is plotted against the number of PCR cycles required to generate a certain threshold of fluorescent products (indicated by the orange line in Figure 33.8), the GMO content of a sample of unknown GMO content can be deduced based upon the number of cycles required to generate that same level of fluorescent products from a series of samples of known GMO content (assuming uniform DNA input in all reactions). An alternative, but less rigorous, approach to quantification by real-time PCR is to run only one concentration of the GMO reference DNA, and generate the standard curve using this one point and assuming an ideal slope of -3.33.

Real-time PCR provides reasonably good quantification over four to five orders of magnitude, and for a sample containing 1% GM DNA precision of analysis should generally be in the range of ± 20%.

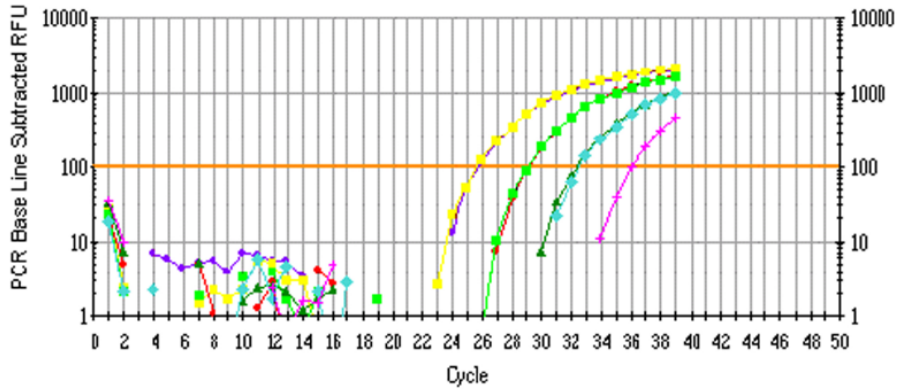

Figure 33.8. Real-time quantitative PCR analysis
Examples of fluorescence profiles generated during real-time PCR, plotted as log fluorescence signal (arbitrary units), versus PCR cycle number. The number of cycles required to generate the threshold level of fluorescence indicated by the orange line is proportional to the log of the initial concentration of the target sequence in the sample. In principle, the threshold specified by this line can be set at any point within the logarithmic portion of the fluorescence profile.

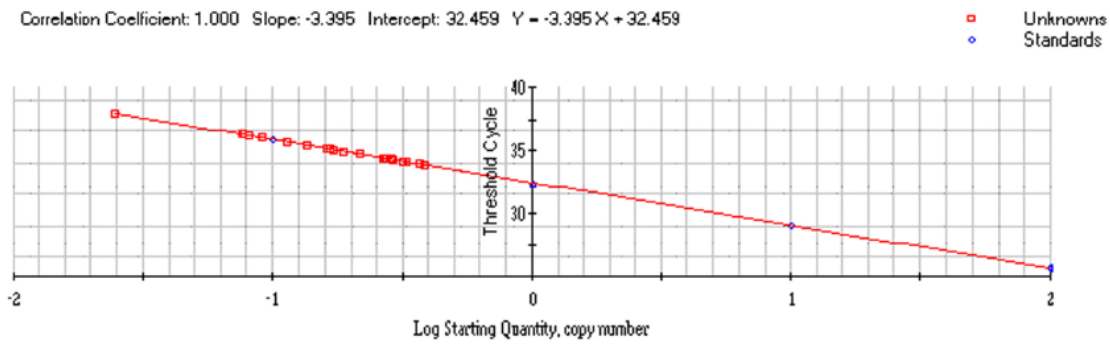

Figure 33.9. Quantification by real-time PCR
When the number of PCR cycles required to generate a fluorescence signal corresponding to the threshold value specified by the orange line in Figure 33.8 is plotted against log of the initial concentration of GMO target sequences in a series of standards (blue circles), a line with slope close to -3.33 is obtained. The GMO concentration in samples of unknown concentration (red squares) can be determined from this plot, based on the number of PCR cycles required for the sample to achieve the fluorescence threshold corresponding to the orange line in Figure 33.8.

The critical design features of a real-time PCR assay for GMOs are similar to those for qualitative PCR in many respects. These include an internal control reaction series, a species-specific reference gene reaction series, and one or more GM-specific reactions series. Some laboratories replace the internal control reaction series, designed to assess the presence/absence of PCR inhibitors, by another approach that assesses the presence of inhibitors through a series of PCR reactions that contain successive dilutions of sample DNA. The cycle number required to reach threshold is compared for PCR reactions containing (a) undiluted sample DNA, (b) sample DNA diluted 1:2, and (c) sample DNA diluted 1:4. If no inhibitors are present, each 1:2 dilution should increase by one the number of cycles required to achieve the threshold. If inhibitors are present, little increase in cycle number, or a non-integral increase will be observed. This dilution approach only provides a qualitative measure of inhibition and, therefore, is not considered as rigorous as

the use of an internal control reaction series, using which, the extent of inhibition can actually be quantified.

Inhibition is generally not a large problem due to the development of efficient DNA purification procedures that remove inhibitors from virtually all sample types, including highly complex multi-ingredient products. The greater limitation to reliable quantification is recovery of sufficient PCR-active DNA to enable quantification in cases where sample DNA is partially degraded. Scaling up purification may improve recovery by 5- to 20-fold; however, some sample types have undergone such extensive processing that it is impractical to recover sufficient intact DNA for a full quantitative analysis. Recoveries of DNA from highly processed materials are highly dependent on the batch of material and cannot be predicted before analysis is carried out. In cases where recoveries are not sufficient for full quantitative analysis, results must be reported in qualitative format only.

In addition to controls for inhibitors and DNA degradation, one must also include controls verifying that the PCR system is operating properly, as well as controls defining and verifying the limit of detection of the method. Analyses must also be run in replicate to verify that analytical results are repeatable and meet pre-established criteria for precision of analysis.

Screening versus Event-specific PCR Analysis

Primer sets that are complementary to DNA sequences unique to a single GMO make it possible to detect specific transgenic crop varieties or events. Such event-specific, or Varietal ID^{sm}, methods can specifically and unambiguously identify each transgenic soy, maize, potato, rice, cotton, etc. variety commercialized to date.

Generally, the importation of GM food or feed is contingent upon approval of these products for specific uses. National and regional differences in approval status for a given GM crop can create substantial challenges for import. Table 33.3 illustrates this situation in the case of maize. The two right-hand columns list all of the transgenic corn events or varieties that have been approved for cultivation in the U.S.A. Of these, the top 15 have actually been produced commercially on a large scale. Of these, four are no longer in commercial use (indicated by ‘terminated’). The remaining 10 events have been authorized, but never commercialized. To the right is the approval status of these products for human use in the EU and Japan, as examples of international markets. All of the currently commercialized events have been approved in Japan, but three of those events have not been approved in the EU.

Differences in approval status create a problem for grain exporters attempting to move maize or maize products into various markets. Not only is it necessary to determine whether GM material is present in order to comply with labeling regulations in these countries, but it is also necessary to insure that a given lot of product does not contain varieties or events that have not been approved for food use in the specific country of import.

The current status of labeling regulations in the European Union (EC Council Regulation 1830/2003, (European Commission 2003) exemplifies the situation encountered in many parts of the globe. Products of unknown composition or products that contain greater than 0.9% GM material must be labeled with a phrase such as ‘Produced through gene modification’. Products containing less than 0.9% GM material do not require labeling, as long as the producer can provide strong traceability documentation demonstrating that positive efforts were taken to avoid GMO admixture. For events not yet approved, but engaged in the approval process and having received a ‘favorable’ safety assessment, there is a transitional threshold (applicable for three

years after the date of application of the Regulation) of 0.5%, whereas, for other unapproved transgenic events, there is zero-tolerance. Thus, importers must demonstrate the absence of GM events that have not been approved for food use in Europe. Event specific methods are designed to provide this key information.

Each event-specific primer set defines an amplicon that spans a sequence junction unique to the transgenic event of interest. These are sites where two sequence elements have been joined in a manner unique to that event. Thus, a positive signal with such a primer set is definitive evidence for the presence of the respective event in the sample of interest.

Figure 33.10 is an example of a recombinant gene that has been created by splicing together five different pieces of DNA, from five different sources. The green bars, which flank sequences 1 and 5, represent maize genomic DNA sequences. The recombinant gene was inserted into a unique site within the maize genome, thus the sequences found at the junctions of the recombinant gene and these flanking sequences are unique to this transgenic event.

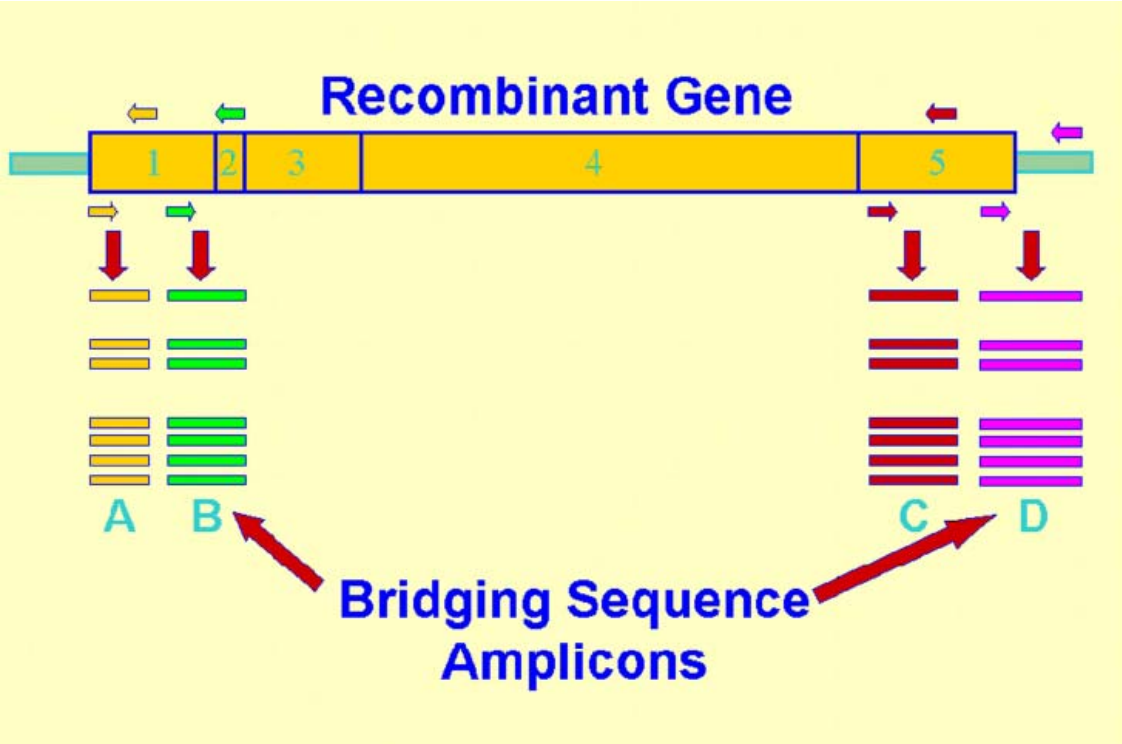

Figure 33.10. Bridging primers allow definitive detection of specific transgenic events
Primer sets B and D bridge junctions between two sequence elements. By selecting primer sets that bridge junctions that are unique to a given transgenic event or variety an assay can be developed that is highly specific for the detection of a single transgenic event. Primer set B bridges the junction between two internal elements in the transgenic construct, allowing construct-specific detection, while primer set D bridges the junction between the transgenic construct and the host genome, allowing event-specific detection.

We show four different primer sets that could be used to detect this gene. Primer sets A and C detect sequences that are wholly within a single sequence element of the transgene. As a consequence, they are unable to distinguish between different transgenic events that contain these genes.

Primer set B, however, consists of one primer that recognizes sequence 1 and another that recognizes sequence 2. These two sequence elements do not exist adjacent to each other in nature, so the only case in which a PCR product will be made from this primer set will be when sequence elements 1 and 2 are juxtaposed, as is the case in the recombinant gene shown in Figure 33.10. Thus, this primer set is specific for DNA isolated from any recombinant organism transformed with this particular recombinant DNA construct. Such a primer set B is termed 'construct-specific.'

In contrast to primer set B, primer set D includes one primer specific for sequence 5 and one specific for the maize genomic sequences flanking the recombinant gene. Because currently available methods insert transgenes randomly into the host genome, the host genomic sequences flanking a transgene will be different in every transformation event. Thus, primer set D will be capable of amplification only when presented with genomic DNA from the transformation event depicted in Figure 33.10. Other transformation events will result in other sites of insertion and their detection will require that the primer specific for sequence 5 be paired with a different primer, which would be homologous to the maize genomic sequences flanking the recombinant gene in that particular event. Thus, primer set D is truly 'event-specific'.

In developing event-specific methods, the specificity of each event-specific primer set must be verified by running PCR reactions containing that primer set and containing DNA isolated from all commercialized transgenic events for that species, as well as reactions containing DNA from all common food plants and gene modification events of other species. It is also important to sequence the event-specific amplicons to verify that the correct, expected transgenic sequences are, in fact, being amplified.

The use of event-specific primer sets is the only approach to definitive quantification of GMO content. The typical approach to quantification that is carried out in GMO analytical laboratories around the world is to quantify based on analysis using one or more broad spectrum primer sets that recognize common transgenic elements, such as the CaMV 35S promoter, the nos terminator, the Cry 1Ab gene, etc. Because these elements are present in different copy numbers in different transgenic events, as illustrated in Table 33.4, they cannot be used for accurate quantification of percent GMO in samples in which more than one event is, or may, be present. Since complex mixtures that contain multiple GMOs in unknown proportions are not the exception but the rule for real-world samples, such broad-spectrum primer sets seldom provide accurate quantification. Only event-specific PCR analysis is capable of providing definitive GMO quantification: event-specific primers can be used to achieve accurate quantification based on definitive quantification of each individual transgenic event present in the sample.

Table 33.4. Copy Number of Common Transgenic Crop Sequences

Variety	35S-P	35S-T	nos-T
Maize			
Mon 810	1	0	1
Bt 11	2	0	2
Mon GA21	0	0	2
Aventis T14	3	3	0
Aventis T25	1	1	0
Aventis CBH-351	4	1	4
DeKalb DBT418	3	0	0
DeKalb DLL25	1 inc.	0	0
Event 176	2	0	2

Soy			
Mon 40-3-2 (RR)	1	0	1
Aventis A5547-127	1	1	0
Aventis GU262	2	2	0
Aventis A2704-12	2	2	0
Aventis A5547-127	1	1	0
Dupont A2396	1	0	1

Maintaining Uniformly High Standards of Performance in the GMO Testing Laboratory

The following section is based on Genetic ID's experience in maintaining the uniformity of quality of GMO analytical services within the laboratories of the Global Laboratory Alliance, which includes three laboratories that Genetic ID owns and operates, in the USA, Germany, and Japan, and 18 additional private and government laboratories around the world that have licensed Genetic ID's GMO testing technology.

Standard operating procedures—To standardize GMO testing and ensure consistency and quality, a thorough and comprehensive system of standard operating procedures (SOPs) is essential. This system must be well embedded within a document control system so that all amendments to methods, and the incorporation of new methods, occur in a uniform, orderly way. It is essential that all individuals that use the methods receive all changes at the same time, so that uniformity can be maintained. SOPs should be established not only for laboratory procedures, but also for analyzing data and reporting results. It is critical that the uniformity of the entire analytical process be maintained.

Laboratory performance assessment—Performance assessment programs or proficiency testing schemes are required to ensure that all procedures are performed consistently, correctly and accurately. Typically, both internal and external performance assessments are conducted. The internal program introduces into the analytical stream of the laboratory blind 'check samples' at a frequency proportional to the total number of commercial samples of that kind analyzed by the laboratory. A range of sample types and analysis types are included in the check sample program, thereby assessing the entire scope of methods used in the laboratory. Technicians should not be aware of which are authentic samples and which are check samples. Results of the check sample program must be reviewed and audited on a regular basis and results shared with both technicians and with management as part of the ongoing quality improvement program of the laboratory. The laboratory should participate in external performance assessment schemes on a frequent basis. Several organizations offer such programs at this time, including the US Department of Agriculture, the UK Food Analysis Performance Assessment Scheme, the American Oil Chemists Society, and the American Association of Cereal Chemists.

Laboratory accreditation—The most widely accepted standard for analytical laboratories is ISO/IEC 17025 (International Organization for Standardization 1999). Regular on-site evaluation of the analytical laboratory by an independent third party accreditation body to verify that the laboratory is operating in compliance with this or an equivalent standard is very important. Accreditation to ISO 17025 includes: (a) inspection of laboratories on a yearly or appropriate basis; (b) evaluation of the laboratory's quality system and technical operations; (c) evaluation of all quality documentation; and (d) evaluation of extensive validation data for each analytical method that is to be included within the scope of accreditation. Accreditation of each method is laboratory-specific and thus a method must be validated independently in each laboratory in which it is used.

The credibility of accreditation is highly dependent on the level of acceptance and recognition of the accreditation body. For instance, reciprocity has not been established between EU and US accrediting bodies. Thus, if a laboratory wishes to provide testing services in Europe, it is prudent to undergo accreditation by an EU-recognized accreditation body.

International standardization of GMO testing methods—Laws and international accords requiring the monitoring of GMOs released into the environment and the labeling of foods consisting of, or containing ingredients derived from, GMOs have created the need for standardization of methods for analysis of GMOs.

The standardization of methods for GMO testing has not progressed at the same pace as the introduction of GMOs into the food system and the enactment of labeling laws. Japan (Japanese Ministry of Health Labour and Welfare 2002), New Zealand (New Zealand Ministry of Agriculture and Forestry 2002), Germany (German-Federal-Foddstuffs-Act 1998), and Switzerland (Scheweizerisches Lebensmittelbuch 1998) have all established official testing methods for some GMOs. Unfortunately, no country has established a comprehensive set of testing methods nor a system for updating methods to assure that they cover all GMOs currently in the marketplace. A global unification and standardization of GMO testing methods needs to be achieved to properly service the food and agricultural industries, which are global in nature. GMO testing services must be available globally that are consistent, reproducible, and reliable in sensitivity, specificity, and accuracy. Only then can exporters be confident that the products released on the basis of test results from one port, will be found acceptable when tested by the buyer in a distant port on the other side of the globe.

Several initiatives are in motion to develop, standardize, and validate methods. Most prominent are the CEN and ISO efforts. In Europe, a network of official government reference laboratories for GMO testing, known as the European Network of GMO Laboratories (ENGL), has been established by the Joint Research Centre of the European Commission. This is a group of more than 50 official control laboratories, each appointed by the national authority of the corresponding EU member state. One of the objectives of this network is to develop and standardize testing methods that will respond to testing needs evolving out of EU legislation on GMO labeling. There are also initiatives in progress within various industries, including the seed, tobacco, and cereals industries.

It is important to note that the aforementioned initiatives will all require years to achieve completion. In the meantime, the food and agricultural industries must find interim strategies to assure consistent compliance with labeling laws and to provide products that are responsive to consumer's expectations. One initiative that is designed to address this need is the Global Laboratory Alliance. This network of more than 18 laboratories from around the world has methods for all commercialized GMOs, and is already operating to uniform standards, with a quality assurance system in place to maintain compliance and assure consistency in testing globally.

Future Technologies

PCR and immunological methods are both too limited to deal with future genetic analytical needs in the food and agricultural industries. We will need technologies that can analyze hundreds of genetic targets simultaneously, quantify accurately, and are highly sensitive. Ideally, these methods should be rapid, inexpensive, effective with diverse food matrices, and field operable. These requirements cannot be fulfilled by either PCR or immunological methods, nor do presently available alternatives meet this need. For example, near infra-red spectroscopy (NIR) is

fast and inexpensive, and can detect GM grain or soybeans, if relatively high levels are present (Hurburgh et al. 2000). However, this approach is not sufficiently sensitive, and its discriminative capability is not sufficient. Moreover, it is not universal in its applicability. Another approach that has been explored, DNA microarrays (Grohmann 2002), is useful for gene discovery, but is not well adapted to GMO analysis. Microarrays can handle many targets simultaneously, but this technology is neither quantitative nor sufficiently sensitive for GMO analysis. Because of their lack of sensitivity, microarrays must be coupled with PCR amplification if they are to be used even for qualitative GMO analysis. Thus, at best, microarrays can only replace the electrophoresis step of current qualitative GMO analysis.

Biosensors are a third technology that may have potential for GMO testing. Biosensors have not found routine use in GMO testing to date. However, three different kinds of biosensors have been evaluated for their suitability. These include surface plasmon resonance (SPR) (Minunni et al. 2001, Feriotto et al. 2002, Mariotti et al. 2002), piezoelectric (Minunni et al. 2001), and electrochemical biosensors (Minunni et al. 2001). When used in conjunction with PCR amplification all three of these approaches were found to provide technically adequate levels of detection (Minunni et al. 2001, Feriotto et al. 2002, Mariotti et al. 2002).

Other features of these biosensors offer advantages for GMO detection. First, they can be multiplexed to screen for many targets simultaneously. Second, the detection process used in these biosensors is nucleic acid hybridization, which is highly selective. Third, other work has shown that these biosensors can function quantitatively. Fourth, because they operate on simple physical principles, detection is rapid and economical. Fifth, commercial instrumentation based on these biosensors should be easy to use and automatable. Finally, in some cases, with further work, portability and field-operability should be achievable.

With currently available biosensors, as with microarrays, sensitivity of detection is the primary limitation, and it may not be possible to upgrade the sensitivity of the biosensors tested to date to achieve the sensitivity required for stand-alone use, independent of PCR. As discussed, elimination of the need for PCR is essential, if a detection method is to constitute a genuinely fundamental advance in GMO detection technology. Although this may not be achievable with currently available biosensors, as research in biosensors continues over the next few years, innovative designs and detection principles may lead to development of novel biosensors having sufficient sensitivity to adequately fulfill future GMO analytical requirements.

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