FOOD COMPOSITION AND ADDITIVES

Polymerase Chain Reaction Technology as Analytical Tool in Agricultural Biotechnology

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The agricultural biotechnology industry applies polymerase chain reaction (PCR) technology at numerous points in product development. Commodity and food companies as well as third-party diagnostic testing companies also rely on PCR technology for a number of purposes. The primary use of the technology is to verify the presence or absence of genetically modified (GM) material in a product or to quantify the amount of GM material present in a product. This article describes the fundamental elements of PCR analysis and its application to the testing of grains. The document highlights the many areas to which attention must be paid in order to produce reliable test results. These include sample preparation, method validation, choice of appropriate reference materials, and biological and instrumental sources of error. The article also discusses issues related to the analysis of different matrixes and the effect they may have on the accuracy of the PCR analytical results.

The class of plant products developed through modern biotechnology has been described as genetically modified (GM), genetically engineered (GE), genetically modified organism (GMO), transgenic, biotech, and recombinant. For the present discussion, the term "genetically modified" (GM) will be used for its simplicity and broad recognition. This discussion will address polymerase chain reaction (PCR) technology as it applies to food biotechnology in the soybean and corn (maize) industries only, though the principles are applicable to other crops as well. PCR is only one of the techniques that are used for the detection of GM material in a product. Although protein-based test technology is available and applied to testing (1), especially in the seed and grain industry, the remainder of the article will focus exclusively on PCR technology.

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A number of countries have adopted, or are in the process of developing, legislation related to the approval of GM products. Authorities in many countries require that DNA sequence information be provided as part of the registration package. In addition, a PCR detection method that is specific to the event may also be requested. The term "event" is used to describe a plant and its offspring that contain a specific insertion of DNA. Such an event is distinguishable from other events by its unique site of integration of the introduced DNA. A PCR method that can distinguish such an event from all other events is described as being "event-specific" and generally is based on the detection of a junction fragment between the original plant DNA and the introduced DNA.

Uses of PCR Technology

The agricultural biotechnology industry applies PCR technology at numerous steps throughout product development, much as it does with immunoassays (1). The major uses of PCR technology during product development include gene discovery and cloning, vector construction, transformant identification, screening and characterization, and seed quality control. Commodity and food companies, as well as third-party diagnostic testing companies, rely on PCR

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technology to verify the presence or absence of GM material in a product or to quantify the amount of GM material present in a product. Quantitative PCR technology also has been used to estimate GM copy number and zygosity in seeds and plants (2–4).

The grain handling and grain processing industry uses PCR to certify compliance with contracts between buyer and seller. PCR testing is used for 4 specific purposes in the grain handling/processing industry:

PCR testing for unapproved events.—In countries that have a defined approval process for GM crops, an event may be approved for use in the country of production but not yet approved for use in an importing country. In these instances, the importing country often requires that the grain shipment be tested for the presence of specific GM events to ensure that the grain shipment does not contain these unapproved events. Such testing often relies on qualitative PCR because the detection of these events, in most cases, is at a zero-tolerance threshold.

PCR testing for GM content.—Most countries that have adopted mandatory labeling rules for food or feed have set tolerances for the adventitious presence of GM material in grain products or the final foods based on a percent GM (weight-to-weight) content. In these countries, food and feed manufacturers and retailers often choose to originate/obtain grain and grain products below the defined regulatory threshold to avoid labeling their products. In this case, grain must come from a non-GM identity preservation program and be certified to contain GM grains at a level below the threshold specified in the contract. To meet this need for testing, several laboratories currently are adopting quantitative PCR for percent GM determinations.

PCR testing for non-GM labeling.—In some cases, food manufacturers and retailers wish to use positive labeling for their non-GM products. These companies hope to gain market share among consumers who wish to avoid GM products. In most cases, the use of positive labeling requires that the grain and grain products originate from a non-GM identity preservation program and test negative or at least below a certain threshold for GM DNA. Qualitative PCR testing is most often used to certify compliance with a non-GM contract.

PCR testing for presence of a high-value commodity.—In certain cases, it is desirable to show that a commodity is made up of a specific crop commodity (e.g., low phytate maize, soybean with altered oil profile). PCR could be used for this purpose by testing for the GM trait that conveys the characteristic, although the grain may also be tested by quantifying the improved quality of the commodity.

The PCR Process

The PCR process mimics in vitro the natural process of DNA replication occurring in all cellular organisms in which the DNA molecules of a cell are duplicated prior to cell division. In contrast to natural DNA replication, the DNA reproduction during PCR does not cover the entire sequence of the original DNA molecules but is restricted and targets a specific, relatively short, region of the template DNA molecules. Short, single-stranded, synthetic DNA molecules called the primers give the specificity of the reaction. They are designed to be complementary to their intended binding site. Most commonly, 2 primers are involved and the DNA section in between the distal ends of their binding sites are replicated during the reaction.

A single cycle of the PCR and the corresponding temperature profile are typically divided into the 3 phases: denaturation, annealing, and elongation. At the end of this procedure, the targeted DNA region has been replicated into 2 copies of the original double-helix molecule. This process of selective duplication is repeated multiple times in a cyclic reaction. The repetitive DNA duplication is driven solely through quick and precise shifts in the reaction temperature, facilitated by the thermocycler instrument. DNA replication is catalyzed by heat-stable DNA polymerases, previously isolated and characterized from *Thermus aquaticus* (5). The kinetics of the DNA reproduction resemble an exponential amplification in which the replicas of distinct length (amplicons) accumulate quickly and outnumber the original template molecules. The distinct size of the amplified copies allows them to be detected by gel electrophoresis in the background of nonamplified DNA.

Sample Preparation

Sampling

If applying PCR to test for GM material, one must carefully conduct the sampling in a manner that avoids erroneous results. When PCR-based diagnostic assays are used to test for the presence of GM material in seed or grain, a number of sampling steps occur (Figure 1): (*1*) *Sampling* the consignment of seed or grain to obtain the bulk sample. (*2*) *Sampling* the bulk sample to obtain the laboratory sample. (*3*) *Subsampling* the laboratory sample to obtain the test sample. (*4*) *Sampling* the meal that results from grinding the test sample to obtain the analytical sample. (*5*) *Sampling* the DNA solution that results from extraction of the meal sample to obtain the test portion.

Typically, sampling considerations are limited to the laboratory and test sample with regard to the desired threshold of detection. The subsequent sampling step (meal) is typically driven by the traditional procedures of a particular laboratory or the capacity of the equipment used. It is incumbent on the analytical laboratory to have characterized and standardized the production of the meal sample as part of the method development. The final sampling step (DNA to be used in a single PCR) is typically constrained to <200 ng DNA by the limitations of the technique and the equipment used.

Each of the sampling steps has the potential to introduce error that may impact the detection of GM material at the desired threshold. As such, designing a sampling strategy that will be suitably representative requires knowledge of the particle size characteristics of the test sample and the meal, the genome size of the species in question, and the limit of

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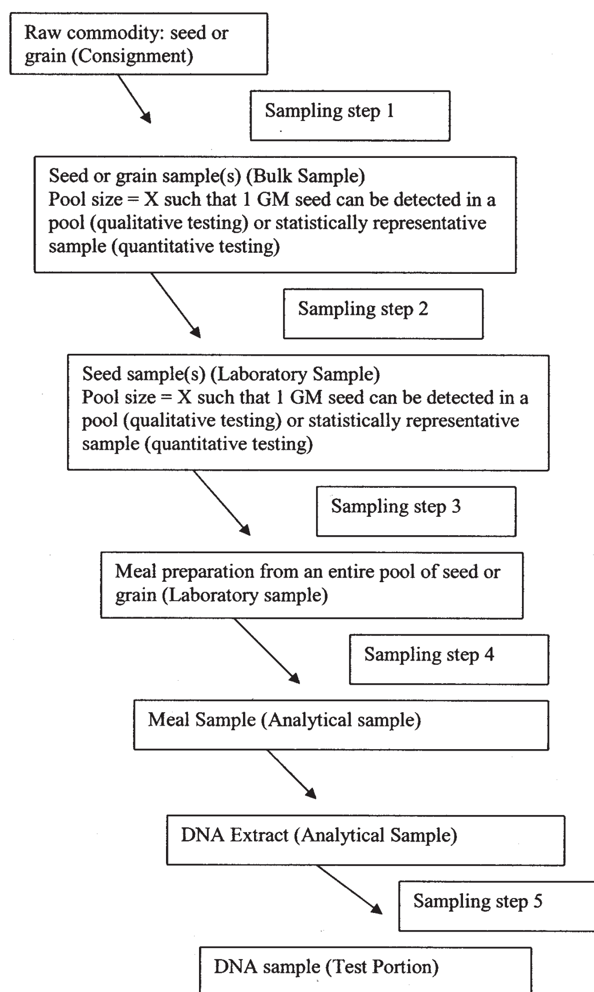

Figure 1. Sampling steps for PCR-based diagnostic assays for GM detection.

detection (LOD) or range of quantification (ROQ) of the analytical technique. Sampling considerations have been addressed in a number of recent papers (6, 7).

Although it is often overlooked, the overall sampling method must be carefully designed and characterized. Particular attention should be given to understanding the limitations of the analytical technique as it relates to the sampling plan when testing for GM material at low thresholds of detection.

The following is an example of a theoretical exercise that demonstrates the impact of sampling on the ability of a qualitative detection method to detect small quantities. To frame the exercise, we must first make some basic assumptions about the biological material to be analyzed: (*1*) the objective of the analysis is to detect trace amounts of GM material in maize; (*2*) the GM material is in the hemizygous state (and no information is available on the genetic background/variety); (*3*) the PCR sample size is approximately 40 000 maize genomes, which equals ~109 ng (8); (*4*) to compensate for sampling error at the DNA sampling stage, the minimum nominal number of target sequences must be 20 per reaction as suggested by Kay and Van den Eede (9); (*5*) the target gene is present as a single copy per haploid genome and, thus, with the same relative abundance as the endogenous control gene.

The first calculation estimates the minimum level of percent GM (on a weight/weight basis assuming all kernels have equal weight) that would be detected in the sample and is called the relative LOD. To calculate this value, we need to divide the minimum number of genome copies that can be reliably detected in a qualitative analysis (20 copies) by the total number of copies in the reaction tube (40 000 copies). The result of this must be multiplied by 2 to account for the fact that in the hemizygous state, the GM gene is present in only 1 of the 2 parental genomes. The result of the calculation: $20 \div 40\,000$ (ratio of GM genomes to total) $\times$ 2 (hemizygosity correction factor) $\times 100 = 0.1\%$. This represents the lowest concentration of GM that can be reliably detected using qualitative PCR.

In this example, the meal sample from which the DNA preparation is extracted must be at least 0.1% GM in order to achieve reliable detection. This fact imposes constraints on the choice of pool size and meal sample size. In this example, a seed pool containing a single GM seed must result in a meal sample that is at least 0.1% GM with high confidence.

If one accepts the limitations of the analytical technique outlined above (i.e., LOD equal to 20 target sequences per reaction, equivalent to 0.1% GM), the absolute maximum pool size for which one can expect reliable detection of the presence of a single positive kernel in the pool is 1000 seeds, if all the seeds in the pool are ground to meal and the entire resulting meal preparation is used for DNA extraction (thereby eliminating sampling error at the meal sample stage). Using a pool size >1000 will result in situations in which some positive pools will have GM levels below the LOD of the analytical technique. In order to test to a very low threshold, multiple sample approaches must be adopted to increase the overall sample size. For example, assuming a binomial distribution and testing a single pool of 998 seeds with a negative result provides 95% confidence that GM content is <0.3%. In order to have 95% confidence that GM content is <0.1%, one must test 2995 seeds, whether in a single pool or as multiple pools, with no pools testing positive (http://www.seedtest.org/upload/cms/user/seedcalc6.zip).

DNA Extraction and Matrix Effects

The performance of an analytical method will vary with the nature of the sample under study. Typically, a method will be developed and validated for only 1 sample type or a very restricted set of different matrixes. Modifications to the method may be required to accommodate other matrixes, thereby creating a different method/procedure.

This section outlines aspects that apply to effects of the sample matrix on the performance of the method. Although the considerations focus on effects from evidently different matrixes, it must be noted that the term "sample matrix" may not be clearly defined. There may be ambiguity, and the analyst must decide if the unknown sample falls in the same

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category as the samples used during validation of the method. A detailed scope of the method will minimize such ambiguity but cannot entirely prevent it.

PCR methods for detection of GM traits are commonly developed and validated with samples of ground seed because validation studies, proficiency schemes, and check sample programs with large samples of whole seed are not practical. However, initiating validation and proficiency programs with ground seeds ignores the variability in the preparation process. Some organizations, notably the International Seed Testing Association (ISTA), have carried out proficiency programs using whole seed.

In routine applications, the analyst will frequently use PCR to analyze grain. However, even a matrix that appears simple from the method developer's point of view, i.e., maize kernels, may be challenging in a laboratory situation. For example, a seed sample may have been treated with chemicals such as fungicides, which were not present in the samples used for method validation. These compounds may interfere with or inhibit the PCR, particularly if the DNA extraction procedure is not tailored for their effective removal. If the method does not include appropriate controls, this inhibition can lead to false-negative results. In addition, any 2 maize samples are unlikely to be exactly the same with regard to such characteristics as moisture, fiber, starch, and residues of chemicals, and at some point the assumption must be made that they belong to the same matrix, although the boundaries of this category cannot be exactly defined.

A prudent approach to this challenge is not to rely solely on method validation for a particular matrix or to assume all samples that are considered the same matrix will behave in exactly the same way. Controls that monitor performance should be developed as part of the method to detect potential effects originating from the individual unknown sample under study. The effectiveness of these controls should be demonstrated during method development and, ideally, also during validation.

(**a**) *PCR inhibitors*.—Various compounds in plant material and food products can be co-extracted with genomic DNA and inhibit the PCR. This may lead to false-negative results because of failure of the PCR (10). Likewise, partial inhibition is very likely to bias results from a relative quantitative PCR assay using 2 PCR systems in parallel, because the 2 PCR systems will rarely be affected to exactly the same extent.

PCR inhibitors from plants and processed foods include polysaccharides, proteins, phenolic compounds, and other uncharacterized plant secondary metabolites (11–13). Moreover, covalent cross-linking of proteins to DNA through carbohydrates can render the DNA unsuitable as a PCR template (14, 15). The inhibitors may vary in the extent to which they affect individual PCRs using different primer pairs in separate or multiplex PCRs (16, 17).

Although it is necessary to use or develop a DNA extraction method that sufficiently removes PCR inhibitors from the matrixes that fall within the scope of the method, it is not practical to identify and characterize all potential inhibitors. A more feasible approach is to test DNA extracts for the presence of inhibitors and then modify the DNA isolation protocol or the PCR conditions to reduce the effects of PCR inhibitors, if necessary (16–19). Generally, a positive control DNA (spike) is added to the PCR. Specific amplification of the control DNA is tested in the presence and absence of DNA extracts to see if the extract inhibits amplification. This should preferably be done with DNA extracts from known negative samples so that the spike DNA is the only source of a constant amount of target DNA in the PCR. There are several examples of DNA extraction methods that target removal of specific inhibitors (12, 13, 18, 20, 21).

An inhibition control in the method is a requisite to monitor potential inhibition arising from an individual sample in routine application of the method. Such a control will considerably lower the risk of false-negative results caused by PCR inhibition. During method development and validation, the fitness of an inhibition control can be assessed by analyzing samples with small amounts of analyte close to the anticipated or intended detection limit. In PCRs that contain purposely added inhibitory compounds or crude DNA extracts that are known to contain inhibitors, the failure of the PCR (false negative) should be indicated by the malfunction of the inhibition control. Holden et al. (10) have shown that the amplification of an endogenous sequence does not always fulfill this requirement. The sensitivity of this type of reaction as an inhibition control is restricted, probably because the presence of the endogenous target is greater than the presence of the GM target; therefore, it still can be amplified successfully in partially inhibited reactions where amplification of the GM target fails. Ideally, accept/reject criteria for negative results for an individual sample consider the outcome of the corresponding inhibition control and are described under the appropriate sections of method validation.

(**b**) *DNA degradation, fragmentation, and extractability.*—Processing of raw agricultural commodities to food ingredients and finished food products usually comprises steps that extract, fragment, or otherwise compromise the DNA molecules. Complex sample matrixes may also require multiple consecutive DNA extraction and purification steps that lower the DNA yield of the overall procedure. Reduced size of the DNA molecules that can be extracted from a processed matrix is of concern if a considerable portion of the fragments cannot function as PCR templates because their insufficient size does not span the entire target sequence. Also, certain types of DNA damage during food processing may interfere with the DNA's ability to serve as a PCR template. Whereas it may not be required or feasible to address the exact nature of these limitations, validation studies will reasonably show their impact on the applicability of a method to a processed matrix.

These effects imply that a detection or quantification limit established for a given method is restricted to the matrix used during validation (most commonly, raw material, i.e, ground seeds) and cannot be projected to any other matrix outside the scope of the method. Decreasing the amount or otherwise compromising the integrity of DNA that can be extracted from

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the sample and amplified in PCR reciprocally increases the detection and quantification limits expressed in terms of percent GM DNA relative to plant species DNA. In extreme cases, only minute amounts of DNA can be extracted from certain matrixes (e.g., refined oil, modified starch, and soybean lecithin). If the DNA yield is extremely low, or only an insignificant portion of the extracted DNA is present in amplifiable fragments larger than the PCR target sequence, attempts to establish the LOD of a PCR method by validation likely will fail. With significant lack of extractable and amplifiable DNA, relative quantification will fail as well, with poor reproducibility and very high or entirely unknown quantification limits. Modifications to DNA extraction procedures may compensate for extremely low DNA yield from such matrixes to some extent and may result in an improvement of the detection limit, but are still not likely to result in methods that are practicable in the real world.

In contrast to the decrease of DNA content by most food processing, it is possible that some procedures such as freeze-drying may concentrate the DNA in the matrix and, thereby, decrease the lower limits of the method's applicability. The detection limit will remain unknown for the processed matrix unless established by appropriate validation.

Because the concept of relative quantitative PCR involves the measurement of 2 analytes in parallel, removal, degradation/fragmentation, or restricted extractability of DNA have the potential to shift the ratio between these 2 analytes if they are not equally subject to the adverse effects. Variation in nuclease susceptibility of different regions of the genome is a well-established phenomenon particularly with respect to Dnase I sensitivity of actively transcribed chromatin regions (22–25). Differences in nuclease susceptibility may arise as a result of a number of factors. Some of these factors may be relatively global in scope, such as higher-order chromatin structure, whereas others may be quite local, such as methylation state of specific sequences, nucleosome placement, or level of transcriptional activity. Whatever the root cause, differential nuclease susceptibility between the endogenous control gene and the target sequence can result in biased results particularly when dealing with processed materials. For example, processing that preferentially eliminates the DNA sequence used to measure the amount of plant species DNA would increase the result given in percent GM DNA relative to plant species DNA in the processed matrix. Although results that are expressed in terms of GM DNA relative to plant species DNA are, strictly speaking, still correct, the restriction that may arise to their interpretation is evident. They do not necessarily reflect the relative composition of the original material.

(**c**) *Mixed products with DNA from multiple sources*.—The specificity of a PCR assay for its intended target analyte is determined by the likelihood that sequences that are similar to, but not the same as the target sequence are not amplified and therefore do not lead to false-positive results. Besides the ability of the primers and PCR conditions to discriminate between the intended and unintended detection of closely related sequences, the composition of the DNA in the sample matrix plays a major role. The minimum requirement for the specificity of a PCR assay for GM detection is that it does not cross-react with any of the DNA sequences that are present in the non-GM plant genome. Furthermore, it is critical that sequences from other GM events in the same species do not interfere with the assay in a manner that could lead to positive signals that do not originate from the intended GM analyte. However, PCR analyses that are targeted to a sequence present in many different events (e.g., the 35S promoter) will react with those multiple events.

Compliance with the minimum specificity requirements can be established during development and validation of a method. However, the scope of such method would be fairly limited and not necessarily suitable for routine application in daily laboratory practice. For example, a method for detection of a GM maize event may show a satisfactory specificity on maize samples prepared for a validation study; however, this does not demonstrate the method's suitability for analysis of commercial maize samples that contain foreign materials, such as soybean or wheat. Therefore, it is advisable to design and validate a method so that it is specific for the intended GM event, even in the presence of DNA from other plant species that are likely to be present as adventitious material in routine grain or processed samples.

The same argument applies to methods that are intended for use in more complex food matrixes that may have even more sources of DNA from different organisms than a grain or flour sample. Experimental assessment of the method's specificity on DNA from various sources will define the scope of the methods that must be considered during method validation.

PCR methods that target artificial transitions between DNA sequences that naturally do not occur in juxtaposition are believed to be less problematic with regard to their specificity than PCR methods that target a sequence located within only 1 genetic element that has been used in genetic engineering of plants. For example, the detection of cauliflower mosaic virus (CaMV) 35S promoter sequences is not necessarily conclusive evidence that the DNA detected originated from a GM plant. The virus itself may be present (the CaMV is a common plant virus affecting e.g., *Brassica* species) and lead to a positive result, and the likelihood of its presence could vary considerably with the sample matrix, i.e., the ingredients of a finished food product. PCR assays that detect CaMV itself by targeting a viral nucleotide sequence other than 35S promoter can be useful to investigate the probability of 35S promoter positive results originating from CaMV (26). In other words, the presence of the 35S promoter can be caused by a naturally occurring plant virus and its presence per se cannot be linked to the presence of GM plants without further additional information. Similarly, the detection of a gene derived from *Bacillus thuringiensis* may be the consequence of traces of DNA from soil bacteria present in a grain sample. If possible, these limitations should be considered in the scope of the method and instructions given for interpretation of results.

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The presence of CaMV in considerable amounts can also affect results from quantitative PCR that measure the ratio between 35S promoter DNA and a species-specific DNA sequence. Although the result would probably still reflect the ratio of the 2 types of DNA correctly, it may defy reasonable interpretation because the contributions from CaMV sequences and GM DNA cannot be distinguished.

A further major restriction to the interpretation of 35S promoter quantification in complex matrixes is GM DNA from different crop plants that could contribute to the value obtained for the 35S promoter DNA; most commonly, only 1 plant species at a time is targeted in the species-specific PCR in parallel. Multiple significant contributions to the numerator of the percent GM calculation, without the contribution of all respective plant species to the denominator, would consequently yield an overestimate that does not reflect percent GM.

Applications of PCR

PCR can be used in 2 primary ways in the detection of GM DNA in plants. These are termed quantitative PCR, which yields an estimate of the amount of the specific analyte present, and qualitative PCR, which yields a yes/no answer as to the presence of GM material.

Quantification of DNA

The analyst must be aware of the measurement uncertainty in the determination of the amount or concentration of DNA used in an experiment. The following list contains a number of factors that contribute to this uncertainty (27). This list is not considered exhaustive:

The following factors are known to influence the accuracy and precision of DNA quantification by UV spectrometry: (*1*) presence of other components absorbing at 260 nm, e.g., proteins, RNA; (*2*) ratio of single-stranded vs double-stranded DNA; their absorption coefficients differ; (*3*) size distribution of DNA in solution.

The following factors are known to influence the accuracy and precision of DNA quantification by fluorescence-spectrometry: (*1*) size distribution; (*2*) in cases of dyes that bind exclusively to double-stranded DNA, single-stranded DNA, if present, will not be determined at all.

Currently, all DNA concentration quantification techniques have limitations in their use and application. Spectrophotometric analyses (i.e., A260-A280) require a relatively large amount (2.5–5.0 μg) of DNA of almost pure quality. DNA extracted from certain food matrixes is unlikely to meet this requirement. Spectrophotometric assays are also unable to differentiate between single- and double-stranded DNA or between DNA and RNA. Fluorometric assays require that a DNA standard of a comparable size, and in the case of Hoescht assays, adenine and cytosine content be used (28, 29).

Thus, all DNA quantification methods have their strengths and weaknesses, although the spectroscopic determination with absorbance at 260 nm is commonly used. Regardless of method choice, the analyst must recognize that the uncertainty of the DNA determination will be an inherent part of the total uncertainty of the method and, thus, be reflected in each analytical result. No method will be more precise and accurate than the estimates of the concentrations of its calibrators.

For some applications, DNA is diluted exhaustively (through sequential dilution the number of PCR target molecules is decreased) to determine the copy number of the limit of a method, or to quantify the amount of DNA by measurement of an endogenous gene. For very low numbers of molecules, stochastic effects will predominate.

The best estimate of the absolute quantity of DNA in a given reaction volume can be determined using one of the techniques described above. This value is multiplied by the genome size (8) as given in literature to express the quantity as copy number or genome equivalent. The uncertainty of this value is determined by the uncertainty of the DNA measurement technique and the genome size, as well as dilution and any absorption by the apparatus. However, we are not aware if any good estimates of uncertainty for the biological variation of the genome size as expressed in equivalent/mole or equivalent/g are available. Thus, the analyst needs to be cautious about using copy number calculations in reporting results.

Qualitative PCR

In a qualitative analytical setup, the PCR components are combined with DNA extracted from the unknown sample. If the DNA sample contains the target DNA sequence in question, this sample DNA will function as the template DNA that can be amplified successfully. Together with appropriate negative controls, detection of the correct PCR product indicates the presence of the targeted DNA sequence in the original sample. Absence of PCR product in conjunction with suitable positive controls implies the DNA target was not present in detectable amounts.

Qualitative PCR products are commonly analyzed by agarose or polyacrylamide gel electrophoresis (30). Applying a voltage will cause the negatively charged DNA to migrate and will separate DNA fragments according to their length. The very large numbers of identical DNA molecules that are the product of the PCR form a distinct band that can be visualized as UV fluorescence using the fluorophor ethidium bromide or other means.

At the end of the PCR, the intensity of the signals may vary between samples. However, the signal does not necessarily correlate in a linear way to the amount of target DNA that was present in the beginning of the reaction, primarily because PCR of this type stall and enter a plateau phase after large amounts of PCR product have been made, usually due to exhaustion of one or more substrate(s). Analyzing PCR products after the reaction is finished restricts the results to merely detected or not detected (positive or negative).

Qualitative PCR assays are used in 2 main ways. The first way is a simple test to determine whether the sequence in question is present in a bulk sample (usually flour or other processed material). The second way is semiquantitative. If the sample is made up of seeds or grain, or other discrete units,

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a number of test samples can be used to estimate the number of particles (seeds or grains) that contain the target analyte. As an example, instead of testing a single bulk of 1000 seeds or grains, the analyst can make 10 pools of 100 seeds or grains. The number of pools that test positive for the analyte gives an estimate of the number of positive seeds in the lot. This method works only when the percentage of positive seeds in the sample is low (typically below 5%). For example, if 5 out of 10 pools each containing 100 seeds test positive, then the calculated level of the adventitious presence is 0.69% and the 95% confidence limits are 0.21 to 1.66% (31). This is much more information than can be gained by testing a single pool, in which the answer would be "positive."

Another advantage of the semiquantitative approach is that the method can be applied in a range that is well above the LOD; thus, the likelihood of false-positive or -negative results will be significantly lower. Nevertheless, care has to be taken that contamination with fragments of seed, grain, or dust does not cause false-positive results.

Quantitative PCR

There are various approaches to quantification of GM material in a sample using PCR. In all cases, quantification by PCR determines the amount of GM DNA vs a reference DNA target (e.g., maize or soy DNA). This is not a direct weight-to-weight measurement. The following is a discussion of various real-time PCR chemistries, as well as different approaches for standard curve generation and data analysis.

Real-time PCR technology allows for the monitoring of fluorescence associated with amplification products throughout the PCR process. This technology is available with different types of fluorescent chemistries. Examples are fluorogenic probes (i.e., TaqMan®, FRET), Scorpion™ primers, and SYBR® Green.

With TaqMan fluorogenic probes, an additional oligonucleotide, located between the 2 primers required for amplification, is added. This probe is labeled with a fluorescent reporter dye and a corresponding quencher dye. The quencher dye absorbs the fluorescence from the reporter dye, and when they are in close proximity, no fluorescent signal is emitted. During the extension phase of the PCR, the polymerase breaks down the probe, thus physically separating the quencher dye from the reporter dye. This results in the reporter dye emitting a fluorescent signal upon excitation. The amount of fluorescence can be measured in real-time and used for quantification purposes.

With Scorpion primers, no additional probe is added, but 1 of the 2 PCR primers is specially configured and labeled with a fluorescent dye and a corresponding quencher, as with the aforementioned fluorescent probe. The primer is arranged to form a hairpin loop structure, enabling the quencher to be in close proximity with the reporter dye. During the extension phase, the Scorpion primer unfolds to hybridize with the new daughter strand, and the hairpin loop within the Scorpion primer disassociates, separating the fluorescent reporter dye from the quencher.

SYBR Green quantification is a completely different approach. It uses a dye (SYBR Green) that binds to double-stranded DNA and quantifies the amount of double-stranded DNA produced. SYBR Green will quantify both specific and nonspecific PCR products. In contrast, Scorpion primers and fluorogenic probes will only hybridize with specific PCR products downstream in the PCR, resulting in higher specificity and lower background noise than SYBR Green assays.

Using real-time PCR equipment, such as the ABI Prism® 7700 or 7900 (Applied Biosystems, Foster City, CA), LightCycler® (Roche Diagnostics, Indianapolis, IN), I-Cycler® (Bio-Rad, Richmond, CA), or the Mx3000P™ (Stratagene, La Jolla, CA), fluorescence can be monitored and analyzed through this process via computer interface. As previously mentioned, fluorescent signals will eventually plateau after a number of PCR cycles; therefore, end point fluorescence is not suitable for quantification. However, by plotting the fluorescence vs cycle number (Figure 2), and assigning a threshold within the exponential phase of the fluorescence amplification, the corresponding cycle number at which fluorescence crosses this threshold will be inversely proportional to the amount of DNA target in the sample. Therefore, this cycle number, referred to as cycle threshold (C_T), can be used to quantify target DNA amounts.

With the resulting quantitative data, real-time PCR techniques can be used to determine the percentage of a GM DNA sequence in a sample. Values can be determined for a GM target DNA and compared to total target species DNA, which is determined by use of a species-specific, preferably single-copy reference gene (an endogenous control gene). Quantification units will, therefore, be the amount of the GM sequence expressed as a percentage of the reference DNA (example, 1% RR soy DNA vs total soy DNA). PCR for GM and reference sequences can be performed either in separate tubes, or in the same reaction using reporter dyes that fluoresce at different wavelengths (multiplex). With multiplex reactions, individual component concentrations (i.e., primers, probes, deoxynucleotides) have to be carefully titrated and validated to protect against competitive effects between the 2 target amplifications. Multiplex PCR also introduces some errors into the final results because of limitations in fluorescence multicomponent analysis (32).

Quantification can be achieved with 2 approaches: One approach is to construct a standard curve and interpolate values into the standard curve to extract data. The second approach is the comparative C_T method (32).

There are 2 common ways of constructing a standard curve for quantitative analysis. One common way is the use of a serial dilution of DNA of known concentration and GM content. GM DNA from certified reference materials, plasmids, or hybrid amplicons (33) can be used for the standard curve. In any case, the target DNA copy number must be precisely quantified beforehand. Examples of DNA concentration quantification techniques are fluorometry (i.e., using Hoechst or Picogreen® dyes), melting curve analysis

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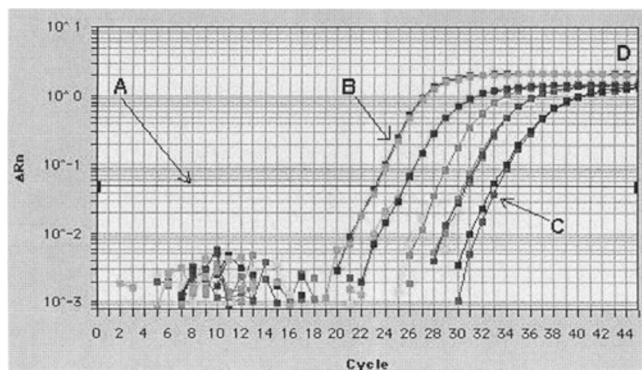

Figure 2. Amplification plot from Applied Biosystems ABI Prism 7700 showing serial dilution of sample. Each dilution was run in duplicate. The *y*-axis represents normalized, logarithmically converted fluorescence intensity data. The *x*-axis represents the cycle number. (A) Set threshold across samples' exponential phase; (B) sample with highest concentration has lowest C_T (point at which amplification curve crosses threshold); (C) sample with lowest concentration has highest C_T; (D) plateau phase; all samples have very similar fluorescence; quantification is not possible here.

(i.e., using SYBR Green), or spectrophotometric analysis. These were discussed previously (*see* above).

Plant GM DNA concentration can be converted to copy number equivalents by using conversion factors as reported by Arumuganathan and Earle (8) or by referring to the Web site "C Value" (34). However, genome sizes may vary depending on the variety within the species, so varietal information, if available, should be considered. If using certified reference materials (CRMs) of a certain percent GM, that percentage must be taken into consideration when calculating GM copy number equivalents as well.

With this technique, reference and GM C_T values from the sample can be interpolated against the corresponding standard curve to determine the copy number equivalents: Dividing the calculated GM copy numbers by the reference copy numbers will yield the result in percent GM target DNA versus total species DNA. Factors such as ploidy and copy numbers per genome of GM and reference gene have to be taken into account in order to relate DNA fraction to mass fraction.

A second common approach in construction of standard curves is the use of CRMs of different percent GM, measuring the difference in C_T values from the GM and reference target genes for each standard. This approach is referred as the ΔC_T standard curve method. ΔC_T values will be inversely proportional to percent GM, and the ΔC_T of the sample can be interpolated against the ΔC_T of the standards to approximate the sample's percent GM. Certified reference standards of some GM varieties are commercially available at 0, 0.1, 0.5, 1, 2, and 5% concentrations.

An alternative to standard curves is the comparative C_T method. This method is also referred to as the $\Delta\Delta C_T$ method (32). In this method, the amount of target, normalized to an endogenous reference and relative to a calibrator, is given by the following formula:

$$\text{Amount of target DNA} = 2^{-\Delta\Delta C_T}$$

in which

$$\Delta\Delta C_T = \Delta C_{T,\text{sample}} - \Delta C_{T,\text{calibrator}}$$

For this calculation to be valid, the efficiency of the reference and target amplifications must be equivalent.

Each of the 3 quantitative approaches discussed has its own advantages and limitations. The serially diluted standard curve method requires the smallest amount of validation and optimization. It is also less sensitive to variations in PCR efficiency and more rugged in its application with various sample matrixes. In contrast, it may be affected by dilution errors and may be biased against the particular standard used. The ΔC_T standard curve method uses more standards, and therefore reduces bias, but is more sensitive to variations in PCR efficiency and requires a greater amount of validation. It is very useful when DNA is consistently analyzed from the same sample matrix. Of all the quantification methods, the comparative C_T method requires the greatest amount of validation and optimization and is most prone to errors arising from changes in PCR efficiency. Nevertheless, in the event of a successful validation, this could be the most cost-effective and highest-throughput method, as it eliminates the need for a standard curve and also eliminates the error arising from standard curve dilutions. However, the inherent problems associated with the comparative C_T method usually restrict its application to 1 or very few sample matrixes and extraction methods, because it is unlikely that PCR efficiencies are constant.

Method Validation

There is sometimes confusion about the terms used to describe interlaboratory studies for the purpose of method performance, material performance, and laboratory performance studies. The reader is reminded that these are 3 distinct activities. The International Union of Pure and Applied Chemistry (IUPAC) classifies (35) interlaboratory studies into the following 3 categories: (*1*) *Method performance*.—Determines the bias and precision of an analytical method. (*2*) *Material performance*.—Assigns a value and an uncertainty (or reliability) to a characteristic (usually concentration) of a material. (*3*) *Laboratory performance*.—Permits the evaluation of each participant against preset criteria or criteria estimated from the study itself.

Although the procedures regarding statistical data evaluation from these 3 types of interlaboratory studies may be identical, the use and interpretation of the resulting statistical estimates will be determined by the primary purpose of the study.

The validation of methods consists of 2 phases. The first is an in-house validation of all of the parameters except

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reproducibility. The second is a collaborative trial, the main outcome of which is a measure of the repeatability and reproducibility in order to estimate the transferability of methods between laboratories. It is our experience that a small-scale collaborative trial should be performed to test the ruggedness of a particular method before the expense of organizing a large-scale trial is incurred. In case any improvement of the method or the method description is needed, only limited costs are incurred through the pretrial, whereas a failure of a full interlaboratory method validation because of an ambiguous method description is a very costly failure. Implementation of an already validated method in a laboratory needs to include the confirmation that the implemented method performs as well under local conditions as it did in the interlaboratory method validation.

A method must be validated by using the protocols and reaction conditions under which it will be performed. For example, the protocol should not be changed using higher or lower numbers of amplification cycles. These and other changes, as well as the application to a different matrix, are likely to affect method characteristics such as the specificity or sensitivity. PCRs may have a tendency of unspecific background amplification at low rates, which can be tolerated if the specified conditions and number of cycles do not result in analytical artifacts as demonstrated by validation. However, they can be expected to result in artifacts if operated with more cycles and/or under nonoptimal conditions.

This document deals primarily with the use of interlaboratory studies for the assessment of method performance. However, before use at a particular location, any method must be subjected to an in-house validation procedure.

The results of a determination are often expressed in terms of percent of a sample that contains a particular biotechnology-derived sequence. In a quantitative test, this measurement actually involves 2 PCR-based determinations: that of the primary analyte (e.g., an inserted gene sequence) and that of the endogenous or comparator sequence (e.g., an endogenous maize gene). Each of these determinations has its own uncertainties, and the 2 are likely to have different measurement characteristics. In most applications, the primary analyte will be present at low concentrations and the comparator will be present at concentrations 10–1000 times higher. Thus, it is important that both measurements are properly validated. In cases in which the measurement is expressed directly as a percentage (as in the use of ΔC_T), these factors must be considered when validating the method.

Validation Parameters

The method performance study or method validation establishes the performance characteristic for a specific method application, i.e., a specific analytical procedure for a well-defined scope. In the following text, the spirit of the most relevant terms has been captured by a simple definition for use in this document (Table 1). For a more detailed discussion and explanation of the definitions, refer to the Procedural Manual of Codex Alimentarius (36).

The concepts of a LOD, limit of quantitation (LOQ), and ROQ are not yet explicitly defined by Codex Alimentarius. These parameters can be considered to pertain to the applicability of the method. However, as they are useful for some applications, definitions are also given in Table 1.

The LOD is the amount of analyte at which the analytical method detects the presence of the analyte at least 95% of the time (<5% false-negative results). This and the false-positive rate are the only parameters required for a qualitative method, other than specificity.

Determination of an LOD is not necessarily needed to establish the validity of a method for a given qualitative application if it can be shown that the false-negative rate is <1% in the range of the application. For example, the precise determination of the LOD to be 1 ng/kg does not add much value when the scope of the method validation extends only for concentrations ranging in g/kg. Similar considerations apply for the LOQ. However, the range over which a method is applicable (ROQ) should always be established and included in the validation study.

Many quantitative methods are applied and have their most linear response near the LOQ. In such cases, it is important to know the LOD and LOQ in order to determine whether an observed result is, in fact, significantly different from the background, and can be satisfactorily quantified. In a quantitative method, it is common practice to assume that LOD is the signal strength of a blank increased by 3 times the standard deviation of the blank. However, this method gives at best an estimate, relies on normal Gaussian distribution of the blank measurements around zero, and may give a lower value than the actual LOD. Its use is not valid in methods such as quantitative PCR, in which the distribution of measurement values for blanks is typically truncated at zero and thus is not normally distributed. Thus, the LOD needs to be experimentally determined unless the targeted concentrations are well above the LOD, and the LOD, therefore, becomes irrelevant.

For a quantitative method, it is also important to know whether the LOQ for a particular matrix is close to the values to be measured. Again using a traditional approach, the LOQ has to be expressed as the signal strength of a blank increased by 6–10 times the standard deviation of the blank. These data must be experimentally determined, as discussed previously, unless it is known from other sources that the measured values range so high above the LOQ that this information becomes irrelevant. However, this method to determine the LOQ leads only to an estimate of the LOQ that may be an artificially high or low approximation.

In practice, 2 procedures have been used to determine the LOQ. The first approach is to assay a number of negative samples that have been supplemented (spiked) with known amounts of analyte. The LOQ is then the level at which the variability of the result and percent recovery of the analyte meet certain pre-set criteria. For small molecules, these criteria have typically been a coefficient of variation of 20 and 70–110% recovery (37). DNA recovery, however, may be difficult from some matrixes, e.g., starches or ketchup, and

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Table 1. Parameters for method validation

Accuracy	The closeness of agreement between the reported result and the accepted reference value. Accuracy describes how close the measured value is to the actual value of the known reference sample.
Precision	The closeness of agreement between independent test results obtained under stipulated conditions. Precision describes how well the results agree between repeated analyses of the same material. Less precision is reflected by a larger standard deviation of the combined results than the individual results.
Sensitivity	Change in the response divided by the corresponding change in the concentration of a standard (calibration) curve, i.e., the slope of the analytical calibration curve.
Specificity	The ability of a method to respond exclusively to the characteristic or analyte. Specificity describes how often the analyte is not detected.
Ruggedness (robustness)	The ability of a method to resist changes in results when subjected to minor changes in environmental and procedural variables, laboratories, personnel, etc.
Applicability	The analytes, matrixes, and concentrations for which a method of analysis is validated that may be used satisfactorily.
Repeatability	Precision under repeatability conditions. These are conditions in which independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time.
Reproducibility	Precision under reproducibility conditions. These are conditions in which test results are obtained with the same method on identical test items in different laboratories with different equipment and operators.
Limit of detection (LOD)	The lowest amount of analyte in a sample that can be detected with suitable confidence but not necessarily quantified as an exact value as determined by method validation.
Limit of quantitation (LOQ)	The lowest amount of analyte in a sample that can be quantified with suitable accuracy and precision as determined by method validation.
Range of quantification (ROQ)	The range within which the amount of analyte in a sample can be quantified with suitable accuracy as determined by method validation.

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lower recovery efficiencies may have to be accepted. When recovery efficiencies are low, this must be stated in the validation data and in the analytical report. The second, more complete approach is to test the method using a number of samples that contain known amounts of the GM material. This is more complicated, as it requires access to significant quantities of reference materials that contain a known range of concentrations of the GM event of interest. Procedures for assessing LOD and LOQ during the validation of qualitative and quantitative PCR methods are described in the following text.

Parameters Common to Qualitative and Quantitative Methods

A number of the parameters measured in a validation are common to both qualitative and quantitative methods; some are more applicable to the quantitative methods. Those common to both types of methods will be discussed first.

(**a**) *Specificity*.—Specificity is the starting point for a method and needs to be considered during primer design. Primers should be checked against the known sequence of the event insert and pertinent databases for possible matches. Experimental confirmation of the specificity must be performed. The following suggests a reasonable approach; the experiments should be performed during development or in-house validation of an assay before a larger validation is conducted.

For event-specific assays: (*1*) analyze a total of at least 10 sources, including nontarget GM events and any non-GM plants that may commonly be found as contaminants in the commodity; (*2*) test 1 sample from each source (total of at least 10 DNA samples); (*3*) analyze 2 replicates for each DNA sample.

Test results shall clearly indicate that no significant positive signal is observed.

For assays on plant endogenous genes: (*1*) analyze a total of at least 10 different plant samples that comprise different varieties of the same plant species as well as other plant species important for food production (such as wheat, rice, maize, potato, and soybean) and that may commonly be found as contaminants in the commodity; (*2*) test 1 sample from each source (total of at least 10 DNA samples); (*3*) analyze 2 replicates for each DNA sample.

Test results shall clearly indicate that no significant positive signal is observed.

(**b**) *Applicability*.—It is not feasible to provide reference materials for every one of the thousands of food matrixes available, so the use of a seed-derived or other such matrix reference will usually be necessary. The use of the method in a new matrix will need to be validated at a minimum via

in-house validation, which may be done using spike and recovery experiments, and the reference material used should be described on the report to the customer.

Validation of a Qualitative PCR Method

The following section concentrates on issues surrounding validation of qualitative methods. However, many of the principles also apply to quantitative methods, especially when they are used in a qualitative way. The sensitivity of a qualitative method must be shown to be such that it can reliably detect 1 positive particle (e.g., seed) in a pool and does not give rise to a significant number of false positives. A concept of using false-positive and -negative rates to describe the accuracy and precision of a qualitative assay has been developed for microbial assays (38). This concept can be applied to qualitative PCR assays. A critical issue in the validation of this type of method is the availability of test materials that are known to be positive and negative. The provision of negative reference materials is particularly important and critical in the case of a qualitative method. Any impurities present must be only at levels so low that they become negligible. Development of reference materials is covered later in this article.

(**a**) *False-positive and -negative results*.—By their very nature, qualitative tests result only in yes/no answers. The measures of precision and accuracy are the frequencies of false-negative and/or false-positive results. False-negative results indicate the absence of a given analyte when in fact the analyte is present in the sample; false-positive results indicate the presence of an analyte that is not present in the sample. Because of the inherent nature of the analytical technique, an increase in false-negative results will be observed when the amount of analyte approaches the LOD of the method. Like the LOD for quantitative methods, the LOD for a qualitative method can be defined as the concentration at which a positive sample yields a positive result at least 95% of the time. This results in a rate of false-negative results of 5% or less. During validation of a qualitative PCR assay, it is also important to determine the frequency of false-positive results (a positive result obtained using a sample that is known to be negative). Both false-positive and -negative results are expressed as rates (38).

(*1*) *False-positive rate.*—This is the probability that a known negative test sample has been classified as positive by the method. The false-positive rate is the number of misclassified known negatives divided by the total number of negative test samples (misclassified positives plus the number of correctly classified known negatives) obtained with the method.

For convenience, this rate can be expressed as percentage:

$$\%\ \text{false - positive results} = \frac{\text{number of misclassified known negative samples}}{\text{total number of negative test results [incl. misclassified]}} \times 100$$

(*2*) *False-negative rate.*—This is the probability that a known positive test sample has been classified as negative by the method. The false-negative rate is the number of misclassified known positives divided by the total number of positive test samples (misclassified positives plus the number of correctly classified known positives) obtained with the method.

For convenience this rate can be expressed as percentage:

$$\text{false - negative results, \%} = \frac{\text{number of misclassified known positive samples}}{\text{total number of positive test results [incl. misclassified]}} \times 100$$

In order to demonstrate the false-negative rate for a qualitative assay, a series of samples (e.g., grain/seed pools) with a constant, known concentration of positive material in a pool of negative material (e.g., 1 positive kernel in 199 conventional maize kernels) has to be analyzed and the results evaluated. The concept of confidence intervals and statistical uncertainty needs to be applied to the risk of false-positive and/or false-negative results as well. The desired level of confidence determines the size and number of pools that need to be tested. For example, 100 positive test results obtained from 100 independent measurements on truly positive samples lead to the conclusion that the level of false-negative results is below 4.5% at a confidence level of 99% for the tested concentration of positive kernels (expressed as the number of positive kernels in a pool of negative kernels).

(**b**) *Ruggedness*.—As with any validated method, reasonable efforts must be made to demonstrate the ruggedness of the assay. This involves careful optimization and investigation of the impact of small modifications that could occur for technical reasons.

(**c**) *Acceptance criteria and interpretation of results*.—A validated method includes criteria from which an observed measurement result can be accepted as valid. It is important to follow these criteria and to observe the rules for data interpretation. It is, therefore, important to ensure that the result of the positive DNA target control is positive. Similarly, the amplification reagent control (a control containing all the reagents, except extracted test sample template DNA, which is replaced by a corresponding volume of nucleic acid-free water or buffer) must be negative.

In addition to these controls, it is desirable to conduct a parallel reaction on the same DNA sample using a primer set that detects an endogenous single copy sequence. This reaction is performed on every DNA sample and can either be in the same reaction (multiplexed) or as a separate reaction. In the case of multiplexed reactions, it is important for the endogenous reaction not to out-compete the event-specific reaction for reagents, as the endogenous sequence is likely to be present at a rate up to 1000-fold the amount of the target sequence. The control reaction with the endogenous sequence can give an indication of the quality of the DNA as a template for the PCR.

The fact that qualitative PCRs are typically performed in duplicate introduces a further complication: The duplicates may not agree. It is common practice to repeat PCRs once on DNA samples that are rejected because of conflicting (indeterminate) results. A repeated indeterminate result indicates that the analyte cannot be reliably detected (Table 2) and that the assay is operating below the LOD as, by definition, a 95% or better detection rate would be achieved at

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the LOD. The sample is, therefore, scored negative. Similar criteria apply if more replicates are performed on each DNA sample.

Validation of a Quantitative PCR Method

A harmonized ISO/IUPAC/AOAC protocol was developed for chemical analytical methods. This defines the procedures necessary to validate a method (39). All the principles and rules of the harmonized protocol are applicable to quantitative PCR methods. The parameters involved in validation of the performance of a quantitative PCR assay include LOD, LOQ, ROQ, accuracy, precision, sensitivity, and ruggedness. Other important factors are acceptance criteria and interpretation of results, and the units in which the results are expressed.

A quantitative PCR assay typically consists of 2 assays: one determines the amount of DNA specific for the GM product; the other is specific for the amount of plant-specific DNA. Each of these assays is considered separately, because they are independent analytical procedures. Thus, all parameters listed below, including specificity and sensitivity, have to be assessed individually for each of the assays involved. A method validation for the whole assay cannot be appropriately performed unless both assays are validated individually.

(**a**) *Limits of detection and quantitation (LOD and LOQ)*.—If validation of the quantitative PCR assay shows that the assay can measure GM plant DNA at the required concentration with acceptable trueness and precision, then it is often not necessary to determine the LOD and LOQ, as the method is only being applied above the range where these are relevant. However, if the method is being used at concentrations close to the LOD and LOQ (typically 0.1–0.5%), then the assessment of the LOD and LOQ will become part of the validation procedure.

(**b**) *Range of quantification (ROQ)*.—The ROQ of the method defines the concentration range over which the analyte will be determined. Typically, the range for a GM product will run from a tenth of a percent up to a few percent; the endogenous control range will be close to 100%, unless the testing of complex mixtures is envisioned. This desired concentration range defines the standard curves, and a sufficient number of standards must be used to adequately define the relationship between concentration and response. The relationship between concentration and response should be demonstrated to be continuous and reproducible and should be linear after suitable transformation.

The quantitative method is designed to operate in the range of 0.1–100% (DNA %, w/w). However, it is common to validate a method for a range of concentrations that is relevant to the ROQ of the application. If a method is validated for a given range of values, the range may not be extended without validation. For certain applications (e.g., seed or grain analysis), the use of genomic DNA for the preparation of the standard curve (*see* discussion on the use of plasmid DNA below) may be considered. Although it is easy to establish a nominal 100% standard (limited only by the purity of the materials used), it is difficult to reliably produce standard solutions <0.1%. This is due to the uncertainties involved in measuring small volumes and the error propagation if serial dilution steps are applied. Additionally, the number of target sites (DNA sequence to be amplified) becomes so small that stochastic errors will begin to dominate and no reliable analysis is possible (9, 40). If genomic DNA is chosen to be used as calibrator, it is important that this calibrator be traced back (in its metrological meaning) to a reference of highest metrological order, e.g., a CRM. The range will be established by confirming that the PCR procedure provides an acceptable degree of linearity and accuracy when applied to samples containing amounts of analyte within or at the extremes of the specified range of the procedure.

(**c**) *Accuracy and precision*.—The accuracy of a method should be compared to known values derived from reference materials. This may be a challenge in this field because of the limited availability of such materials. However, the accuracy must be compared to the best available reference material. Precision will be determined in the usual way from single-laboratory (repeatability) and multilaboratory (reproducibility) studies.

(**d**) *Sensitivity*.—A linear relationship of the C_T as a function of the logarithm of the concentration of the target should be obtained across the range of the method. The correlation coefficient, *y*-intercept, slope of the regression line, and percent of residual should be reported. The percent of residual for each of the calibrators should preferably be ≤30%.

In order to obtain a standard curve for event-specific quantitative assays, standard DNA mixtures can be prepared by combining purified genomic DNA from GM and non-GM plant material such as seed or leaves. The content of GM plant DNA in the mixtures might be 100, 50, 10, 5, 1, 0.5, 0.1, and 0% or as appropriate for a smaller concentration range. Three replicates should be analyzed for each point on the standard curve.

For quantitative assays on plant endogenous genes, standard DNA mixtures can be prepared by combining purified genomic DNA from the target plant species and that of a nontarget plant species. For example, for validation of a maize ADH1 quantitative assay, the target plant species is maize and the nontarget plant species could be soybean or another species. The content of DNA of the target plant species in the mixtures is typically 100, 90, 80, etc., and 0% or

Table 2. Criteria for scoring duplicate qualitative PCR analyses

Lane 1	Lane 2	Scoring of test
+	+	Positive
–	+	Repeat/indeterminate
+	–	Repeat/indeterminate
–	–	Negative

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as appropriate. Three replicates should be analyzed for each point on the standard curve. When the ΔC_T method is used, it will be the responsibility of the analyst to ensure that the overall amount of DNA is within the range for which the assay was validated.

(**e**) *Ruggedness*.—The evaluation of ruggedness (robustness) demonstrates the reliability of a method with respect to inadvertent variations in assay parameters. Variations that may be included are reaction volumes (e.g., 25 vs 30 μL), annealing temperature (e.g., plus and minus 1°C), and/or other relevant variations. The experiments need to be performed at least in triplicate, and the recovery needs to be calculated. The response of an assay with respect to these small changes should not deviate more than ±30% from the response obtained under the original conditions.

(**f**) *Acceptance criteria and interpretation of results*.—A validated method also includes criteria on which the observed measurement result can be accepted as valid. It is important to follow these criteria and to observe the rules for data interpretation. If a case calls for the deviation from said criteria and rules, a new method validation study would be needed to demonstrate the validity of the new rules and procedures.

At a minimum, the following acceptance criteria are common to all quantitative PCR methods and applicable to each PCR run: (*1*) the result of the positive DNA target control with, for example 1% GM DNA, the mean of the replicates deviates <3 standard deviations from the assigned value. A target DNA control is defined as reference DNA or DNA extracted from a certified reference material or known positive sample representative of the sequence or organism under study. The control is intended to demonstrate the result of analyses of test samples containing the target sequence. (*2*) The amplification reagent control is negative. The amplification control is defined as a control containing all the reagents, except extracted test sample template DNA. Instead of the template DNA, a corresponding volume of nucleic acid-free water is added to the reaction. (*3*) The percent of residual for each of the standards should be <30%.

To accept the result of an unknown sample, the relative standard deviation of the sample replicates should be <30%.

Reference Materials

General Considerations

Reference materials play a number of roles in development, validation, and troubleshooting of PCR-based diagnostics, as well as in the routine conduct of such assays. In the context of assay validation, positive reference materials are used to establish the accuracy, precision, sensitivity, LOD, and false-negative rate in quantitative assays. Negative reference materials are very important in determining false-positive rates and specificity.

Reference materials can be of several levels of metrologic quality: (*1*) *A certified or standard reference material (CRM or SRM®)* is accompanied by a specific certificate. This certificate states that one or more of the property values of the reference material is certified by a procedure that establishes the value's traceability to an accurate realization of the unit in which the property value is expressed; in addition, the certificate states a level of confidence of uncertainty (41). Such reference materials are usually issued by National Metrology Institutes such as the Institute of Reference Materials and Measurement (IRMM) of the Joint Research Center of the European Union and the National Institute of Standards and Technology (NIST) in the United States. (*2*) *A reference material (RM)* is a reference material or substance one or more of whose properties are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials (ISO definition). (*3*) *A working standard (WS)* is a secondary standard in regular use. This working standard is equivalent to RM if it is quantified/characterized by comparison with the CRM/SRM.

Quality Standards for Reference Materials

A certificate of analysis will accompany each reference material. The certificate will describe the characteristics of the material, both as to the presence of the target material and the absence of other possibly interfering materials. In addition, a reference material may even be restricted as to the method for which it can be used or is validated.

A certificate of analysis for a GM reference material will address the following factors: (*1*) target event or sequence; (*2*) adventitious presence of other events; (*3*) strength and purity; (*4*) genetic background.

Target event or sequence.—The first consideration is whether the material is a reference for a particular event. In most detection processes, this will be the case. However, it is foreseeable that many reference materials or working standards used in screening methods will be for a certain sequence, such as the 35S promoter. Event reference materials can be used for this purpose, providing it is known how many copies of the sequence are present in the event.

Adventitious presence of other events.—An important factor that will influence the true concentration of the samples prepared for the validation experiments is, of course, the level of impurity in the reference materials. The starting material used for the preparation of the reference materials needs to be characterized for purity with respect to the desired analyte. To do so, a representative subsample must be analyzed for the absence or presence of the analyte in both negative and positive pools. The sample size and measurement error will determine the confidence level of the results.

Positive reference materials are considered to be those that contain the event or sequence of interest (e.g., MON810, T25, 35S, NOS). Negative reference materials are materials that do not contain the stated event or sequence, at least not at a detectable level. A negative reference material will not have a value of 0% assigned. The known presence or absence of other events that may interfere with the analysis should be stated. As with all analytical blanks or negative and positive reference materials, an uncertainty about the assigned values needs to be expressed. Ideally, this uncertainty includes 0%

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for the negative materials. For example, regarding Roundup Ready® soybeans, currently available negative reference materials (Joint Research Center, IRMM, Geel, Belgium) are assigned to contain <0.03% Roundup Ready soybeans (42). This value reflects the uncertainty of the methods and sampling procedures used for the certification exercise of the materials. This is particularly the case for any material prepared from grain, as it is not feasible to test every grain kernel before using it in a reference material. Thus, a reference material certificate will typically state that it contains less than a particular amount (e.g., <0.03%) of other events, which should be listed explicitly, with a known certainty (e.g., 99%). Similar restrictions or considerations are applicable for all reference materials used in analytical sciences.

Strength and purity.—Strength and purity are 2 measurements that normally are determined for a reference material. However, how the strength and purity of a GM reference material is defined is not yet clear.

(*1*) *Strength*.—In some cases, it can be clearly stated that a sample is of 100% strength. For example, if DNA is prepared from a single homozygous plant, this DNA sample can be considered to have a strength of 100%, if a procedure has been used that produces essentially uncontaminated material. If the DNA was produced from a heterozygous plant, then it can be argued that the strength is 50%. This, however, ignores the effect of chloroplast DNA, which may dilute the sample to below 100 or 50%, depending on the definition. Special considerations may apply for hemizygous maize grain material in which the strength may depend on whether the applicable gene was introduced via the male or female parent, resulting from variations in zygosity of the tissues in the kernel.

(*2*) *Purity*.—Classically, purity is the percentage of the sample that consists of the material. For example, if a sample contains >99% DNA, then its purity would be >99%.

Strength and purity also may depend on loss of particulate material during any sieving process, unless such material is reground and added back to the sample. These factors may be expressed in alternative terms, such as the mass fraction (w/w) of the stated material.

Genetic background.—Many reference materials are prepared by mixing GM with non-GM materials. Comparison of the 2 sources as to whether they consist of near-isogenic lines may be included, if desired.

Choice of Reference Material

There are a number of matrixes that can be used to develop reference materials or working standards for methods of detection of GM products. Each has its own advantages and disadvantages for particular purposes. The 3 reference materials discussed here include: (*1*) grain or seed or seed-derived powders; (*2*) GM DNA of plant origin; (*3*) plasmid DNA or amplicons containing the target sequence.

For purposes of this discussion, the term "seed" will indicate both grain and seed. Seed is the preferred reference material for testing of grain and oilseed commodities. When dealing with hybrid crops, seed is preferred, as grain will be segregating for the trait. Both hybrid and inbred seed may be acceptable, provided one is cognizant of potential bias that can be introduced by both approaches. In the case of hybrid seed, the presence of a triploid endosperm in maize and other cereals will make a variable contribution to the number of PCR targets, depending on whether the GM parental line was the male or female parent. Use of inbred seed as a positive reference standard for a hybrid crop (e.g., maize) will result in underestimation of GM content that is present as hemizygous material if not corrected appropriately.

In addition to these reference materials, there may be reference materials produced that consist of other plant organs, or refined and finished products. Examples of such materials may be leaves, starch, and lecithin. It is beyond the scope of this paper to discuss these types of materials.

(**a**) *Grain or seed or seed-derived powders*.—Given the present commercial and trade situation and the present target crops for genetic modification, seed is the most commonly used reference material. Unfortunately, seed may be heterogeneous and the uncertainty factor caused by sampling is significant at all levels. This is particularly true when the reference materials contain <1% of the event in question, unless very large samples are supplied. For example, testing for the presence of any GM seed in a sample of maize or soybeans at a level of 0.1% would require testing 10 000 kernels (or roughly 3 kg) to achieve a 99% confidence in the analytical result. Even in this case, there will be uncertainty in the actual number of positive kernels in any sample. To use so much material for each reference point is not practical, and thus, seed powders are used widely.

However, seed mixtures are particularly useful for validation of qualitative methods. These validations can be made using pools of seeds. When preparing the pooled samples for a validation study, the following errors must be taken into consideration: (*1*) the positive kernel(s) may contain a small amount of negative kernels with respect to the desired analyte; (*2*) the negative kernels may contain a small amount of positive kernels.

Consequently, the pools that are prepared for use in the validation study could be impacted in the following ways: (*1*) the number of spiked kernels is smaller than calculated, i.e., 1 or more of the spiked positive kernels was actually negative; (*2*) the number of spiked kernels is larger than calculated, i.e., the negative kernels contained 1 or more positive kernels.

Table 3 summarizes the probability that a given negative seed bulk contains at least 1 positive (GM trait) kernel. Table 3 shows that, for large seed pools and high impurity levels in the negative material, the chance of observing at least 1 positive kernel in the pool is essentially 100%. Consequently, when operating with large pools, the purity of the negative materials needs to be extremely high. In order to assess all variations of the true value for a spiked pool, the impurity of the positive material (the spike) must therefore be taken into consideration.

Table 4 gives examples of the effect of purity in the situation in which seed pools are spiked with GM material to

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Table 3. Probability that a given negative seed bulk contains at least 1 GM trait seed

	Conventional reference material impurity level		
Seed builk size	0.01%	0.10%	1.00%
100	1	10	63
200	2	18	87
250	2	22	92
300	3	26	95
500	5	39	99
600	6	45	100
1000	10	63	100

achieve a target of 1% level of GM seeds in non-GM seeds (6). It illustrates the high level of purity of the negative material that is needed if large seed pools with a small number of kernels carrying the GM trait need to be prepared, as may be the case when very sensitive qualitative methods are tested. The purity of the negative material is much more important than the purity of the positive material because much more negative material than positive material is used to build the pools.

For example, pools of 300 kernels targeted to have 1% GM kernels are composed of 3 positive and 297 negative kernels, pools of 600 kernels are composed of 6 positive and 594 negative, and pools of 1000 kernels contain 10 positive kernels and 990 negative kernels. In constructing the table, a variation of approximately 20% was assumed. Thus, the table shows the frequency of pools of 300 kernels containing at least 2, but at most 4 positive kernels. (For a pool size of 600, 5–7 positive and for a pool size of 1000, 8–12 positive kernels were tolerated.)

Seed powders are a compromise that best mimics the genuine test material and will be processed in a manner similar to the test material, while avoiding the need to prepare large amounts of mixtures of positive seeds in pure negative seeds. Thus, matrix effects and extraction-related artifacts should be similar between control and test samples. They can be prepared with a known particle size and can be tested for homogeneity and accuracy with respect to the expected value. Seed powders do have considerable preparation and storage costs and must be tested for stability. Some seed-based reference materials of this type are currently commercially available (IRMM, Geel, Belgium).

Preparation of reference materials from seed is complicated by the particulate nature of such material, which necessitates statistical considerations as described above. The starting material can be a sample that contains a certain number of negative kernels and a certain number of positive kernels. The purity of the sample of negative kernels is subject to limitations because of the inability to completely test the sample; the estimate of purity is based on a subsample of a larger sample from which the negative material also is derived. Thus, it is never possible to be 100% sure that a supposed negative reference material is negative. It can only be established that the amount of positive material is less than the LOD of the method used to establish that the sample is negative and will be additionally restricted by statistical

Table 4. Number of pools (out of 100) that contain the given number of GM seeds when pools are prepared at a 1.0% target concentration

		Pool size		
		300	600	1000
GM trait, reference material impurity, %	Non-GM, reference material impurity, %	2–4 GM seeds	5–7 GM seeds	8–12 GM seeds
4.0	0.01	100[a] (98, 100)	98 (95, 100)	100 (98, 100)
4.0	0.10	97 (93, 99)	89 (84, 94)	94 (90, 98)
4.0	1.00	23 (16, 30)	3 (1, 6)	1 (0, 2)
2.0	0.01	100 (99, 100)	100 (98, 100)	100 (99, 100)
2.0	0.10	97 (93, 99)	89 (83, 94)	93 (89, 97)
2.0	1.00	21 (15, 29)	2 (0, 5)	0 (0, 2)
1.0	0.01	100 (99, 100)	100 (99, 100)	100 (100, 100)
1.0	0.10	97 (93, 99)	89 (83, 94)	93 (88, 97)
1.0	1.00	21 (14, 28)	2 (0, 5)	0 (0, 2)
0.5	0.01	100 (100, 100)	100 (99, 100)	100 (100, 100)
0.5	0.10	97 (93, 99)	88 (83, 93)	93 (88, 96)
0.5	1.00	20 (14, 27)	2 (0, 4)	0 (0, 1)

[a] Median and 5th and 95th percentiles (in parentheses) from distribution of number of seed bulks with specified number of GM trait seeds.

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sampling considerations, most notably by the size of subsample(s) analyzed.

A further complication in comparing a sample to a reference material is present when material is analyzed for the presence of maize or other monocotyledonous material. The various tissues of the maize kernel are of maternal origin, or receive different contributions from the male and female parent. This will give rise to a different result when the proportion of GM DNA is measured in a sample, depending on whether the GM component came from the male or female parent. This is particularly relevant when testing for adventitious presence of small amounts of GM material, in which the adventitious material may have arisen by incoming pollen, or by seed mixing. The analyst should be aware that these 2 sources would give rise to different results, even though the analyst can do little or nothing to solve the problem. In addition, processed samples arising from starchy endosperm, the germ, or the pericarp can be subject to similar issues. The issue of parental contribution to the DNA content of different tissue must be addressed when preparing reference material, and ideally, the type of material (inbred, hybrid material with description of the cross direction) should be included on the certificate of analysis.

Preparing reference material often involves the preparation of materials containing a small percentage of GM in a background of non-GM material. Some authorities prefer to use mixtures of the GM seed with what they define as near-isogenic seed. Near-isogenic lines are ideally lines that differ only in the gene of interest, although this is impossible to achieve in practice because of the nature of plant breeding and the seed multiplication steps that are necessary to produce enough material for reference seed production. This approach could lead to obtaining seed that is alike in size and composition. However, seed for use in the preparation of reference material must also be of very high purity, and therefore the GM and non-GM plants must be grown in isolation from each other, either physically, or in time. To do so is particularly important for an outcrossing crop such as maize. Thus, environmental effects will likely overshadow most possible benefits that could be obtained by using a near-isogenic line. In a real-life situation, however, the presence of any GM-seed in a conventional seed lot can be the result of mechanical mixing or cross-pollination. Both effects will invariably lead to GM kernels that are not isogenic to the bulk of the material with which they are commingled.

(**b**) *GM DNA of plant origin*.—Using GM DNA derived from plant materials other than seed provides somewhat more flexibility than using seed or powders as a reference material. The storage of DNA solutions may also be easier.

One approach to using GM DNA is to use spiked mixtures of seed as a starting point for a positive control. Seed that has been tested and shown to contain below a threshold of the accepted level of adventitious material is used as a starting point for the negative control. This approach has some of the same sampling issues that are associated with using seed, at least concerning the initial material. However, the actual reference material can be tested for homogeneity, and relatively small amounts are required.

Another approach to using GM DNA is to use fresh plant (leaf) tissue as the source of the DNA. Negative controls can be derived from a known non-GM plant; positive controls are derived from mixtures of positive and negative GM DNA to simulate various percentages of GM presence in grain. In this case, it is possible to establish that a particular plant is in fact negative, so that a true negative control can be obtained by use of plant DNA.

The disadvantage of directly using DNA as reference material is that it cannot be used to control for extraction and matrix-related artifacts. Additional biological factors may also play a role; as discussed elsewhere in this article, different tissues may exhibit differences in their genetic makeup. For most agriculturally relevant crops, leaves would not be a relevant commodity, as typically grain is used for food (e.g., maize, soy, canola, wheat, barley). And, as with any reference material, stability may be an issue; the stability of a DNA-based reference material should be tested and documented in the certificate of analysis.

Plasmid DNA or Amplicons Containing the Target Sequence

Using a plasmid or amplicon (or cloned fragment) containing the cloned target sequences may be attractive for certain purposes, such as protocol optimization and troubleshooting, as well as for an additional positive control. Those using plasmid or amplicon DNA as reference material usually validate the assay using the matrix of interest and only then use the plasmid as reference. As with GM DNA, use of plasmid or amplicon DNA ignores matrix effects compared to the test material. The reference DNA is compared to the reference material and is therefore traceable back to the physical standard. However, this type of DNA has some special characteristics.

It is possible that plasmid DNA may behave differently in a reaction if presented as a closed circular, relaxed, or linear form. In addition, measuring the actual copy number of plasmid or amplicon DNA added to a reaction poses special challenges. There is no accurate method to measure such small amounts of DNA, so the amount added must be inferred from a dilution series, which may have to take into account the absorption of low concentrations of DNA onto surfaces. In addition, the presence of concentrated plasmid solutions in laboratories poses a potent contamination hazard.

Summary of Reference Material Considerations

In summary, the analyst conducting PCR to detect the presence of a GM material in seed or processed materials must make a number of decisions. A key decision is the type of reference material to use. This decision will be influenced by the availability of reference materials and any consideration of matrix effects. In any case, each method should be validated in the laboratory using a reference material of the highest metrological standard available (SRM or CRM if possible).

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The laboratory may then use a reference material or working standard that has been calibrated back to the CRM/SRM.

Sources of Errors

Biological Sources of Errors

In determining the percent GM value for an unknown sample, the laboratory must convert the analytical result (copies of the GM gene/copies of the endogenous gene) into a percent GM value (weight to weight). This conversion assumes there is a direct 1:1 relationship between the endogenous control gene and the GM gene. However, there are many biological factors that can affect this 1:1 relationship and, as such, this basic assumption is not valid in many circumstances. Of most significance is the effect of biological factors on the 1:1 relationship. This effect is most pronounced in maize and wheat grains and grain products, but soybeans and cotton are not exempt from the basic physiological issues discussed below. In this discussion, we will focus on the major factors that impact the 1:1 ratio assumptions. A number of these impacts appear easy to account for or to develop an adjustment factor for. However, it is important to remember the test portion used for testing likely contains a mixture of GM events, and there is no understanding of the relative contributions of these events in the test portion. As such, the use of conversion factors to account for differences in copy numbers is not readily possible. In addition, there are a number of issues that arise from plant breeding and physiology that can impact the conversion factors. In this discussion, maize and maize products will be used as the basis for discussion.

(**a**) *Hybrid status*.—A large proportion of maize grown in the world is produced by using hybrid seed. However, the grain produced for this seed and used for the production of food and feed does not maintain the homogeneous genotype and segregates the traits based on simple Mendelian inheritance patterns. The relationship between the GM gene and the endogenous gene can be significantly affected, depending on whether the GM gene comes from the maternal or paternal parent, or both. The most significant effects of hybrid status appear in the endosperm fraction of maize products, based on its triploid condition, and are discussed under tissue type effects below.

(**b**) *GM gene copy number*.—Several laboratories use the 35S and NOS screen to quantify the presence of GM in grain and grain products. With this approach, the testing laboratory assumes that the endogenous gene and the GM gene are present in a 1:1 relationship in the GM grains present in the sample. This relationship is correct in some of the GM events currently on the market. However, it does not hold true for all of the GM events that could be present in a grain or grain products. For example, the maize event Bt11 contains 2 copies of the 35S promoter for every 1 copy of the maize (endogenous) gene in the DNA extract. In this specific case, the percent GM level in this sample could be overestimated by a factor of 2×. In general, the assumption that there is a 1:1 ratio generally leads to the overestimation of the amount of GM present in the sample (43).

(**c**) *DNA degradation*.—There is an assumption in the testing of grain and grain products that the DNA present in the grain is of good quality and present in all cells. The goal of the analytical laboratory is to remove DNA in as intact a form as possible and subject it to the PCR analysis. All DNA testing laboratories acknowledge that DNA quality is a critical element in performing a rugged analytical test. The presence of high-quality DNA in mature grain, however, may not be an accurate assumption. There is evidence that the cells of the endosperm undergo apoptosis, or programmed cell death, during the development of the maize kernel (44, 45). One hallmark of apoptosis is the degradation of the nuclear DNA into small fragments. These studies show that most of the DNA in mature kernels is degraded to some degree.

(**d**) *DNA endoreduplication*.—Endoreduplication results when replication is not coordinated to the cell cycle. Endosperm development in maize is characterized by a period of intense mitotic activity followed by a period in which mitosis is essentially eliminated and the cell cycle becomes one of alternating S and G phases, leading to endoreduplication of the nuclear DNA. This leads to the polyploidization, in which a single cell can contain several copies of the genome. This process is initiated with the onset of starch and storage protein synthesis and results in polyploidy values ranging from 6C to 96C. (The DNA amount in the unreplicated gametic nucleus of an organism is referred to as its C-value, irrespective of the ploidy level of the taxon; 46, 47). This biological process combined with apoptosis challenges the assumption that maize kernels are a good source of genetic material for quantification of percent GM, as it impacts the relative contribution of germ and endosperm representation in ground maize kernels. This complexity in genetic contribution and copy representativeness must be taken into account when quantitative PCR is used.

(**e**) *Outcrossing vs inbreeding*.—In the production of maize grain in the field, there are 2 sources of pollination. Within any field, a majority of the kernels will be fertilized with pollen by plants within the same field. In this case, the plant population will maintain the same average genetic constitution as original seed materials. In the production of non-GM maize, external pollination from neighboring fields of GM maize contributes to the production of maize with adventitious GM material. In this case, the resulting maize grain does not contain the same ratio of GM copies to the endogenous control.

(**f**) *Variability in the genome*.—One major assumption during the development of the PCR testing metrics for GM is the calculation of the number of copies in the PCR. This value is calculated to determine the lowest GM concentration that can be estimated from the test portion subjected to PCR analysis. Generally, the size of a genome is estimated for each species and this value is used to calculate such terms as the theoretical LOD. The assumption that maize varieties are consistent in genome size (pg/2C) is not supported by the

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published literature. For example, the maize genome size is shown to vary by up to 40% (8, 48). This is not restricted to widely disparate maize accessions, and significant variation can exist within a single seed company's breeding program (49). A high degree of variability is not restricted to maize, and similar variability can be found in soybeans (50, 51).

(**g**) *Effects of grain processing*.—One of the first steps in processing maize into a food ingredient involves the separation of embryo (germ) and endosperm. The germ is further processed for the extraction of maize oil and production of maize gluten feed. The endosperm is processed to produce starches, syrups, and maltodextrins.

The embryo and endosperm are significantly different at the genetic level. During the development of a seed, one of the 2 pollen nuclei fuses with the egg cell. This diploid cell type continues to develop into the embryo. The second pollen nucleus fuses with 2 polar bodies, producing a triploid cell type; this forms the endosperm of the developing seed. As such, the embryo maintains a diploid status (1:1 relationship), but the endosperm does not, and the proportion of endogenous gene to GM gene can be either 2:1 or 1:2, depending on hybrid status. Based on this fact, it may be inappropriate to assume a 1:1 ratio of the GM gene:endogenous gene when testing food ingredients or food products for the percent GM level.

Analytical/Instrumental Sources of Errors

Total analytical error (or measurement error) refers to assay errors from all sources derived from a data collection experiment. The accuracy and precision of a PCR method for GM detection or quantification are subject to influences of total analytical error. Total analytical error is of paramount importance in judging the acceptability of PCR-based GM detection or quantification methods. Errors in PCR assays can be classified as follows: (*1*) random (indeterminate); (*2*) systematic (determinate).

(**a**) *Random error.*—Because the intrinsically uncertain nature of the measurement technique is the source of random error, this kind of error occurs in every analysis and is not predictable. The amount of random error can be greatly increased or decreased by a variety of factors in PCR-based GM detection or quantification methods. Such factors include the number and complexity of steps in the method, the number of replicates, the skill of the analysts who perform the assay, and the laboratory conditions. For example, 2 analysts with different skills in PCR technique in the same laboratory or 2 analysts with similar skill in 2 different laboratories could produce different results using the same sample and same assay procedure. Therefore, a rugged PCR assay is a very important factor in reducing random errors. Random errors can be reduced by increasing the number of data points and calculating the mean of these data. The average of a large number of data points that are affected only by random error is always accurate.

In a single qualitative PCR, a proper number of replicates per sample is essential to reduce random errors. In the quantitative PCR method, 3 repeats per sample are the minimum acceptable for the collection of each data point. Repeatability and reproducibility standard deviations are usually used to estimate random errors. Good statistical practices, such as the measurement of coefficient of variation (%CV) or the repeatability standard deviation (RSD_r), need to be implemented in a quantitative PCR method and will aid the analyst in the evaluation of detection methods and the performance of such a method under local conditions. Generally, a CV value of replicates should not exceed 30%.

(**b**) *Systematic error.*—Systematic errors cause results to deviate from the expected or true values in a constant manner. Sources include improper instrument calibration procedures, insufficient purity of reagents, and improper operation of the measurement instrument. Generally, systematic errors cannot be reduced by the application of statistical methods, such as taking the average of replicate measurements. This kind of error may often be identified by careful validation and data analysis and subsequently minimized by modifying the analytical procedure.

The quality of reagents used in DNA extraction, PCR amplification, and labeled probes in a quantitative PCR assay can affect the test results. DNA quantification is one of the key steps in a PCR-based assay. Instrument errors caused by a malfunctioning UV-visible spectrophotometer or fluorescence spectrophotometer (for instance, an expired bulb for emission/excitation light sources), or improper operation of the instruments will affect the accuracy of DNA measurements, resulting in errors in downstream applications. Routine use of pipet devices in PCR-based assays is another source of instrumental errors. All pipet devices require calibration on a regular basis.

The most critical instrument in PCR-based methods is a thermal cycler. Because temperature change, especially in the annealing step, can alter PCR amplification efficiencies, temperature verification of a thermal cycler is recommended. Temperature changes in the heat and cooling block of a thermal cycler can be checked by using a verification system, and many manufacturers will offer this as a service.

Real-time PCR technology has been extensively developed in recent years. Several different types of instrument are commercially available. They differ in design and many specifications, including the heating and cooling system, source of excitation/emission light, detection range of fluorescence, and calculation algorithm (software). For this reason, a quantitative method using real-time PCR technology should be specific to certain types of instruments (e.g., block cycler). Application of an assay across instruments of different platforms without calibration or validation will result in errors.

(**c**) *Quality control*.—In order to reduce the total analytical error, quality control steps such as training of analysts, standard operation protocols (SOP), and regular instrument maintenance should be implemented throughout the assay process. Good laboratory practice (GLP), quality assurance systems according to ISO17025 (52), or other equivalent quality assurance management systems are highly recommended. Quality assurance schemes may be required in laboratories or facilities where PCR-based methods are used

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for GM detection and quantification in order for the results to be accepted in some countries. Tools from statistical process control (SPC) can be used to objectively evaluate the nature (random error vs systematic error) and amount of measurement uncertainty.

Conclusions

PCR technology is often used for the detection of products of agricultural biotechnology. It is critical that such methods are reliable and give the same results in laboratories across the world. This can be achieved by proper validation of the methods. The choice of the appropriate reference material will impact the reliability and accuracy of the analytical results. It is important that analysts pay proper attention to the effect of specific matrixes on the methods. In addition, numerous biological and analytical factors need to be taken into account when reporting results. This is particularly important when interpreting quantitative data.

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