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POLYMERASE CHAIN REACTION (PCR) TECHNOLOGY: DETECTION OF GM DNA & AGRICULTURAL BIOTECHNOLOGY PRODUCTS

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ABSTRACT

The principle of a PCR reaction can be generalized as the, *in-vitro* replication of small fragment of template DNA upto a desired size (< 10 kb). The DNA product formed after PCR are known as amplicons. Amplicons are increased in number exponentially after completion of each cycle. It includes separation of two opposite DNA strand by heat denaturation instead of enzymatic unwinding occurs in cells. 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. PCR can be used in two 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. Polymerase Chain Reaction 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 only be achieved by proper validation of the methods.

Key words: PCR reaction, protein-based test technology, GM DNA, agricultural biotechnology.

INTRODUCTION

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. In 1983, Karry Mullis and co-workers have developed PCR technique to replicate fragments of DNA by semi-conservative method but under in-vitro condition. The use of this invention for crop improvement was widely accepted in the areas of research like detection of plant pathogens, development of genetic or physical map of crop genome, cloning and characterization of existing or new trait responsive genes and their expression pattern analysis, genetic diversity analysis and marker assisted breeding for development of new crop varieties [1-2].

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 [1-3].

Principle of PCR

The principle of a PCR reaction can be generalized as the, in-vitro replication of small fragment of template DNA upto a desired size (< 10 kb). The DNA product formed after PCR are known as amplicons. Amplicons are increased in number exponentially after completion of each cycle. It includes separation of two opposite DNA strand by heat denaturation instead of enzymatic unwinding occurs in cells [3-7].

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, two primers are involved and the DNA section in between the distal ends of their binding sites is replicated during the reaction [4-7].

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A single cycle of the PCR and the corresponding temperature profile are typically divided into the 3 phases [7]:

1. denaturation,
2. annealing, and
3. elongation.

At the end of this procedure, the targeted DNA region has been replicated into two 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. The kinetics of the DNA reproduction resembles 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.

Applications of PCR Technology

The agricultural biotechnology industry applies PCR technology [5-7] at numerous steps throughout product development, much as it does with immunoassays. 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 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. The grain handling and grain processing industry uses PCR to certify compliance with contracts between buyer and seller.

PCR testing is used for four specific purposes in the grain handling/processing industry [4-7]:

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.

Applications of PCR technology in crop improvement

Applications of PCR

PCR can be used in two 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. This list is not considered exhaustive.

The following factors are known to influence the accuracy and precision of DNA quantification by UV spectrometry [5-7]:

(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 mg) of DNA of almost pure quality. DNA extracted from certain food matrixes is unlikely to meet this requirement.

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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.

Thus, all DNA quantification methods have their strengths and weaknesses, although the spectroscopic determination with absorbance at 260 nm is commonly used.

Various approaches to quantification of GM material in a sample using PCR are used in different laboratories. In all cases, quantification by PCR determines the amount of GM DNA versus a reference DNA target (e.g., maize or soy DNA,). This is not a direct weight-to-weight (of seed) measurement. The manuscript discusses in detail the various Real-Time PCR chemistries, as well as different approaches for standard curve generation and data analysis with their respective benefits and drawbacks. It is the responsibility of the analyst to understand the limitation of each method and select the most appropriate analytical approach [6-7].

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 poly acrylamide gel electrophoresis. 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.

Qualitative PCR assays are used in two 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 semi-quantitative, where analysis of multiple samples from the same lot of seeds or grain can be used to estimate the number of kernels that contain the target analyte. An advantage of the semi-quantitative approach that is the method can be applied in a range that is well above the LOD (limit of detection); thus, the likelihood of false positive or false negative results can be significantly reduced. Nevertheless, care has to be taken that contamination with fragments of seed, grain, or dust does not cause false positive results [5-7].

Choice of reference materials

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). The laboratory may then use a reference material or working standard that has been calibrated back to the CRM/SRM™ [7].

Method validation

A method must be validated using the protocols and reaction conditions under which it will be performed. Changes, as well as the application to a different matrix, are likely to affect method characteristics such as the specificity or sensitivity. PCR may have a tendency to produce artifacts if operated outside the scope of the method as it was validated [7].

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 if required by the customer). 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. 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. The manuscript discusses the major factors that impact the 1:1 ratio assumptions: hybrid status, copy cumber, DNA degradation, DNA endo-reduplication, out crossing vs. inbreeding, effects of grain processing and variability in the genome [5-7].

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 and are discussed as random (indeterminate) error and systematic (determinate) error [5-7].

1. To increase the number of very few number of DNA template.
2. Isolation of an orthologous gene sequence by using degenerate primers designed from closely related plant species gene sequence alignments.
3. Amplification and isolation of potential trait responsive genes from crops and their transcript expression pattern analysis under different stress conditions.
4. Synthesis of complementary DNA (cDNA) from RNA isolated from the crop using modified process of PCR called Reverse-transcriptase PCR.
5. Amplification of gene from cDNA.
6. PCR is used for DNA sequencing to determine unknown PCR-amplified sequences, which helps in gene discovery.
7. PCR remains as an integral tool for cloning, vector construction, and transformant identification into bacterial cells.

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8. Identification of genetically modified crops for the presence of transgene using PCR.
9. PCR amplification and sequencing for marker genes like 16S and 18S ribosomal RNA genes in prokaryotes and eukaryotes, respectively for phylogenetic analysis.
10. Screening and differentiation of diseased with viral pathogen and healthy plants using viral gene specific primers.
11. Screening and characterization of genotypes during marker assisted breeding for development of new varieties in different crop plants use PCR.
12. Seed quality control: Commodity and food companies, as well as third-party diagnostic testing companies, rely on PCR technology to verify the presence or absence of transgene material in a product or to quantify the amount of transgene material present in a product. The copy number of transgene and zygosity is determined by quantitative PCR technology.

CONCLUSIONS

Polymerase Chain Reaction 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 only 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 matrices on the methods. In addition, there are numerous biological and analytical factors that need to be taken into account when reporting results.

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