The iCycler iQ™ and Amplifluor Detection Systems for Analysis of Genetically Modified Organisms in Foodstuffs

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Introduction

One of the most topical issues in the food industry is the use of genetically modified organisms (GMOs) in agriculture and food production. Demands from consumers, particularly in Europe, for clear labeling of products that contain genetically modified (GM) plants or their derivatives have given rise to a requirement for analytical methods that can detect GM material. Within Europe, producers of foodstuffs must comply with European regulations regarding labeling of products, including a recent regulation, (EC) 49/2000, that sets a maximum threshold of 1% on certain GM ingredients. With the introduction of the threshold, quantitative methods of GMO detection have become increasingly important.

There are 2 basic analytical methods for the detection of GM plants: detection of the novel DNA used in the genetic modification, or detection by ELISA of novel proteins produced as a result of the modification. The polymerase chain reaction (PCR*) has remained the method of choice for GMO detection, because DNA is more likely to survive food processing than proteins. The introduction of real-time PCR has made the accurate quantitation of the starting amount of DNA targets possible and its application to the quantitation of GMOs in food is becoming more important. Aside from quantitation, real-time PCR offers another advantage for the analytical molecular biology lab because it requires no postamplification handling of samples, reducing the risk of contaminating the laboratory and analytical samples with PCR products.

In real-time PCR, a fluorescent reporter molecule is used to track the progress of the PCR reaction. The fluorescence of a reaction increases as the amount of amplified DNA increases until a point is reached where the level of fluorescence rises significantly above background. The point at which this occurs is known as the threshold cycle. Since at this point PCR is operating at close to maximum efficiency, the threshold cycle number bears a linear relationship to the amount of target DNA in the reaction. The amount of target DNA in a test sample can be calculated by comparing the sample's threshold cycle to a standard curve obtained from the threshold cycles of reference standards.

Many systems exist to introduce the fluorescent reporter molecule to the reaction. The Amplifluor universal amplification and detection system (Intergen) utilizes a molecular energy transfer system that uses an acceptor moiety to quench fluorescence from an excited fluorophore (Nazarenko et al. 1997). The fluorophore (fluorescein) and quencher (DABSYL) are located on the same oligonucleotide primer. The UniPrimer primer has a universal format, as it will bind to any amplified DNA containing a unique recognition sequence that can be added to existing PCR primers. The UniPrimer will only emit fluorescence when incorporated into amplification products; unincorporated UniPrimer does not fluoresce. It is also possible to obtain custom primers with the UniPrimer sequence already included. In this application note, we describe the combined use of the Amplifluor universal amplification and detection system's chemistry and the iCycler iQ real-time PCR detection system's instrumentation for the detection and quantitation GMOs in foodstuffs.

Methods

Determination of Relative Soy DNA Content

A master mix was prepared to quantitate the relative amounts of soy DNA in commercially available and in-house Roundup Ready GM soy standards. Primers were chosen to amplify part of the soy lectin gene. The master mix contained:

90 µl 10x PCR buffer (Life Technologies)

90 µl dNTPs (2.5 mM)

90 µl lectin gene forward primer (2.5 µM)

90 µl lectin gene reverse primer (including recognition sequence) (0.5 µM)

90 µl Amplifluor UniPrimer primer (5.0 µM, Intergen)

9 µl Platinum *Taq* polymerase (5 U/µl, Life Technologies)

27 µl $MgCl_2$ (50 mM)

342 µl H_2O (tissue-culture grade, Life Technologies)

The master mix was thoroughly mixed and stored on ice

prior to use. DNA was prepared from an in-house Roundup Ready GM soy standard and diluted to 20 ng/µl to make a "100% soy" stock. This stock was then further diluted in a 10-fold dilution series to a "0.1% soy" stock. A 2 µl aliquot of each diluted soy DNA stock was placed in each of 3 wells on a 96-well optical plate. DNA was also prepared from commercially available Roundup Ready GM soy standards to use as "unknowns". Standards were obtained from Fluka (series 410R) and containined 0%, 0.1%, 0.5%, 1%, 2%, and 5% Roundup Ready GM soy. DNA from each of the commercial standards was diluted to 20 ng/µl and 2 µl of these stocks was added to each of 3 wells on the same 96-well plate. Three control reactions containing 2 µl of tissue-culture grade water were also prepared. To each tube containing DNA or water, 23 µl of master mix was then added. The plate was covered with a sheet of optical sealing film. PCR conditions were 95°C for 3 min, followed by 50 cycles of 95°C for 15 sec, 55°C for 20 sec, and 72°C for 40 sec. Fluorescent data were collected during the 55°C step.

Determination of GMO Content

Primers were chosen to amplify part of the construct used in Roundup Ready GM soy. The following master mix was prepared:

90 µl 10x PCR buffer (Life Technologies)

90 µl dNTPs (2.5 mM)

90 µl forward primer (4 µM)

90 µl reverse primer (directly labeled with UniPrimer sequence and energy transfer system) (4 µM)

9 µl Platinum *Taq* polymerase (5 U/µl, Life Technologies)

27 µl $MgCl_2$ (50 mM)

432 µl H_2O (tissue-culture grade, Life Technologies)

DNA and controls and the 96-well plate were prepared as above and run using the same thermal cycling protocol.

Results

Determination of Relative Soy DNA Content

Background-corrected data were brought down to the PCR baseline. The standard curve generated by the in-house standards showed a correlation coefficient (r) of 0.991 (Figure 1). The correlation coefficient was not improved by extension of the number of cycles to be included in the baseline. The data showed that soy DNA could be quantitated at all dilutions used (Figure 2), and it was possible to calculate the amount of soy DNA in the commercial standards relative to the in-house standards.

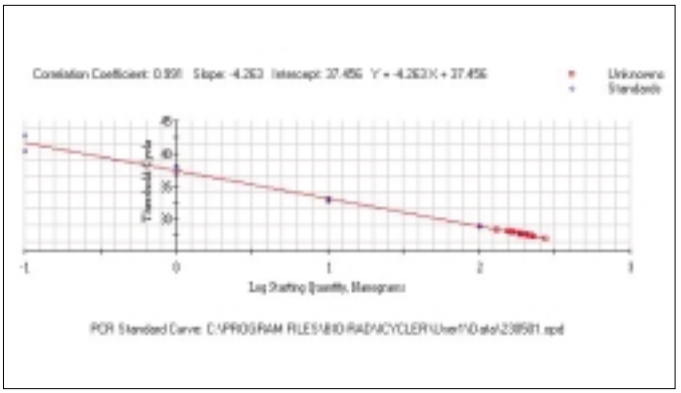

Fig. 1. Standard curve generated by the iCycler software from data shown in Figure 2.

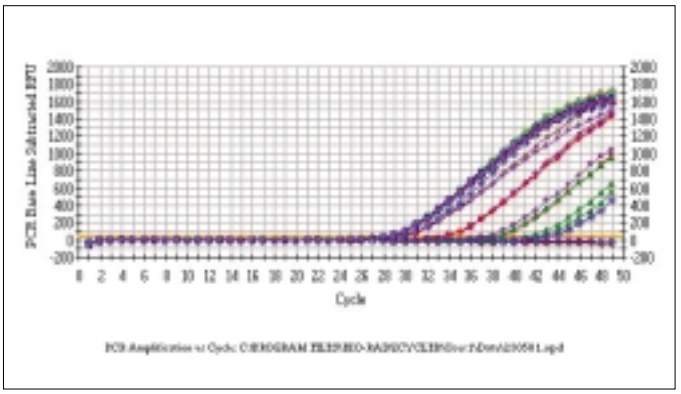

Fig. 2. iCycler screen view showing results of a soy DNA dilution series amplified using the Amplifluor universal amplification and detection system. The dynamic range covers 100% soy to 0.1% soy.

Determination of GMO Content

Background-corrected data were brought down to the PCR baseline. The standard curve generated by the in-house standards showed a correlation coefficient (r) of 0.996. This could be improved to 0.998 when the number of background cycles was increased to 22 (Figure 3). The data showed that GM soy could be detected in both the in-house and commercial standards (Figure 4). Results from the GM quantitation of the commercial standards ("unknowns") were corrected for the total soy DNA present in the sample using data obtained from the total soy DNA determination described above. The results are shown in the table.

Table. Comparison of actual GM soy content in standards to values calculated by real-time PCR detection.

Commercial Standard GM Soy Content	GM Soy Content Calculated from In-House Standards
0%	0%
0.1%	0.1%
0.5%	0.4%
1.0%	1.0%
2.0%	1.7%
5.0%	4.5%

Discussion

Using the iCycler iQ and the Amplifluor universal amplification and detection systems, it was possible to detect and quantitate GM soy DNA present in both in-house and commercially available standards to a high degree of accuracy. Extension of this technology to the determination of GM soy content in foodstuffs is straightforward, and by changing the primers used in each step of the process, levels of GM maize or other GMOs can be determined. Indeed, since the iCycler iQ and the Amplifluor systems are multiplex compatible, the development of assays to detect 2 or more targets simultaneously is an area we are actively pursuing.

References

Nazarenko IA et al., A closed tube format for amplification and detection of DNA based on energy transfer, Nucleic Acids Res 25, 2516–2521 (1997)

Regulation (EC) 49/2000, Official Journal of the European Communities, L6, vol 43, 13–14, Brussels, Belgium (2000)

* Practice of the patented polymerase chain reaction (PCR) process requires a licence. The iCycler iQ system includes a licensed thermal cycler and may be used with PCR licenses available from Applera. Its use with authorized reagents also provides a limited PCR license in accordance with the label rights accompanying such reagents. Some applications may require licenses from other parties.

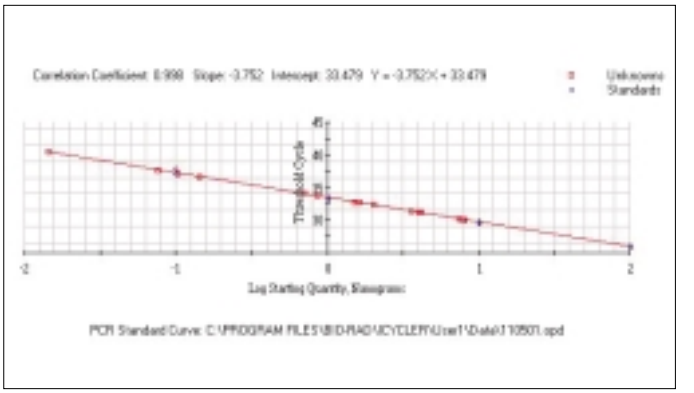

Fig. 3. Standard curve generated by the iCycler software from data shown in Figure 4.

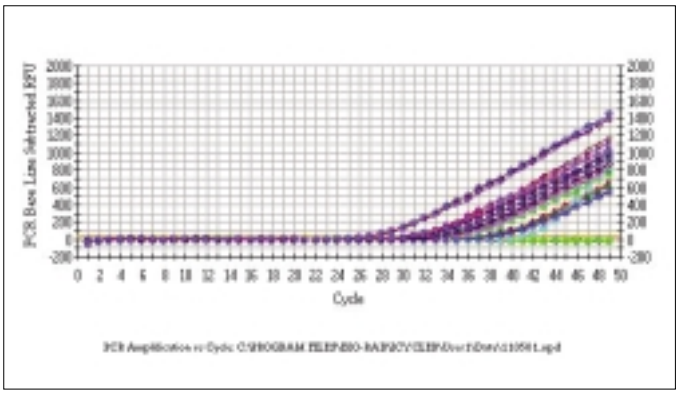

Fig. 4. iCycler screen view showing results of a GM soy DNA dilution series amplified using the Amplifluor universal amplification and detection system. The dynamic range covers 100% GM soy to 0.1% GM soy.

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