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GMOs Detection With Minimal Reaction Volume Real-Time PCR For Environmental Conservation

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Abstract Detection of GMOs with Real-time PCR is the standard technology for tracing their presence in different sample types ranging from food to plant materials. GM plant seeds are one of the main sources for GM dispersal in the environment, affecting genetic stability and biodiversity. A certified protocol of minimal Real-time PCR reaction volume with low cost is presented, that offers the ability to identify trace amounts of GMOs in seeds, maintaining high specificity, sensitivity, efficiency and robustness (≥95%). The proposed methodology can serve also sampling for environmental monitoring, where handling of increased sample numbers is difficult or/and the sample amount is very low and precious.
Keywords: GMO, Real-time PCR, biodiversity

1. Introduction

With the advent of genetic engineering in modification technologies of nucleic acids, a number of food commercial products, especially from plants, appeared in the markets containing nucleic sequences not previously present in the genomes using classical breeding and selection methods. The result from the introduction of these genetically modified organisms (GMOs) and their products was that many countries around the world decided that a legislative framework needs to be designed about the GMOs production. In parallel it was also necessary to set analytical methods that will permit the detection of these products qualitatively and quantitatively in accordance to the legislative framework per country. The initial ELISA tests were mainly designed to identify genetically modified proteins, but have been proved inadequate because of the presence of genetic sequences that were not expressed as proteins. They have also reduced sensitivity due to variability of the modified protein structures. As a consequence of technology progress Real Time PCR was adopted as the most accurate methodology for tracing GMOs.

Nowadays, there is a quite rich number of methods in use for GMOs detection and the main driving forces in their design are: the simplicity of application, the ability to detect a wide range of different modifications using generic nucleic sequences and the cost.

In this presentation a certified protocol of minimal Real-time PCR reaction volume is presented, that offers the opportunity to identify trace amounts of GMOs in seeds with low cost, maintaining high specificity, sensitivity, efficiency and robustness (≥95%).

2. Materials and Methods

Detection of genetic modifications in seeds or flour samples is performed according to the presented flow chart. Commonly, for each sample analysis for GMO presence there are five steps:

A. Extraction of genetic material (DNA),

B. DNA quantification,

C. Detection of plant DNA by real-time PCR,

D. Qualitative detection of 35S & NOS with real-time PCR,

E. Analysis of fluorescence results of real-time PCR products.

Real Time PCR detection of genetic modifications is performed with Taqman technology, where amplification is accomplished in a duplex reaction using specific 35S/NOS primers and corresponding fluorescent probes. The detection is qualitative, detection or non-detection of the certain genetic elements. The PCR primer–probe sequences are based on Eurofins Test kit "GMOScreen RT 35S / NOS IPC (LR)" as 35S and NOS are generic element sequences present in many GMOs. Plant DNA detection is based on "SpeciesScreen RT PLANT IPC" Eurofins test kit. Reaction times and volumes of Real Time PCR were determined after a series of experiments in order to achieve optimum detection complying with ISO 17025 guidelines for method sensitivity, specificity, efficiency and robustness at the minimum cost of PCR materials. In the tests performed the critical value of 10 GMO copies per PCR were used. The final in-house protocol resulted after changes of the initially proposed by Eurofins protocol with significant reduction in the reaction volumes and times, adjusted to be compatible with the instrument PCRmax ECO 48 (Cole Palmer). Real Time PCR volumes were 5, 10 and 15 μλ of Master mix plus 10 GMO copies each time under the parameters of Eurofins protocol and the following ones: 10min initial denaturation at 95 °C followed by 50

cycles of 10sec denaturation at 95 °C and 1 min amplicon annealing-extension at 60 °C. Two independent experiments with three replications each for different mastermix volume and PCR conditions were performed for plant DNA, 35S and NOS detection.

3. Results and Discussion

In all tested cases it was observed that 5μλ master mix volume is the most efficient one, producing the highest fluorescence intensity per volume unit (μl). Modified PCR conditions of In-house vs Eurofins method did not affect outcomes. Consequently, use of reduced master mix volume 5μλ with modified PCR conditions for faster PCR results was adopted for future sample analysis as 100% robustness of the method was maintained. The reduced master mix volume dropped the cost of PCR reactions by four times. The proposed method can alleviate demands in cases of environmental sampling in open areas with increased sample numbers, reducing the analytic cost and the sample size.

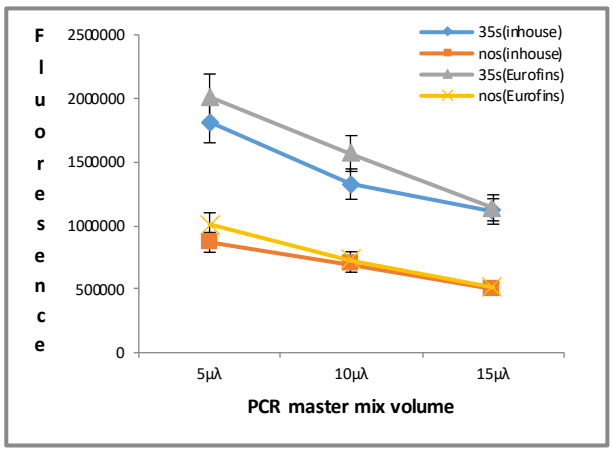

Figure 1. PCR master mix volumes and conditions effect in the produced fluorescence intensity between In-house and Eurofins protocols. GMO presence was assessed during detection at the critical value of 10 GMO copies (LOD), using primers-probe for 35S and NOS nucleic sequences.

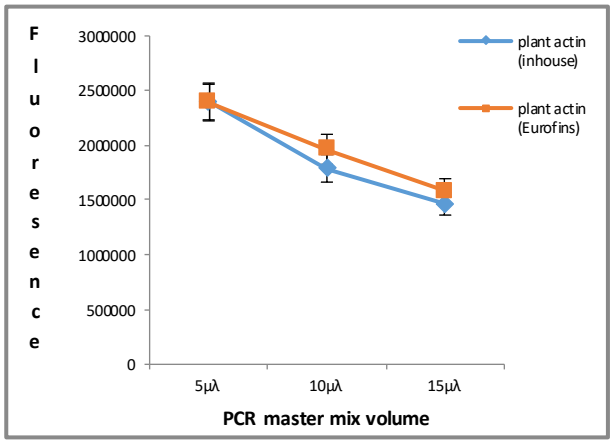

Figure 2. PCR master mix volumes and conditions effect in the produced fluorescence intensity between In-house and Eurofins protocols. Plant presence was assessed during detection at the critical value of 10 GMO copies (LOD), using primers-probe for plant actin nucleic sequences.

The produced duplex Real time PCR method was further evaluated with certified reference materials of 0%, 0.1% and 1% GMO content and samples of two ring trials. From the analysis of the results sensitivity, specificity and efficiency of the In-house GMO detection method were verified as presented in Table 1.

Table 1: Validation of the In-house GMO detection method

Amplicon Targets	Specificity CN/(CN+FN)	Sensitivity CP/(CP+FP)	Efficiency (CN+CP)/TN
35S	51/52 98.1%	50/50 100%	101/102 99%
NOS	51/52 98.1%	50/50 100%	101/102 99%
Plant Actin	34/34 100%	46/46 100%	80/80 100%

CN: Correct Negative Samples
CP: Correct Positive samples
FN: False Negative samples
FP: False Positive samples
TN: Total Number of tested samples

4. Conclusions

The presented procedure, in accordance to ISO 17025, resulted in the verification of an In-house Real time PCR method for GMO detection with the following characteristics:

1. The PCR master mix volume was reduced to 5 μl (4x reduction),
2. Sensitivity and specificity ≥ 95%,
3. Efficiency ≥ 99%,
4. Robustness ≥ 100% as all replicates of reference material with GMO content at LOD (0.1%) gave positive results.

The proposed protocol is a reliable and of low cost analytical tool, extremely useful in handling large number of samples and/or minimal amount of samples.

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