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Comparative Analysis of Verification Parameters of Event-Specific Methods in GMO Maize

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Abstract

The second most dominant genetically modified (GM) crop is maize. Increasing number of GM maize events puts significant pressure on GMO testing laboratories to achieve the level of competence necessary to fulfill legal requirements. In the European Union, Polymerase Chain Reaction (PCR) is the method of choice for identification and quantification of GMOs. We performed verification of validated methods for identification of four GM maize events. Additionaly we aimed to explore the option of designing a method for simultaneous detection of these events in a multiplex PCR reaction. DNA was extracted from certified reference materials (CRM) using validated CTAB extraction protocol. Concentration of DNA was measured using Qubit dsDNA Broad Range Assay. Amplification of taxon specific marker for maize and all event-specific methods was performed according to the JRC Compendium of Reference Methods for GMO Analysis. Absolute limit of detection (LOD_{abs}) was determined for taxon specific and four event specific RealTime PCR based methods. DNA extracted from CRMs showed sufficient concentration for downstream analyses and preparation of dilutions for determination of LOD_{abs}. Determined LOD_{abs} for all tested methods meet acceptance criteria. As expected, the methods performance with respect to the repeatability and precision decline with the decrease in concentration of the target. Event-specific GA21 and NK603 methods show high Ct values at the determined LOD_{abs}. However, by adjusting the concentrations of primers and probes sensitivity of these two methods should be improved. Considering that the amplicons for all five methods are quite short (<120 bp) optimization of multiplex reaction conditions for simultaneous amplification should be feasible.

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Introduction

Maize is the second most widely grown biotech crop in the world. According to ISAAA, 2016, in 2015 biotech maize was cultivated on 60 million hectares worldwide, which accounts for a third of total maize production. Cultivation of GM maize is steadily

increasing since 1996, with 13% increase in 2016 (ISAAA, 2016). Maize is also the most diverse biotech crop when it comes to the number of modifications. ISAAA lists 229 maize GMO events, 42 of those are single transgenes. At the moment of publication of this paper, EU has over 60 authorized GMO events with another 11 pending applications. Among those, 15 authorizations pertain to unique GMO events. Over 50 authorizations are stacked events, some of those containing as many as six authorized transgenes (EU Register of authorized GMOs - http://ec.europa.eu/food/dyna/gm_register/index_en.cfm). In the countries with regulated GMO such abundance of maize events puts significant pressure on testing laboratories to achieve level of competence necessary to fulfil their regulatory roles.

In environment with developed legal framework for GMO, ideal situation would be that GMO testing laboratories are able to detect, identify and, in most cases, quantify GMO present in a sample submitted for the analysis in order to establish its regulatory status. In order to achieve these requirements the laboratories would need to implement hundreds of analytical methods. Implementation of a method means that testing laboratories must verify that the method can be used for its intended purpose. Therefore verification procedure is carried out according to the published guidelines.

In the EU, Polymerase Chain Reaction (PCR) is the method of choice for the identification and quantification (Hougs et al., 2017). In addition to that, official testing laboratories must implement and maintain quality requirements according to ISO17025 (International Organization for Standardization, 2005). This analytical machinery is extremely expensive to implement and maintain thus leading to high prices of analysis which, in the end, is paid by final consumer. Already now, strategies like simplex or multiplex RealTime PCR targeting known junction sequences are not rational and it is also extremely expensive and time consuming. With the number of authorized GM events increasing and occasional emergence of unauthorized GM events for which no target sequences are available, the approaches based solely on RealTime PCR will easily become unattainable (Broeders et al., 2012).

Therefore, in the world of GMO testing laboratories, there is an ongoing search for more economical and more efficient solutions that would ensure food safety at more affordable level. A number of approaches to the detection of multiple targets were designed with different level of accomplishment. PCR capillary gel electrophoresis, DNA microarray, Luminex are some of those approaches (Fraiture et al., 2015). However, some of them require not widely affordable equipment as well as high level of technical competence (DNA microarray, Luminex). Most of the GMO testing laboratories have elaborated a screening strategy which includes a minimum set of PCR tests targeting specific genetic elements that allow detection / exclusion of as many GM events as possible. Only samples tested positive for particular set of GM events undergo identification analysis (Broeders et al., 2012).

PCR and fluorescent capillary gel electrophoresis are ubiquitous methods and most laboratories already posses necessary equipment. Therefore, a number of screening strategies based on PCR with fluorescent capillary electrophoresis have been developed for maize (Garcia-Canas et al., 2004; Heide et al., 2008; Nadal et al., 2006). When screening targets are carefully selected and multiplex system is well optimized, good coverage in current environment of GM maze authorizations may be achieved. The study reported on in this paper was conducted with the following aims:

a) To perform verification of validated methods for identification of four authorized maize GM events that are present in high proportion of stacked events;

b) To explore the option of designing a system for simultaneous detection of these events in single multiplex PCR reaction and to analyze limiting factors.

Materials and methods

Certified reference materials (CRM) were obtained from Joint Research Center – Institute for Reference Materials and Measurements via authorized vendor. All CRMs were supplied as pulverized maize flour (10% TC1507, 0% TC1507, 10% MON810, 0% MON810, 4.3% GA21, 0% GA21, 5% NK603, 0% NK603).

Table 1. Primers and TaqMan probe sequences used in RealTime PCR reactions

Target / amplicon size	Primers and TaqMan probe sequences (Applied Biosystems, Foster City, CA)	Original reference
hmg (79 bp)	F: 5'-TTGGACTAGAAATCTCGTGCTGA-3' R: 5'-GCTACATAGGGAGCCTTGTCCT-3' 5'-FAM-CAATCCACACAAACGCACGCGTA-TAMRA-3'	Van Den Eede (2011)
NK603 (108 bp)	F: 5'-ATGAATGACCTCGAGTAAGCTTGTTAA-3' R: 5'-AAGAGATAACAGGATCCACTCAAACACT-3' 5'-FAM-TGGTACCACGCGACACACTTCCACTC-TAMRA-3'	Mazzara et al. (2005b)
GA21 (112 bp)	F: 5'-CTTATCGTTATGCTATTTGCAACTTTAGA-3' R: 5'-TGGCTCGCGATCCTCCT-3' 5'-FAM-CATATACTAACTCATATCTCTTTCTCAACAGCAGGTGGGT-TAMRA-3'	Paoletti et al. (2005)
MON810 (92 bp)	F: 5'-TCGAAGGACGAAGGACTCTAACGT-3' R: 5'-GCCACCTTCCTTTTCCACTATCTT-3' 5'-FAM-AACATCCTTTGCCATTGCCCAGC-TAMRA-3'	Mazzara et al. (2009)
TC1507 (58 bp)	F: 5'-TAGTCTTCGGCCAGAATGG-3' R: 5'-CTTTGCCAAGATCAAGCG-3' 5'-FAM-TAACTCAAGGCCCTCACTCCG-TAMRA-3'	Mazzara et al. (2005a)

DNA was isolated from 0,2 g of reference material using CTAB precipitation protocol validated by EURL-GMFF (European Union Reference Laboratory for GM Food and Feed) for seeds and flour (ISO 21571:2005; Annex A, part A3). The extractions were performed in duplicate with accompanying reagent blank control. DNA concentration was determined fluorometrically using Qubit dsDNA Broad Range Assay (ThermoFisher Scientific (Invitrogen), Waltham, MS, USA). Standard genome size for maize of 1C = 2,275 pg (Arumuganathan & Earle, 1991) was used as a reference for preparation of DNA dilutions series containing precise number of target copies for each maize GMO event (70C, 35C, 17C, 8C, 4C, 2C).

Taxon specific marker for maize, high mobility group (hmg) and all event-specific methods have been adopted from JRC Compendium of Reference Methods for GMO Analysis (2011).

The methods have been validated in a collaborative trial according to the principles and requirements of ISO5725 and/or IUPAC protocol (Thompson et al., 2002). Primer and TaqMan probe sequences used in RealTime PCR reactions for the selected targets are given in the Table 1.

Verification of the methods was performed according to the published guidelines (Hougs & Žel, 2011; Hougs et al., 2017). Absolute Limit of Detection (LOD) was defined as the lowest amount or concentration of analyte in a sample, which can be reliably detected but not necessarily quantified. Methods should detect the presence of the analyte at least 95% of the times at the LOD, ensuring ≤5% false negative results. For each method we have performed 10 replicas of RealTime PCR reactions at all concentrations in prepared dilution series. The lowest concentration where all 10 replicas are positive is the estimated LOD_{abs}.

Table 2. RealTime PCR reaction conditions

Reagent	*hmg*	NK603	GA21	MON810	TC1507
TaqMan Universal Master Mix *Applied Biosystems*	1 x	1 x	1 x	1 x	1 x
Primer Fwd	0.3 µmol/L	0.15 µmol/L	0.15 µmol/L	0.3 µmol/L	0.3 µmol/L
Primer Rev	0.3 µmol/L	0.15 µmol/L	0.15 µmol/L	0.3 µmol/L	0.3 µmol/L
TaqMan probe	0.16 µmol/L	0.05 µmol/L	0.05 µmol/L	0.18 µmol/L	0.15 µmol/L
DNA dilution vol*	5 µl	5 µl	5 µl	5 µl	5 µl
Rxn volume	25 µl	25 µl	25 µl	25 µl	25 µl

*For CRM certified for absence of analyte 200 ng DNA was used

Reference material certified for the absence of DNA target (0%) was also analyzed in order to ensure absence of false positive results. RealTime PCR reaction conditions are given in Table 2. The reactions were performed using Sequence Detection System 7300 (Applied Biosystems, Foster City, CA) with the following cycling parameters: UNG decontamination at 50°C for 2 min, activation of Taq DNAPol at 95°C for 10 min and 45 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min. Reagent blank reactions accompanied each series of amplifications.

Results and Discussion

DNA extracted from 0,2 g of CRM resulted in sufficient concentration required for subsequent analysis and the preparation of dilutions for LOD_{abs} (Table 3). As RealTime PCR analysis was performed on a series of twofold dilutions the results of those were used to verify the absence of inhibition. As ΔCt_{av} values of 10 RealTime PCR reaction replicas between two sequential twofold template dilutions (70 C and 35 C) were well within the range $0,5 \leq \Delta Ct_{av} \leq 1,5$ (Hougs & Žel, 2011; Žel et al., 2012) the presence of inhibitors was excluded and extracts were suitable for determination of $LOD_{abs.}$

All the CRMs certified for the absence of target resulted in negative amplification thus eliminating the possibility of false positive results. Since all the methods were previously validated in collaborative trials, specificity does not need to be reexamined in the verification process (Hougs & Žel, 2011; Hougs et al., 2017).

LOD_{abs} was determined as the lowest concentration of an analyte with false negative rate <5%. False negative rate is the probability that a known positive test sample is classified as negative by the method. False negative rate increases as the amount of analyte approaches the LOD of the method (Hougs & Žel, 2011; Hougs et al., 2017). Considering that our verification procedure was based on 10 RealTime PCR reaction replicas, failure of one reaction replica to produce results already presents false negative rate >5%. Thus, LOD_{abs} was determined as the lowest template concentration for which all 10 replicas were positive (Table 4).

Table 3. Concentration of DNA obtained from 0,2 g of CRM

Maize CRM	Average concentration (ng/µl)
TC1507 0%	131
TC1507 10%	133
GA21 0%	148
GA21 4,3%	139
NK603 0%	159
NK603 5%	140
MON810 0%	149
MON810 10%	137

According to the Hougs and Žel (2011) acceptance criteria, LOD_{abs} resulting from verification procedure should be in line with the reported LOD_{abs} of the method. However, validation reports for hmg, NK603 and GA21 methods do not contain information on LOD_{abs} so no comparison is possible. The latest guidelines by Hougs et al. (2017) extend the acceptance criteria to minimum performance requirements (MPR; <25 copies with a level of confidence of 95%). Thus, the LOD_{abs} determined for these three methods meet the acceptance criteria.

Table 4. Verification parameters of the five tested methods obtained from 10 replicas of RealTime PCR reactions on a series of twofold template dilutions

Target	LOD_{abs} (No. target copies)	$Ct_{av} \pm SD$ (10 replicas at LOD_{abs})	Ct range at LOD_{abs} ($Ct_{max} - Ct_{min}$ for 10 replicas)	Reported method LOD_{abs}
hmg	4 C	36.28 ± 0.731	2.56	not reported
NK603	8 C	41.49 ± 0.826	2.27	not reported
GA21	17 C	42.45 ± 1.072	2.8	not reported
MON810	2 C	37.14 ± 0.845	2.85	5 C
TC1507	4 C	36.97 ± 1.259	3.49	1.25 C*

*LOD reported by the method developer, not assessed in the collaborative trial (Bonfini et al., 2012)

Validation report for TC1507 sets LOD_{abs} of the method at 1.25 copies, however this value is not the result of experimental procedure. It was adopted from the method developer (Bonfini et al., 2012). According to the latest guidelines (Hougs et al, 2017) LOD_{abs} cannot be lower than 3 copies per reaction. Thus, LOD_{abs} determined for TC1507 method meets the criteria. Reported method LOD_{abs} for MON810 is slightly higher than the one determined in our verification procedure and is in line with MPR. However, the most recent criteria do not allow LOD_{abs} lower than 3 copies per reaction. Therefore, LOD_{abs} for our purposes should be set at the next higher dilution i.e. 4 copies per reaction.

Closer evaluation of the results shown in Table 4 reveals additional input. As expected, the methods performance with respect to the repeatability and precision decline with the decrease in concentration of the target. That is evident from Ct range at LOD_{abs} ($Ct_{max} - Ct_{min}$ for 10 replicas) and $Ct_{av} \pm SD$ (10 replicas at LOD_{abs}) for all methods. Identification method for TC1507 has particularly high Ct range at LOD_{abs} of 3,49 at 4 C dilution. When we reexamine row data it becomes evident that precision declines drastically below 17 C per reaction (Ct range at LOD_{abs} = 2,14; $Ct_{av} \pm SD$ =34,56 ± 0,592) so it may be prudent to set LOD_{abs} at this level. Testing laboratories should be more concerned with repeatability and precision than with sensitivity. Also, the methods for GA21 and NK603 exhibit unusually high Ct values at the determined LOD_{abs} which translates into low sensitivity. When reaction conditions recommended by the method developer and confirmed in collaborative trials are examined (Table 2), it becomes evident that the concentrations of primers and probes are significantly lower for these two methods. By adjusting the concentrations of primers and probes to those used for other methods (*hmg*, MON810 and TC1507) sensitivity of these two methods may be improved.

Determination of practical LOD is out of scope of verification procedure and is of the major concern for testing laboratories as it is sample rather than the method dependant. It should be evaluated for each type of sample separately.

Considering that the amplicons for all five methods are quite short (≤ 112 bp) the design of reaction conditions for simultaneous amplification of all five targets should be feasible. Coupled with fluorescent labeling and capillary gel electrophoresis it may be powerful tool for GMO screening in maize as has been shown before (Garcia-Canas et al., 2004; Heide et al., 2008; Nadal et al., 2006). This particular combination of GMO events could detect close to 80% of stacked maize events currently authorized in the EU. However, certain limitations must be taken into account: sensitivity of GA21 and NK603 methods is rather low and detection limits for TC1507 and GA21 methods are higher than expected. These limitations must be considered when dealing with matrices with low maize content or heavily processed samples. Therefore, guidance given for verification of multiplex reactions (Hougs et al., 2017) must be carefully observed in developing multiplex screening strategy.

Conclusions

LOD_{abs} determined during verification procedure for four event specific RealTime PCR methods and taxon specific RealTime PCR method for maize (*hmg*) meet acceptance criteria. However, prudence implies that repeatability and precision be given precedence over sensitivity. For practical purpose LOD_{abs} for MON810 and TC1507 are set at 4C and 17C respectively. By adequate optimization and careful observation of verification guidance, development of multiplex PCR screening strategy for GMO maize may be feasible.

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