Eur Food Res Technol (2010) 230:383–393
DOI 10.1007/s00217-009-1170-5

ORIGINAL PAPER

SYBR®Green qPCR screening methods for the presence of "35S promoter" and "NOS terminator" elements in food and feed products

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Received: 30 June 2009 / Revised: 28 September 2009 / Accepted: 11 October 2009 / Published online: 7 November 2009

Abstract The Cauliflower Mosaic Virus "35S promotor" (p35S) and the *Agrobacterium* "Nopaline Synthase" terminator (tNOS) are the most represented generic recombinant elements in commercial genetically modified crops to date. A set of four new SYBR®Green qPCR methods targeting the "p35S" and "tNOS" core elements have been developed. These qPCR methods generate short amplicons of 147 and 75 bp for the "p35S" element and 172 and 69 bp for the "tNOS" element. Single target plasmids containing these amplicons were constructed and allow determining the nominal melting temperature (T_m value) of each amplicon. The four methods are specific for their respective targets, and moreover, three of them are highly sensitive (up to 1–2 copies detectable) at a PCR efficiency ranging between 95 and 100%. The latter methods can detect their respective targets at 0.1% (w/w) gDNA levels and are suitable for detecting low levels of genetically modified materials containing the "p35S" and/or "tNOS" elements.

Keywords Real-time PCR · Food and feed analysis · GMO detection · 35S promoter · NOS terminator · SYBR®Green

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Introduction

In the European Union, the development of genetically modified organisms (GMO) is subject to a complex legal framework. The most important GMO EC legislations are the environmental directive EC/2001/18 [1], the GM Food/Feed regulations EC/2003/1829 [2] and EC/2003/1830 [3], the EC Recommendation EC/787/2004 [4] and the Enforcement regulation EC/882/2004 [5]. Within these legislations, the detection of GMO represents an important element for compliance with the conditions set in the authorizations. Molecular characteristics (especially DNA sequence information) represent the most important identification criterion and legal basis for the presence of a particular GMO in a product [2–4].

Consequently, the EU enforcement framework is primarily based on molecular DNA methodology. Within the GM Food/Feed legislation, authorizations of new GM products require the availability of validated (quantitative) product-specific detection methods. Most elaborate in this respect are the so-called event-specific detection methods for GM crops validated by the Community Reference Laboratory for Genetically Modified Organisms (CRL-GMO) of the EC-JRC (Ispra, Italy) [6].

Table 1 Specificity assessment of the four SYBR®Green qPCR methods: "p35S-long" and "p35S-short", "tNOS-long" and "tNOS-short"

A. Determination of the nominal T_m value of each amplicon applying "Sybricon" plasmids as DNA template

Sample name	Origin/BCCM number	200 copies	
		C_t	T_m (°C)
Sybricon010 (p35S-long)	LMBP5460	32.04	80
Sybricon017 (p35S-short)	LMBP5662	29.81	76.5
Sybricon001 (tNOS-long)	LMBP5451	28.56	73.3
Sybricon006 (tNOS-short)	LMBP5456	29.51	72.5

B. Specificity assessment of the four SYBR®Green qPCR methods: "p35S-long" and "p35S-short", "tNOS-long" and "tNOS-short" using gDNA from EU-authorized GM events as template

Sample name	Species	GM percentage (m/m)	Origin	p35S-target presence	p35S long		p35S-short		tNOS-target presence	tNOS-long		tNOS-short	
					C_t	T_m	C_t	T_m		C_t	T_m	C_t	T_m
Sybricon010 (p35S-long)	NA	NA	BCCM	Yes	+	+	+	+	No	–	–	–	–
Sybricon017 (p35S-short)	NA	NA	BCCM	Yes	–	–	+	+	No	–	–	–	–
Sybricon001 (tNOS-long)	NA	NA	BCCM	No	–	–	–	–	Yes	+	+	–	–
Sybricon006 (tNOS-short)	NA	NA	BCCM	No	–	–	–	–	Yes	–	–	+	+
A 2704-12	Soyabean	100	AOCS	Yes	+	+	+	+	No	–	–	–	–
GTS40-3-2	Soyabean	5	IRMM	Yes	+	+	+	+	Yes	+	+	+	+
MON 89788	Soyabean	1	AOCS	No	–	–	–	+	No	–	–	–	+
Wt Maize	Maize	0	IRMM	No	–	–	–	–	No	–	–	–	–
Bt11	Maize	5	IRMM	Yes	+	+	+	+	Yes	+	+	+	+
Bt176[c]	Maize	5	IRMM	Yes	+	+	+	+	No	–	–	–	–
DAS59122	Maize	9.86	IRMM	Yes	+	+	+	+	No	–	–	–	+
GA21	Maize	1	IRMM	No	–[a]	+	–[a]	+	Yes	–	+	+	+
MIR 604[b]	Maize	10	IRMM	No	–	–	–	–	Yes	+	+	+	+
Mon810	Maize	5	IRMM	Yes	+	+	+	+	Yes/no	–	–	–	+
MON863	Maize	9.85	IRMM	Yes	+	+	+	+	Yes	–	+	+	+
NK603	Maize	1	IRMM	Yes	+	+	+	+	Yes	+	+	+	+
T25	Maize	100	Bayer	Yes	+	+	+	+	No	–	–	–	–
TC1507	Maize	9.86	IRMM	Yes	+	+	+	+	No	–	–	–[a]	+
Wt Oilseed rape	Oilseed rape	0	AOCS	No	–	–	–	–	No	–	–	–	–
GT73	Oilseed rape	1	Bayer	No	–	–	–	+	No	–	–	–[a]	+
Rf1[c]	Oilseed rape	100	Bayer	No	–	–	–	+	Yes	+	+	+	+
Rf2[c]	Oilseed rape	100	Bayer	No	–[a]	+	–[a]	+	Yes	+	+	+	+
Rf3	Oilseed rape	1	Bayer	No	–	–	–	–	Yes	+	+	+	+
T45	Oilseed rape	1	Bayer	Yes	+	+	+	+	No	–	–	+	+
MS1[c]	Oilseed rape	100	Bayer	No	–	–	–	–	Yes	+	+	+	+
MS8	Oilseed rape	100	Bayer	No	–[a]	+	–[a]	+	Yes	+	+	+	+
Topas 19/2[c]	Oilseed rape	100	Bayer	Yes	+	+	+	+	No	–	–	–[a]	+
Wt Rice	Rice	0	AOCS	No	–	–	–	–	No	–	–	–	–
LL62[b]	Rice	100	Bayer	Yes	+	+	+	+	No	–	–	–	–
LL62[b]	Rice	100	AOCS	Yes	+	+	+	+	No	–	–	–	–
Wt Cotton	Cotton	0	IRMM	No	–	–	–	–	No	–	–	–[a]	+
MON 1445	Cotton	100	AOCS	Yes	+	+	+	+	Yes	+	+	+	+
MON 531	Cotton	100	AOCS	Yes	+	+	+	+	Yes	+	+	+	+
MON 15985	Cotton	100	AOCS	Yes	+	+	+	+	Yes	+	+	+	+
LL25	Cotton	100	Bayer	Yes	+	+	+	+	Yes	+	+	+	+
Wt Sugarbeet	Sugarbeet	0	IRMM	No	–	–	–	–	No	–	–	–	–

Table 1 continued

Sample name	Species	GM percentage (m/m)	Origin	p35S-target presence	p35S long		p35S-short		tNOS-target presence	tNOS-long		tNOS-short	
					C_t	T_m	C_t	T_m		C_t	T_m	C_t	T_m
H7-1	Sugarbeet	100	IRMM	No	–	–	–	–	No	–	–	–	–
Wt Potato	Potato	0	IRMM	No	–[a]	+	–[a]	+	No	–	–	–	–
EH92-527-1[b]	Potato	100	IRMM	No	–	–	–	–	Yes	+	+	+	+
NTC	NA	NA	NA	No	–	–	–	–	No	–	–	–	–

Yes target is present, *No* target is absent, *NA* not applicable. For the C_t values: "+" means (exponential) amplification and a C_t value above the LOD, "–" means no amplification or amplification below the LOD. For the T_m values: "+" means that the observed T_m value equals the T_m of the complementary Sybricon ±1 °C, while a "–" means that the observed T_m value differs more than ±1 °C from the T_m of the complementary Sybricon

[a] Weak-positive signal, [b] GM event not authorized in EU, [c] GM event only tolerated below 0.9% in EU

In 2007 on a global basis, about 114.3 million hectares GM crops were cultivated, especially soy, maize and oilseed rape [7]. The most common recombinant elements in these GM crops are the so-called "35S" promoter and "NOS" terminator sequences [8]. The 35S promoter (p35S) and NOS terminator (tNOS) are both transcription-regulating sequences [9, 10]. To date, many EU-authorized GMOs (17/24) contain either the "p35S" (15/24) or the "tNOS" (15/24) or both (9/24) [8, 11, 12] (for more details see Table 1). In order to assess the presence of GM material in a product, screening by "p35S" and/or "tNOS" PCR is very often performed [13]. Several detection methods have already been published for "p35S" and "tNOS" detection in a broad range of matrices. In most cases, either end-point detection on agarose gel or real-time qPCR with TaqMan® probe technology is applied [13–19].

In only a few cases, SYBR®Green qPCR methods were developed for detecting GM targets [e.g., 20, 21]. "SYBR®Green I", is an asymmetrical cyanine dye [22] which has been reported to specifically detect the presence of double-stranded (ds) DNA [23]. Two criteria are routinely taken into account when assessing the outputs of PCR amplification by SYBR®Green qPCR analysis: the threshold cycle value (C_t) and the melting temperature (T_m). The C_t value of qPCR amplification represents the time-point at which a PCR reaction reaches a prior-set threshold level for the reaction. This threshold level takes into account fluctuations in the background level during early reaction steps and the start of measurable exponential amplification [24, 25]. As such, the lack of a measurable C_t value in a qPCR is to be interpreted as the absence of any (exponential) amplification above background level. The T_m value represents the temperature at which 50% of the SYBR®Green fluorescence is lost due to denaturation and strand separation of the PCR end product. The T_m is a physical parameter inherent to the sequence of the amplified product (esp. the GC content) and influenced by chemical factors that affect double-strand DNA stability (e.g., salt concentration, DMSO, formamide, etc.) [26].

In a GMO screening approach, SYBR®Green qPCR offers a number of advantages over other fluorescence-based PCR methods: (1) SYBR®Green qPCR monitors the increase in total fluorescence throughout the amplification, allowing to estimate the presence of non-specific amplification, (2) the melting temperature analysis allows post-PCR identification of the amplification not only of the expected target but also scoring the presence of closely related target(s), (3) the SYBR®Green technology is (rather) cost-effective as no dye-labeled oligonucleotide probes are required.

In this study, four SYBR®Green qPCR methods were developed allowing detecting core "p35S" and "tNOS" DNA sequences. Representative amplicons for each method were cloned in pENGL™-like vectors and characterized by DNA sequencing. The nominal T_m value of the amplicons was determined by using these plasmids as template DNA with each of the SYBR®Green qPCR methods. The specificity of the methods was tested on a range of commodity crop species and on all EU-authorized GMO (date March 2009). Their respective sensitivity was estimated by applying different low-level detection criteria on various GM reference materials.

Materials and methods

Materials

Plant materials

To study the specificity of the different SYBR®Green qPCR methods, genomic DNA (gDNA) from either Certified

Reference Materials (CRM) or from in-house grown plants is used. The CRM are obtained from the Institute of Reference Materials and Methods (IRMM) (Geel, Belgium), American Oil Chemists' Society (AOCS) (Urbana, USA) or Bayer CropScience (Ghent, Belgium). In-house leaf material is produced from seeds obtained from the Biotech Companies or from the local commercial market. All plants are grown in a Snijders Scientific (Tilburg, The Netherlands) S1084 plant growth chamber under standard conditions (16/8 h day/night regime at 25 °C/80% humidity). A list of all applied materials is given in Table 1.

Chemicals, PCR reagents and PCR primers

All applied chemical products are analytical grade (NaCl, EDTA, Tris, boric acid, HCl, CTAB, chloroform, isopropanol, ethanol). The applied enzyme products are: Ribonuclase A (Sigma–Aldrich), Proteinase K (Sigma–Aldrich), EcoRI (Invitrogen) and T4 DNA polymerase (Invitrogen). DNase and RNase free water was purchased from ACROS organics.

All kit-products were used according to the manufacturer's recommendation: Quant-iT^{TM} PicoGreen$^{®}$ dsDNA Assay Kit, TOPO TA Cloning$^{®}$ Kit, pCR$^{®}$ 2.1-TOPO$^{®}$ vector, TOP10F′ competent cells (Invitrogen); Genome lab, Dye Terminator Cycle Sequencing (DTCS) kit (Beckman Coulter), QIAGEN Plasmid Midi kit (QIAGEN).

Pre-casted "Ready Agarose^{TM} 96 Plus Gel (3%)" (BioRad) gels and "EZ Load HT molecular weight markers (100 bp–2 kb)" (Biorad) were used for agarose gel analysis.

In the PCR reactions, Amplitaq Gold DNA polymerase (Applied Biosystems), Oligold$^{®}$ oligonucleotides (Eurogentec), and SYBR$^{®}$Green PCR Mastermix [Diagenode (ref: GMO-GS2X-A300)] were used.

Methods

The CTAB gDNA extraction, the qPCR analysis, the agarose gel analysis methods, the applied criteria and the analytical procedures were accredited under ISO-17025 by the official Belgian ISO accreditation organisation "Belac" (2006)

Bioinformatic development of primer pair

All bioinformatic analysis of DNA sequences are performed using the wEMBOSS software package [27–29]. Relevant DNA sequences were collected from public data bases (NCBI and EMBL), patents and scientific literature as well as from in-house DNA sequencing. A uniform primer design approach was applied in the development of primer pairs for the respective targets. A first step consists of identifying regions with high DNA sequence homology within the "p35S" and "tNOS" regions from the different GM events or retrieved DNA sequences. Next, several different primer pairs, comprised within the common target region(s), are designed using the "Primer Express" program from Applied Biosystems (version 3.0) using standard program configuration. An in silico specificity analysis for each primer is performed by probing it against several public and GMO DNA sequence dbases [30, 31] as well as the available in-house sequence information. Any primer showing homology with a non-relevant DNA sequence is discarded from further analysis. The remaining primers are organized in pairs, where as much as possible the primer pairs proposed by Primer Express are retained, and tested experimentally.

Extraction of genomic DNA

A CTAB-based extraction method was applied for the extraction of genomic DNA from all test matrices.

Prior to extraction, leaf tissue is homogenized to powder in a mortar and pestle after liquid nitrogen freezing. Small amounts of seeds (<30 g) are homogenized by crushing in a blender (Kika-Werke Corp.).

Genomic DNA (gDNA) is extracted using a CTAB-based method adapted from Dellaporta et al. 1983 [32]. To a particular powder mass, four volumes (w:v) of CTAB extraction buffer (NaCl 1.4 M, EDTA 0.02 M, Tris–Hcl 0.1 M, CTAB 2%), supplemented with Ribonuclase A (at a final concentration of 15 ng/µl) is added, mixed and incubated for 30 min at 65 °C. Next, Proteinase K (at a final concentration of 100 ng/µl) is added and incubated for 45 min at 65 °C. Upon centrifugation (20 min at 13,000*g*), 0.2 volume of chloroform is added to the supernatant. After mixing and centrifuging (20 min at 13,000*g*), the upper phase is collected and two volumes of CTAB precipitation buffer (NaCl 0.04 M, CTAB 0.5%) are added. After gently mixing, the gDNA is precipitated by incubation at room temperature for 1 h. Upon centrifugation (10 min at 13,000*g*), the gDNA pellet is resuspended in 700 µl NaCl (1.2 M) and 700 µl chloroform, mixed and centrifuged for 15 min at 13,000*g*. The aqueous phase is collected and 0.6 volume isopropanol is added, mixed and centrifuged (10 min at 13,000*g*). The pellet is washed with 500 µl of 70% ethanol and centrifuged after washing (10 min at 13,000*g*). Washing is repeated and the cleaned pellet is dried for 30 min at 28 °C in a dry bath (Fisher Bioblock). Finally, the pellet is resuspended in 200 µl of DNase and RNase free water and allowed to dissolve overnight at 4 °C under agitation. The extracted gDNA is quantified using a VersaFluor^{TM} Fluorometer (Biorad) using the Quant-iT^{TM} PicoGreen$^{®}$ dsDNA Assay Kit. Finally, the gDNA is stored at −20 °C.

Real-time PCR

All qPCR assays are performed on an ABI 7300 PCR System (Applied Biosystems) in 25 µl reaction volume containing 5 µl of template (10 ng/µl gDNA), 1× SYBR®Green PCR Mastermix, and 250 nM of each primer. The following thermal program is applied: a single cycle of DNA polymerase activation for 10 min at 95 °C followed by 40 amplification cycles of 15 s at 95 °C (denaturing step) and 1 min at 60 °C (annealing-extension step). Subsequently, melting temperature analysis of the obtained amplification products is performed by gradually increasing the temperature from 60 to 95 °C in 20 min (±0.6°/20 s). The fluorescent reporter signal is normalized against the internal reference dye (ROX) signal and the threshold limit setting is performed in automatic mode, according to the ABI Sequence Detection Software version 1.4, unless manual adjustment is considered necessary.

Amplicon cloning, sequencing and plasmid deposit

PCR fragments obtained by "classical" PCR amplification using Bt11 leaf gDNA as template are cloned in a pUC18 plasmid applying common "Good Laboratory Cloning Practices" [33]. The respective amplification products are subcloned in pCR®2.1 TOPO using the TOPO TA Cloning® Kit and characterized by restriction analysis. Plasmid DNA from a correct clone is then prepared (QIAGEN Plasmid Midi kit), and the corresponding gel-separated EcoRI fragment isolated and T4-ligated into pUC18 vector DNA (Invitrogen). These plasmids are designated as "Sybricons", standing for "SYBR®Green amplicon".

The respective amplicons are characterized by dideoxy-sequence analysis on a CEQ8000 Genetic Analysis System (Beckman Coulter) with the Genome lab, Dye Terminator Cycle Sequencing (DTCS) Quick start Kit. Each obtained sequence is verified by DNA sequence analysis using the alignment ClustalW2 program [34].

The Sybricon plasmids are registered under "Safe Deposit" or "Patent deposit" at the "Belgian Culture Collection for Micro-organisms" in the "Plasmid and DNA Library Collection" ([35] (BCCM/LMBP) (Ghent, Belgium) (see Table 1). Authenticity testing for each plasmid is performed by the BCCM/LMBP prior to acceptance and certification.

SYBR®Green qPCR assay specificity assessment

Primer pair specificity is assessed by testing amplification of reference materials for target-containing and target-lacking GM events (for an overview see Table 1). Four criteria were set to define what is considered as a "specific signal" generated in SYBR®Green qPCR analysis: (1) an (exponential) amplification above the threshold level is obtained with template DNA comprising the target sequence(s), while negative controls [the so-called "No Template Controls" (NTC) and the gDNA from wild-type crop plants] do not yield such amplification; with all target-containing template DNA, the obtained PCR product(s) represents (2) a single peak upon melting analysis with a unique T_m value corresponding to the nominal T_m value obtained with the respective Sybricon as template DNA (with an acceptable SD ± 1 °C), while no specific peaks are detectable in the negative controls, and (3) a single band on agarose gel analysis with (4) a molecular weight corresponding to the predicted size (SD ± 10 bp).

In each analysis, 50 ng of DNA template is applied. "No Template" controls (NTC) are included in each assay to assess primer dimers formation or specific background fluorescence.

SYBR®Green qPCR assay sensitivity assessment

In this study the sensitivity of the assays was estimated according to the former AFNOR Norm XP V03-020-2 [36] and the IUPAC guidelines [37]. The so-called "LOD_6" of a qPCR method for detection of a particular target represents the estimated haploid genome equivalent (HGE), at which level within a linear serial dilution analysis, each of the six repeats provides a positive signal ($n = 6$; 6/6 specific signals).

In this study, gDNA obtained from leaf tissue of Roundup Ready® soy GTS40-3-2 (RRS) is used as the model system. The calculation of the target copy numbers of "p35S" and "tNOS" in RRS genomic leaf tissue DNA took into consideration the following: (1) an estimated 1.25 pg Haploid Genome Weight for soy as described by Arumugunathan and Earle [38], (2) the homozygous status for the GTS40-3-2 locus in the applied reference material (gDNA from leaf tissue of homozygous seeds (Monsanto Company)), and (3) the available information on the inserted DNA present in RRS [8, 11, 12, 39, 40]. Based on these data, the "Roundup Ready GTS 40-3-2" locus comprises 1 copy of "p35S" and 1 copy of "tNOS" per haploid genome.

The SYBR®Green qPCR assay sensitivity is assessed by (1) serial dilution (in water) of leaf tissue DNA from homozygous Roundup Ready® soy GTS40-3-2 (RRS) (40.000–0.1 HGE), and (2) a dilution of the same leaf tissue DNA RRS in leaf tissue DNA Wt Soybean at 100, 1 and 0.1% RRS. All analyses are repeated sixfold and the LOD_6 is determined. From these analyses, also the PCR efficiency (E) for each of the methods can be calculated according to: [41]

$$E = \left(10^{-1/\text{slope}}\right) - 1$$

The PCR efficiency (E) could be expressed in percentage:

$$E = \left(\left(10^{-1/\text{slope}}\right) - 1\right) \times 100$$

Agarose gel analysis

Agarose gel electrophoresis (3% precast gels, Biorad) is performed using 0.5× TBE (45 mM Tris–borate–1 mM EDTA) at 100 V for 15 min, including a 100 bp–2 kb Molecular Marker (BioRad).

Results and discussion

Identification of core target DNA regions in the "p35S and "tNOS" elements present in the "EU-authorized GMO" Universe (March 2009), primer design and selection

Most EU-authorized GMOs contain either the "p35S" or the "tNOS" element, or both of them (see Table 1) [8, 11, 12]. In order to develop primer sets that specifically amplify all the "p35S" or the "tNOS" elements as present in the EU-authorized GM plants, a Bioinformatics DBase was compiled containing all the available relevant DNA sequences. Within both elements, a highly conserved core region could be identified: a 366-bp sequence for the "p35S" (reference GenBank: V00141.1, position 7,072–7,437) and a 256 bp for "tNOS" (reference GenBank: V00087.1, position 1,844–2,099). A common strategy for the development and selection of primer sets for both core elements was then applied (see "Materials and methods"). Several primer pairs were developed and a limited assessment of their amplification efficiency, selectivity, and specificity on gDNA of several target-containing GMO was performed (data not shown). The primer pairs listed in

Fig. 1 DNA sequence of the "p35S-long", "p35S-short", "tNOS-long" and "tNOS-short" amplicons obtained by SYBR®Green qPCR using "Sybricon" reference plasmids as template DNA. **a** Sybricon010 (p35S-long qPCR). **b** Sybricon017 (p35S-short qPCR). **c** Sybricon001 (tNOS-long qPCR). **d** Sybricon006 (tNOS-short qPCR). The reverse and forward sequencing primers are indicated in *bold*

Table 2 performed best in this assessment. The corresponding qPCR methods are further designated as "p35S-long", "p35S-short", "tNOS-long", and "tNOS-short", respectively. To guarantee that these qPCR methods amplified the correct target sequences, so-called "Sybricon" plasmids containing the respective amplification products are constructed using gDNA from Bt11 maize leaf tissue as template DNA. The DNA sequences of the cloned amplicons are shown in Fig. 1. The obtained sequences match perfectly with the sequence from which the primers were designed. "p35S-long" amplicon matches reference GenBank: V00141.1 (position 7,249–7,395), "p35S-short" amplicon matches reference GenBank: V00141.1 (position 7,323–7,397), and "tNOS-long" amplicon matches reference GenBank: V00087.1 (position 1,850–2,021). The "tNOS-short" amplicon matches reference GenBank:

Table 2 Primer pairs and amplicon size for each SYBR®Green qPCR method

SYBR®Green qPCR method name	Target	Primer name	Primer sequence	Amplicon size (bp)	References
"p35S-long"	CaMV 35S promoter	35S-3	GACAGTGGTCCCAAAGATGG	147	[42]
		35S-6	GTCTTGCGAAGGATAGTGGG		
"p35S-short"	CaMV 35S promoter	35S_N3Fwd	AAAGCAAGTGGATTGATGTGATA	75	This study
		35S_N3 Rev	GGGTCTTGCGAAGGATAGTG		
"tNOS-long"	tNOS trait specific	tNOS NEW Fwd1	CGTTCAAACATTTGGCAATAAAG	172	This study
		tNOS NEW Rev1	AAATGTATAATTGCGGGACTCTAATC		
"tNOS-short"	tNOS trait specific	tNOS_NN_Fwd	GATTAGAGTCCCGCAATTATACATTTAA	69	This study
		tNOS D REV	TTATCCTAGKTTGCGCGCTATATTT[a]		

[a] K represents a degenerate nucleotide equaling a G or T at that position

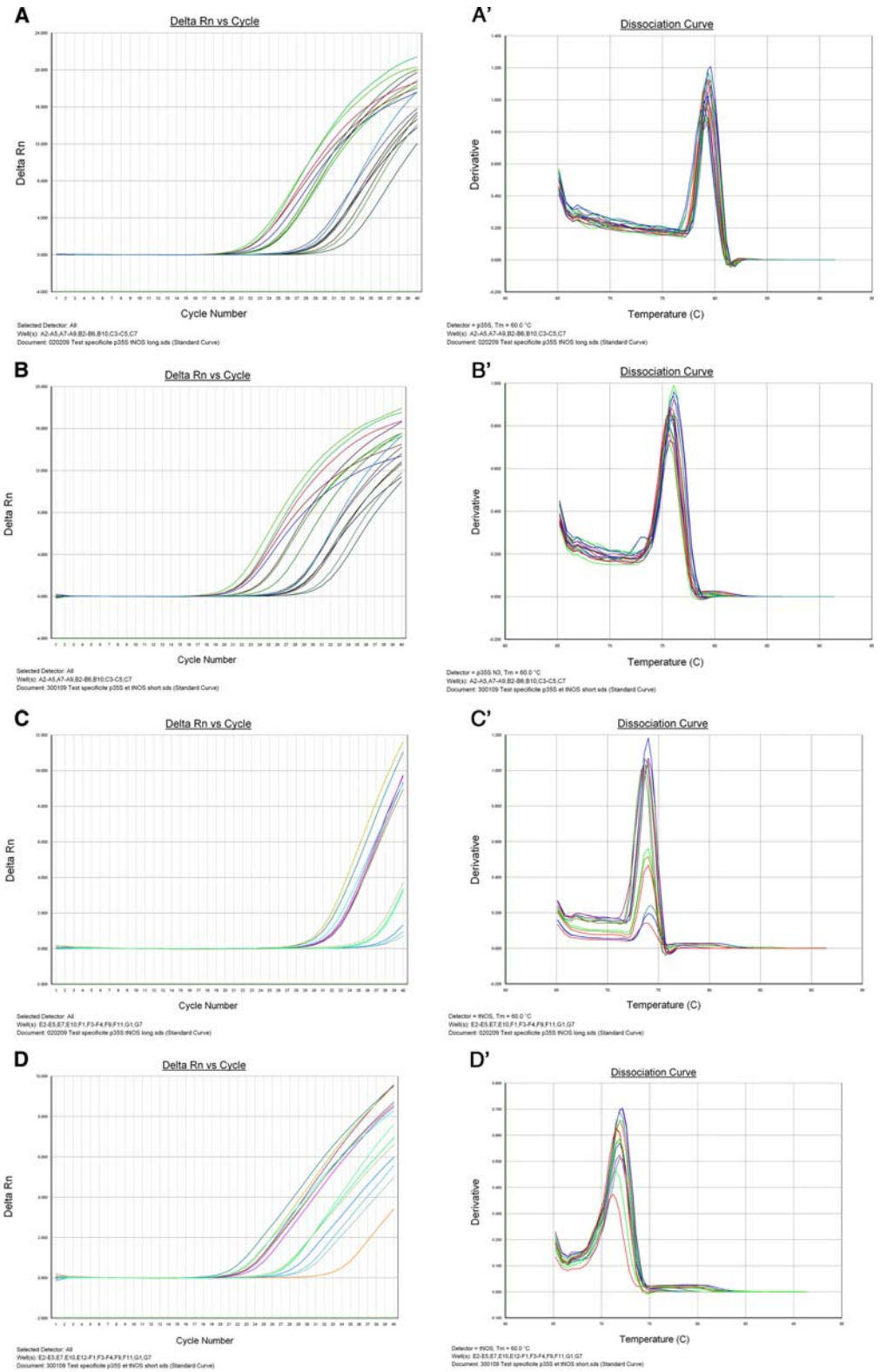

Fig. 2 Linear amplification plots (panels **a**–**d**) and melting curves (panels **a**'–**d**') obtained by SYBR®Green qPCR analysis of the target-containing GMO listed in Table 1. The different qPCR methods applied are the p35S-long qPCR in panel **a** and **a**', the p35S-short qPCR in panel **b** and **b**', the tNOS-long qPCR in panel **c** and **c**' and the tNOS-short in panel **d** and **d**'. In the amplification curves (panels **a**–**d**), the cycle number is plotted on the *X*-axis versus the measured fluorescence increase (expressed as ΔRn) on the *Y*-axis. In the melting curve analysis (panels **a**'–**d**'), the temperature (°C) is plotted on the *X*-axis versus the inverse of the first derivate of the best-fitted curve of the measured fluorescence decrease on the *Y*-axis

V00087.1 (position 1,996–2,064) with a single mismatch in position 2,055 (A → C) due to a degenerate nucleotide in the reverse primer (Table 2). The respective sequences match perfectly the expected ones as notified for Bt11 maize and recognize all to date "p35S" and "tNOS" containing EU-authorized GMO [as evaluated through blast analysis of the CCSIS Bioinformatics data analysis [40] (data not shown).

T_m value determination for the "p35S" and "tNOS" SYBR®Green qPCR amplicons with "reference plasmids" as DNA template

To minimize bias due to the genetic background in determining the nominal value of the melting temperature for each target, the "Sybricon" plasmids containing the respective amplification products were used to generate each of the "p35S" and "tNOS" amplicons. The T_m values for the different "p35S" and "tNOS" amplicons are distinct from each other with a T_m value at 80 and 76.5 °C for the "p35S-long" and "p35S-short", respectively, and at 73.3 and 72.5 °C for the "tNOS-long" and "tNOS-short", respectively (Table 1A). It is generally accepted that the T_m obtained with SYBR®Green qPCR could vary between 0.5 and 1 °C for the same amplicon [43, 44]. Therefore, to cover slight deviations in the T_m value between reference materials (Sybricons) and samples due to analyte impurities, a standard deviation of ±1 °C on the nominal T_m value will be applied, as the acceptance range, in further analysis.

Determination of "p35S" and "tNOS" SYBR®Green qPCR specificity

Using the 4 SYBR®Green qPCR methods, all target-containing GM-event samples give specific signal for "p35S" and/or "tNOS" (Table 1B). All NTC samples are negative and also all WT crop templates do not yield any specific signals. It can thus be concluded that all four methods are specific for their targets. In several CRM (8 out of 35 materials), however, weak-positive signals are detectable (indicated with "–[a]" in Table 1B.). These weak-positive signals are most probably due to the presence of low amounts of GMO impurities in the reference materials because the C_t levels of the signals reside at or below the LOD of the methods (see below) and a $\Delta C_t > 6$ between these aberrant signals and any target-positive element is observed. The CRM are certified for the presence of a specific target at a particular mass% but are not certified for the absence of any other GM targets that could be present at low level [45, 46]. Due to the very low quantities present, the nature of these impurities was not further investigated.

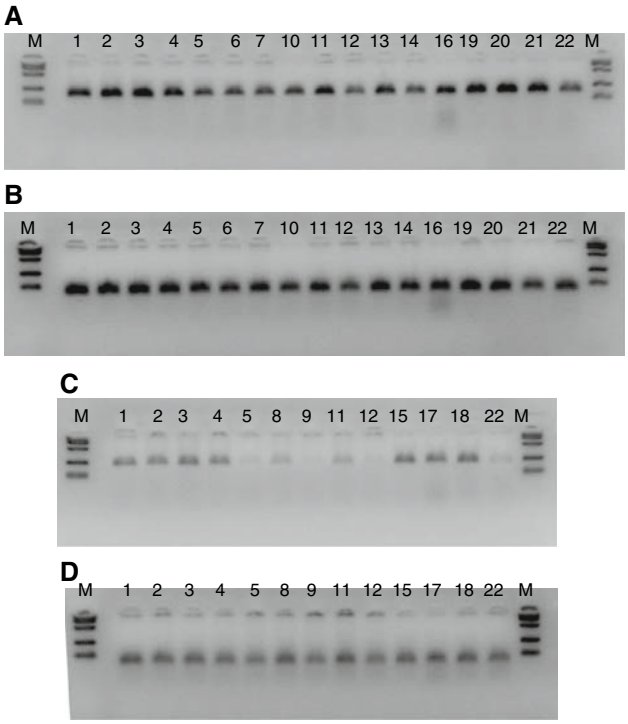

Fig. 3 Agarose gel electrophoresis of the "p35S" and "tNOS" PCR products amplified by SYBR®Green qPCR from gDNA extracted from reference material containing these elements. The respective qPCR methods applied were: panel **a** the p35S-long qPCR (expected amplicon length: 147 bp), panel **b** the p35S-short qPCR (expected amplicon length: 75 bp), panel **c** the tNOS-long qPCR (expected amplicon length: 172 bp), and panel **d** the tNOS-short qPCR (expected amplicon length: 69 bp). Tested GMO events containing these elements are: *1* "MON1445", *2* "MON531", *3* "MON15985", *4* "LL25", *5* "BT11", *6* "BT176", *7* "DAS 59122", *8* "GA21", *9* "MIR604", *10* "MON810", *11* "MON863", *12* "NK603", *13* "T25", *14* "TC1507", *15* "RF3", *16* "T45", *17* "MS8", *18* "EH92-527-1", *19* "LL62" (Bayer material), *20* "LL62" (AOCS material), *21* "A2704-12", *22* "GTS 40-3-2". *M* EZ load HT molecular marker, 100 bp–2 kb (5 bands: 100, 200, 500, 1,000, 2,000 bp)

All specific signals in the target-containing GMO generate a unique peak in melting analysis and the T_m values of the PCR products differ less than 1 °C from the nominal T_m value of the corresponding Sybricon plasmids (see Table 1B and Fig. 2). No additional peaks were observed in these analyses. Thus, the 4 SYBR®Green qPCR reactions generate a single specific signal without major additional amplification products.

Agarose gel analysis of the respective PCR products yields a single band at the expected molecular weight in all target-containing GMO (147 bp for "p35S-long, 75 bp for "p35S-short", 175 bp for "tNOS-long" and 69 bp for "tNOS-short"). Again, no major additional amplification products are observed (Fig. 3).

Table 3 Sensitivity assessment of the four SYBR®Green qPCR methods: "p35S-long" and "p35S-short", "tNOS-long" and "tNOS-short"

	pg gDNA RRS 100%/assay	50,000	5,000	500	50	25	12.5	6.25	2.5	1.25	0.625	0.125	NTC
	Theoretical copy number/assay	40,000	4,000	400	40	20	10	5	2	1	0.5	0.1	0
"p35S-long"	Signal ratio	6/6	6/6	6/6	6/6	6/6	6/6	6/6	*6/6*	5/6	6/6	1/6	0/6
	C_t mean	18.53	22.06	25.4	29.65	30.75	31.78	33.09	*35.17*	34.5	34.96	35.16	NA
	C_t standard deviation	0.23	0.38	0.22	0.21	0.45	0.43	0.71	*1.27*	0.42	0.95	NA	NA
"tNOS-long"	Signal ratio	6/6	6/6	*6/6*	2/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
	C_t mean	27.30	30.63	*35.17*	39.77	NA	NA	NA	NA	NA	NA	NA	NA
	C_t standard deviation	0.32	0.51	*0.57*	0.23	NA	NA	NA	NA	NA	NA	NA	NA
"p35S-short"	Signal ratio	6/6	6/6	6/6	6/6	6/6	6/6	6/6	*6/6*	5/6	4/6	0/6	1/6
	C_t mean	19.21	22.55	26.08	29.56	30.79	31.60	32.82	*34.45*	35.45	35.49	NA	36.75
	C_t standard deviation	0.10	0.09	0.05	0.30	0.31	0.35	0.51	*0.96*	0.46	0.70	NA	NA
"tNOS-short"	Signal ratio	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	*6/6*	4/6	2/6	0/6
	C_t mean	18.28	21.58	25.18	28.85	30.07	30.65	31.94	33.66	*34.46*	34.50	35.80	NA
	C_t standard deviation	0.19	0.12	0.09	0.23	0.15	0.42	0.33	1.12	*0.83*	1.62	0.29	NA

Average C_t values from a six repeats sensitivity assessment on gDNA from GM-soy event GTS40-30-2 (RRS). The LOD_6 is italicized. The signal ratio is expressed as specific signal/total number of reaction: as a positive control PCR, a soybean-specific lectin SYBR®Green qPCR was applied (data not shown)

NA Not applicable

Table 4 Sensitivity assessment of 4 SYBR®Green PCR methods: "p35S-long" and "p35S-short", "tNOS-long" and "tNOS-short", with 3 percentages of GM-soy event GTS40-3-2

	Template (50 ng)	GTS40-3-2 0.1%	GTS40-3-2 1%	GTS40-3-2 100%	NTC
p35S					
Long	Signal ratio	6/6	6/6	6/6	0/6
	Average C_ts	33.05	30.01	23.29	NA
	SD C_ts	0.21	0.17	0.16	NA
Short	Signal ratio	6/6	6/6	6/6	1/6
	Average C_ts	28.40	25.53	18.72	36.95
	SD C_ts	0.24	0.10	0.07	NA
tNOS					
Long	Signal ratio	2/6	6/6	6/6	0/6
	Average C_ts	39.18	37.13	29.07	NA
	SD C_ts	0.60	0.83	1.08	NA
Short	Signal ratio	6/6	6/6	6/6	0/6
	Average C_ts	28.97	26.13	19.29	NA
	SD C_ts	0.17	0.13	0.29	NA
PCR control		nt	nt	+	–

NA Not applicable, + positive signal, – no signal, *nt* not tested

Sensitivity of the 4 SYBR®Green qPCR methods for "p35S" and "tNOS" analytes on "model" reference materials

The results for the LOD_6 determination for the 4 SYBR®Green qPCR methods by serial dilution of leaf DNA from RRS is shown in Table 3. For the "p35S-long", "p35S-short" and "tNOS-short" qPCR methods, the LOD_6 can be set at 1–2 estimated HGE of the respective targets (Table 3). In the dilution series of the "p35S-long" analysis, the one copy dilution showed an initial deviation from the 6/6 positives, what would make the 2-copy level the LOD_6. However, at the consecutive estimated 0.5-copy dilution in this particular series, again 6/6 positives were found and a single positive was found at 0.1 copy. To clarify this statistically highly improbable observation, the latter dilution series was repeated and this time, the 1-copy dilution yielded 6/6, the 0.5 copy 3/3 and the 0.1 copy a 0/6 positives, respectively (data not shown). This allows to conclude that the LOD_6 for the "p35S-long" method is

indeed to be set at 2 copies. These three qPCR methods meet as such the criteria set by Kay and Van den Eede [47] (LOD < 20 copies) and by the ENGL method performance guidelines (2008) [41]. The PCR efficiencies of these 3 SYBR®Green qPCR methods (92.4, 94.2 and 96.1% for the "p35S-long", "p35S-short" and "tNOS-short" qPCR methods, respectively), also meet the ENGL acceptance criteria (accepted PCR efficiency between 89.6 and 110.2%) [41]. The performance of the "tNOS-long" method is however not acceptable with respect to both its sensitivity ($LOD_6 > 400$ estimated copies) and its PCR efficiency (75.4%). With the "p35S-short" SYBR®Green qPCR method one false positive is observed in a NTC; this weak signal ($C_t = 36.75$) is probably the result of aerosol contamination (e.g., from the co-analyzed RRS samples).

Finally, the performance of the 4 SYBR®Green qPCR methods on admixed leaf tissue gDNA preparation at 0.1, 1 and 100% RRS (w/w) was evaluated (Table 4). The "p35S-long", "p35S-short" and "tNOS-short" methods reliably detect 0.1% RRS, whereas the "tNOS-long" method fails at the 0.1% level (only 2/6 detected). Again, one weak-false positive signal was observed with the "p35S-short" SYBR®Green qPCR method in a NTC sample ($C_t = 36.95$). The lesser PCR sensitivity of the "tNOS-long" method is also reflected in a much larger ΔC_t with the "tNOS-short" method ($\Delta C_t = 10$), compared to the ΔC_t between both "p35S" methods ($\Delta C_t = 4.5$), Together, these results confirm that only three of the developed SYBR®Green qPCR methods are suitable in detecting low levels of GM material comprising "p35S" or "tNOS" elements.

Conclusion

Four different SYBR®Green qPCR methods for detecting "p35S" and "tNOS" elements, currently the two major targets in GMO screening analysis, have been developed. All four methods perform reliably with respect to target specificity, as (1) only target-positive DNA templates generate an exponential amplification, (2) the melting temperature analysis of the generated amplicons represents a single peak at the expected temperature, (3) a single band is visualized by agarose gel analysis with target-containing GM-event samples, and (4) the MW and DNA sequence of the respective amplification products matches the expected size and predicted DNA sequence. Three SYBR®Green qPCR methods ("p35S-long", "p35S-short" and "tNOS-short") have a high PCR efficiency (between 91 and 96%,) and are highly efficient at detecting low target concentrations [LOD < 20 HGE; 0.1% RRS (w/w)]. These three SYBR®Green qPCR methods offer a new valuable tool in screening for GMO presence in products. Combining these methods for generic targets with appropriate methods for GMO discriminating targets such as trait and/or endogenous markers, may enable the development of a cost-efficient GMO screening platform.

Acknowledgments The authors would like to greatly thank Els Vandermassen and Dirk van Geel for their technical assistance. Gilbert Berben (CRA-W, Belgium) and his team are acknowledged for providing the "p35S-long" primers sequences prior to publication. This study was financially supported by the European Commission through the Integrated Project Co-Extra, Contract No. 007158, under the 6th Framework Program, and by the GMODETEC project (RT-06/6) of the Belgian federal ministry of "Health, Food Chain safety and Environment".

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