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Comparison of Real-Time PCR Detection Chemistries and Cycling Modes Using Mon810 Event-Specific Assays as Model

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The most widely accepted methods for accurate quantitative detection of genetically modified organisms rely on real-time PCR. Various detection chemistries are available for real-time PCR. They include sequence-unspecific DNA labeling dyes such SYBR-Green I and the use of both universal (e.g., AmpliFluor) and sequence-specific double-labeled probes, the latter comprising hybridization (e.g., Molecular Beacon) and hydrolysis (e.g., TaqMan or MGB) probes. Also, new real-time PCR devices and reagents allowing fast cycling reactions exist. Five Mon810 real-time PCR assays were developed in which the event specificity was based on the detection of transgene and plant rearranged sequences found to 3′ flank the insertion site. Every assay was specifically designed for one particular detection chemistry, that is, AmpliFluor, Molecular Beacon, MGB, TaqMan, and SYBR-Green I. When possible, the assays were adapted to fast cycling mode. All assays displayed satisfactory performance parameters, although Molecular Beacon, MGB, and TaqMan chemistries were the most suitable for quantification purposes in both conventional and fast cycling modes.

KEYWORDS: Real-time PCR; genetically modified organism (GMO); detection chemistry; fast cycling mode; Mon810

INTRODUCTION

Real-time Polymerase Chain Reaction (PCR) has emerged as a powerful technique of DNA identification and quantification with a wide number of applications in molecular biology owing to its great sensitivity, precision, and accuracy. It is widely used, for example, in biomedical research to monitor gene expression, genotyping, and detection of single-nucleotide polymorphisms or in clinical diagnosis for quick detection of pathogenic microorganisms and viruses. In the fields of food science and agriculture real-time PCR is considered to be the most suitable technique for the detection and quantification of genetically modified organisms (GMO) in food and feed (*1*−*5*). Real-time PCR allows monitoring of the reaction in real time through fluorescence (*6*).

Different fluorogenic systems (chemistries) have been developed with a focus on either improving specificity and sensitivity or decreasing economic cost. Most published protocols rely on the use of probes dual-labeled with a reporter and a quencher dye [interacting via energy transfer resonance (FRET)] (*7*). (i) Both conventional (TM) and minor groove binding (MGB) TaqMan sequence-specific probes are dual-labeled with a fluorophore dye at 5′ end (reporter) and a quencher dye at 3′ end [in addition, MGB probes also include a minor groove binder group to increase the melting temperature (T_m)]. Specific amplification entails hydrolyses of the probe, which permits the detection of the reporter fluorescence (*8*−*10*). (ii) Molecular beacons (MB) are single-stranded sequence-specific probes with a hairpin-shaped structure with complementary ends (stem) that maintain the fluorophore and the quencher in close proximity. The loop region is complementary to the target sequence. During amplification, molecular beacons hybridize to the target sequence and unfold, thus allowing the emission of fluoresce (*11*). (iii) One of the alternative chemistries permitting lower costs is the Amplifluor (AF) technology, based on the unique dual-labeled hairpin primer UniPrimer, which emits fluorescence upon incorporation into the PCR product (unfolding). The UniPrimer contains a 3′ Z tail sequence that is also present at the 5′ end of one of the target-specific primers: this allows UniPrimer to act as a universal primer (*12*). (iv) SYBR-Green I (SYB) is the most commonly used real-time PCR chemistry that is not based on a probe. SYB is an intercalating dye having fluorescence that significantly increases upon interaction with double-stranded DNA (*13*). Hence, fluorescence emission is independent of the DNA sequence. Consequently, it is recommended to confirm the origin of the signal through a dissociation curve (identification of the product by T_m) (*14*).

The widespread use of real-time PCR in routine testing has prompted the development not only of new chemistries but also of new rapid real-time PCR protocols that take advantage of the fast thermal cycling capabilities of the new PCR machines.

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These new assays are certainly promising because they can reduce the reaction time by half. However, the stability of their performance due to rapid thermal cycling is still unclear (*15*).

Here we report a comparative study of five different real-time PCR fluorogenic systems (i.e., AF, MB, MGB, TM, and SYB) and two different cycling modes (i.e., conventional and fast) for use in GMO detection and quantification. The GM maize event Mon810 (Yieldgard, Monsanto) is here used as model. Being widely commercialized, a number of varieties derived from the original transgenic line are available, which allow testing the capacity of real-time PCR assays to equally detect different Mon810 varieties. Three Mon810 event specific real-time PCR assays based on TM chemistry and the 5′- and 3′-flanking sequences, respectively (*16*−*18*), have been published, the three of them displaying adequate performance values according to the JRC and CRL (the definition of minimum performance requirements for analytical methods of GMO testing are listed in http://gmo-crl.jrc.it/guidancedocs.htm, version 25-01-2005). Transgene−host plant DNA flanking sequences are the only real-time PCR target sequences so far described to grant event-specific detection and quantitation of GMOs (*2*, *19*, *20*).

Our approach to the comparison of different chemistries and cycling programs was as follows: we first defined a target sequence of around 210 bp corresponding to the 3′ junction region between the Mon810 insert and the plant host genome. We next designed five real-time PCR assays (one for each chemistry mentioned above) targeting the selected sequence, with the only condition that one primer targeted the transgene and the other one the host plant genome. For each chemistry, we used the appropriate software and took into account the most relevant parameters of each particular chemistry, according to the recommendations of each manufacturer. We selected the best in silico primers and probes and independently optimized each assay, according to the conventional protocols. When possible, the assays were designed by the developing company. Finally, the assays were transferred to fast real-time PCR without modifications other than the reagents specifically recommended. Linearity, limits of detection and quantification, and specificity of the assays were compared. Costs, practicality, and reliability of the different chemistries are also discussed.

MATERIALS AND METHODS

Plant Material and DNA Isolation. Powdered certified reference material (CRM) for GM maize lines Mon810 (ref ERM-BF413A,B,D,F), NK603 (ref ERM-BF415F), GA21 (ref ERM-BF414F), Bt176 (ref ERM-BF411F), Bt11 (ref ERM-BF412F), 1507 (ref ERM-BF418D), Mon863 (ref ERM-BF416D), and GM Roundup Ready soybean (ref ERM-BF410F) were purchased from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium), commercialized by Fluka (Fluka-Riedel, Geel, Belgium). Powdered CRM for GM cotton MON531, MON1445, and MON15985 and GM canola GT73 were purchased from the American Oil Chemist's Society (AOCS, www.aocs.org). Leaves of *Zea mays* cultivar W64A, *Hordeum vulgare*, *Brassica napus*, and *Helianthus annuus* were from plants cultivated in the greenhouses at the IBMB-CSIC. Seeds of 10 Mon810 commercial varieties were obtained in the Spanish market. Genomic DNA of T25 maize was provided by Bayer CropScience AG (Monheim am Rhein, Germany), and 1% CBH-351 genomic DNA was purchased from Fluka. Genomic DNAs were isolated from 0.2 g of plant material using the Nucleospin food kit (Macherey-Nagel Int., Easton, PA). DNA concentration was quantified by UV absorption at 260 nm using a NanoDrop ND1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE), and aliquots at 50 ng/μL were prepared for each sample.

Real-Time PCR Oligonucleotide Design. Real-time PCR assays were designed within a 210 bp sequence that included the 3′ region of the Mon810 transgene (bp 1−112) and the flanking host plant genome (bp 113−210), GenBank Accession no. AF490398 (*16*). To allow event-specific GMO identification and quantification, forward primers were placed within the transgene and reverse primers within the host plant genome.

The design of each assay was conducted following the conventional procedures recommended for this particular chemistry. For MB and SYB assays the software BeaconDesigner 5.0 (www.premierbiosoft-.com) was used with default parameters and an optimal primer length of 20 bp, GC content range from 40 to 80%, and maximum amplicon length of 150 bp. Only primer pairs not exhibiting dimerization were considered in order to avoid loss of sensitivity due to primer−dimer formation. The molecular beacon was labeled with 6-FAM as 5′ reporter and DABCYL as 3′ quencher with a conventional stem of 7 bp. The previously published TM primers and probe (*16*) were used, and a MGB assay was custom-designed by Applied Biosystems (Custom TaqMan Gene Expression Assays, Applied Biosystems, Foster City, CA). TM and MGB probes were also 5′ labeled with 6-FAM, whereas each one presented the most widely used 3′ quencher dye, that is, TAMRA (conventional TM probe) or the black MGBNFQ (MGB). The AF assay was designed by the developer corporation (Chemicon International Inc., Temecula, CA). Note that the forward primer included the Z sequence at the 5′ end. The AF hairpin primer (UniPrimer) was labeled as commonly distributed, that is, with JOE (reporter) and DABCYL (quencher).

Real-Time PCR Amplifications. Real-time PCR assays were carried out in a final volume of 20 μL using the Universal PCR Master Mix UPM (Applied Biosystems) 1× final concentration (except for AF reactions that were performed in 1× final concentration of SNP Buffer S+) and 4 μL (50 ng) of DNA template. The sequences of primers and probes are shown in **Table 1**. Optimization of the concentrations of primers and probes was carried out through a matrix approach (*21*) with genomic DNA extracted from 1% Mon810 CRM. In a first set of reactions, different concentrations of primers (50−900 nM) were run in triplicate in combination with a fixed concentration of probe (150 nM). The combination giving the lowest threshold cycle (C_T) and the highest final fluorescence values was selected and tested with different concentrations of probe (100−300 nM). For SYB, primer concentrations above 300 nM were omitted due to the detected unspecific signals produced in nontemplate control reactions. In the optimized reactions the primers and probes were used at the following concentrations: 200 nM each MonF/MonR and MonP (TM reactions); 900 nM each MonMGBF/MonMGBR and 200 nM MonMGBP (MGB reactions); 200 nM each MonMBF/MonMBR and 500 nM of MonMBP (MB reactions); 30 nM MonAFF and 300 nM each MonAFR and UniprimerMon (AF reactions); and 100 nM each SYBMonF/SYBMonR (SYB reactions). SYB reactions additionally included 1× SYB dye (Sigma-Aldrich Química, S.A., Madrid, Spain).

Real-time PCR amplifications were carried out in an ABI 7300 Real-Time PCR thermocycler (Applied Biosystems). MB, MGB, TM, and SYB reactions started with 2 min at 50 °C (UNG-Glycosylase activity) and 10 min at 95 °C (polymerase activation and UNG-Glycosylase inactivation). Subsequently, MB, MGB, and TM reactions were carried out under the same thermal cycling conditions, that is, 45 cycles of 15 s at 95 °C and 1 min at 60 °C with data collection at the annealing step. Conversely, SYB reactions were subjected to 45 cycles of 15 s at 95 °C, 30 s at 64 °C, and 30 s at 72 °C with data collection at the extension step, followed by a dissociation analysis: 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C (temperature ramp, 0.2 °C/s). AF reactions were as follows: 3 min at 95 °C and 45 cycles of 10 s at 95 °C, 20 s at 55 °C, and 40 s at 72 °C with data collection at the annealing step.

Fast real-time PCR assays (for MB, MGB, and TM chemistries) were performed as conventional reactions except for the specific Fast Universal PCR Master Mix (Fast-UPM, Applied Biosystems) that was used instead of UPM. Fast reactions were carried out on an ABI7500 Fast Real-Time PCR thermocycler (Applied Biosystems) with the following rapid thermal cycling program: 20 s at 95 °C and 45 cycles of 3 s at 95 °C and 30 s at 60 °C.

Unless otherwise stated, all reactions were performed at least in a total of 10 replicates in three independent experiments.

Table 1. Primers and Probes Used in the Real-Time PCR Assays

real-time PCR chemistry	name	sequence	amplicon length (bp)
AF	MonAFF	5′-Z sequence-TCACCGACCTGAACGAGGAT-3′	78
	MonAFR	5′-GCTCGCAAGCAAATTCGGAAAT-3′	
	UniPrimer	labeled with JOE at 5′ and DABCYL at 3′	
MB	MonMBF	5′-CCACCACAGCCACCACTTC-3′	100
	MonMBR	5′-CTCGCAAGCAAATTCGGAAATG-3′	
	MonMB[a]	5′-FAM-**CGCGATC**TGCACCGACCTGAACGAGGAC**GATCGCG**-DABCYL-3′	
MGB	MonMGBF	5′-ACCACTTCTCCTTGGACATCGA-3′	87
	MonMGBR	5′-CGCAAGCAAATTCGGAAATGAAAGA-3′	
	MonMGBP	5′-FAM-CTGAACGAGGACTTTCG-MGBNFQ-3′	
TM	MonF	5′-CAAGTGTGCCCACCACAGC-3′	104
	MonR	5′-GCAAGCAAATTCGGAAATGAA-3′	
	MonP	5′-FAM-CGACCTGAACGAGGACTTTCGGTAGCC-TAMRA-3′	
SYB	SYBMonF	5′-CCACCACAGCCACCACTTC-3′	100
	SYBMonR	5′-CTCGCAAGCAAATTCGGAAATG-3′	

[a] The stem sequence of the Molecular Beacon is highlighted in bold.

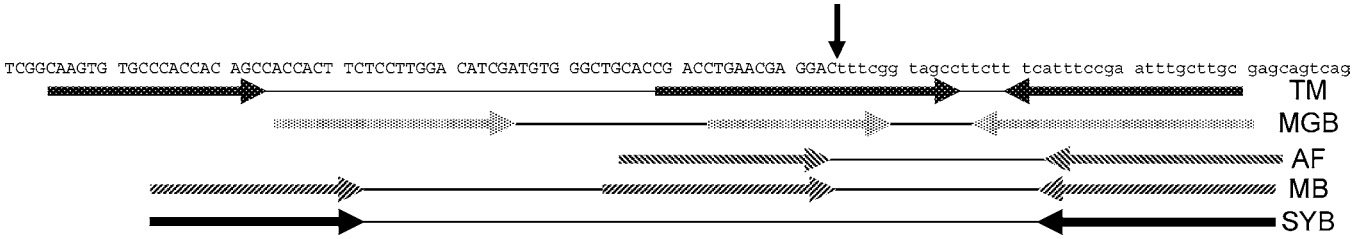

Figure 1. Schematic representation of the position of real-time PCR primers and probes along the target Mon810 3′ flanking sequence. Capital letters, *cryIA(b)* sequence; lower case letters, plant genome sequence. The vertical arrow shows the insertion point. Horizontal arrows indicate the primers and probes in a 5′→3′ direction. AF, MB, MGB, TM, and SYB indicate real-time PCR chemistries.

Specificity, Linearity, Efficiency, and Detection (LOD) and Quantification (LOQ) Limits. Specificity tests were performed with 100 ng of DNA corresponding to (i) 5 or 1% (w/w) CRM from GMO events other than Mon810, (ii) 5, 1, and 0.1% (w/w) Mon810 CRM, and (iii) other plant species. The following commercially available Mon810 varieties were also assayed: Aristis Bt (Limagrain Ibérica), Campero Bt (Limagrain Ibérica), Cuartal Bt (Arlesa Semillas), DKC6575 (Monsanto), Helen Bt (Limagrain Ibérica), Jaral Bt (Semillas Fitó), PR33P67 (Pioneer Hi-Bred), Protect (Koipesol), and SF1112T (Semillas Fitó). Genomic DNA extracted from each Mon810 variety was diluted in genomic DNA extracted from conventional maize (CRM Mon810 isogenic line, JRCC, Geel, Belgium) to achieve 1% GMO solutions. For each sample, at least five replicates were tested.

Linearity, efficiency, LOD, and LOQ were assayed using genomic DNA extracted from 5% (w/w) Mon810 CRM and serially diluted to 2000, 500, 125, 31, 8, 4, and 2 target molecules per 4 μL. This was calculated by considering the size of the maize haploid genome (*22*) and the molecular weight of double-stranded DNA. The same DNA samples were used as template to assay the five different chemistries (AF, MB, MGB, TM, and SYB) and the two different cycling modes (real-time PCR and fast real-time PCR). For each chemistry and cycling mode, a total of 10 replicates of each DNA concentration were run in three independent experiments. For each experiment and chemistry, a regression curve was calculated, which correlated the initial number of target DNA molecules and the C_T mean. The linearity was estimated by R^2 values. The efficiency (E) of the reaction was calculated using the formula $E = [10^{(-1/s)}] - 1$, s being the slope of the regression curve and 1 the value resulting from optimal efficiency. Acceptance criteria are $R^2 \geq 0.98$ and $-3.1 \geq s \geq -3.6$ (http://gmo-crl.jrc.it/guidance-docs.htm, version 25-01-2005). The absolute LOD (95%) was calculated as the lowest number of target molecules giving a positive signal with a 95% probability (http://gmo-crl.jrc.it/guidancedocs.htm, version 25-01-2005). The relative LOD was assessed by analysis of Mon810 CRM at 5, 1, 0.1, and 0% (w/w). The LOQ was placed at the lowest copy number exhibiting linear correlation with the C_T, with non-overlapping standard deviations (SD) among contiguous DNA dilutions. The unit of measurement has an impact on GMO quantity estimates. This is discussed in extensive detail in refs *19*, *20*, and *23*. Here we used the most widely applied reference materials for GMO testing purposes, that is, matrix-attached CRM produced from ground kernels by the IRMM. These CRMs have been produced on the basis of mass ratios.

Statistical Analyses. The slope, R^2, and Y-intercept values of the regression curves obtained with each chemistry (for each cycling mode) were compared by a one-way ANOVA test with P value below $\alpha = 0.05$. In case the differences were significant, a Tukey HSD post-hoc test was run to assess individual-pair differences.

The impact of the chemistry and the cycling mode on the slope, R^2, and Y-intercept values was also analyzed by a two-way ANOVA test (significance level, 0.05). For each parameter an F statistic was calculated, which indicated the significance of the differences ($p < 0.05$ indicates statistically significant differences).

RESULTS

Real-Time PCR Design Strategies. With the aim of reliably comparing the performance of five of the most commonly used real-time PCR detection chemistries, we selected a single target DNA (i.e., the Mon810 GM event 3′ flanking sequences) and independently designed and optimized the best possible real-time PCR assay using each technology: AF, MB, MGB, TM, and SYB. To make the assays easy, universal reagent mixes and universal cycling conditions were used when possible (i.e., MB, MGB, and TM). **Table 1** lists the selected primers and probes, and **Figure 1** shows their positions in the target sequence. All assays produced short amplicons ranging from 78 to 104 bp long.

Specificity of the Real-Time PCR Assays. The capacity of the real-time PCR assays to distinguish between target and nontarget samples was confirmed using as template genomic DNA from 14 different GMO events (i.e., 9 maize, 1 soybean,

Table 2. Specificity of Mon810 Real-Time PCR Assays Using as Template 50 ng of Genomic DNA from Different Commercial Maize Varieties[a]

	real-time PCR[b]					fast real-time PCR[b]		
Mon810 variety	AF	MB	MGB	TM	SYB	MB	MGB	TM
Aristis Bt	31.25 ± 0.11	30.17 ± 0.16	28.80 ± 0.05	28.16 ± 0.04	28.95 ± 0.11	28.08 ± 0.01	27.05 ± 0	31.56 ± 0.01
Campero Bt	31.62 ± 0.21	31.13 ± 0.01	28.30 ± 0.20	28.77 ± 0.40	28.36 ± 0.35	28.69 ± 0.25	27.63 ± 0.34	31.64 ± 0.33
Cuartal Bt	30.89 ± 0.67	30.31 ± 0.30	28.54 ± 0.13	28.21 ± 0.06	28.26 ± 0.08	28.14 ± 0.04	27.21 ± 0.03	31.29 ± 0.14
DKC6575	31.23 ± 0.03	30.12 ± 0.01	28.50 ± 0.10	28.19 ± 0.05	29.17 ± 0.55	28.12 ± 0.03	27.13 ± 0.01	31.22 ± 0.14
Helen Bt	31.42 ± 0.21	29.97 ± 0.15	28.56 ± 0.01	28.26 ± 0.04	29.29 ± 0.06	28.32 ± 0.07	27.13 ± 0	32.06 ± 0.19
Jaral Bt	31.28 ± 0.12	30.76 ± 0.05	28.97 ± 0.01	28.46 ± 0.17	28.97 ± 0.08	28.38 ± 0.14	27.50 ± 0.14	31.34 ± 0.07
PR33P67	31.67 ± 0.16	30.30 ± 0.21	28.55 ± 0.32	28.16 ± 0.04	28.64 ± 0.02	28.03 ± 0.10	27.68 ± 0.01	31.92 ± 0.01
Protect	31.27 ± 0.35	30.32 ± 0.15	28.69 ± 0.21	28.25 ± 0.18	28.24 ± 0.10	28.36 ± 0	27.15 ± 0.05	31.46 ± 0.39
SF1112T	31.03 ± 0.27	30.37 ± 0.42	28.42 ± 0.19	28.24 ± 0.03	28.76 ± 0	28.08 ± 0.04	27.11 ± 0.01	31.24 ± 0.05
Mon810 CRM	30.31 ± 0.23	30.23 ± 0.03	28.34 ± 0.10	28.89 ± 0.11	28.34 ± 0.22	28.37 ± 0.10	27.24 ± 0.11	31.32 ± 0.12
non-GM isogenic CRM	ND[c]	ND	ND	ND	ND	ND	ND	ND
mean values	31.29 ± 0.23	30.38 ± 0.23	28.59 ± 0.14	28.30 ± 0.11	28.74 ± 0.15	28.25 ± 0.22	27.22 ± 0.22	31.53 ± 0.32
% RSD values	7.5	0.53	0.47	0.39	0.52	0.78	0.80	1.03

[a] Simulated mixtures of 1% Mon810 genomic DNA were prepared with the CRM Mon810 isogenic conventional line and 10 commercial Mon810 varieties. Mean C_T and SD values are indicated. [b] For each particular chemistry and cycling mode, none of the obtained C_T values was statistically different (ANOVA, $p < 0.05$). [c] Not detected.

Table 3. Real-Time PCR Values Obtained with Decreasing Amounts of Genomic DNA Extracted from Mon810 CRM[a]

	(**A**) real-time PCR (conventional cycling mode)[a]					(**B**) fast real-time PCR[a]		
approx template molecules	AF	MB	MGB	TM	SYB	MB	MGB	TM
2000	30.55 ± 0.17	27.59 ± 0.11	25.67 ± 0.09	26.61 ± 0.06	24.59 ± 0.30	27.25 ± 0.18	26.86 ± 0.11	27.43 ± 0.25
500	32.95 ± 0.38	29.64 ± 0.17	27.70 ± 0.07	28.51 ± 0.05	27.15 ± 0.16	29.35 ± 0.17	28.87 ± 0.11	29.50 ± 0.36
125	34.76 ± 0.91	31.67 ± 0.27	29.74 ± 0.19	30.59 ± 0.16	29.45 ± 0.51	31.25 ± 0.49	31.03 ± 0.20	31.40 ± 0.35
31	35.24 ± 2.46	33.91 ± 0.35	32.01 ± 0.49	32.71 ± 0.28	31.87 ± 1.07	33.32 ± 0.58	33.21 ± 0.35	33.79 ± 0.59
8	36.13 ± 2.05	36.08 ± 0.73	33.72 ± 0.63	34.78 ± 0.65	33.81 ± 1.00	35.31 ± 0.74	35.17 ± 0.40	36.05 ± 0.70
2[b]	37.54 ± 1.23	38.44 ± 0.73	35.44 ± 1.09	37.50 ± 0.87	36.86 ± 1.23	37.02 ± 1.45	37.21 ± 0.33	37.75 ± 1.24
slope	–3.26 ± 0.11	–3.45 ± 0.06	–3.46 ± 0.01	–3.44 ± 0.15	–3.87 ± 0.31	–3.37 ± 0.16	–3.49 ± 0.11	–3.51 ± 0.13
R^2	0.98 ± 0.01	0.99 ± 0.01	0.99 ± 0.01	0.99 ± 0.01	0.99 ± 0.01	0.99 ± 0.01	0.99 ± 0.01	0.99 ± 0.01
efficiency	1.03 ± 0.06	0.95 ± 0.03	0.94 ± 0.01	0.95 ± 0.07	0.81 ± 0.12	0.98 ± 0.09	0.93 ± 0.03	0.93 ± 0.06

[a] Mean value and standard deviation of three independent real-time PCR runs corresponding to a total of 10 replicates are indicated. [b] Negative values were excluded from calculations.

3 cotton, and 1 canola events) and 10 non-GM species frequently found in food products. As expected, the five assays produced positive results only with Mon810 samples, demonstrating the selectivity of the assays. Moreover, all 10 tested Mon810 commercial varieties gave a positive signal (**Table 2**). When approximately the same numbers of target copies were analyzed, C_T values lay within one cycle (RSD below 7.5, 0.53, 0.47, 0.39, and 0.52 for AF, MB, MGB, TM, and SYB assays, respectively). This suggested that all five assays were adequate to analyze different commercial varieties.

Performance of Real-Time PCR Assays with Five Different Chemistries. Comparison of the five chemistries was carried out in replicate experiments performed with serial dilutions of 5% Mon810 genomic DNA ranging from 2000 to 2 target molecules per reaction. All reactions containing down to 8 target molecules produced positive amplification, and only 30−50% of the reactions containing 2 target molecules were positive. These results placed the absolute LOD (i.e., the lowest target copy number that can be reliably detected with a probability of 95%) between 8 and 2 target molecules for all chemistries and were consistent with statistical studies that considered the error associated with serial dilution processes [Poisson distribution of target molecules (*2*) and Monte Carlo simulations (*16*)]. The relative LOD of the five assays was tested through reactions with 5, 1, and 0.1% Mon810 CRM. All reactions produced positive amplification.

Table 3A summarizes the linearity and performance parameters obtained. Correlation coefficients (C_T vs initial amounts of target molecules) were ≥0.98, indicating high linearity and thus adequate quantification capacity of the reactions. The five R^2 values were similar ($p < 0.05$, **Table 4**). AF, MB, MGB, and TM assays exhibited efficiencies of 0.93−0.98, that is, close to the optimal value. However, one-way ANOVA ($p < 0.05$) and Tukey HSD tests (**Table 4**) showed that SYB reactions displayed significantly lower efficiency ($E = 0.81$).

For MB, MGB, and TM assays relative SD (RSD) values were below 1.5% down to 31 target molecules. AF and SYB reactions displayed higher RSD values over the whole range of

Table 4. Statistical Analysis of Regression Curves Calculated from Real-Time PCR Assays

	parameter[a]	one-way ANOVA (*F*)	*P* value	Tukey HSD test[b]
A[c]	slope	5.666	0.012	SYBR-Green, rest of chemistries <0.01
	R^2	0.008	0.204	
B[d]	slope	1.138	0.388	
	R^2	0.288	0.758	
C[e]	slope	0.016	0.926	
	R^2	1.616	0.225	

[a] Mean values for slope and correlation coefficient of three real-time PCR runs were assayed for significant differences in a one-way ANOVA with $\alpha = 0.05$. [b] Estimated difference for Tukey HSD analysis of chemistry pairs. [c] AF, MB, MGB, TM, and SYB chemistries in conventional cycling mode. [d] MB, MGB, and TM chemistries in fast mode. [e] Conventional vs fast cycling modes (MB, MGB, and TM chemistries).

target molecules. Taking into account the SD values, the LOQ could be placed around 31 target molecules for the former three assays, around 125 target molecules for SYB assays, and around 500 target molecules for AF assays.

MB, MGB, and TM Assays Can Be Easily Adapted to Fast Real-Time PCR. The new fast real-time PCR allows shortening real-time PCR assays through a fast cycling mode. Mon810 MB, MGB, and TM assays were run in fast mode with the only modification of a special universal PCR master mix. All three fast real-time PCR assays displayed adequate specificity, as indicated by the lack of amplification of DNA from nontarget samples (i.e., GMO events other than Mon810 and non-GM species) and positive results with all tested Mon810 varieties (**Table 2**). Fast reactions displayed performance parameters similar to the ones obtained with conventional reactions and close to the theoretical optimum values (**Table 3B**). The absolute LODs were between 8 and 2 target molecules, and the LOQs were around 31 target molecules. R^2 values were above 0.99 and the efficiencies between 0.93 and 0.98.

R^2 (linearity) and slope (efficiency) values corresponding to fast versus conventional cycling modes exhibited no statistical differences (**Table 4C**) and so was true for MB versus MGB versus TM chemistries in either conventional (**Table 4A**) or fast (**Table 4B**) mode. Further analyses were conducted by two-way ANOVA test considering two possible sources of variation (i.e., real-time PCR chemistry and cycling mode) on the same performance parameters (i.e., slope and R^2 of the regression curves). Although the chemistry or cycling mode did not statistically influence R^2 and slope values (significance level, 0.05), they contributed differently to the variation of these parameters, with the cycling mode exhibiting higher contribution on the linearity (11.28% of the total variation, whereas chemistry, 1.43%; and the interaction, 3.28%) and the chemistry and interaction exhibiting higher impact on the slope (8.79 and 11.59%, respectively; and cycling mode, 0.06%).

DISCUSSION

Real-time PCR assays allow sensitive specific detection and accurate quantitation of DNA sequences. They are nowadays common assays in research but are also routine in laboratories such as those carrying out GMO analysis. Due to its broad implementation in many areas, various detection technologies have been developed and are commercially available. At the same time, universal mixtures of PCR reagents have been optimized that facilitate the setup of the reactions, thus reducing the probability of errors. In the past few years, a novel development arose consisting of real-time PCR thermocyclers (and specific reagents) capable of running fast real-time PCR amplifications in conventional 96-well polypropylene microtube format. Fast reactions are also available using carousel-based PCR devices in a 32 glass capillary format and specific reagents (LightCycler, Roche Molecular Systems, Pleasanton, CA). Although all are based on fluorescence emission derived from amplicon synthesis or accumulation, the different chemistries display different mechanisms of action that may result in differences in the specificity and performance of the reactions. The choice of the most suitable detection chemistry will depend upon the intended use of this particular assay and should take into account parameters such as the performance characteristics of the reaction and also the economic cost and difficulty of carrying out the assays. To assist user laboratories in the selection of chemistries and PCR devices, we compared five of the most widely used real-time PCR chemistries using universal PCR mix reagents and a single DNA target sequence, that is, the 3′ flanking sequence of GM maize Mon810 event, which allows event-specific detection and quantification of this GMO. Furthermore, and with the only modification of using a special universal PCR mix, the reactions were evaluated in fast cycling mode. Such quick adaptation from conventional to fast cycling mode is important for the implementation of this technology because most real-time PCR assays optimized to date have been developed and validated in conventional cycling conditions.

Development of a real-time PCR assay aiming at the specific detection and quantification of a GMO event requires demonstration of its lack of cross-reactivity with DNA from other GMO events and non-GM plant species, especially those frequently found in feed and food products. Our Mon810 assays based on AF, MB, MGB, TM, and SYB technologies (for both conventional and fast cycling modes) did not show cross-reactivity with DNA from other GMOs or other plant species, thus proving adequate exclusivity. These types of tests have been published for most real-time PCR assays for GMO analysis (*23*, *24*). However, a second specificity requirement is inclusivity (i.e., the capacity of the assay to detect all possible target varieties). GM events are as a rule commercialized in a number of varieties adapted to the different target geographical and climatologic conditions, as well as to the different purposes (e.g., food or feed) of the culture. This is normally achieved through crossings with appropriate varieties followed by a series of backcrossings and results in GM varieties of a single event that display different agronomic properties (*25*). Ten Mon810 varieties produced and commercialized by different seed companies and widely cultured in Spain were initially tested. All of our Mon810 real-time PCR assays were capable of equally detecting all analyzed commercial varieties. In addition, they were capable of quantifying Mon810 percentages in combination with adequate validated species-specific reference assays such as *adh1* [Community Reference Laboratory, (CRL, http://gmo-crl.jrc.it) (*26*)]. As an example, the results here presented (**Table 2**) together with the *adh1* endogenous reference real-time PCR assay (not shown) produced the expected results (1.16 ± 0.19, 1.02 ± 0.11, 1.02 ± 0.05, 1.01 ± 0.07, and 0.95 ± 0.21% for AF, MB, MGB, TM, and SYB, respectively, and 1.02 ± 0.08, 0.99 ± 0.06, and 1.03 ± 0.04% for fast MB, MGB, and TM, respectively). Therefore, we concluded that event-specific real-time PCR assays could be designed using any of the tested chemistries and cycling modes.

Real-time PCR assays optimized with all five tested chemistries displayed satisfactory performance parameters, although some differences were observed. The absolute LODs (95%) were placed below 8 target molecules, i.e., they were in the range of previous real-time PCR publications (*23*, *24*, *27*−*32*). Relative LODs were at least 0.1%, further proving that these assays fulfill the LOD requirements of the European Union legislation (i.e., a threshold at 0.9% approved GMO is established above which labeling is compulsory). Quantification of target DNA was possible with all tested chemistries, that is, linearity values were statistically similar. But SYB assays showed the lowest efficiency values, that is, below the ENGL accepted limit. MB, MGB, and TM assays displayed lower LOQ values than SYB and mainly AF. Especially MB, MGB, TM, and SYB LOQs were adequate considering other published assays and statistical approaches (*33*) and fit with the ENGL acceptance criteria. The high LOQ exhibited by AF assays might be linked to this particular target sequence: examples exist of AF real-time PCR assays displaying LOQs around 100 copies (*34*). In a related study performed by partners of the Co-Extra

project these four chemistries performed equally in the event-specific detection and quantification of Roundup Ready soybean (*35*), although MB assays were somewhat less efficient and sensitive. Therefore, it is likely that the sequence and structural properties of the target DNA have an influence on the behavior of the molecules involved in each chemistry.

The new fast real-time PCR cycling mode allows real-time PCR to be run in <1 h. For MB, MGB, and TM chemistries fast reagents are available, whereas for AF and SYB adequate reagents are lacking and therefore their use in rapid thermal cycling mode is currently not recommended. We successfully adapted Mon810 MB-, MGB-, and TM-based reactions to fast cycling mode while maintaining their performance and with minimal optimization requirements, that is, only the special universal PCR master mix. Another advantage of fast PCR cycling might be that the polymerase (and other reagents) has to spend less time at high temperatures. Prolonged heating will reduce the activity of the enzyme, and fast cycles might be why MB assay preformance seems to slightly improve when a fast protocol is used.

The choice of detection chemistry is largely dependent upon the particular use of real-time PCR, personal experience, and the resources available. The reliability of real-time PCR increases significantly with probes (this is discussed in ref *3*), but probe design can be difficult, and probe chemistries render probes highly expensive. SYB assays use a universal dye, and AF chemistry relies on a universal probe, making these assays more economical than MB, TM, and especially MGB if small quantities of different reactions are to be simultaneously run in a laboratory (this is discussed in ref *35*). The possibility of decontamination approaches such as uracyl-*N*-glycosylase (UNG) can be very relevant. Notably, the AF cannot use this strategy. Finally, MB, MGB, and TM assays can be run in fast cycling mode. This increases the throughput, although a fast thermal PCR thermocycler is needed.

Therefore, when this particular target sequence was used and after the design of the best possible assay for each chemistry, MB, MGB, and TM technologies performed equally well and were easily adapted to fast cycling conditions. Conversely, although being more economical, AF and SYB proved to be less adequate for quantification purposes.

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