Multiplex real-time PCR-based detection and quantification of genetically modified maize events employing SYBR® Green I and TaqMan® chemistries

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Multiplexing in real-time PCR offers advantages over singleplex real-time PCR by saving time and resources. SYBR® Green I-based duplex and triplex real-time PCR assays targeting event-specific sequences of three genetically modified (GM) maize events, namely *Bt*11, *Bt*176 and MON89034, and taxon-specific endogenous *Adh1* gene were developed to simultaneously identify multiple events. Duplex real-time PCR assay based on TaqMan® chemistry targeting *Bt*176 event-specific sequence and *Adh1* was also optimized for quantification purpose. Limit of detection of developed assays was up to 0.05% and limit of quantification of the reported TaqMan® based real-time PCR was up to 0.5%.

Keywords: Dyes, genetically modified maize, GM detection and quantification, multiplex real-time PCR, primers and probes.

GENETICALLY modified (GM) maize, an important food and feed crop, is the second largest commercially cultivated GM crop globally with the highest number of approved GM events, i.e. 138, covering 55.2 m ha of global area under cultivation of GM crops[1,2]. With the increasing number of GM maize events, development of efficient, sensitive and reliable assays for detection and quantification is required.

PCR is the method of choice for GM detection due to high specificity, sensitivity and robustness[3–6]. Real-time PCR is the robust technique being employed for GM detection and quantification, with high-throughput testing and minimum risk of contamination due to elimination of post-PCR analysis. Multiplexing in real-time PCR offers advantages over conventional and singleplex real-time PCR by saving time and resources[7–10]. SYBR® Green I and TaqMan® chemistries are being employed for real-time PCR. In multiplex real-time PCR using SYBR® Green I chemistry, differentiation of events can be done based on melting curve analysis. These assays could be utilized for screening of GM events, which can be further quantified using singleplex or multiplex TaqMan® real-time PCR assay. TaqMan®-based real-time PCR approach is more specific, robust and can be used for quantification of GM content.

Real-time multiplex PCR assays employing TaqMan® chemistry have been reported for screening of genetically modified organisms (GMO). Tetraplex real-time PCR assay targeting commonly used screening elements, *Cauliflower Mosaic Virus 35S* promoter (*P-35S*), *Figwort Mosaic Virus* promoter (*P-FMV*), *Agrobacterium tumefaciens nos terminator* (*T-nos*) and *Cauliflower Mosaic Virus 35S* terminator (*T-35S*) has been developed[11]. Hexaplex real-time PCR for detection of *P-35S*, *P-FMV 34S*, *T-nos*, two construct-specific sequences and a positive internal control has been developed[10]. Quadruplex real-time PCR assay for *P-35S*, *T-nos* and endogenous reference genes for soy and maize has also been developed[12]. Few more duplex real-time PCR assays targeting *P-35S* and *T-nos* have been reported[7,9,13].

The present study discusses the development of event-specific multiplex PCR assays for detection of GM maize events, *Bt*11, *Bt*176, MON89034 along with endogenous *Adh1* gene in different multiplex formats employing SYBR® Green I chemistry. TaqMan®-based duplex real-time PCR targeting event-specific sequence for *Bt*176 event and *Adh1* gene was optimized for quantification purposes. Efficiency and practical utility of the two chemistries being employed were also compared.

Materials and methods

Test samples and DNA extraction

Certified reference material (CRM) of GM maize events *Bt*11 (ERM-BF412f) and *Bt*176 (ERM-BF411f) was procured from the Institute for Reference Materials and Measurements (IRMM), European Commission-Joint

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Table 1. Primers and probes employed in multiplex real-time PCR assays

Target	Primer	Primer sequence (5′–3′)	Expected amplicon size (bp)	Reference
Using SYBR® Green I chemistry				
*Bt*176	bar3/*Bt*176 plant	F-AAGCACGGTCAACTTCCGTAC R-TCGACTTTATAGGAAGGGAGAGG	570	15
MON89034	MON89034 Primer1/Primer2	F-TTCTCCATATTGACCATCATACTCATT R-CGGTATCTATAATACCGTGGTTTTTAAA	77	16
*Bt*11	T25 1–5′/*Bt*11 PR	F-GCCAGTTAGGCCAGTTACCCA R-CAAAAATCCAAGAATCCCTCCAT	580	15
Adh1	*Adh1* F/R	F-CCTTCTTGGCGGCTTATCTG R-CCAGCCTCATGGCCAAAG	70	17
Using TaqMan® chemistry				
*Bt*176	*Bt*176F/R	F-GGCCGTGAACGAGCTGTT R-GGGAAGAAGCCTACATGTTTTCTAA Probe: FAM-AGCAACCAGATCGGCCGACACC-TAMRA	82	18
Adh1	*Adh1* F/R	F-CCTTCTTGGCGGCTTATCTG R-CCAGCCTCATGGCCAAAG Probe: TET-CTTAGGGGCAGACTCCCGTGTTCCCT-TAMRA	70	17

Research Centre, through Sigma Aldrich. DNA sample of GM maize event MON89034 imported through ICAR-National Bureau of Plant Genetic Resources for research purposes was used while molecular testing.

Genomic DNA from 100 mg of homogenized seed powder of the CRMs was extracted using HiPurA™ Plant Genomic DNA Miniprep Purification Kit (HiMedia Laboratories Private Limited, Mumbai, India) according to the manufacturer's instructions. Quantity and quality of purified DNA samples were evaluated using a UV spectrophotometer (Eppendorf, Hamburg, Germany). DNA samples were diluted to a final concentration of 40 ng/µl. DNA samples of *Bt*11, *Bt*176 and MON89034 were used for duplex real-time PCR with the respective event-specific primers along with endogenous gene *Adh1*. Equimolar mixes comprising (i) DNA samples of *Bt*176 and MON89034, and (ii) DNA samples of *Bt*11, *Bt*176 and MON89034 were used for duplex and triplex SYBR® Green I-based real-time PCR respectively.

Oligonucleotide primers and TaqMan® probes

Two fluorescent channels were employed, using the fluorescent reporter dyes 6-carboxyfluorescein (FAM) and tetrachlorofluorescein (TET) to label the 5′-ends of the probes targeting *Bt*176 and *Adh1* respectively. Tetramethylrhodamine (TAMRA) was used as quencher dye for FAM and TET. The primers and fluorescence-labelled probes were synthesized and purified by Pivotal Marketing, India. Table 1 provides details of primers and probes for real-time PCR.

Real-time multiplex PCR assays

Using SYBR® Green I chemistry: Event-specific real-time PCR assays were optimized in duplex formats targeting one of these events, *Bt*11, *Bt*176 or MON89034, along with endogenous reference *Adh1* gene. After optimizing the reaction conditions, event-specific duplex and triplex real-time PCR assays were developed simultaneously targeting (i) *Bt*176, and MON89034 and (ii) *Bt*11, *Bt*176 and MON89034 respectively. Real-time PCR assays were performed on 7500 Real-time PCR System (Applied Biosystem, CA, USA). PCR was carried out in 25 µl of reaction mix containing 300 ng of template DNA, 1X QuantiTect SYBR® Green PCR master mix (Qiagen, Germany) and 0.2 µM each of the forward and reverse primers of each target. The following thermal profile was used: 2 min at 50°C, single cycle of DNA pre-activation for 10 min at 95°C followed by 40 amplification cycles of 15 sec at 95°C (denaturation step), and 1 min at 60°C (annealing–extension step). After PCR amplification, melting curve analysis was performed using Dissociation Curve Analysis software v2.0.6 (Applied Biosystems). The PCR products were heated to 95°C for 15 sec, cooled at 60°C for 1 min and then slowly heated back to 95°C at a rate of 1% ramp rate.

Limit of detection (LOD) of developed *Bt*176 event-specific duplex real-time PCR assay with *Adh1* was tested by preparing dilutions to obtain test samples with 5% (5504 copies), 2.5% (2752 copies), 1% (1101 copies), 0.5% (550 copies), 0.1% (110 copies), 0.05% (55 copies) and 0.01% (11 copies) GM content.

Using TaqMan® chemistry: TaqMan®-based real-time duplex PCR assay was carried out on the 7500 Real-time PCR System. PCR was performed in 25 µl of reaction mix containing 300 ng of template DNA, 1X Qiagen multiplex PCR master mix (Qiagen, Germany), primers and probe of *Bt*176 (0.1 µM of forward primer, 0.6 µM of reverse primer and 0.2 µM of probe), endogenous gene

Adh1 primers and probe (0.15 μM of forward and reverse primers and 0.1 μM of probe).

Performance of quantitative duplex TaqMan® real-time PCR assay targeting Bt176 and Adh1

For estimation of LOD and limit of quantification (LOQ) of the developed real-time PCR assays, DNA sample of CRM of *Bt*176 with mass fraction 5% equivalent to 5504 copies was diluted to obtain reference samples with 2.5%, 1%, 0.5%, 0.1%, 0.05% and 0.01% corresponding to 2752, 1101, 550, 110, 55 and 11 copies of the target respectively. Repeatability of standards was determined from data of triplicate reactions. To calculate amplification efficiency, a standard curve of Ct (cycle threshold) values was plotted against the log of estimated GM concentration in the sample. Method acceptance criteria and method performance requirements according to the Codex Alimentarius Commission guidelines (CAC/GL 74-2010) were considered. Squared correlation coefficient (R^2) was calculated as correlation coefficient of the standard curve obtained by line regression analysis. Parameters including accuracy in terms of precision and trueness, LOD and LOQ of the method were evaluated by comparing experimental mean value with theoretical value of GM content.

Practical applicability of the developed duplex real-time PCR assays

Unknown samples were also run for quantification to verify the practical applicability. Reactions were run under optimized conditions. CRMs of 2%, 1%, 0.5% and 0.1% *Bt*176 were used as test samples unknown to the operators for determination of applicability of developed real-time multiplex assay.

Results and discussion

SYBR® Green I-based duplex real-time PCR assays targeting GM maize events and endogenous Adh1 gene

SYBR® Green I-based three duplex real-time PCR assays targeting GM maize events, *Bt*11, *Bt*176 and MON89034 along with the endogenous *Adh1* gene were optimized for identification of the respective GM events. The endogenous *Adh1* gene was included as the internal control to ensure efficiency of PCR assays by eliminating false negatives. In the assays, *Bt*11, *Bt*176 and MON89034 events were differentiated from their non-GM counterparts using melting curve analysis. Initially, melting curves for event-specific and endogenous *Adh1* gene-specific primer pairs were analysed using singleplex real-time PCR. Calculated *T*m values for *Bt*11, *Bt*176, MON89034 and *Adh1* primers were 74.84°C, 88.08°C, 71.94°C and 78.98°C respectively. In duplex real-time PCR, melting curves of both the event and *Adh1*-specific targets were detected in each sample, with the corresponding melting temperature, whereas only endogenous gene-specific melting curve was observed in the sample of non-GM maize (Figure S1, see Supplementary Material online).

Event-specific SYBR® Green I-based duplex and triplex real-time PCR assays

Event-specific SYBR® Green I-based duplex PCR assay targeting *Bt*176 and MON89034 events was developed, discriminating two events based on the difference in melting curves of their products. Amplification signals with expected melting temperatures were detected in duplex event-specific PCRs with *Adh1* (Figure 1 *a*). Triplex real-time PCR assay was developed for simultaneous identification of three GM maize events, viz. *Bt*11, *Bt*176 and MON89034. Amplification signals were observed with their respective melting temperatures, i.e. 74.84°C in *Bt*11, 88.08°C in *Bt*176 and 71.94°C in MON89034 (Figure 1 *b*).

Sensitivity of duplex real-time PCR assays targeting Bt176 and Adh1 using SYBR® Green I and TaqMan® chemistries

In SYBR® Green I-based real-time PCR, fluorescent signals and melting peaks corresponding to both targets (*Bt*176 and *Adh1*) were detected in all the six replicates of test samples with 5%, 2.5%, 1.0%, 0.5%, 0.1% and 0.05% GM content (Figure 2). Sensitivity in terms of copies was also calculated using the 1C (nuclear DNA content) value corresponding to the weight in picograms per haploid host plant genome[14]. LOD of the developed assay was established up to 0.05%, equivalent to 55 copies of the target.

TaqMan®-based duplex real-time PCR assay was optimized to quantify *Bt*176 event in the unknown sample. *Adh1* was included as the reference gene for absolute quantification. Calibration curves for event and endogenous gene-specific targets were prepared using standards with 5504, 2752, 1101, 550, 110 and 55 copies of *Bt*176 event per reaction, and 110,080, 55,040, 22,016, 11,008, 2201 and 1101 copies of endogenous gene per reaction respectively. All six levels of both the standards gave fluorescent signals (Figure S2, see Supplementary Material online). Repeatability and reproducibility were calculated from data of triplicate reactions. Values of relative standard deviation for repeatability (RSD_r) and relative standard deviation for reproducibility (RSD_R) ranged from 0.055% to 0.1% and 0.1% to 0.36% respectively (Table S1, see Supplementary Material online).

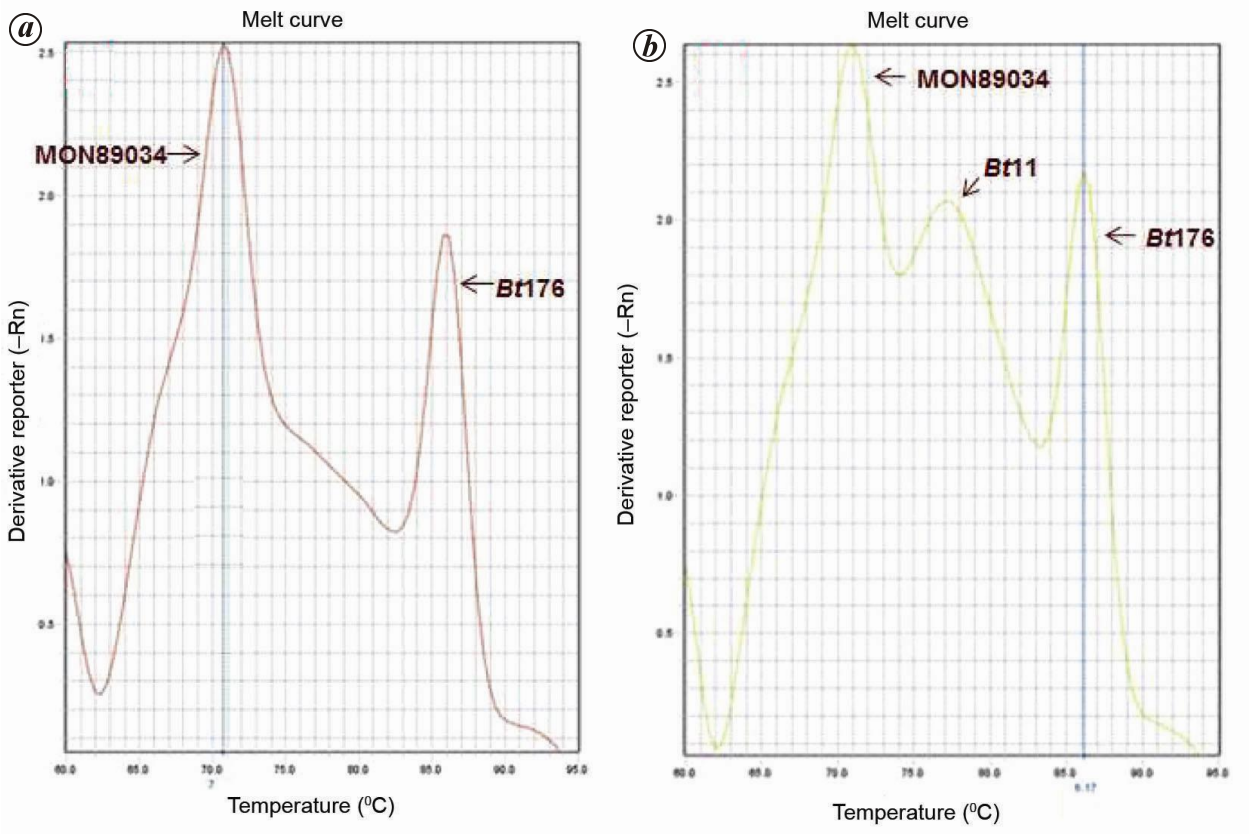

Figure 1. Event-specific SYBR® Green I-based multiplex real-time PCR assays for simultaneous identification of GM maize events. ***a***, Duplex real-time PCR targeting events *Bt*176 and MON89034. ***b***, Triplex real-time PCR targeting events *Bt*11, *Bt*176 and MON89034.

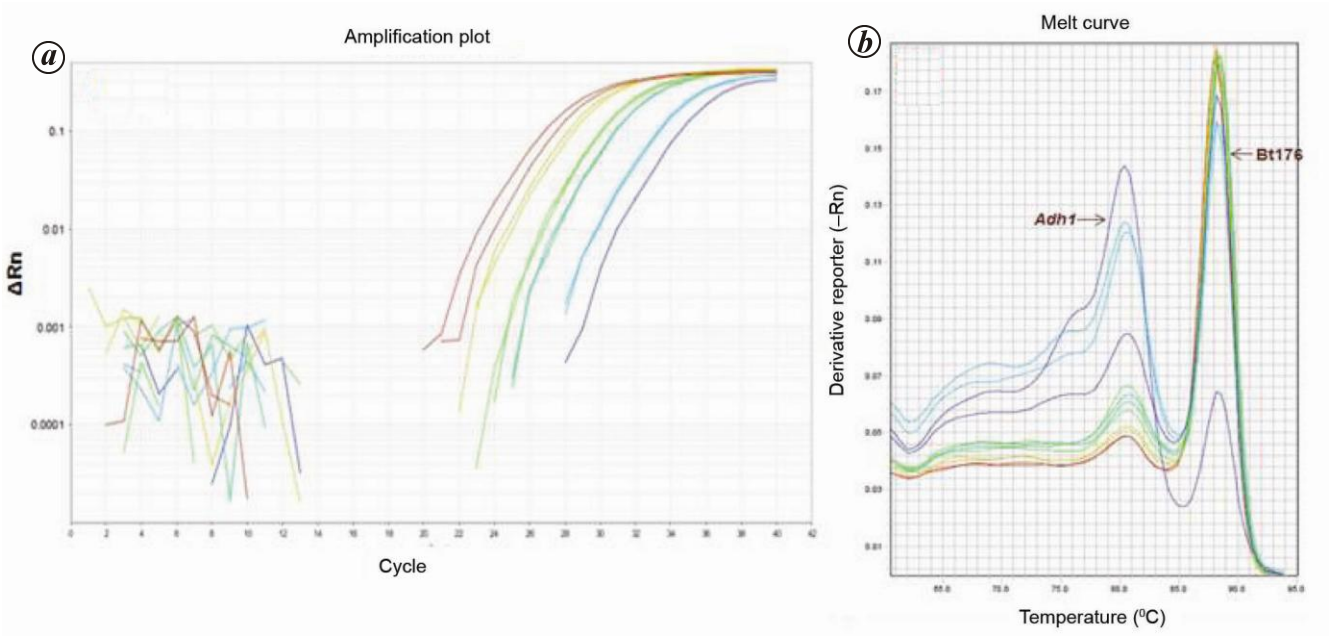

Figure 2. Sensitivity results of the developed SYBR® Green I-based duplex real-time PCR assay targeting *Bt*176 and endogenous *Adh1* gene for identification of GM maize event *Bt*176 and differntiation of GM maize from non-GM counterpart. (***a***, ***b***) Amplification (***a***) and melting (***b***) profiles for the test samples with 5%, 2.5%, 1.0%, 0.5%, 0.1% and 0.05% GM content to test limit of detection of developed duplex PCR assays (reactions were run in triplicate). In (***a***) and (***b***) representation of colours of the amplification curves for GM content is as follows: red curve: 5%, yellow: 2.5%, light green: 1%, green: 0.5%, light blue: 0.1%, purple: 0.05%.

Table 2. Accuracy in terms of trueness and precision for quantitative estimation of GM maize event *Bt*176 using TaqMan®-based duplex assay

	Trueness		Precision	
True value	Mean GM content (%)	*Bias (%)	SD	**RSDr
2	2.1	5.00	0.04	1.90
1	1.09	9.00	0.03	2.75
0.5	0.46	–8.00	0.075	16.30
0.1	–	–	–	–

*Deviation from true value (Bias true value in %) was calculated using the following formula:
[(Experimentally determined GM value – true GMO value)/true GMO value] × 100.
**RSDr, Relative repeatability standard deviation values obtained by dividing the standard deviation by mean value, and given in %.
–, No amplification was detected.

Both the standard curves showed nearly 100% efficiency with slopes of –3.101 and –3.361 for *Bt*176 and *Adh1* respectively. LOD for the developed assay was up to 0.05%, detecting 55 copies (Figure S2, see supplementary material online). To confirm the practical utility of the developed assay for quantification, CRMs were provided as unknown test samples to the operators; and results showed the positive amplification with approximate GM content present in each sample, i.e. 2%, 1% and 0.5% with the RSD_r values less than 25% (Table 2). The results of the reported assay are in line with the acceptance criteria for quantification of specific GM events according to the Codex Alimentarius Guidelines.

Comparison of developed multiplex real-time PCR assays employing SYBR® Green I and TaqMan® chemistries

The developed SYBR® Green I-based duplex real-time PCR assays, simultaneously targeting event and endogenous gene-specific sequences, can be used to detect specific GM events with more accuracy due to elimination of chances of false negatives. In comparison with the TaqMan® approach SYBR Green Chemistry, it is more cost-efficient, which can be used to detect the target with similar efficiency and sensitivity. SYBR® Green I-based multiplex assays simultaneously targeting two or more GM events can be efficiently used to identify multiple GM events in a run. However, there is a limitation in that GM content cannot be quantified as a single fluorescent dye is used for all the targets. In multiplex TaqMan®-based real-time PCR assay, a specific reporter dye is used for each target and hence, can be employed for quantification for multiple GM events. TaqMan®-based multiplex real-time PCR assay is more specific and can be used to quantify GM content up to 0.5%.

The duplex and triplex approaches reported here may be employed to screen three GM maize events simultaneously; other event-specific assays could also be multiplexed for identification of more GM events based on the differences in their melting temperature. The reported TaqMan®-based duplex real-time PCR assay can be precisely used to quantify GM content of specific events in an unknown sample in a cost-effective manner compared to the respective singleplex assays.

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