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A Real-Time Quantitative PCR Detection Method Specific to Widestrike Transgenic Cotton (Event 281-24-236/3006-210-23)

STEFAN BAEUMLER,† DÖRTE WULFF,† LAURA TAGLIANI,‡ AND PING SONG*,‡

GeneScan Analytics GmbH, Eurofins, Engesserstrasse 4, D-79108 Freiburg, Germany; and Biotechnology Regulatory Science, Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, Indiana 46268

In compliance with global regulations on transgenic crops, a real-time quantitative PCR method specific to Widestrike transgenic cotton (event 281-24-236/3006-210-23, OECD Unique Identifier DAS-24236-5/DAS-21023-5) was established on the basis of the DNA sequences in the junction between the transgene insert and cotton genome. The optimized method consists of a DNA extraction method for cotton seeds and three PCR systems corresponding to a cotton-specific endogenous reference DNA sequence SAH7 (Sinapis Arabidopsis Homolog 7) and specific detection of event 281-24-236 and event 3006-210-23. The method performance including specificity, sensitivity, accuracy, and precision was determined at a dynamic range of Widestrike DNA levels from 0.04% to 5.0%. The limits of detection (LOD) and quantification (LOQ) were ≤0.04% and ≤0.09%, respectively, at 100 ng of DNA sample per reaction. The quantification results using either the event 281-24-236 or 3006-210-23 system were consistent, and the relative deviation from the expected (true) value was in the range of ±25%. The robustness of the method was demonstrated by a series of tests with deviations from the optimized assay parameters such as annealing temperature, extension time, PCR instrument, interlaboratory transferability, etc. All the measurements from these tests met the criteria set by EU JRC-CRL (European Commission Joint Research Centre-Community Reference Lab). This real-time quantitative PCR method is accurate and robust, and is recommended as a global benchmark method for the detection and quantification of Widestrike cotton. The method including description, protocol, and performance results is available on the JRC-CRL website (http://gmo-crl.jrc.it/statusofdoss.htm).

KEYWORDS: Widestrike cotton; GMO detection; quantitative real-time PCR

INTRODUCTION

In recent years, acreage of transgenic crops has increased globally, reaching more than 90 million hectares in 2005 with a market value of $5.25 billion U.S. dollars (*1*). Since the first transgenic tomato (FlavrSavr) was approved by regulatory authorities in 1994, many transgenic crops have been sold commercially, including maize (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), soybean (*Glycine max* L.), canola (*Brassica napus* L.), squash (*Cucurbita pepo* L.), and papaya (*Carica papaya* L.). Meanwhile, transgenic crops such as rice (*Oryza sativa* L.), the most important food crop in the world, are undergoing precommercialization trials (*1*). Due to the concerns for food and feed safety, consumer protection, and product stewardship, a number of countries have adopted, or are in the process of developing, legislation specifically related to the approval of transgenic crops. In addition, the Cartagena Protocol on Biosafety (BSP), which has been ratified by over 100 countries, includes specific provisions for handling, transport, packaging, and identification of GMOs (genetically modified organisms). Consequently, labeling of food and feed products containing ingredients from GMOs has become an important part of the regulatory framework in many countries and regions such as the EU, Japan, and Korea, although the specific labeling thresholds are different (*2*−*5*).

In alignment with the labeling policies, regulatory authorities in many countries require that a detection method be provided as a part of the registration package. At present, the most widely used detection method in compliance with labeling policies is PCR because of its simplicity, specificity, and sensitivity (*6*). To date, TaqMan based real-time PCR has been the method of choice for GMO quantification, especially for compliance with labeling laws (*6*, *7*). With commercialization of transgenic crops carrying the same traits produced by different developers, event-specific quantitative PCR methods are required to differentiate between different events of the same trait. Quantitative event-specific methods are now requested or recommended by regulatory authorities in many regions and countries. Based on the DNA sequence located in the junction region between the transgene insert and the host genome, many event-specific quantitative PCR methods have been developed and become available in the past few years (*8*, *9*, *10*). Due to the nature of

* Corresponding author (tel 317-337-3434; fax 317-337-3235; e-mail psong@dow.com).
† GeneScan Analytics GmbH.
‡ Dow AgroSciences LLC.

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Table 1. DNA Sequence of Primers and Probes[a]

name	orientation	sequence 5′–3′	size (bp)	specificity	amplicon size (bp)
SAH7-uni-f1	forward	AGT TTG TAG GTT TTG ATG TTA CAT TGA G	28	cotton	115 for A-subgenome
SAH7-uni-r1	reverse	GCA TCT TTG AAC CGC CTA CTG	21	SAH7 DNA	123 for B-subgenome
SAH7-uni-s1	probe (forward)	AAA CAT AAA ATA ATG GGA ACA ACC ATG ACA TGT	33	sequence	
281-f1	forward	CTC ATT GCT GAT CCA TGT AGA TTT C	25	event	
281-r2	reverse	GGA CAA TGC TGG GCT TTG TG	20	281-24-236	111
281-s2(rev)	probe (reverse)	TTG GGT TAA TAA AGT CAG ATT AGA GGG AGA CAA	33		
3006-f3	forward	AAA TAT TAA CAA TGC ATT GAG TAT GAT G	28	event	
3006-r2	reverse	ACT CTT TCT TTT TCT CCA TAT TGA CC	26	3006-210-23	90
3006-s2(rev)	probe (reverse)	TAC TCA TTG CTG ATC CAT GTA GAT TTC CCG	30		

[a] Primer and probe sequences specific to event 281-24-236 were derived from the 3′ junction between the insert and cotton genomic DNA; primer and probe sequences specific to event 3006-210-23 were derived from the 5′ junction between the insert and cotton genomic DNA.

PCR technology, scientifically sound method development and validation are critical to the application of event-specific quantitative PCR methods. To accomplish this goal, EC-JRC (European Commission-Joint Research Centre) assisted by ENGL (European Network of GMO Laboratories) has established the Community Reference Laboratory (CRL) in the context of the EU GM Food and Feed Legislation to implement a validation procedure. Codex Alimentarius Commission (Committee of Methods of Analysis and Sampling) has issued a draft guideline on methods for the detection and identification of foods derived from biotechnology (*11*). ISO (International Standardization Organization) is also very active in development of standards for GMO detection methods using PCR. All these efforts have contributed significantly to the global harmonization of GMO detection methods.

Cotton is the major fiber crop worldwide, with commercial production in 86 countries in 2004. Cotton seeds are also a common source for cooking oil and feedstuff. Transgenic cotton has been adopted by millions of farmers in the United States, China, and India (*1*, *12*, *13*). Widestrike (trademark of Dow AgroSciences LLC) cotton was developed by Dow AgroSciences through crossing two independent transgenic cotton events, 281-24-236 and 3006-210-23 (OECD Unique Identifier DAS-24236-5/DAS-21023-5), generated by *Agrobacterium*-mediated transformation and expressing Cry1F and Cry1Ac insecticidal proteins from *Bacillus thuringiensis* (http://www.agbios.com/main.php; http://www.dowagro.com/widestrike/index.htm). This new two-gene combination provides Widestrike cotton varieties with excellent season-long protection against a broad spectrum of insect pests. The two transgenic events have been deregulated, and commercial production in the United States began in 2005. To comply with regulations in other countries and to support the use of PCR technology in GMO testing, a scientifically sound, validated detection method specific to Widestrike cotton is essential. With this in mind, we have developed and validated a real-time quantitative PCR method based on unique DNA sequences in the junction regions between the inserts and cotton genome of the two transgenic events in Widestrike cotton.

MATERIALS AND METHODS

Seed Materials. Mature cotton seeds were used as the source of DNA. Widestrike cotton variety PHY440W contains two independent events, 281-24-236 and 3006-210-23, in the homozygous state. Cotton variety PSC355 is the nontransgenic counterpart of PHY440W. One sister event of 3006-210-23 and another event 278-13-122 generated with a gene construct similar to the one in event 281-24-236 were also used. These sister events were generated with the same gene constructs as the selected events, but were independent transformation events each containing their own unique insertion site. DNA samples were also extracted from seeds of 18 other cotton varieties, representing different genotypic backgrounds in North America. In addition, previously extracted DNA samples from soybean, canola, rice, wheat (*Triticum aestivum* L.), potato (*Solanum tuberosum* L.), sugar beet (*Beta vulgaris* L.), tomato (*Lycopersicon esculentum* Mill.), maize, mallow (*Malva sylvestris* L.), marshmallow root (*Althaea officinalis* L.), and hollyhock (*Alcea rosea* L.) were used for the validation of the cotton reference gene system, and genomic DNA from several commercially available transgenic crops including maize, soybean, and canola were used for specificity tests.

DNA Extraction and Quality Control. A CTAB-based method (*14*) combined with the Genomic-tip 20/G kit (Qiagen, Hilden, Germany, Catalog No. 10223) was used. Generally, DNA was extracted from ground seeds with the CTAB-based method, followed by purification using Genomic-tip 20/G essentially according to the manufacturer's protocol. DNA concentration was measured using the PicoGreen dsDNA quantitation kit (Invitrogen, Carlsbad, CA). DNA samples were dissolved in 1× TE buffer and stored in a freezer for future use. DNA fragmentation was checked by 1.5% agarose gel (TAE buffer system) electrophoresis along with a defined amount of calf thymus DNA, followed by ethidium bromide solution stain (1 μg/mL in distilled water) for 30 min and visualization on an UV transilluminator. DNA quality was further checked for the presence of inhibitors by spiking each extract with a specified quantity of plasmid DNA carrying a specific target sequence (HMG; a fragment of the gene coding for the maize high mobility group protein A, GenBank Accession No. AJ131373). Subsequently, 4-fold serial dilutions (1:4, 1:16, 1:64, 1:256) of each extract were prepared with 0.2× TE buffer and analyzed using a real-time PCR system specific to the target sequence (HMG) on the spiked plasmid (*15*).

Primers and Probes. Based on the DNA sequences in the junction between the inserts and cotton genome, primers and probes specific to events 281-24-236 and 3006-210-23 were designed (**Table 1**) to generate amplicons of appropriate size for current real-time PCR technology (~100 bp). For the cotton-specific reference system, primers and probes specific to SAH7 (Sinapis Arabidopsis Homolog 7) sequences (*19*) from *Gossypium raimondii* L. (the best living model of the D-subgenome progenitor, GenBank Accession No. AY117066) and *Gossypium herbaceum* L. (the closest relative of A-subgenome, GenBank Accession No. AY117065) were designed with their sequences completely matching to both subgenome gene copies to generate amplicons specific to cotton. TaqMan probes were labeled with the fluorescent 6-carboxyfluorescein (FAM) at the 5′ end and with the quench dye 6-carboxytetramethylrhodamine (TAMRA) attached to the 3′ end. Primers and probes were synthesized by MWG Biotech AG, Germany.

Real-Time PCR. PCR reactions were performed in 25 μL volumes containing 5 μL of DNA sample. **Table 2** lists all the components and their concentrations for amplicons specific to SAH7, event 281-24-236, and event 3006-210-23 sequences. PCR reactions were run on an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) with the following standard program suggested by the

Table 2. PCR Reagent Composition in Three PCR Systems for Widestrike Cotton Quantification

reagent		final concn and vol (μL) in 25 μL reaction					
		cotton SAH7		event 281-24-236		event 3006-210-23	
name	stock concn	concn	vol	concn	vol	concn	vol
10× PCR buffer II[a]	10×	1×	2.5	1×	2.5	1×	2.5
$MgCl_2$[d]	100 mM	6.0 mM	1.50	5.0 mM	1.25	6.0 mM	1.5
50× Rox reference dye[b,e]	50	0.7×	0.35	0.7×	0.35	0.7×	0.35
dATP[c]	10 mM	200 μM	0.5	200 μM	0.5	200 μM	0.5
dCTP[c]	10 mM	200 μM	0.5	200 μM	0.5	200 μM	0.5
dGTP[c]	10 mM	200 μM	0.5	200 μM	0.5	200 μM	0.5
dUTP[c]	20 mM	400 μM	0.5	400 μM	0.5	400 μM	0.5
glycerol[d]	20%	0.8%	1.0	0.8%	1.0	0.8%	1.0
Tween 20[d]	1%	0.01%	0.25	0.01%	0.25	0.01%	0.25
forward primer	10 μM	350 nM	0.88	350 nM	0.88	400 nM	1,0
reverse primer	10 μM	250 nM	0.63	450 nM	1.13	400 nM	1.0
probe	10 μM	175 nM	0.44	175 nM	0.44	150 nM	0.38
AmpliTaq gold polymerase[a]	5 units/μL	1 unit/reactn	0.2	1 unit/reactn	0.2	1 unit/reactn	0.2
sterile H_2O			10.26		10.01		9.83

[a–d] Supplier: [a]ABI, [b]Invitrogen, [c]Amersham Biosciences, [d]Sigma. [e] The concentration of Rox reference dye may exhibit significant variations between production lots. For proper function of reporter signal normalization the Rox signal intensity has to be clearly higher than the background signal. This has to be checked in multicomponent view for each production lot, especially when using ABI 7900HT instrument series which reveals lower sensitivity to Rox. In the case of low Rox signal strength the volume of reference dye solution has to be increased from 0.35 μL to, e.g., 0.50 μL per reaction. In the case of using ABI 7500 system or Stratagene Mx Series which both excite Rox much more efficiently, a reduction of the Rox concentration in the final mix (e.g., 0.05–0.10 μL per reaction) is advisable.

manufacturer: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. To test the robustness of the method, PCR reactions were performed under conditions that deviated from the optimized parameters, such as altered annealing temperature, time for denaturation, annealing and extension, concentration of reaction reagents, and sample matrix background. The final PCR reaction protocol was also tested in an ABI Prism 7900HT sequence detection system and an ABI 7500 real-time PCR system for transferability across different instruments.

Quantification of Widestrike Cotton DNA Content. Widestrike cotton DNA content was determined by interpolation with a standard curve of CT values generated from DNA samples of known concentration. The DNA calibration stock was produced by preparing a solution of 20 ng/μL of total DNA containing 10% Widestrike cotton DNA (event 281-24-236/3006-210-23) in nontransgenic cotton DNA (PSC355). This DNA sample was subsequently diluted with 0.1× TE into final concentrations of 4 ng/μL, 0.8 ng/μL, and 0.16 ng/μL. Thus, by using 5 μL of DNA solution, the amount of DNA per reaction for the generation of the standard curve was 100, 20, 4, and 0.8 ng, respectively. Each standard point had 3 replicates in each run. Five DNA test samples at a concentration of 20 ng/μL but containing 5%, 2%, 0.9%, 0.5%, and 0.09% of DNA from Widestrike cotton seeds, respectively, were prepared and analyzed in replicates of 3, to evaluate the performance of the method. This concentration range was designed based on 1/10× to 5× the 0.9% EU labeling threshold and served as the dynamic range in evaluation. Reference (SAH7) and Widestrike cotton CT values from test samples were interpolated against the corresponding standard curve to determine the Widestrike cotton DNA content. This was accomplished by dividing the calculated Widestrike DNA (event 281-24-236 or 3006-210-23) amount by the SAH7 DNA amount, resulting in the percentage of Widestrike cotton DNA versus total cotton DNA.

RESULTS AND DISCUSSION

Validation of DNA Extraction Method. For DNA quantification using a real-time PCR system, it is important to ensure that the DNA sample is pure and free from PCR inhibitors. To achieve this, six different samples of homogenized cotton seeds were extracted using the CTAB/Genomic-Tip 20/G method. Five samples were extracted in duplicate, and one sample was extracted six times. In the sample extracted six times, an average of 59 μg of DNA was extracted from 1 g of homogenized cotton seeds with a relative standard deviation of 12%, indicating the consistency of the extraction method. From a total of 16 extractions, an average of 56 μg of DNA could be extracted from 1 g of homogenized cotton seeds. By comparison with calf thymus DNA, gel electrophoresis showed that the molecular weight of extracted DNA ranged from medium to high (data not shown).

In order to assess purity and confirm the absence of PCR inhibitors, the extracted DNA solutions were adjusted to a concentration of 30 ng/μL and spiked with a defined copy number of plasmid molecules carrying a specific target sequence coding for part of the maize high mobility group (HMG) protein A. Subsequently 4-fold serial dilutions of each extract were prepared with 0.2× TE buffer (1:4, 1:16, 1:64, 1:256) and analyzed using the maize-specific HMG real-time PCR system to detect the target sequence on the spiked plasmid (*15*). To measure inhibition, the CT values of the four diluted samples were plotted against the logarithm of the dilution and the CT value for the "undiluted" sample (30 ng/μL, 150 ng/reaction) was extrapolated from the equation calculated by linear regression. Then the extrapolated CT for the "undiluted" sample was compared with the measured CT. With this approach, if the measured CT value for the "undiluted" sample was suppressed by >0.5 cycle from the calculated CT value, *i.e.*, the difference (ΔCT) between the measured CT and calculated CT, then it was concluded that PCR inhibitors were present in the sample. All the samples in the test had a ΔCT value less than 0.5, demonstrating the absence of PCR inhibitors (**Table 3**). Using the same protocol, genomic DNA from cotton seeds of PSC355, PHY440W, and PH00A.303 (a nontransgenic cotton variety) was extracted in another independent laboratory and the same DNA quality testing described above was applied to these 3 DNA samples. Like the first experiment, no PCR inhibitors were found in these 3 samples either (data not shown).

Factors such as plant tissue matrix, extent of DNA degradation, and presence of PCR inhibitors coextracted with genomic DNA, will affect the results of PCR analysis, especially in the case of real-time quantitative PCR assays (*6*, *16*). A study on the effect of four different DNA extraction methods on GMO quantification by real-time PCR indicated that Roundup Ready soybean flour and MON810 corn flour required different DNA extraction methods to obtain significant correlation between the

Table 3. Comparison of Calculated (Extrapolated) CT Values versus Measured CT Values[a] in DNA Quality Study[d]

DNA sample	extraction replicate	CT calculated	CT measured	ΔCT[b]	R^2 [c]
PH98M.3199	I	25.26	24.83	0.43	0.9984
	II	24.77	24.88	0.11	0.9994
3006-48-81	I	26.19	25.93	0.25	0.999
	II	26.03	26.05	0.02	0.9991
PHY440W	I	25.97	26.13	0.16	1.0000
	II	25.89	25.84	0.04	0.9994
278–13-122	I	25.88	26.08	0.20	0.9986
	II	25.69	26.02	0.33	0.9994
PSC355.112	I	26.22	26.06	0.16	0.9991
	II	26.05	26.03	0.01	0.9999
PSC355	I	25.71	26.09	0.38	0.9949
	II	25.94	25.99	0.05	0.9983
	III	25.79	25.64	0.14	0.9995
	IV	24.87	25.16	0.30	0.9993
	V	25.89	25.87	0.01	0.9953
	VI	25.66	25.93	0.28	0.9984

[a] CT values were the mean of triplicates. [b] ΔCT = abs(CT calculated − CT measured) [c] Coefficient between the CT values of the diluted samples and the logarithm of the target concentration in the diluted samples. [d] For assessment of DNA purity, all DNA solutions, after adjustment to 30 ng/μL, were spiked with a defined copy number of plasmid molecules. Subsequently 4-fold serial dilutions were prepared and analyzed using the HMG real-time PCR system. To measure PCR inhibition, the CT values of the four diluted samples were plotted against the logarithm of the dilution and the CT value for the "undiluted" sample was extrapolated from the equation calculated by linear regression. Afterward the calculated (extrapolated) CT for the "undiluted" sample was compared with the measured CT.

expected and measured values (*17*). It is well-known that cotton seeds are rich in protein, oil, and phenolic compounds, which make it difficult to extract pure genomic DNA. The results presented here demonstrate that a regular CTAB method combined with Genomic-Tip purification was able to produce pure genomic DNA suitable for GMO quantification.

Validation of SAH7 Gene as a Cotton-Specific Endogenous Reference. One of the common algorithms for GMO DNA quantification using real-time PCR is dividing the measured target DNA amount or copy number by the amount or copy number of an endogenous reference gene or DNA sequence. An ideal endogenous reference gene or DNA sequence should be species-specific and have single or low copy number per genome, as well as low heterogeneity across genotypes within the species. A validated reference gene system is critical because factors such as ploidy and copy numbers per genome can influence the quantitative relationship of the target DNA fraction to the mass fraction (*15*, *18*).

Cotton is an allotetraploid species (AADD) consisting of one A-subgenome and one D-subgenome per haploid genome (or two A-subgenome and two D-subgenome copies in the allotetraploid state) (*19*, *20*). Therefore it is important to make sure that the primers and probes used for the detection of cotton DNA either discriminate between the respective gene copies of the A- and D-subgenome or match perfectly to both subgenome gene copies. After intensive search of available databases of cotton genomic sequences, a putative SAH7 (Sinapis Arabidopsis Homolog 7) gene sequence was identified. Using SAH7 sequences from *Gossypium raimondii* L. (the best living model of the D-subgenome progenitor, GenBank Accession No. AY117066) and *Gossypium herbaceum* L. (the closest relative of A-subgenome, GenBank Accession No. AY117065), primers were designed to achieve cotton-specific amplification. Using these primers, A-subgenome- and D-subgenome-specific SAH7 gene fragments from three different *G. hirsutum* lines (PSC355, PH98M.3199, PH00A.309) were amplified, purified, and sequenced. The A-subgenome-specific SAH7 copy differs from the D-subgenome-specific copy by several single or double nucleotide substitutions and small deletions/insertions. Based on an alignment of the resulting sequences, various putative A-subgenome- and D-subgenome-specific primer−probes were identified and tested; however, none of these systems was able to perfectly discriminate between A- and D-subgenome sequences in tests with A- and D-subgenome templates.

Table 4. Specificity Tests with the SAH7 Cotton-specific PCR System in Different Species

plant species	result
Glycine max L.(soybean)	−
Brassica napus L. (rapeseed)	−
Oryza sativa L. (rice)	−
Triticum aestivum L. (wheat)	−
Solanum tuberosum L. (potato)	−
Beta vulgaris L. (sugar beet)	−
Lycopersicon esculentum L. (tomato)	−
Zea mays L. (maize)	−
Malva sylvestris L. (mallow)	−
Althaea officinalis L. (marshmallow root)	−
Alcea rosea L. (hollyhock)	−
Gossypium hirsutum L. (cotton variety PSC355)	+

Additionally sequencing of the primer/probe target region of the putative SAH7 protein gene was done by amplification of A-subgenome- and D-subgenome-specific SAH7 fragments from 11 different cotton lines with external primers and subsequent sequence analysis of the PCR products. The sequences of all A-subgenome-specific fragments as well as the sequences of all D-subgenome-specific fragments were identical. None of the 22 SAH7 sequences produced showed any sequence deviations in primer/probe binding sites.

Consequently, a universal SAH7 primer−probe system was designed within the intron sequences of the putative SAH7 gene where primers and probe sequences are identical to both subgenome gene copies, but the size of the amplicons resulting from A- and D-subgenome differ slightly due to sequence deviations within the amplified region (**Table 1**). Experimental tests of this real-time system displayed satisfying performance with respect to CT values, delta Rn values, and the shape of the amplification plots; therefore, this universal system was chosen for further testing and optimization.

The specificity of the SAH7 reference system was confirmed by database search and by PCR analysis with DNA samples from different plant species. A BLAST search of the DNA sequence derived from the SAH7 amplicon against GenBank did not generate significant similarity with other entries in the database, other than SAH7 DNA sequences in *Gossypium* species. Genomic DNA samples extracted from twelve common crop species and close-by relatives were tested with the SAH7 real-time PCR system. Each sample was run in duplicate with at least 1000 genome copies per reaction. CT values of ≥45 were defined as negative (−), lower than 45 as positive (+). All the samples, except cotton, were negative (**Table 4**).

Generally, an endogenous reference gene should be present at a consistently low copy number, without allelic variations across different genotypes or varieties within the same species. To verify whether the SAH7 reference system met this criteria of stability across genotypes, SAH7 real-time PCR was performed using genomic DNA extracted from 22 cotton varieties,

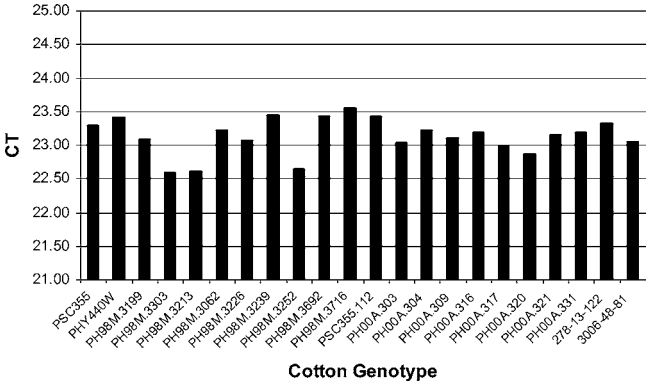

Figure 1. CT values of cotton varieties or genotypes generated from SAH7 real-time PCR system (values were the average of triplicates).

including Widestrike cotton variety PHY440W. The varieties tested represent a broad spectrum of cotton genetic backgrounds in North America. At an amount of 50 ng per reaction with 3 replicates, the DNA of multiple cotton varieties exhibited uniform CT value ranging from 22.59 to 23.55 with an average of 23.13, indicating "stability" of the respective PCR target in those varieties or lines. The deviation of the minimum and maximum CT values from the mean (23.13) was 0.54 and 0.42, respectively (**Figure 1**).

Performance of the Real-Time PCR Systems Specific to Widestrike Transgenic Cotton. Since Widestrike cotton is a breeding stack of two independent transgenic events, validated PCR systems were required for each event, as well as correlation of the results of each system in the combined trait product.

Specificity. The DNA sequence located in the junction region between a transgene insert and host genomic DNA serves as a cornerstone for event-specific PCR detection. After several rounds of testing and optimization, two sets of primers and probes specific to events 281-24-236 and 3006-210-23 were identified from the 3′ border and 5′ border junctions, respectively, and used as real-time PCR systems specific to Widestrike transgenic cotton DNA. Sequences of the two PCR amplicons were searched against the GenBank database using the Blastn program. As expected, DNA sequences from the amplicon specific to events 281-24-236 and 3006-210-23 had no significant similarity hits except a match of partial vector sequence that was used in the gene construct for cotton transformation and the cotton genome sequences flanking the inserts (data not shown).

Specificity tests were also conducted using DNA samples from different species and commercially available transgenic crops including cotton, maize, soybean, and canola. Reactions with PCR systems specific to events 281-24-236 and 3006-210-23 were performed in duplicate with at least 1000 copies per reaction of genomic DNA. As in the validation of the SAH7 reference system, CT values of ≥45 were defined as negative (−), lower than 45 as positive (+). None of the tested samples was positive, except for the corresponding transgenic cotton events, thereby confirming the specificity of the Widestrike cotton PCR systems (**Table 5**).

Sensitivity. The event 281-24-236 and 3006-210-23 PCR systems were investigated with respect to their ability to detect single target copies of event 281-24-236 and 3006-210-23 cotton respectively, under the assumption that one haploid *G. hirsutum* genome is equivalent to 2.33 pg of *G. hirsutum* genomic DNA (*21*). In order to prove that the two systems are "single copy sensitive", a dilution series of 100% Widestrike cotton genomic DNA ranging from 20 to as low as 0.313 genome copies per reaction was prepared with 0.2× TE and analyzed in 6 replicates. A reaction with a CT ≤45 was scored positive. At 5 genomic copies per reaction, all the reactions were positive in both systems. At 1.25 genomic copies per reaction, 4 out of 6 and 5 out of 6 were positive for event 281-24-236 and 3006-210-23 systems, respectively (data not shown).

Table 5. Specificity Tests with Widestrike Cotton Specific PCR Systems

		PCR system	
DNA source	transgene	281-24-236	3006-210-23
soybean	none	–	–
rapeseed	none	–	–
rice	none	–	–
wheat	none	–	–
maize	none	–	–
cotton (PH98M.3199)	none	–	–
cotton (PSC355.112)	none	–	–
cotton (PH00A.303)	none	–	–
Bt176 maize	Bt Cry1Ab	–	–
Bt11 maize	Bt Cry1Ab/ PAT	–	–
GA21 maize	EPSPS	–	–
DAS-59122-7 maize	Bt Cry34/ 35Ab1/PAT	–	–
Roundup Ready soybean	EPSPS	–	–
Roundup Ready rapeseed	EPSPS	–	–
potato New Leaf	none	–	–
Mon810 maize	Bt Cry1Ab	–	–
event 3006-48-81	Bt Cry1Ac	nd[a]	–
event 278-13-122	Bt Cry1F	–	nd
event 3006-210-23	Bt Cry1Ac	–	+
event 281-24-236	Bt Cry1F	+	–
Widestrike cotton (PHY440W)	Bt Cry1Ac/ Cry1F	+	+

[a] Not determined.

Table 6. Regression Parameters and PCR Efficiencies of the Standard Curves Derived from Widestrike Cotton Specific PCR Systems[a]

system	slope	intercept	R^2	efficiency
SAH7	–3.48	38.81	0.99973	0.94
281-24-236	–3.40	39.72	0.99877	0.97
3006-210-23	–3.46	38.78	0.99955	0.95

[a] All numbers were the mean of 6 independent runs.

Amplification Efficiency and Correlation. To assess amplification efficiency and coefficient of correlation in all three PCR systems, the standard curves of 6 independent runs were evaluated and the regression parameters including slope, intercept, and R^2 were determined. The efficiency of the reaction was calculated by the following equation: $E = [10(-1/\text{slope})] - 1$. The results indicated that the average value of the slope was in the range from −3.40 to −3.48 and the average value of R^2 was ≥0.99 with amplification efficiency ≥0.94 (**Table 6**).

Precision, Accuracy, Dynamic Range, and Limits of Detection (LOD) and Quantification (LOQ). Precision, accuracy, LOD, and LOQ are very important essential parameters for a validated analytical method. For the determination of precision, accuracy, dynamic range, LOQ, and LOD, the following experiment was carried out in 6 independent runs.

The assay format made use of standard curves for each of the three PCR systems comprising four standard points each in duplicate. Four negative controls (NTC) per system were run to verify purity of reagents. Each sample (containing different ratios of Widestrike cotton DNA in non-GM cotton DNA) was

Table 7. Accuracy and Precision for Widestrike Cotton Specific PCR Systems

			accuracy		precision	
event	true value (%)	replicates	mean (%)	rel deviation from true value (%)[a]	SD	RSD[b]
281-24-236	5.0	18	5.345	6.90	0.664	12.42
	2.0	18	2.134	6.70	0.354	16.59
	0.9	18	0.944	4.88	0.131	13.88
	0.5	18	0.481	−3.80	0.064	13.31
	0.09	17[c]	0.091	1.10	0.021	23.08
3006-210-23	5.0	18	5.045	0.90	0.651	12.90
	2.0	18	2.128	6.40	0.296	13.91
	0.9	18	0.937	4.11	0.117	12.49
	0.5	18	0.503	0.60	0.107	21.27
	0.09	18	0.099	10.0	0.021	21.21

[a] Deviation from true value was calculated with the formula [(experimentally determined GM value − true GMO value)/true GMO value] × 100. [b] RSD (relative standard deviation) values were obtained by dividing the standard deviation by mean value, and given in %. [c] One of the reactions failed for unknown reasons.

analyzed at 100 ng per reaction. Data analysis was performed using a baseline setting of 3−15 and a threshold value of 0.3 on the ABI Prism 7700 sequence detection system.

Each of the 6 independent runs included 3 replicates, resulting in 18 test results in total for every percentage of Widestrike cotton DNA mixture. For each of the 5 samples (ranging from 5.0, 2.0, 0.9, 0.5, and down to 0.09% Widestrike cotton DNA in non-GM cotton DNA), the mean, the relative deviation from the expected (true) values, the standard deviation, and the relative standard deviation (RSD_r) of the quantification results were calculated to determine accuracy and repeatability.

Table 7 summarizes the performance results of the Widestrike cotton PCR systems. The relative deviation of mean value from expected (true) value in all samples ranged between −3.8% and 6.9% in event 281-24-236 and between 0.7% and 9.8% in event 3006-210-23 quantification system, across the whole dynamic range. In this case, the dynamic range is based on 1/10× to 5× the 0.9% EU labeling threshold. Precision values measured by relative standard deviation for all samples between 5.0% and 0.09% concentration ranged from 12.4% to 23.4% in event 281-24-236 and from 12.5% to 21.6% in event 3006-210-23 quantification system, less than commonly recognized 25% (*6*). The dynamic range (0.09−5% Widestrike cotton DNA level) covered 1/10× and 5× the EU labeling threshold (0.9%) with an acceptable level of accuracy and precision.

Generally, LOD is the lowest amount or concentration of analyte in a sample, which can be detected reliably, but not necessarily quantified, and LOQ is the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy. At 0.04% (≤1/20× the EU labeling threshold) Widestrike DNA level with 100 ng per reaction, all the data points generated with event 282−24-236 and 3006-210-24 system were positive, indicating that the LOD is below 1/20× the EU labeling threshold (data not shown). The LOQ is ≤0.09% since at this level both quantitative PCR systems were able to determine the content with acceptable accuracy and precision.

Consistency. Widestrike cotton is marketed as a combined (stacked) trait for insect resistance, suggesting that the copy numbers of events 281-24-236 and 3006-210-23 are identical in every sample containing Widestrike cotton DNA. Therefore, test results generated with event 281-24-236 and 3006-210-23 specific quantification PCR systems should be in agreement with each other over the whole dynamic range. To assess the consistency of test results from these two systems, test results of the 6 independent runs have been evaluated by measuring samples containing 0.09%, 0.5%, 0.9%, 2%, and 5% Widestrike DNA as well as plotting the mean values directly to expected (true) values. As shown in **Figure 2**, the mean values were proportionally correlated to the true values in both systems with the values of R^2 and slope close to 1. Similarly, the mean values obtained with the event 281-24-236 specific PCR system correlated with that obtained with the event 3006-210-23 specific PCR system at the five tested levels of Widestrike DNA (**Figure 3**). These data support the use of either of the two PCR systems for quantitative detection of Widestrike cotton.

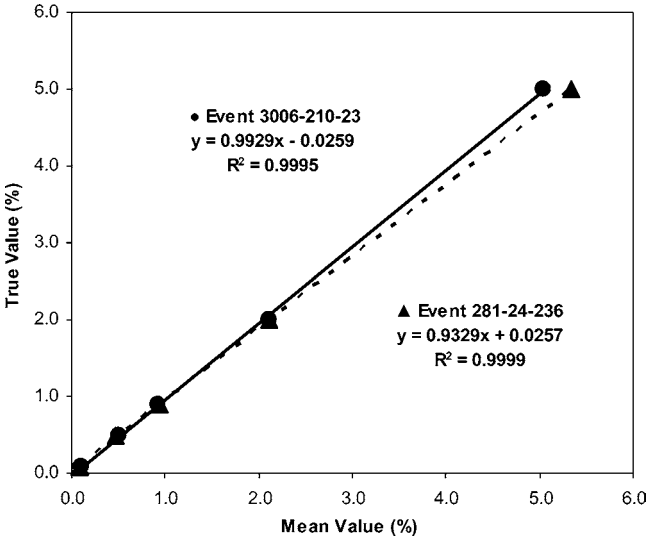

Figure 2. Correlation between the tested values and true values of Widestrike cotton (data derived from 6 runs with 3 replicates in each run).

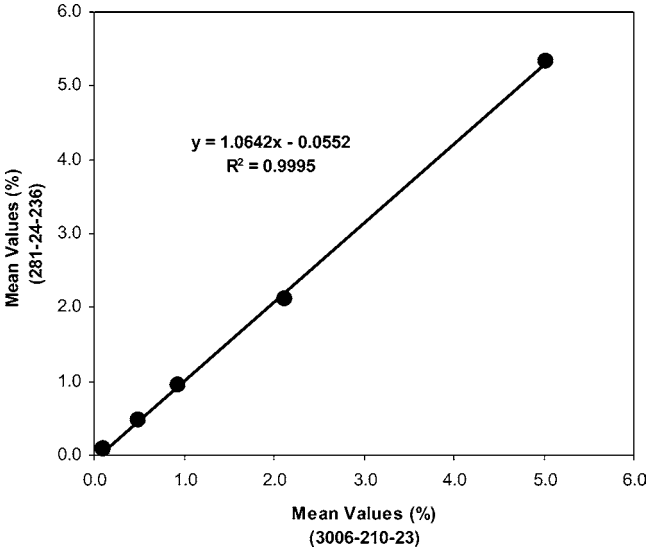

Figure 3. Correlation of tested values between event 281-24-236 and 3006-210-23 systems (data derived from 6 runs with 3 replicates in each run).

Robustness. The evaluation of robustness demonstrates the reliability of a method with respect to inadvertent variations in assay parameters (*6*). To achieve this, the following variations from the SAH7 reference and two event-specific PCR systems were tested at 3 levels of Widestrike DNA (0.09%, 0.9%, and 5.0% in nontransgenic cotton DNA): (1) ±20% deviation from

Table 8. Tested Values Produced with Assay Parameter Deviations from Optimized Widestrike Specific PCR Systems

	tested values (%)					
	0.09[a]		0.9[a]		5[a]	
variation	281-24-236 system	3006-210-23 system	281-24-236 system	3006-210-23 system	281-24-236 system	3006-210-23 system
master mix +20%[b]	0.09	0.09	0.80	0.85	4.6	4.6
master mix −20%[b]	0.11	0.08	1.09	0.97	5.8	5.7
UNG + dUTP[c]	0.09	0.10	0.90	0.80	5.5	5.4
dTTP[d]	0.07	0.14	0.97	1.12	5.8	5.7
profile A[e]	0.10	0.11	0.82	0.91	4.8	4.7
profile B[f]	0.09	0.09	0.98	0.83	4.3	4.3
58 °C annealing and extension	0.08	0.10	0.89	0.94	5.1	5.6
62 °C annealing and extension	0.11	0.10	1.09	1.00	6.2	5.2
ABI 7900HT	0.09	0.11	0.75	0.92	3.5	3.8
ABI 7500	0.11	0.11	1.12	1.16	5.7	5.1
STDEV	0.01	0.01	0.13	0.12	0.8	0.6
RSDr	15.6%	12.2%	14.7%	13.1%	16.7%	12.9%

[a] Expected (true) value (% of Widestrike). [b] ±20% deviation from optimized reagent concentration in 25 μL reaction (**Table 2**). [c] Addition of the enzyme UNG (uracil-DNA *N*-glycosylase) to the reaction mix (including dUTP). [d] Replacing the dUTP in standard dNTP mixture (200 μM each for A, C, and G and 400 μM for U, final concentration) with an equimolar amount of dTTP. [e] 95 °C/20 s; 60 °C/80 s × 45 cycles after activation of DNA polymerase. [f] 95 °C/10 s; 60 °C/45 s × 45 cycles after activation of DNA polymerase.

Table 9. Effect of Different Sample Matrices on Performance of Widestrike Cotton Specific PCR System[a]

	SAH7 system		281-24-236 system		3006-210-23 system	
sample	CT	ΔCT	CT	ΔCT	CT	ΔCT
0.2× TE	31.29	0	32.53	0	31.60	0
maize kernels	31.24	0.05	32.51	0.02	31.65	0.05
soybeans	31.57	0.28	32.87	0.34	31.71	0.11
starch	31.90	0.61	32.72	0.19	31.40	0.20
maize grits	31.67	0.38	32.27	0.26	31.67	0.07
rapeseeds	31.32	0.03	32.03	0.50	31.56	0.04

[a] Each sample contained 100 copies of Widestrike cotton DNA.

the concentration of all reaction components in optimized PCR reaction (**Table 2**); (2) replacing the dUTP in the standard mixture (200 μM each for A, C, and G and 400 μM for U, final concentration) with 200 μM of dTTP; (3) addition of the enzyme UNG (uracil-DNA *N*-glycosylase) to the reaction mix (including dUTP); (4) aberrant cycling conditions on ABI Prism 7700 system; (5) aberrant annealing temperatures of 58 °C and 62 °C on ABI Prism 7700 system; (6) ABI Prism 7700 vs 7900HT system. For each sample, 100 ng of DNA per reaction was used with triplicates at each run. In all cases, the relative deviations from the expected (true) values fell into the range of ±30% (data not shown) and the overall RSDr was less than 30% (**Table 8**).

In addition to the above assay parameter deviations, the Widestrike cotton specific PCR system was assessed with respect to matrix influences on the CT values of the measurements. DNA samples extracted from five different matrices (maize kernels, soybeans, starch, maize grits, and canola seeds) were spiked with 100 copies of Widestrike cotton DNA (281-24-236/3006-210-23), respectively, and then tested in triplicate. The CT difference (ΔCT) between each sample (mean of triplicates) and the control (100 copies of Widestrike DNA in 0.2× TE buffer instead of sample DNA, mean of triplicates) was evaluated for five different sample types. The results indicated that ΔCT values were less than 0.7 for all matrices tested (**Table 9**).

To further demonstrate the robustness in the aspect of reproducibility, different samples containing 0.09−5.0% Widestrike DNA in nontransgenic cotton DNA were analyzed at 100 ng of DNA per reaction on an ABI 7500 real-time PCR system in an independent laboratory by following the assay optimized on an ABI Prism 7700 system. The quantification results for each level of Widestrike DNA in both event 281-24-236 and 3006-210-23 systems appeared very close to the expected (true) values with relative deviation from true values within the −11.5% to 15.9% range, suggesting reproducibility and interlaboratory transferability in spite of different PCR instrumentation (data not shown).

Due to the nature of PCR technology and the principles of DNA based quantification, it is critical that PCR based GMO quantification methods are reliable and generate the same results in laboratories across the world. The Widestrike cotton specific PCR quantification system was developed on the basis of the unique DNA structure in the junction region between the transgene insert and cotton genomic DNA, and comprehensively tested with a broad spectrum of deviations from the optimized assay. As described above, the validation test results met the currently most stringent criteria for GMO quantitative PCR method set by JRC-CRL, demonstrating the reliability and credibility of this method. As such, this quantitative PCR method specific to Widestrike cotton serves as a global benchmark method for the detection and quantification of Widestrike cotton.

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