

## Equal Performance of TaqMan, MGB, Molecular Beacon, and SYBR Green-Based Detection Assays in Detection and Quantification of Roundup Ready Soybean

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We have tested and compared the performance of 12 different assays representing four different real-time polymerase chain reaction (PCR) chemistries in the context of genetically modified organism detection. Several different molecular beacon, SYBR Green, TaqMan, and MGB assays were designed for the event specific detection and quantification of the 3' integration junction of GTS 40-3-2 (Roundup Ready) soybean. Sensitivity as well as robustness in the presence of background DNA were tested. None of the PCR-based approaches appeared to be significantly better than any of the other, but the molecular beacon assays had the lowest efficiency and also seemed more sensitive to changes in experimental setup.

**KEYWORDS:** Event specific detection; genetically modified organisms; GMO; method performance; real-time PCR; reliability; RoundupReady soybean

### INTRODUCTION

A global status report from 2005 states that the global area planted with genetically modified (GM) crops has increased for a full decade, and in 2005 alone, the growth rate was 11% (1). The most commonly grown GM plants are soybean, maize, cotton, and canola. Several gene constructs may be introduced in GM plants, and commonly introduced elements include the cauliflower mosaic virus promoter (P35S), the gene coding for CryIA(b) toxin from *Bacillus thuringiensis*, the 5-enol-pyruvylshikimat-3-phosphate synthase gene (*EPSPS*), and the *Agrobacterium tumefaciens* nopaline synthase (*NOS*) terminator. In 2005, biotech soybean occupied approximately 60% of the global soybean market (1). GM soybeans that tolerate the use of the herbicide glyphosate (Roundup Ready soybean; RRS) were commercially introduced in 1996 and have since become increasingly more widespread. RRS (event GTS 40-3-2; see <http://www.agbios.com>) contains P35S, *EPSPS*, and *NOS* elements. A characterization of the junction region between insert DNA and plant DNA in RRS showed genomic rearrangements in the 3' (*NOS* terminator) end of the construct used to do the initial transformation, where a part of the *EPSPS* gene was found flanking what was initially reported as the 3'-end of the insert (ref 2 and Figure 1). A 534 bp fragment of unknown origin was found between this *EPSPS* fragment and the soybean genomic DNA (3). This information made it possible to design

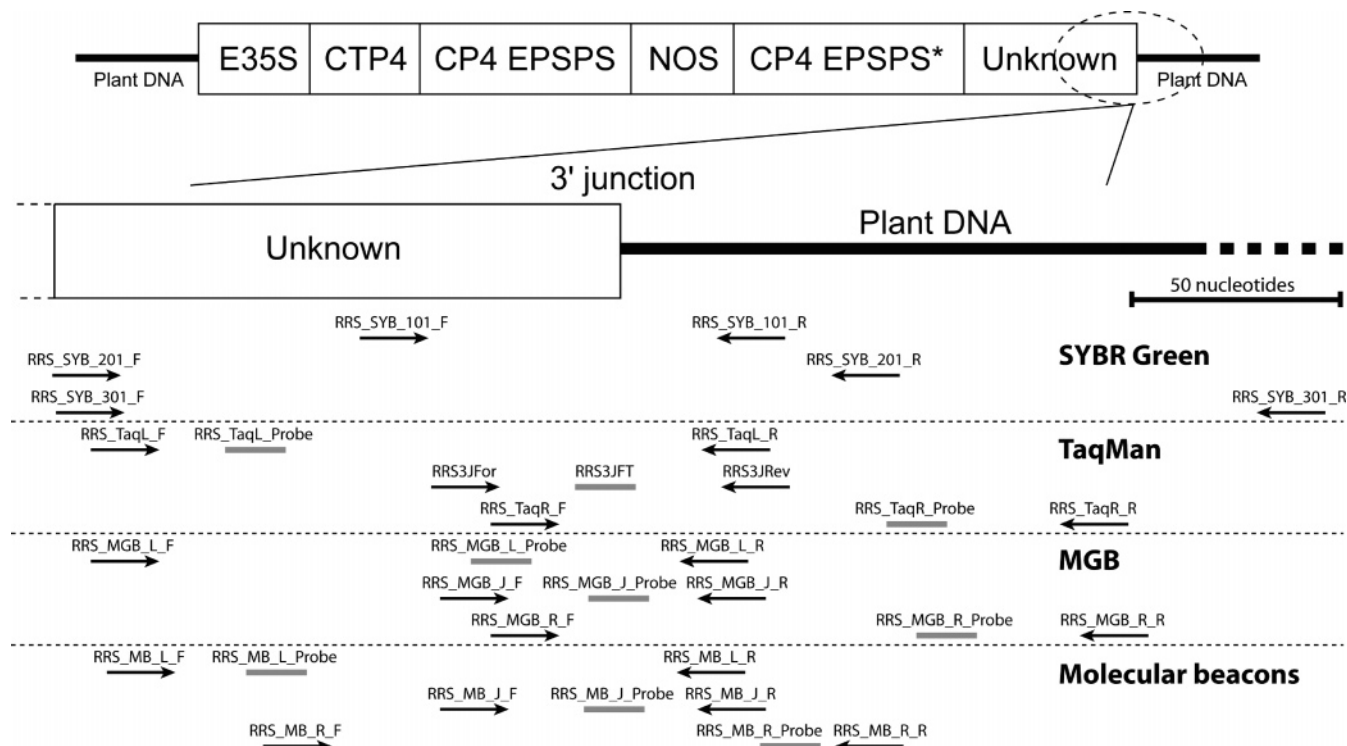
event specific detection methods detecting the junction region between the DNA fragment of unknown origin and the plant DNA (4). Such a junction region is unique for a single transformation event, and only one or two copies per diploid genome are present depending on whether the GM plant line is heterozygous or homozygous for the introduced element. Assays targeting such unique junctions are believed to represent the most specific genetically modified organism (GMO) detection methods (5). For a more comprehensive discussion of this, see ref 6.

The majority of the currently used molecular detection methods designed to detect genetic modifications in plants rely on the use of real-time polymerase chain reaction (PCR) (5). In real-time PCR, the amplification reaction is monitored in "real time". In the linear part of the PCR amplification, the cycle number ( $C_T$ ) where the amplification reaches a certain threshold is inversely correlated with the log of the number of nucleic acid target sequence copies initially present in the sample (7), and this approach can thus be used in a quantitative manner. In the early days of real-time PCR, nonspecific DNA binding dyes such as ethidium bromide were simply added to the PCR mix and the level of amplification could be monitored by observing the fluorescent properties of the reaction vessel (8). Later, ethidium bromide was replaced by SYBR Green giving an increase in sensitivity of the assays (9). Both ethidium bromide and SYBR Green are intercalating dyes that bind to double-stranded DNA (dsDNA) hereby giving an increase in fluorescence proportional to the amount of dsDNA present. When using intercalating dyes, the observed fluorescence may originate from

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**Figure 1.** Map of target sequence and primers/probes. E35S, CaMV 35S promoter; CTP4, chloroplast targeting sequence; CP4 EPSPS, 5-enol-pyruvylshikimat-3-phosphate synthase open reading frame; NOS, nopaline synthase terminator; CP4 EPSPS\*, truncated fragment of CP4 EPSPS; and unknown, DNA of unknown origin. A map of the construct is modified from a figure available as part of RRS data on AGBIOS webpage (<http://www.agbios.com/>), and primers and probes are shown in correct distance relative to the 3'-junction (see scale bar).

both the intended products (specific) and/or the unspecific products due to the fact that these dyes bind to all DNA present in the reaction. By using a subsequent melting curve analysis, one can distinguish specific and nonspecific PCR products based on their specific melting temperatures, although this can sometimes be problematic since the melting dynamics are dependent on not just the overall GC content but also the distribution of GC/AT in the PCR product (for examples and discussion, see ref 10).

To avoid the problems associated with nonspecific PCR amplification products being detected, one or more fluorescent probes can be added to the reaction mix. The probe(s) are designed to be complementary to a sequence motif in the target amplicon, and a PCR product will only be detected if the amplified sequence is complementary to the annealing probe. Exonuclease assays, 5'-3' (now commercially marketed as TaqMan assays), utilize dually labeled probes with a reporter fluorophore covalently attached to one end of the oligonucleotide and a quencher attached to the other (11). When the probe anneals to the PCR product during the elongation step of the amplification reaction, the 5'-exonuclease activity of the polymerase cleaves the probe releasing the reporter dye, allowing it to move away from the quencher. As a result, the fluorescence of the reporter dye can be detected. To improve the specificity of such hydrolyzing probes, minor groove binding (MGB) probes have been developed. MGB probes are similar to TaqMan probes in both design and function, but they are conjugated with a minor groove binder group that will increase the melting temperature ( $T_m$ ) of the oligonucleotide, thereby improving the specificity of the hybridization (12). The increased  $T_m$  will also allow the design of shorter probes, and this can sometimes be advantageous in the design of detection assays.

TaqMan and MGB probes rely on fluorescence resonance energy transfer (FRET) between the dye and the quencher. The

quencher and the dye molecules are separated by relatively long distances (possibly tens of nucleotides), and in such a system, the quenched signal will be dissipated as light. In other systems, the quencher and the fluorescent reporter are in closer proximity, allowing the quenched signal to be dissipated as heat. There should thus be essentially no fluorescence when the signal from the reporter is quenched (for details, see ref 13). An example of this technology is the molecular beacons (14). Molecular beacons have a hairpin structure with a sequence specific loop region flanked by two inverted repeats. These repeat regions can form a stem, giving the probes their characteristic secondary structure. One end of the probe is labeled with a fluorophore, and the other end is labeled with a quencher. In the presence of a complementary target sequence, the beacon binds and unfolds, leading to increased fluorescence due to the separation of the fluorophore from the quencher.

The international standard ISO 21570:2005 (15) provides an overall framework for GMO detection in foodstuffs. It presents validated target taxon specific (16), screening (17), construct, and event specific (18) real-time PCR methods. Among the most popular real-time chemistries are TaqMan and MGB probes, molecular beacons, and PCR with SYBR Green. When deciding upon which real-time PCR technology to use for a specific detection assay, many variables have to be taken into account. Reagent costs and availability of real-time thermocyclers, the structure of the target sequence, and the specificity and sensitivity needed are just a few important elements. In order to provide guidelines for method developers and to investigate the limitations and advantages of different real-time PCR approaches, we have tested the performance of the four most commonly used technologies for detection of the RRS 3'-junction. Three different assays were tested for each chemistry type, and sensitivity as well as robustness in the presence of background DNA were assessed.

**Table 1.** Primers and Probes Used in This Study

detection method	primer/probe name	sequence	comments
SYBR Green	RRS SYB 101 F	5'-ATAAAAAGAATAAAAATAGCATCTACATATAGCTTC-3'	-61 <sup>a</sup>
	RRS SYB 101 R	5'-ACCAGGCCATTCGCCTC-3'	40 (101 bp product)
SYBR Green	RRS SYB 201 F	5'-TAAGATTAAGCTTCAACATGTGAAGGA-3'	-134
	RRS SYB 201 R	5'-ACGAACTTCTCGACGATGGC-3'	67 (201 bp product)
SYBR Green	RRS SYB 301 F	5'-AAGATTAAGCTTCAACATGTGAAGGA-3'	-133
	RRS SYB 301 R	5'-ACAGAGAGAAAAATGGACGTGG-3'	168 (301 bp product)
TaqMan	RRS TaqL F	5'-GCTTCAACATGTGAAGGAGTAGTACA-3'	-125
	RRS TaqL R	5'-GGCCATTCGCCTCAAACAGT-3'	36
TaqMan	RRS TaqL probe	5'-6-FAM-AGTGACCCTAATAGGCAACAGCATGAAAAAA-TAMRA-3'	-93 (downstream of 3'-junction)
	RRS3JFor <sup>b</sup>	5'-TAGCATCTACATATAGCTTC-3'	-44
TaqMan	RRS3JRev <sup>b</sup>	5'-GACCAGGCCATTCGCCTCA-3'	41
	RRS3JF <sup>b</sup>	5'-6-FAM-ACAAAATTTGGGATCGGAGAAGA-TAMRA-3'	-10 (3'-junction)
TaqMan	RRS TaqR F	5'-AGCTTCTCGTTGTAGAAAAACAAA-3'	-30
	RRS TaqR R	5'-TGGTGAACCTTTCTGTTGGAAGAG-3'	121
MGB	RRS TaqR probe	5'-6-FAM-TCGCGCCATCGTCGAGAAGTT-TAMRA-3'	64 (upstream of junction)
	RRS MGB L F	5'-GCTTCAACATGTGAAGGAGTAGTACA-3'	-125
MGB	RRS MGB L R	5'-TTCGCCTCAAACAGTCTTCTC-3'	31
	RRS MGB L probe	5'-6-FAM-TACATATAGCTTCTCGTTGTAGAA-MGB-3'	-37 (downstream of junction)
MGB	RRS MGB J F	5'-GCATCTACATATAGCTTCTCGTTGTAG-3'	-42
	RRS MGB J R	5'-GCCATTCGCCTCAAACAGT-3'	35
MGB	RRS MGB J probe	5'-6-FAM-AAACTATTTGGGATCGGAGAA-MGB-3'	-7 (3'-junction)
	RRS MGB R F	5'-AGCTTCTCGTTGTAGAAAAACAAA-3'	-30
MGB	RRS MGB R R	5'-TTGCGTGGTGAACCTTCTGTTG-3'	126
	RRS MGB R probe	5'-6-FAM-CGTGAGAAAGTTCTGTTGAA-MGB-3'	71 (upstream of junction)
molecular beacon	RRS MB L F	5'-CAACATGTGAAGGAGTAGTACA-3'	-121
	RRS MB L R	5'-TCGCCTCAAACAGTCTTCT-3'	30
molecular beacon	RRS MB L probe	5'-6-FAM- <b>CGCGAT</b> CCCTAATAGGCAACAGCATGAAA <b>ATCGCG</b> -Dabcyl-3' <sup>c</sup>	-88 (downstream of junction)
	RRS MB J F	5'-GCATCTACATATAGCTTCTCGTTGTAG-3'	-42
molecular beacon	RRS MB J R	5'-GCCATTCGCCTCAAACAGT-3'	35
	RRS MB J probe	5'-6-FAM- <b>CGCGATA</b> AAAACCTAATTTGGGATCGGAGAAGAA <b>ATCGCG</b> -Dabcyl-3' <sup>c</sup>	-8 (3'-junction)
molecular beacon	RRS MB R F	5'-AATAGGCAACAGCATGAAA-3'	-84
	RRS MB R R	5'-CACGAACCTTCGACGAT-3'	68
	RRS MB R probe	5'-6-FAM- <b>CGCGATA</b> AGAACCTGTTTGGAGCGAATGG <b>ATCGCG</b> -Dabcyl-3' <sup>c</sup>	34 (upstream of junction)

<sup>a</sup> Distance in nucleotides from the 3'-junction to the 5'-end of the primer/probe. <sup>b</sup> Ref 4. <sup>c</sup> Boldface letters for molecular beacon probes indicate stem sequences.

## MATERIALS AND METHODS

**Soybean Samples.** DNA was extracted from 100% RRS leaf material and non-GMO soybean flour using a CTAB-based protocol (19). The leaf material was freeze-dried and crushed prior to DNA extraction while the soybean flour was extracted directly. DNA concentrations were estimated by using a NanoDrop ND-1000 V3.1.0 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and DNA dilutions were made in a 0.1 mg/mL bovine serum albumin (Tamro MedLab AS, Oslo, Norway) solution. The number of RRS and wild-type soybean genomes was estimated based on the DNA concentration and the diploid genome size of soybean ( $1.115 \times 10^9$  bp) (20).

**Assay Design.** The assays were designed using the RRS 3'-junction sequence deposited in EMBL sequence database by Windels et al. (3) (EMBL accession number AJ308515). A total of 11 different assays were designed as follows: three molecular beacon assays, three MGB assays, two TaqMan assays, and three SYBR Green assays (Table 1).

All primers, as well as TaqMan and MGB probes, were designed using the software Primer Express Version 2.0.0 (Applied Biosystems, Carlsbad, CA). TaqMan probes [5'-label, 6-FAM (fluorescein); 3'-label, TAMRA], MGB probes [5'-label, 6-FAM; 3'-label, MGBNFQ (minor groove binder/nonfluorescent quencher)], and their corresponding PCR primers were designed using default parameters and a defined optimal primer melting temperature (55 °C), optimal primer length (20 bases), minimum GC content [30% (maximum 80%)], and maximum amplicon melting temperature (85 °C). By changing the optimal amplicon length parameter, SYBR Green assays corresponding to three different amplicon lengths (101, 201, and 301 base pairs, respectively) were also designed using the parameters and software described above. All SYBR Green assays had primers flanking the 3'-junction (Table 1 and Figure 1). TaqMan/MGB assays were designed that had probes upstream of the 3'-RRS junction, and similar assays were made with probes downstream of the junction. In addition, an MGB assay was made with the probe targeting the junction locus itself (Table 1 and Figure 1). Changes in probe target locus were accomplished by providing the Primer Express software with different fragments of the junction sequence for the primer and probe design; to design assays with the probe annealing upstream of the junction, the terminal part of the target

sequence was excluded; for assays targeting the junction itself, only the central part of the target sequence was used; and for the assays with the probe downstream of the junction, the beginning of the sequence was not included. A previously published TaqMan assay targeting the 3'-RRS junction was also used (4).

Molecular beacon probes were designed using the guidelines outlined at the molecular beacons web page (<http://www.molecular-beacons.org>). The beacons used 6-FAM as reporter dyes (5'-end) and Dabcyl as quenchers (3'-end). Oligonucleotide properties were calculated using the Primer Express software, and primers were designed to have optimal melting temperatures of 50 °C (other parameters were as described above). Using the strategy described above, three different molecular beacons were designed as follows: one with a sequence specific loop complementary to the 3'-junction, one with the beacon annealing upstream of the junction, and one where the beacon should hybridize to the sequence downstream of the junction (Table 1 and Figure 1).

All PCR primers, TaqMan probes, and molecular beacons were purchased from DNA technology (Århus, Denmark). The MGB probes were purchased from Applied Biosystems. All probes were ordered as RP-HPLC (reverse phase high-pressure liquid chromatography) purified oligonucleotides, whereas the primers were ordered with standard purification (ethanol precipitation).

**Real-Time PCR.** All PCRs were run on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems), and data were analyzed using version 2.2.1 of the SDS software (Applied Biosystems). During the first cycles of the amplification, the SDS software generated a baseline that it used to normalize the signal intensities across all samples in a run. For all of the analyses, the "threshold value" was manually set to 0.06. This corresponded to the early part of the linear phase for all of the amplifications and allowed direct comparison of threshold cycle ( $C_T$ ; PCR cycle number where the signal from a sample is defined as positive by the software) values between different assays and experiments.

The SYBR Green PCR assays contained 1× SYBR Green PCR Master Mix (Applied Biosystems), 150 nM concentration of each primer, and 5 μL of DNA in a total volume of 25 μL. The PCR program consisted of an initial 2 min at 50 °C [the master mix contained uracil-

**Table 2.** Threshold Cycle Numbers ( $C_T$  Values) for the Different Assays When Using Different Amounts of Template DNA in the Presence/Absence of Background DNA

no. of diploid RRS genomes used as template	RRS_SYB_101	RRS_SYB_201	RRS_SYB_301	RRS_TAQ_L	RRS_TAQ_3J	RRS_TAQ_R	RRS_MGB_L	RRS_MGB_J	RRS_MGB_R	RRS_MB_L	RRS_MB_J	RRS_MB_R
$10^4$	24.23 (0.23) <sup>a</sup>	29.71 (0.44)	28.65 (0.33)	33.07 (0.73)	25.77 (0.23)	26.93 (0.30)	30.61 (0.60)	25.68 (0.04)	26.63 (0.36)	31.73 (0.33)	29.60 (0.47)	29.18 (0.29)
$10^4$ + WT soybean <sup>b</sup>	24.10 (0.16)	29.52 (0.18)	28.97 (0.13)	33.61 (0.49)	25.95 (0.02)	26.76 (0.31)	31.01 (0.52)	25.80 (0.07)	26.56 (0.18)	33.11 (0.18)	30.44 (0.27)	29.40 (0.00)
$10^3$	27.52 (0.15)	33.74 (0.84)	33.05 (1.38)	36.69 (0.21)	29.14 (0.09)	30.16 (0.24)	34.01 (0.21)	29.38 (0.11)	30.16 (0.22)	36.00 (0.56)	33.84 (0.21)	33.23 (0.26)
$10^3$ + WT soybean	27.50 (0.17)	33.41 (0.89)	32.35 (0.74)	38.28 (0.29)	29.24 (0.00)	30.75 (0.33)	34.00 (0.20)	29.25 (0.32)	29.92 (0.07)	37.85 (0.13)	34.02 (0.18)	33.36 (0.39)
$10^2$	30.88 (0.34)	38.94 (0.98)	36.50 (0.97)	41.30 (0.36)	32.76 (0.23)	34.50 (0.25)	38.47 (0.44)	32.71 (0.17)	33.61 (0.31)	41.75 (0.64)	39.19 (0.63)	38.26 (0.83)
$10^2$ + WT soybean	31.10 (0.32)	36.36 (0.89)	36.10 (0.40)	42.07 (0.69)	32.61 (0.28)	34.20 (0.18)	37.77 (0.47)	32.89 (0.49)	33.15 (0.06)	0/2 (N/A)	39.09 (0.53)	38.35 (0.41)
50	32.02 (0.30)	39.13 (0.67)	36.95 (0.26)	41.98 (0.49)	33.53 (0.22)	35.05 (0.34)	39.04 (0.23)	34.15 (0.21)	35.12 (0.48)	42.74 (0.68)	39.89 (0.60)	39.50 (0.45)
50 + WT soybean	31.91 (0.16)	38.43 (0.11)	37.10 (0.25)	41.74 (0.09)	33.68 (0.48)	35.08 (0.35)	39.73 (0.04)	33.73 (0.17)	34.52 (0.59)	0/2 (N/A)	1/2 (N/A)	38.60 (0.11)
10	35.09 (1.19)	44.08 (1.94)	40.64 (0.54)	44.92 (1.53)	36.97 (0.92)	38.43 (0.64)	42.82 (1.42)	36.45 (0.45)	37.39 (0.97)	2/5 (N/A)	2/5 (N/A)	4/5 (N/A)
10 + WT soybean	34.12 (0.28)	40.04 (0.06)	40.05 (1.16)	44.23 (0.32)	36.59 (0.22)	38.88 (1.24)	0/2 (N/A)	36.02 (0.02)	36.58 (0.58)	0/2 (N/A)	0/2 (N/A)	0/2 (N/A)
5	36.05 (0.48)	4/5 (N/A)	40.43 (2.17)	45.53 (1.07)	36.97 (0.50)	38.44 (0.70)	4/5 (N/A)	38.23 (0.83)	4/5 (N/A)	1/5 (N/A)	1/5 (N/A)	2/5 (N/A)
5 + WT soybean	35.57 (0.21)	38.92 (0.18)	39.54 (0.90)	1/2 (N/A)	37.50 (0.93)	39.36 (2.47)	0/2 (N/A)	37.29 (0.90)	37.81 (0.37)	0/2 (N/A)	0/2 (N/A)	0/2 (N/A)
slope <sup>c</sup>	-3.61	-4.32	-3.73	-3.88	-3.54	-3.67	-4.23	-3.71	-3.71	-4.40	-4.68	-4.43

<sup>a</sup> Average  $C_T$  value and standard deviation (X/X; number of positives/number of experiments. Standard deviation was not calculated when some of the duplicates failed to amplify). <sup>b</sup> DNA corresponding to  $2 \times 10^4$  copies of the diploid wild-type soybean genome was added. <sup>c</sup> The assay efficiency slope was calculated based on the  $C_T$  values from the experiments using only RRS DNA.

DNA glycosylase (UNG), and this step was included to degrade any contaminating PCR products] and 10 min at 95 °C (denaturing of dsDNA and inactivation of UNG) followed by 50 cycles of 15 s at 95 °C and 1 min at 55 °C with data collection at the annealing step. After the 50 cycles, we included a dissociation/melting curve stage with 15 s at 95 °C, 15 s at 55 °C, and 15 s at 95 °C and a temperature ramp rate set to 2%.

For the TaqMan and MGB PCR assays, we used 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), 750 nM concentration of each primer, 200 nM concentration of the probe, and 5 μL of DNA in a total volume of 25 μL. The PCR program consisted of an initial 2 min at 50 °C and 10 min at 95 °C followed by 50 cycles of 15 s at 95 °C and 1 min at 55 °C with data collection at the annealing step.

In the molecular beacon assays, we used a PCR setup consisting of 1 × TaqMan buffer A, 4 mM MgCl<sub>2</sub>, 0.2 mM each of dATP, dGTP, dCTP, and dUTP, 0.2 U of UNG, and 1 U of AmpliTaq Gold DNA polymerase (TaqMan PCR Core Reagents; Applied Biosystems), 750 nM concentration of each primer, and 200 nM concentration of the probe in a total volume of 25 μL. The PCR program consisted of an initial 2 min at 50 °C and 10 min at 95 °C followed by 50 cycles of 30 s at 95 °C, 1 min at 50 °C, and 30 s at 72 °C with data collection at the annealing step.

Five technical duplicates of DNA dilutions corresponding to  $10^4$ ,  $10^3$ ,  $10^2$ , 50, 10, and five copies of diploid RRS genomes (representing twice as many copies of the target sequence) were done for each assay. Experiments were also done to assess the effect of background wild-type soybean DNA. This was done by adding  $2 \times 10^4$  diploid copies of the soybean genome to the reaction mix. These experiments were all done in duplicate.

**Southern Blot.** Approximately 10 μg of genomic DNA from RRS and wild-type soybean was digested to completion using *Bam*HI and *Bgl*III (New England Biolabs, Ipswich, MA). Fragments were separated by 0.8% agarose gel electrophoresis and blotted on to a Hybond N+ membrane (GE Healthcare, Uppsala, Sverige). The blot was hybridized with a probe covering the 534 bp fragment of unknown origin (probe PCR primers available upon request). The probe was labeled with <sup>32</sup>P dCTP and generated using the Rediprime II Random Prime Labeling System (GE Healthcare) according to the manufacturer's recommendations.

## RESULTS AND DISCUSSION

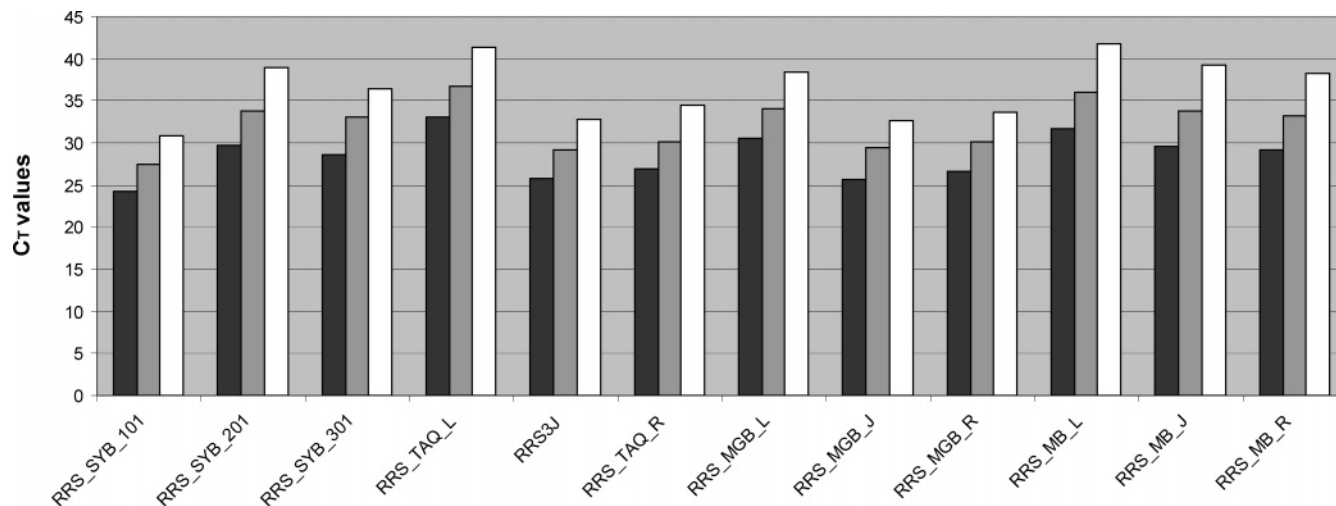
Many different real-time PCR assays have been published for the detection of RRS. These include event specific assays using TaqMan and FRET probes as well as Scorpion primers (4, 21, 22). The choice of detection strategy may reflect personal preferences as well as available funding and hardware. Costs

associated with the different assay types tested here did not vary significantly. Reagent costs (PCR reagents, primers, and probes) per assay were estimated to be about 1.4 Euros for the molecular beacon assays, 1.5 Euros for the TaqMan assays, 1.7 Euros for the MGB assays, and 1.2 Euros for the SYBR Green assays (prices do not include plasticware and optical covers since these are the same for all of the assays). All of the assays could be designed in-house without additional cost. SYBR Green is the least expensive since there is no need for a probe, and MGB probes are slightly more expensive than TaqMan probes, making this the most expensive approach.

The RRS locus that we selected (sequence AJ308515 in EMBL) can be said to represent a general case where a junction sequence is the target of interest. The junction between a transformed organism's genome and a genetic element introduced using molecular techniques represents an event specific target that uniquely defines that particular transformation event, making this a particularly suitable target for GM detection (6). To detect this target, we selected four of the most popular real-time PCR chemistries and designed 11 novel detection assays (Table 1 and Figure 1). All of the assays of a particular type were designed using the same software with identical settings. For each of the four real-time PCR technologies, we should thus have a set of different assays designed to have the same optimal reaction conditions. This set of assays allowed us to objectively evaluate the performance of the different real-time strategies when it comes to the detection and quantification of an event specific junction sequence.

Data from our SYBR Green assays indicated that the assay with the shortest amplicon length had the highest sensitivity, and this amplification product also gave the cleanest melting curve (data not shown). It is expected that a shorter amplicon in general will make any real-time PCR more efficient than if the amplified locus is longer. However, we saw no indication of such a correlation for our set of 12 assays, and more assays would have to be compared to see if this is a consistent trend when amplicons are as short as the ones included here (300 base pairs or less).

Our results in general imply that none of the real-time approaches tested are significantly more sensitive than any of the other (Table 2 and Figure 2).  $C_T$  values for experiments with  $10^4$  copies of the diploid RRS genome varied from 24.23 to 33.07 (Table 2), but none of the assay types seemed to be consistently more sensitive than any of the others. Similarly,



**Figure 2.** Comparison of  $C_T$  values for RRS detection using different real-time chemistries. Black,  $10^4$  copies of the (diploid) RRS genome; gray,  $10^3$  copies; and white,  $10^2$  copies.

the presence of relatively large amounts of background wild-type soybean DNA did not seem to have a severe and/or systematic effect on any of the chemistries, even though for all of the assays, one of the primers (and in some cases, also the probe) corresponded to a sequence that is present in the wild-type soybean genome as well (Figure 1).

We found that molecular beacon assays were less sensitive than the other types of assays tested, even though  $C_T$  values for  $10^4$  copies of the target sequence were comparable to those of the other chemistries (Table 2). Using data from the serial dilutions, it was shown that the molecular beacon assays did have a steeper slope than the rest, indicating that the efficiency of these assays was somewhat lower (Table 2 and Figure 2). This is consistent with the observed relatively high number of negative reactions for the lowest template concentrations (Table 2) and implies that the sensitivity (limit of detection) for beacon assays was inferior to that of the other chemistries.

Molecular beacons were also somewhat more sensitive to changes in PCR conditions than the other setups, and small changes in PCR cycle parameters as well as the concentration of  $MgCl_2$  seemed to have a more severe effect on beacons than on for instance TaqMan probes (data not shown). The dynamic structure of the beacons during the assay, where the probe is either annealed to the target sequence (signal is emitted), closed with the stem sequences forming a short duplex (no signal), or in transition between the two states (low signal), seems to reduce the ruggedness of this approach. To assess optimal reaction conditions given our stem sequences and loop melting temperatures, pilot experiments were done using different concentrations of  $MgCl_2$  and it was found that a high  $MgCl_2$  concentration (4 mM) was necessary for the beacons to function properly (data not shown). It is also noteworthy that the data collection had to be done at the annealing step only.

It appeared that the assays with probes annealing to the 534 base pair fragment of unknown origin had a lower sensitivity than the others (Table 2 and Figure 2). Sequence similarity searches against genomic databases indicated that this element had features in common with a repetitive element found in the recently completed and mapped rice genome (23). Southern blotting using a probe covering the central ~500 bases of the 534 base pair region indicated that this region is not repetitive in RRS and that the element is not present in wild-type soybean (data not shown). That this element represents a single copy element of unknown origin in RRS is consistent with previously

published results (see ref 3 and documentation for GTS 40-3-2 at the AGBIOS web page; <http://www.agbios.com>). It can thus not be concluded that the presence of similar target sequence in other parts of the soybean genome interferes with this subset of the assays. If this is not coincidental (the total number of assays tested here is quite low), a more likely explanation is that the apparent lowered sensitivity has something to do with structural properties of the DNA in this locus.

In the presented work, we have focused on the detection of a junction sequence as this should be a very specific target (see discussion in Introduction). The specificity of the novel assays described herein should thus be very high even though we have only tested this in the context of (background) wild-type soybean DNA. There are other types of target loci where the different methods included here would most likely have given quite different results. The MGB technology allows for the design of shorter, more specific probes, and if the target sequence is highly similar to a nontarget locus (just a single nucleotide difference in probe regions and identical flanking primer regions, for instance), such an assay would probably outperform both molecular beacons and TaqMan probes in terms of specificity. For a SYBR Green assay to work in this context, the primers would have to be extremely specific and the corresponding melting curves very clean. Molecular beacons seem to have advantages when it comes to detection of nucleic acids in real-time under isothermal conditions and low temperatures. Examples of this includes the NASBA assay (24, 25) and detection of mRNA in live cells (for review, see ref 26).

There is also a growing number of alternative types of assays available, including iso-dC-containing primers (27–29), LUX primers (30), and cycling probes (31). All of the different real-time PCR technologies have their advantages and limitations, and a choice of strategy has to suit the nature of the target locus and the resources available, but we believe that for detection of a GMO junction sequence, several of the most widely used real-time chemistries can be designed to be equally specific, sensitive, and robust.

#### ACKNOWLEDGMENT

The present study is the first in a series of related studies covering several probe chemistries performed by partners of the Co-Extra project.

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Received for review July 17, 2006. Revised manuscript received October 27, 2006. Accepted October 27, 2006. This work was financially supported by the European Commission through the Sixth Framework Program, integrated project Co-Extra (<http://www.coextra.org>; contract FOOD-2005-CT-007158) and by the Research Council of Norway (Grant 170363/D10). This is gratefully acknowledged.

JF061987C