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Detection and Quantification of Genetically Modified Organisms Using Very Short, Locked Nucleic Acid TaqMan Probes

SERGIO SALVI, FABIO D'ORSO, AND GIORGIO MORELLI*

Unità di Nutrizione Sperimentale, Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione, Via Ardeatina 546, 00178 Roma, Italy

Many countries have introduced mandatory labeling requirements on foods derived from genetically modified organisms (GMOs). Real-time quantitative polymerase chain reaction (PCR) based upon the TaqMan probe chemistry has become the method mostly used to support these regulations; moreover, event-specific PCR is the preferred method in GMO detection because of its high specificity based on the flanking sequence of the exogenous integrant. The aim of this study was to evaluate the use of very short (eight-nucleotide long), locked nucleic acid (LNA) TaqMan probes in 5′-nuclease PCR assays for the detection and quantification of GMOs. Classic TaqMan and LNA TaqMan probes were compared for the analysis of the maize MON810 transgene. The performance of the two types of probes was tested on the maize endogenous reference gene *hmga*, the CaMV 35S promoter, and the *hsp70/cryIA(b)* construct as well as for the event-specific 5′-integration junction of MON810, using plasmids as standard reference molecules. The results of our study demonstrate that the LNA 5′-nuclease PCR assays represent a valid and reliable analytical system for the detection and quantification of transgenes. Application of very short LNA TaqMan probes to GMO quantification can simplify the design of 5′-nuclease assays.

KEYWORDS: Event-MON810 maize; GMO; real-time PCR; locked nucleic acid (LNA); standard reference plasmids; limit of detection (LOD); limit of quantification (LOQ)

INTRODUCTION

Many countries have established labeling systems with thresholds for the fortuitous presence of genetically modified (GM) crops, defined as 0.9% in the European Union (*1*), 3% in Korea (*2*), and 5% in Japan (*3*). Validated protocols for transgene detection have been set and approved to perform qualitative and quantitative analysis of transgenic events in foods and feeds. Generally, these protocols use TaqMan 5′-nuclease polymerase chain reaction (PCR) assays with real-time quantitative PCR, although other analytical approaches, such as ligation-dependent probe amplification (*4*) and quantitative-competitive PCR (*5*), were proposed for genetically modified organisms (GMO) quantification.

Problems may arise for the design of optimal TaqMan assays, such as the creation of a sequence-specific 20−25-mer probes having a melting temperature (T_m) around 70 °C and enclosed within an amplifiable fragment whose size should be as short as possible (*6*). This is especially true for the detection of an event-specific insertion or a construct-specific junction. Locked nucleic acid (LNA) TaqMan probes can be used to overcome this problem. LNA oligonucleotides are synthesized with the incorporation into DNA oligomers of nucleotides containing a methylene bridge that connects the 2′-oxygen of ribose with the 4′-carbon. The result is a locked 3′-*endo* conformation that reduces the flexibility of the ribose (*7*). The incorporation of LNA into oligonucleotides results in a significant increase in the thermal stability of duplexes with cDNA (an increase of 3−8 °C of the T_m per modified base). This allows the design of short probes having T_m equivalent to that of conventional TaqMan probes. LNA oligonucleotides and probes are widely used for many research and molecular diagnostic purposes, such as SNP genotyping (*8*) and gene expression studies (*9*). In a recent paper, a 14-*mer* LNA TaqMan probe was designed to specifically detect *Brassica napus BnACCg8* gene as an endogenous reference for the quantification of GM canola (*10*).

Here, we assess the application of very short (8-mer) LNA TaqMan probes as a suitable alternative to the conventional TaqMan chemistry for GMO detection and quantification. To this end, we performed a comparison between conventional TaqMan and LNA TaqMan 5′-nuclease PCR assays at several levels of analysis specificity for the detection of maize with the MON810 transgene, using plasmids as reference materials (*11*).

MATERIALS AND METHODS

Choice of the MON810 TaqMan Assays and LNA TaqMan Probes. To analyze the maize MON810 transgenic construct (**Figure**

* To whom correspondence should be addressed. Tel: +39-6-51494415. Fax: +39-6-51494550. E-mail: morelli@inran.it.

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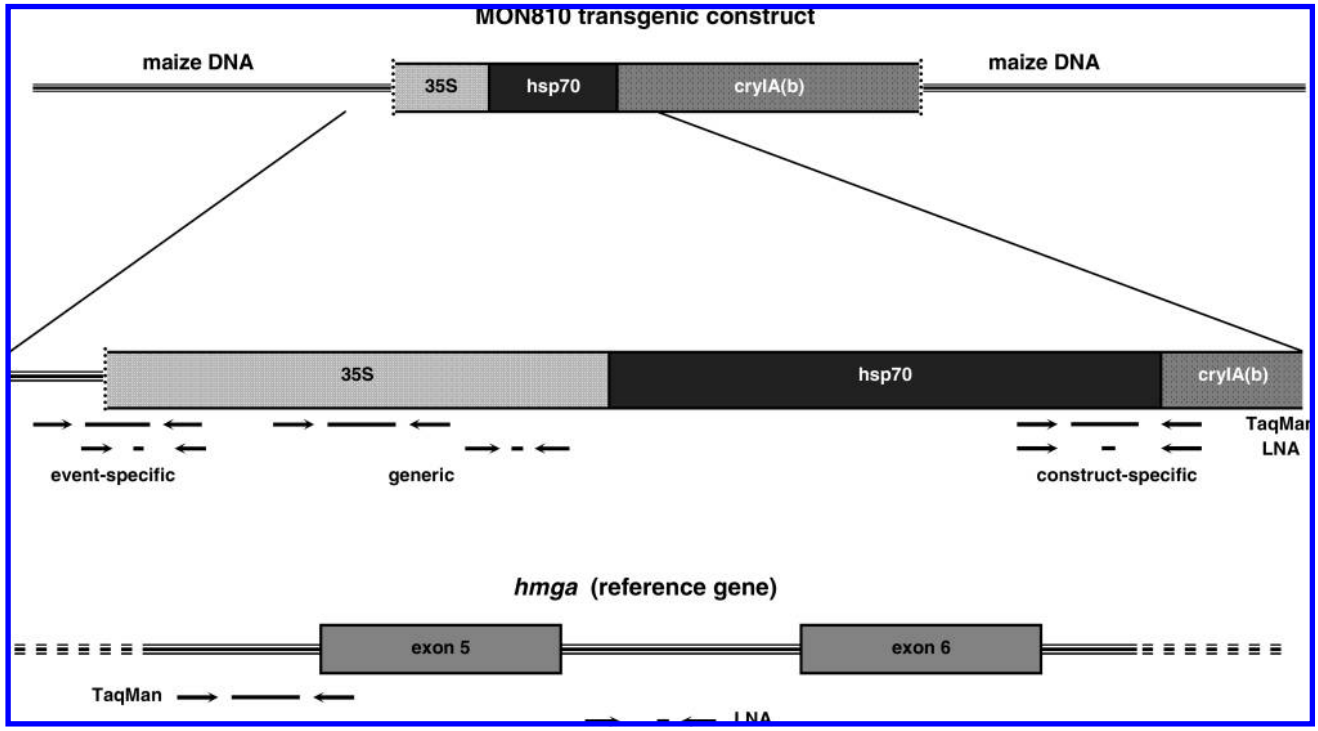

Figure 1. Genomic map of integrated MON810 construct (top) and partial gene structure of *hmga* (bottom). In both diagrams, positions of primers and probes used in TaqMan and LNA 5′-nuclease assays are indicated.

Table 1. Primers and Probes for Real-Time PCR Analysis

target	primer name	sequence (5′−3′)[a]	specificity	amplicon (bp)	reference	GenBank
hmga (TaqMan)	hmga-F	TTGGACTAGAAATCTCGTGCTGA	*hmga*	79	(*14*)	AJ131373
	hmga-R	GCTACATAGGGAGCCTTGTCCT				
	hmga-P[b]	FAM CAATCCACACAAACGCACGCGTA TAMRA				
hmga (LNA)	#68-F	TGCTTCAATTGCGTTGTTACTT		71	this study	
	#68-R	GCATACGTATTTGAATAAATGACCA				
	#68-P	FAM CTGCTCCT[c]				
event-MON810 (TaqMan)	MON810-F	TCGAAGGACGAAGGACTCTAACGT	maize genome/p35S	93	(*14*)	AF434709
	MON810-R	GCCACCTTCCTTTTCCACTATCTT				
	MON810-P	FAM AACATCCTTTGCCATTGCCCAGC TAMRA				
event-MON810 (LNA)	#61-F	GACTCTAACGTTTAACATCCTTTGC		77	this study	
	#61-R	ACCTTCCTTTTCCACTATCTTCAC				
	#61-P	FAM TTGCCCAG				
CaMV p35S (TaqMan)	CaMV p35S-F	GCCTCTGCCGACAGTGGT	p35S	82	(*12*)	AY326434
	CaMV p35S-R	AAGACGTGGTTGGAACGTCTTC				
	CaMV p35S-P	FAM CAAAGATGGACCCCCACCCACG TAMRA				
CaMV p35S (LNA)	#55-F	TTCGCAAGACCCTTCCTCTA		75	this study	
	#55-R	CTGCTAGAGTCAGCTTGTCAGC				
	#55-P	FAM GGAGAGGA				
hsp70/cryIA(b) (TaqMan)	hsp70/cryIA(b)-F	TGACACTATATTGCTTCTCTTTACATACGT	*hsp70/cryIA(b)*	139	(*13*)	AY326434
	hsp70/cryIA(b)-R	GATGTTTGGGTTGTTGTCCAT				
	hsp70/cryIA(b)-P	FAM CTCGATGCCTTCTCCCTAGTGTTGACCA TAMRA				
hsp70/cryIA(b) (LNA)	hsp70/cryIA(b)-F	TGACACTATATTGCTTCTCTTTACATACGT		139	this study (probe only)	
	hsp70/cryIA(b)-R	GATGTTTGGGTTGTTGTCCAT				
	#7-P	FAM CTTCTCCC				

[a] Shared sequences between TaqMan and LNA probes for event-MON810 and *hsp70/cryIA(b)* targets are underlined. [b] The TaqMan *hmga* probe sequence is on the reverse strand of AJ131373. [c] LNA probes have a "dark quencher" (*24*) at their 3′-ends, not indicated in this table.

1), we chose three published TaqMan 5′-nuclease assays used for the analysis at different levels of specificity. The first protocol was chosen for the detection of CaMV 35S promoter (*12*) as a generic target, the second one for *hsp70/cryIA(b)* (*13*) as a construct-specific target, and the last one for the 5′-insertion site as an event-specific target, together with an endogenous reference gene (*hmga*). The last two protocols have been approved by the European Union for the detection of maize event-MON810 (*14*). On the basis of the specific sequences indicated in these protocols, we searched for LNA TaqMan probes (and annexed primers) belonging to the Universal ProbeLibrary (UPL) from Roche, whose sequences were enclosed within those targeted by the conventional TaqMan probes or into regions located very close to them. To make this search, we used the free online ProbeFinder Roche software (*15*). Nucleotide sequences of primers and probes used in the classic TaqMan and LNA TaqMan assays, their target specificity, amplicon size, and GenBank reference sequences are reported in **Table 1**.

Maize Reference Materials. Certified reference standards 0% (w/w) ERM-BF413a and 5% (w/w) ERM-BF413f MON810 GM maize flours, developed at the IRMM (Institute for Reference Materials and Measurements, Geel, Belgium), were purchased from Fluka Chemical Co. and used as sources of total genomic DNA.

DNA Extraction and Quantification. Genomic DNA was extracted from 100 mg aliquots of GM maize flours by using a CTAB-based protocol (*16*). The quality and quantity of DNA samples were evaluated by absorbance measurements at 260 and 280 nm wavelengths using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.,

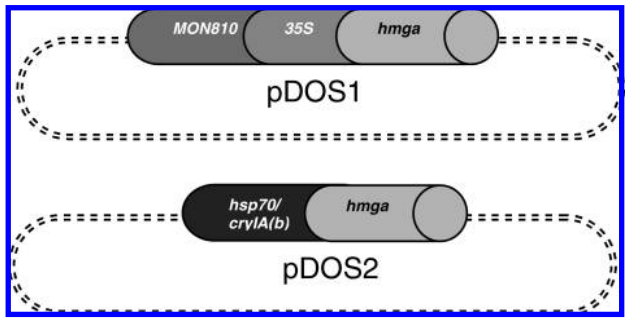

Figure 2. Schematic diagram of the integrated fragments in the pDOS1 (top) and pDOS2 (bottom) reference plasmids containing the target sequences for both TaqMan and LNA 5′-nuclease assays. pDOS1 contains the event-specific MON810, CaMV 35S, and *hmga* targets. pDOS2 contains the construct-specific *hsp70/cryIA(b)* and *hmga* targets.

Montchanin, DE) and by 1% agarose gel electrophoresis. According to several references (*17–19*), the weight of one maize haploid genome copy is 2.6 pg. This value was used to convert genomic DNA concentrations expressed in nanograms per microliter (as determined by spectrophotometer) to haploid genome copies per microliter (which was used in the real-time PCR assays).

Standard PCR Conditions. To synthesize the DNA fragments used to construct the reference plasmids, standard PCR assays were performed in 1× Gold Buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM each primer, 1.25 U AmpliTaq Gold (Applied Biosystems, Monza, Italy) in a final volume of 50 μL. The reactions were run on an GeneAmp PCR System 9700 (Applied Biosystems) under the following conditions: 11 min at 95 °C (hot-start/denaturation); 30−35 cycles of 30 s at 94 °C (denaturation), 30 s at 60 °C (annealing), 30 s at 72 °C (extension); and 7 min at 72 °C (final extension). Amplification products were electrophoresed onto 1% agarose gel in 1× TAE buffer, stained in ethidium bromide, and photographed with an UVIpro (version 12.5) gel documentation system (UVItec Ltd., Cambridge, United Kingdom).

Construction of the Standard Reference Plasmids and Their Quantification. Two different plasmids containing the targets for both TaqMan and LNA TaqMan assays were designed as reference molecules (**Figure 2**). Following the above-mentioned PCR conditions, different PCR steps were performed to assemble the target-containing fragments to be inserted in the recipient plasmids. To construct the insert containing the event-MON810/35S and *hmga* targets, a first PCR step was performed using genomic DNA as a template. For each individual PCR (event-MON810/35S and *hmga*), a specific tailed primer was used (Link MON810-R and Link hmga-F, **Table 2**), with the two tails designed to be reciprocally complementary. After the first PCR step, a second PCR step (asymmetric PCR) was performed using 0.1 volumes of each first PCR product as template, leading to the preferential amplification of two different single-stranded molecules. Then, 0.1 volumes of each asymmetric PCR product (containing the specific tail at 3′ termini) was mixed in a tube with 1× Gold Buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, and 1.25 U AmpliTaq Gold in a final volume of 50 μL, denatured for 11 min at 94 °C, annealed for 5 min at 37 °C, and extended for 5 min at 72 °C. This step allows for the annealing of the starting single-stranded templates through their reciprocally complementary tails, extending the annealed strands from each 3′ termini to complete the transition to a unique, double-stranded product. Finally, 0.1 volumes of this linking product was PCR-amplified, and the final product was inserted into the recipient plasmid vector. A similar PCR procedure was followed to build the second insert, which contained the *hsp70/cryIA(b)* and *hmga* targets. In this case, the specific tailed primers were Link CS-MON810-R and Link hmga-F (**Table 2**).

Both inserts were separately ligated into a pCR2.1-TOPO vector and used to transform *Escherichia coli* TOP10 strain (TOPO TA Cloning kit, Invitrogen). Transformed cells were grown overnight at 37 °C onto LB-agar plates with 50 μg/mL ampicillin. Each plate was previously spread with 40 μL of 40 mg/mL X-Gal to preliminarily select the positive clones by blue/white screening. After this screening step, several positive colonies were picked from plates and further screened

Table 2. PCR Steps and Primers for the Construction of the Cloned Inserts

insert	PCR step	primer name	sequence (5′-3′)[a]	fragment	size (bp)
MON810/35S/*hmga*	genomic	MON810-F	TCG AAG GAC GAA GGA CTC TAA CGT	MON810/35S	392
		link MON810-R	ggattaggccttgatgtgtcaataattaccaCTGCTAGAGTCAGCTTGTCAGC		
		link hmga-F	ggtaattattgacacatcaaggcctaatccaTTGGACTAGAAATCTCGTGCTGA	*hmga*	255
		#68-R	GCA TAC GTA TTT GAA TAA ATG ACC A		
	asymmetric (ssDNA production)	MON810-F	TCG AAG GAC GAA GGA CTC TAA CGT	MON810/35S	392
		#68-R	GCA TAC GTA TTT GAA TAA ATG ACC A	*hmga*	255
	Ann/Ext (mixed ssDNA)	no primers		MON810/35S annealed to *hmga*	617
	final	MON810-F	TCG AAG GAC GAA GGA CTC TAA CGT	MON810/35S/ *hmga*	617
		#68-R	GCA TAC GTA TTT GAA TAA ATG ACC A		
hsp70/cryIA(b)/hmga	genomic	hsp70/cryIA(b)-F	TGA CAC TAT ATT GCT TCT CTT TAC ATA CGT	*hsp70/cryIA(b)*	170
		link CS-MON810-R	ggattaggccttgatgtgtcaataattaccaGATGTTTGGGTTGTTGTCCAT		
		link hmga-F	ggtaattattgacacatcaaggcctaatccaTTGGACTAGAAATCTCGTGCTGA	*hmga*	255
		#68-R	GCA TAC GTA TTT GAA TAA ATG ACC A		
	asymmetric (ssDNA production)	hsp70/cryIA(b)-F	TGA CAC TAT ATT GCT TCT CTT TAC ATA CGT	*hsp70/cryIA(b)*	170
		#68-R	GCA TAC GTA TTT GAA TAA ATG ACC A	*hmga*	255
	Ann/Ext (mixed ssDNA)	no primers		*hsp70/cryIA(b)* annealed to *hmga*	395
	final	hsp70/cryIA(b)-F	TGA CAC TAT ATT GCT TCT CTT TAC ATA CGT	*hsp70/cryIA(b)/hmga*	395
		#68-R	GCA TAC GTA TTT GAA TAA ATG ACC A		

[a] Reciprocally complementary sequences of tailed primers are in lowercase.

Table 3. Plasmid Validation Using TaqMan Probes

template DNA		*hmga*	eent-MON810	*hsp70/cryIA(b)*	p35S
genomic	slope	−3.177	−3.395	−3.443	−3.410
	slope SD	0.074	0.025	0.210	0.152
	Y-intercept	37.624	42.691	44.869	43.586
	R^2	0.996	0.986	0.991	0.988
	E	106.5	95.7	95.8	96.8
plasmid	slope	−3.477	−3.465	−3.517	−3.542
	slope SD	0.118	0.047	0.082	0.091
	Y-intercept	40.681	40.620	41.267	41.665
	R^2	0.993	0.998	0.997	0.998
	E	93.9	94.4	92.5	91.6
plasmid[a]	slope	−3.508	−3.457	−3.542	−3.551
	slope SD	0.156	0.053	0.094	0.136
	Y-intercept	40.525	39.863	41.520	41.019
	R^2	0.992	0.999	0.998	0.999
	E	93.0	95.0	91.6	91.4

[a] Without non-GM soybean DNA.

by standard PCR, using M13 forward and reverse primers (supplied by the TOPO TA Cloning kit) under the following cycling conditions: 11 min at 95 °C (to lysate the bacterial cells and denaturate DNA); 25 cycles of 30 s at 94 °C, 30 s at 50 °C, and 60 s at 72 °C; and 10 min at 72 °C. Plasmid extractions from positive clones were performed with the QIAprep Spin Miniprep kit (Qiagen) according to the supplied extraction protocol. Automated sequencing analysis (performed by Bio-Fab Research, Pomezia, Italy) completed the screening and selection procedures prior to using the extracted standard plasmids in the real-time quantitative PCR assays.

As in the case of maize genomic DNA, plasmid DNA concentrations were converted in copies per microliter for the real-time PCR purposes. From the base pair size of the pCR2.1-TOPO vector (3931 bp) and of each cloned insert (617 bp for event-MON810/35S/*hmga* and 395 bp for *hsp70/cryIA(b)/hmga*), the molecular weight of each standard reference plasmid (pDOS1 and pDOS2, respectively) was calculated from the total number of nucleotide pairs contained in the entire sequence, which was multiplied by the mean molecular weight of 618 (this value was determined taking into account the loss of pyrophosphate groups due to DNA polymerization). To calculate the copy number of the standard reference plasmids, we used the following formula:

$$\text{copies per microliter} = \frac{(C \times N_A)}{(MW \times 10^9)}$$

where C is the concentration, expressed in ng/μL, as determined by spectrophotometer, N_A is Avogadro's number (6.023×10^{23}), MW is the molecular weight of the standard plasmid, and 10^9 is the conversion factor from nanomoles to moles.

Quantitative PCR Conditions. . Both TaqMan and LNA TaqMan 5′-nuclease PCR assays were carried out using ABI Prism 7900HT (Applied Biosystems) and performed according to the following chemical conditions.

For the TaqMan and LNA TaqMan MON810 event-specific assays, we followed a validated protocol (*14*) using 1× TaqMan Buffer A, 6.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, and dGTP, 0.4 mM dUTP, 0.5 U of UNG, and 1.25 U of AmpliTaq Gold DNA polymerase (all reagents purchased from Applied Biosystems), 300 nM concentrations of each primer, and 180 nM concentrations of the probe in a total volume of 25 μL. The same reagent concentrations were used for the *hmga* TaqMan assay, with the exception of the probe, for which a final concentration of 160 nM was used. For the *hmga* LNA TaqMan assay, we used 1× TaqMan Universal PCR MasterMix (Applied Biosystems), 300 nM concentration of each primer, and 160 nM concentration of the probe in a total volume of 25 μL. For all other TaqMan and LNA TaqMan assays, we used 1× TaqMan Universal PCR MasterMix, 250 nM concentration of each primer, and 225 nM concentration of the probe in a total volume of 25 μL.

All PCR reactions were run under the following conditions: 2 min at 50 °C to activate UNG, 10 min at 95 °C to activate AmpliTaq Gold polymerase and deactivate UNG, 45 cycles of 15 s at 95 °C, and 1 min at 60 °C. Data collection and analysis were performed by the ABI Prism Sequence Detection Software (SDS version 2.2, Applied Biosystems). Each sample including all controls was analyzed in triplicate.

Validation of pDOS1 and pDOS2 as Standard Reference Molecules. Plasmid validation was carried out according to a procedure adapted from Toyota et al. (*20*). Calibration curves for *hmga*, event-MON810, *hsp70/cryIA(b)*, and p35S TaqMan targets were made using plasmid and genomic DNA as follows. Plasmids and GM maize DNA were 10- and 2-fold serially diluted, respectively, using molecular biology grade distilled water (Sigma) as a diluent. Real-time PCR was carried out on 5 μL of each plasmid dilution (from 1×10^6 to 1×10^2 final copies in the reaction tubes), and 5 μL of each GM-DNA dilution (2×10^4 to 1.25×10^3 total haploid genome copies in the reaction tubes). Maize DNA and plasmid dilutions were simultaneously processed. To standardize the content of inhibitors and genomic bulk along the entire dynamic range of analysis, each reaction tube contained 50 ng of non-GM soybean DNA. This soybean DNA quantity was determined to be noninhibitory in our real-time PCR assays (not shown).

Each TaqMan assay was independently repeated six times, and each sample was analyzed in triplicate. Mean values of slope, intercept (*Y*-int), correlation (R^2), and efficiency (E) were determined. The efficiency was calculated by the formula $E = 10^{(-1/\text{slope})} - 1$ and expressed in percent (%). All slope values are expressed as means ± standard deviations of the mean. Statistical comparisons between each genomic TaqMan assay and the correspondent plasmidic counterpart were made. More precisely, data from six independent experiments for each genomic TaqMan assay were pooled and used to construct a "mean calibration curve". A similar data pooling was made for each plasmidic TaqMan assay, and the two "mean curves" (genomic vs plasmidic) were compared performing a specific Student's *t* test (*21*). Probability (*P*) values below 0.05 were considered significant.

Performance Comparison of TaqMan and LNA TaqMan Probes Using pDOS1 and pDOS2. Performance comparisons of the TaqMan and LNA TaqMan probes were carried out analyzing *hmga*, event-MON810, *hsp70/cryIA(b)*, and CaMV p35S plasmid calibration curves. Real-time PCR was carried out on 5 μL of each plasmid dilution (from 1×10^6 to 1×10^2 final copies in the reaction tubes), in the presence of 50 ng of non-GM soybean DNA (about 4×10^4 haploid genome copies). Mean values of slope, intercept, correlation, and efficiency were determined. Each assay was repeated three times, and each sample was analyzed in triplicate.

Determination of Detection and Quantification Limits. Limit of detection (LOD) and limit of quantification (LOQ) were considered according to ISO international guidelines (*22*).

The LOD is defined as the lowest copy number that exhibits positive results in at least 95% of the replicates. For absolute LOD determination, maize DNA extracted from 5% (w/w) MON810 GM-flour was 5-fold serially diluted to give 2.6, 0.52, and 0.104 ng of DNA template (corresponding to about 1000, 200, and 40 total haploid genome copies, respectively) in the real-time PCR LNA TaqMan assays. Each dilution was amplified in the presence of 104 ng (4×10^4 haploid genome copies) of maize non-GM DNA to standardize the content of inhibitors and genomic bulk along the entire range of analysis. This non-GM maize DNA quantity was determined to be the highest noninhibitory amount to be used in the real-time PCR LNA TaqMan assays (not shown). Twenty-four replicates of each dilution were analyzed (**Table 5**). Considering the hemizygous contribution of the MON810 construct to the genome copies extracted from the 5% transgenic fraction present in the whole IRMM certified standard, we hypothesized that 2.6, 0.52, and 0.104 ng of genomic DNA would correspond approximatively to 25, five, and one copy of the transgene, respectively. Thus, the relative LOD was estimated by dividing the absolute LOD, expressed in terms of transgenic copies, by 4×10^4 total genome copies and given in percent.

The LOQ is defined as the lowest copy number that can be reliably quantified with an acceptable level of precision and accuracy. As a preliminary to absolute LOQ evaluation of event-MON810 LNA TaqMan assay, a "true value" estimation was performed as follows. Fifty-four replicates of 104 ng of maize DNA (26 ng of genomic DNA extracted from 5% (w/w) GM maize flour, plus 78 ng of genomic DNA extracted from 0% GM maize flour, corresponding to a total of $4 \times$

Table 4. Performance Comparison of TaqMan and LNA Probes

	hmga		event-MON810		*hsp70/cryIA(b)*		p35S	
	TaqMan	LNA	TaqMan	LNA	TaqMan	LNA	TaqMan	LNA
slope	−3.435	−3.588	−3.473	−3.484	−3.493	−3.583	−3.533	−3.381
slope SD	0.023	0.039	0.049	0.066	0.077	0.103	0.043	0.097
Y-intercept	41.253	43.079	41.384	41.071	40.239	40.404	42.311	41.031
R^2	0.996	0.996	0.997	0.994	0.996	0.994	0.997	0.996
E (%)	95.5	90.0	94.0	93.6	93.3	90.1	91.9	97.6

Table 5. LOD Estimations of the MON810 LNA Assays

DNA (ng)[a]	estimated copies	event-MON810	*hsp70/cryIA(b)*	p35S
2.6	25	24/24[b] (100%)	24/24 (100%)	24/24 (100%)
0.52	5	23/24 (95.8%)	23/24 (95.8%)	22/24 (91.7%)
0.104	1	12/24 (50.0%)	9/24 (37.5%)	8/24 (33.3%)

[a] Maize DNA extracted from 5% (w/w) MON810 GM-flour. [b] Positive replicates are expressed in fractions and percent.

Table 6. Trueness and Precision for Event-MON810 LNA Assay

		LNA			
		trueness		precision	
ng	true value	mean pE	error[a] (%)	SD	RSD[b] (%)
104	3116	3607	15.7	170.55	4.73
52	1558	1778	14.1	188.68	10.61
13	389	395	1.5	60.31	15.27
3.25	97	124	27.8	30.22	24.37
1.62	49	33	32.6	15.11	45.79
0.81	24	22	8.3	17.46	79.36

[a] Error was calculated as [(mean value − true value)/true value] × 100. [b] RSD (relative standard deviation) values were obtained by dividing the standard deviation to the mean value and are given in percents.

10^4 copies, viz., the highest noninhibitory amount to be used in the real-time PCR), were analyzed. Threshold cycle (C_t) values were interpolated on a calibration curve made with 10-fold serially diluted pDOS1 standard plasmid (from 1×10^6 to 1×10^2 copies in the reaction tubes) in the presence of 4×10^4 haploid genome copies of non-GM maize DNA. Maize DNA replicates and plasmid dilutions were simultaneously processed. The mean of the intercept values (true value) was expressed in terms of plasmid equivalents (pE). The conversion factor from nanograms of GM maize DNA to pE was determined by dividing the true value by the amount of GM maize DNA (26 ng) contained in the 104 ng of each replicate and expressed as pE/ng. Following a similar procedure, different dilutions of the transgene were produced by mixing different quantities of maize DNA extracted from 5% (w/w) MON810 GM flour with non-GM maize DNA to a final amount of 104 ng (4×10^4 haploid genome copies) (**Table 6**). Nine replicates of each diluted GM maize DNA were assayed against the event-MON810 plasmid calibration curve. The experimentally determined pE mean values were then compared with the correspondent true values (derived from the conversion factor) to calculate the percent error, defined by the following formula:

$$\text{percent error} = \frac{(\text{mean value} - \text{true value})}{\text{true value}} \times 100$$

The absolute LOQ of the MON810 event-specific LNA TaqMan assay was estimated by plotting the best fitting curve for the relationship between relative standard deviation (RSD) and pE copy number, following a procedure adapted from ref *23*. The copy number at which the RSD is equal to 25% was interpolated, and the corresponding value was considered to be the LOQ (error <30%).

The relative LOQ was estimated by dividing the absolute LOQ to the upper limit of 4×10^4 maize genome copies, corrected by means of the conversion factor from transgene copies to pE (true value), and expressed in percent. In consideration of the hemizygous contribution of the MON810 construct to the total genome copy number, we assumed that 26 ng (or 1×10^4 haploid genome copies) of DNA extracted from 5% MON810 GM-flour contained approximatively 250 transgene copies.

RESULTS

Searching for LNA TaqMan Probes Compatible with the Selected TaqMan Assays. To assess the application of LNA TaqMan probes as a suitable alternative to the conventional TaqMan chemistry for GMOs detection and quantification purposes, we selected the MON810 construct because of the availability of (i) the genomic integration map and its DNA sequence and (ii) the published TaqMan real-time quantitative PCR protocols for the detection of MON810 transgenic construct at generic (CaMV 35S), construct-specific [*hsp70/cryIA(b)*], and event-specific (5′ insertion junction) levels (*12*–*14*). We next investigated the commercial availability of LNA TaqMan probes whose sequences were included within those recognized by conventional TaqMan probes used in published protocols for the detection and quantification of the maize MON810 or in nearby regions. The aim of this strategy was to achieve the best coincidence between the two assays in terms of sequence specificity, keeping virtually unchanged the PCR chemical conditions, with particular reference to the final concentrations of primers and probes. To this end, we chose very short (eight-nucleotide long) LNA TaqMan probes belonging to the Universal ProbeLibrary (UPL, Roche) and used the free online ProbeFinder Roche software (*15*) that allowed the automated search for available probes, with annexed primers specific for the targets to be amplified. An important advantage of UPL probes is that no particular PCR master mix formulation is required for their use (*24*).

The #61 and #7 UPL-LNA TaqMan probes (8-mer) had sequences that were located inside the sequences recognized by the conventional TaqMan probes for the event-specific (maize genome/35S) and construct-specific [*hsp70/cryIA(b)*] targets, respectively (**Figure 1** and **Table 1**). The sequences corresponding to the #55 and #68 UPL-LNA TaqMan probes were located very close to the targets specific for the CaMV p35S and *hmga* classic TaqMan assays, respectively, with no overlapping probe sequences identified for (**Figure 1**).

Validation of Standard Reference Plasmids. Plasmids are easy to construct, relatively stable when frozen, and can be produced in large quantities. By introducing a transgenic sequence of interest into a plasmid and producing a linear dilution series, it was demonstrated that the plasmid could be used as a standard to construct a calibration curve for the quantification of GM material in unknown samples (*11*). To perform a comparative analysis of TaqMan and LNA TaqMan probes by real-time PCR, we conceived and constructed two plasmids (pDOS1 and pDOS2, **Figure 2**) carrying sequences derived from a maize endogenous gene (*hmga*) and from the MON810 transgenic construct (see materials and methods for details).

Validation of these constructs was carried out analyzing *hmga*, event-MON810, *hsp70/cryIA(b)*, and CaMV p35S calibration curves according to the selected TaqMan protocols (*12–14*) and using maize DNA and plasmid DNA as templates. The standard curves of pDOS1 and pDOS2 were calibrated by the pDNA series (five concentrations of the plasmids, 1×10^6, 1×10^5, 1×10^4, 1×10^3, and 1×10^2 copies per reaction). The standard curve of the MON810 maize genomic DNA was calibrated by the gDNA series (five concentrations of diluted DNA extracted from 5% MON810 maize flour, 52, 26, 13, 6.5, and 3.25 ng per reaction). Each dilution was amplified in the presence of non-GM soybean DNA.

The results of this comparative analysis are reported in **Table 3**. With the exception of the TaqMan *hmga* genomic assay, whose slope mean value was markedly different than the plasmidic counterpart ($P = 0.002$), the other assays showed no significant differences in terms of efficiency [$P = 0.949$, 0.690, and 0.240 for event-MON810, *hsp70/cryIA(b)*, and CaMV p35S targets, respectively]. The same TaqMan assays repeated on the pDOS1 and pDOS2 plasmids in the absence of non-GM soybean DNA showed no significant differences with respect to the values determined when soybean DNA was added to the reaction tubes (**Table 3**). On the basis of the above data, pDOS1 and pDOS2 were considered suitable to be used as standard reference molecules for the comparative analysis of TaqMan and LNA TaqMan probes.

Performance Comparison of TaqMan and LNA TaqMan Probes Using pDOS1 and pDOS2. *Hmga*, event-MON810, *hsp70/cryIA(b)*, and CaMV p35S targets were analyzed with TaqMan and LNA TaqMan probes to compare the two chemistries, using pDOS1 and pDOS2 DNA as templates in the presence of non-GM soybean genomic DNA as bulk DNA. No marked difference was observed between each TaqMan probe and the correspondent LNA TaqMan probe in terms of efficiency (**Table 4**). Using maize non-GM DNA in place of soybean DNA, no changes were observed concerning the event-MON810 slope value, demonstrating that neither the different complexity of the genomic DNA (maize vs soybean) nor the different quantity of ballast DNA added to the reaction (104 ng of maize DNA vs 50 ng of soybean DNA) affects the efficiency of the event-MON810 LNA TaqMan assay (data not shown).

LOD. As shown in **Table 5**, amplification of at least 95% of the replicates was detected in 0.52 ng of template DNA for event-specific and construct-specific MON810 LNA TaqMan assays and 2.6 ng for p35S. LOD values, expressed in terms of estimated transgenic copies, would correspond to about five copies for event-specific and construct-specific MON810 LNA TaqMan assays and about 25 copies for p35S. The relative LOD of the event-MON810 assay was estimated to be 0.0125%, less than 10% of the threshold value (0.9%) fixed by the European Commission (*1*).

LOQ. As a preliminary to absolute LOQ evaluations, a "true value" estimation by MON810 event-specific LNA TaqMan assay was performed. The true value was fixed to about 778 plasmid copies (pE) for 26 ng of GM maize DNA, corresponding to a conversion factor of about 30 pE per nanogram. Then, nine replicates of six different amounts (104, 52, 13, 3.25, 1.62, and 0.81 ng) of GM maize DNA were amplified in the presence of 4×10^4 maize haploid genome copies as bulk DNA. The related pE mean values, RSD, and other parameters were determined (**Table 6**). The absolute LOQ was estimated to be about 158 pE copies. Estimation trueness was confirmed, with an error that was <30% for all quantities in the range between 3116 and the above-mentioned absolute LOQ (**Table 6**), with equivalence between the true value and the mean value. The relative LOQ was estimated to be 0.127%, less than 20% of the threshold value (0.9%) fixed by the European Commission (*1*).

DISCUSSION

Validated protocols for GMO detection in food matrices are generally based on event-specific real-time PCR 5′-nuclease assays by using conventional TaqMan probes. Many different types of probes from the classic TaqMan system have been developed (*25*) and tested for GMO detection and quantification (*26*). Performance comparisons of LNA TaqMan vs conventional TaqMan (*27*) or MGB (*28*) probes have been published but not evaluated for GMO detection and quantification.

Performance evaluation of LNA TaqMan probes and classic TaqMan probes was carried out using LNA TaqMan probes belonging to the Universal ProbeLibrary collection (Roche). The size of these probes (8-mer) facilitates probe design as compared to conventional TaqMan assays where finding a sequence-specific 20−25-mer probe having the right T_m (around 70 °C) and enclosed within an amplifiable fragment whose size would be as short as possible (50−150 bp) (*6*) is difficult to fulfill, especially in the design of event-specific and construct-specific assays for GMO analysis. For the event-MON810, it was possible to design LNA TaqMan probes having their nucleotide sequences entirely enclosed into the correspondent classic TaqMan probe target sequences in two cases [maize genome/p35S and *hsp70/cryIA(b)*, event- and construct-specific targets, respectively]. These overlapped features allowed us to perform a direct comparison of target specificity. In particular, the same primers in combination with LNA TaqMan or conventional TaqMan probes were used for the analysis of *hsp70/cryIA(b)* junction sequence. To make a more precise comparison of LNA TaqMan probes and classic TaqMan probes, we took advantage of two reference plasmids (pDOS1 and pDOS2, **Figure 2**) that were found to be suitable to detect the MON810 transgenic constructs at different levels of specificity. Previous studies have already demonstrated the potential of GMO plasmids as calibrants for the quantitative determination of GMO presence in complex matrices (*11*).

Although our *hmga* TaqMan assay data of maize genomic DNA are in agreement with those reported in the MON810 validation report (*29*), we observed that the *hmga* TaqMan probe exhibits different slopes using genomic and plasmid DNA (**Table 3**); a similar discrepancy between genomic and plasmidic DNA was also observed using the *hmga* LNA TaqMan assay, which targets a different intron (data not shown). The different efficiency values observed when analyzing *hmga* on genomic and plasmidic DNA might be due to the intrinsic complexity characterizing the *hmga* targets in the maize genome vs plasmidic DNA, for example, the presence of *hmga* extra copies or pseudogenes. This should be excluded on the basis of a recent study demonstrating that *hgma* is present as a single copy gene in the maize haploid genome (*30*). Thus, the use of *hmga* as a reference gene in maize could be problematic when plasmids are employed as reference molecules unless more adequate assays are developed. It is worth mentioning that the *hmga* gene has been selected as maize endogenous reference gene only in three out of nine protocols validated by the European Commission, with *adh1* being preferred. However, the

definition of an endogenous reference gene for maize identification and quantification by real-time PCR is still open (*19, 31*).

Application of very short LNA TaqMan probes revealed performances substantially equivalent to those of conventional TaqMan probes, with similar efficiencies observed for all TaqMan and LNA TaqMan assays, and particularly for the event-specific assay (**Table 4**). The latter result is particularly relevant given that the development of event-specific PCR assays is the primary trend in GMOs detection. In addition, absolute LOD and LOQ estimations of MON810 event-specific LNA TaqMan assay showed values of about five copies and 158 pE, respectively, while relative LOD and LOQ were, respectively, less than 10 and 20% of the threshold value of 0.9% fixed by the European Commission for unintentional mixing of GMO crops in foods. It is important to point out that these results were obtained using validated protocols optimized for TaqMan probes. Therefore, the adoption of specific protocols for the LNA TaqMan probes belonging to the Universal ProbeLibrary (Roche) could further improve the general performances of the 5′-nuclease assays for GMO detection and quantification purposes.

In conclusion, the use of the very short LNA TaqMan probes reducing the amplicon length might facilitate the development of efficient and reproducible 5′-nuclease assays. As a matter of fact, taking advantage of the free online ProbeFinder Roche software (*15*), we were able to find with relative ease several LNA TaqMan probes belonging to the Universal ProbeLibrary that could be used for the quantification of soybean and maize transgenic products (data not shown). The assay-design flexibility of LNA 5′-nuclease assays together with a substantial equivalence between very short LNA TaqMan probes and conventional TaqMan probes in terms of general performances and price range make LNA TaqMan probes a reliable and promising tool for GMO detection and quantification purposes.

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