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Quantitation of Bt-176 Maize Genomic Sequences by Surface Plasmon Resonance-Based Biospecific Interaction Analysis of Multiplex Polymerase Chain Reaction (PCR)

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Surface plasmon resonance (SPR) based biosensors have been described for the identification of genetically modified organisms (GMO) by biospecific interaction analysis (BIA). This paper describes the design and testing of an SPR-based BIA protocol for quantitative determinations of GMOs. Biotinylated multiplex Polymerase Chain Reaction (PCR) products from nontransgenic maize as well as maize powders containing 0.5 and 2% genetically modified Bt-176 sequences were immobilized on different flow cells of a sensor chip. After immobilization, different oligonucleotide probes recognizing maize zein and Bt-176 sequences were injected. The results obtained were compared with Southern blot analysis and with quantitative real-time PCR assays. It was demonstrated that sequential injections of Bt-176 and zein probes to sensor chip flow cells containing multiplex PCR products allow discrimination between PCR performed using maize genomic DNA containing 0.5% Bt-176 sequences and that performed using maize genomic DNA containing 2% Bt-176 sequences. The efficiency of SPR-based BIA in discriminating material containing different amounts of Bt-176 maize is comparable to real-time quantitative PCR and much more reliable than Southern blotting, which in the past has been used for semiquantitative purposes. Furthermore, the approach allows the BIA assay to be repeated several times on the same multiplex PCR product immobilized on the sensor chip, after washing and regeneration of the flow cell. Finally, it is emphasized that the presented strategy to quantify GMOs could be proposed for all of the SPR-based, commercially available biosensors. Some of these optical SPR-based biosensors use, instead of flow-based sensor chips, stirred microcuvettes, reducing the costs of the experimentation.

KEYWORDS: Polymerase Chain Reaction; GMO; biosensors; real-time assays; surface plasmon resonance; multiplex PCR; maize Bt-176

INTRODUCTION

The extensive introduction of genetically modified organisms (GMOs) in agriculture and the increasing number of GMO-derived products recently launched into the food market (*1*−*6*) have led to a strong demand by customers for strict regulations and labeling of such products (*5*). Accordingly, the identification and quantitation of GMOs are becoming issues of great interest and, although several methods for GMO detection have been recently developed (*7*−*12*), the approaches for GMO quantification are still largely based on quantitative Polymerase Chain Reaction (PCR) (*13*−*20*). In fact, real-time quantitative PCR approaches have been recently developed by several laboratories and applied to GMO quantification, because in most countries the content of a given GMO is a very important parameter (*4*−*6*). In this respect, it should be indeed emphasized that quantitative detection methods are needed for enforcement of the recently introduced labeling threshold for GMOs in food ingredients (*2*−*5*). For instance, this labeling threshold is set to 1% in the European Union and Switzerland and must be applied to all approved GMOs (*2*).

As far as the identification of GMO sequences is concerned, the recent development of surface plasmon resonance (SPR) based biosensors (*21*−*23*) enables one to perform biospecific interaction analysis (BIA) for monitoring a variety of molecular reactions in real time. This optical technique detects and quantifies changes in refractive index in the vicinity of sensor chip surfaces to which ligands have been immobilized (*23*), allowing detection of biomolecules (analytes) interacting with the ligand (*22*, *23*). If the ligand is a biotinylated single-stranded DNA, SPR technology could easily monitor DNA−DNA hybridization in real time (*24*, *25*) and has been applied to detect

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Table 1. PCR Primers, TaqMan Probes, and BIA Probes Used in This Study

oligonucleotide	use	sequence
	SPR-Based BIA	
Bt-F	PCR primer	5′-AGCCTGTTCCCCAACTACGAC-3′
Bt-R	PCR primer	5′-TGGTGTAAATCTCGCGGGTC-3′
biot-Bt-F	biotinylated PCR primer	5′-biot-AGCCTGTTCCCCAACTACGAC-3′
Bt-p	probe	5′-GGTGCGGATGGGGTAG-3′
ZM-F	PCR primer	5′-TGCAGCAACTGTTGGCCTTAC-3′
ZM-R	PCR primer	5′-TGTTAGGCGTCATCATCTGTGG-3′
Biot-ZM-F	biotinylated PCR primer	5′-biot-TGCAGCAACTGTTGGCCTTAC-3′
	ZM-p probe	5′-ATCATCACTGGCATCG-3′
	Quantitative TaqMan PCR Analysis	
Bt-F1	PCR primer	5′-GTGGACAGCCTGGACGAGAT-3′
Bt-R1	PCR primer	5′-TGCTGAAGCCACTGCGGAAC-3′
Bt-p1	probe	5′-FAM-AACAACAACGTGCCACCTCGACAGG-TAMRA-3′
ZM-F	PCR primer	5′-TGCAGCAACTGTTGGCCTTAC-3′
ZM-R	PCR primer	5′-TGTTAGGCGTCATCATCTGTGG-3′
ZM-p1	probe	5′-VIC-ATCATCACTGGCATCGTCTGAAGCGG-TAMRA-3′

HIV-1 infection (*26*) and genetic mutations (*27*−*30*). Few reports of SPR-based methodologies to detect GMO have been published (*31*, *32*). In this specific field of GMO detection, we have recently published the SPR-based detection of Roundup-Ready soybeans (*31*). All of the available data demonstrate that SPR-based BIA is an easy, rapid, non-radioactive methodology to detect GMOs. On the other hand, no study has been published so far on the discrimination by SPR-based BIA of materials containing different amounts of GMOs. In addition, to our knowledge, no BIA studies have been published on the analysis of multiplex PCR products.

In the present paper we discuss the design and testing of an SPR-based BIA protocol for the quantitative determinations of GMOs. The protocol is based on the immobilization of a biotin-multiplex PCR product on a single, streptavidin-coated, sensor chip flow cell and injection of specific probes. We immobilized, on different flow cells of a sensor chip, biotinylated multiplex PCR products from nontransgenic maize as well as from material containing 0.5 and 2% genetically modified Bt-176 maize. Multiplex PCR was performed using two sets of PCR primers, one for the maize zein gene and the other for Bt-176 sequences. After immobilization, we injected different oligonucleotide probes recognizing maize zein and Bt-176 sequences. The results obtained were compared with Southern blot analysis and with quantitative PCR assays using the ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA).

MATERIALS AND METHODS

Maize Samples and DNA Isolation. Bt-176 (*5*−*7*, *33*) and conventional maize powders were obtained from Fluka (Buchs, Switzerland). Maize powders contained 0.1, 0.5, and 2% Bt-176 maize. Genomic DNA was isolated using the Wizard Magnetic DNA Purification System for Food (Promega Corp., Madison, WI) as described elsewhere (*31*).

Synthetic Oligonucleotides. The oligonucleotide probes and the PCR primers were purchased from Sigma Genosys (Cambridge, U.K.) and purified by HPLC. The sequences of the oligonucleotides used are reported in **Table 1**. The NCBI accession numbers of DNA sequences used for the design of the PCR primers are I41419 (maize Bt-176) and X07535 (maize zein) (*13*). The PCR primers were designed using Primer Express 1.0 software (PE Applied Biosystems). The Bt-176 and zein probes were designed as described elsewhere (*28*, *29*, *31*) after analysis of the secondary structures of the single-stranded PCR products, derived using the MFOLD software (version 3.0) developed by Zuker et al. (*34*) and Mathews et al. (*35*), the analyses being performed at a temperature of 25 °C and at 0.15 M NaCl.

Polymerase Chain Reaction (PCR). In each PCR reaction 100 ng of genomic DNA from Bt-176 maize and conventional maize was amplified by Taq DNA polymerase using primers amplifying maize zein (ZM-F and ZM-R) and Bt-176 (Bt-F and Bt-R) gene sequences, respectively. The nucleotide sequences of these PCR primers are shown in **Table 1**. PCR was performed in a final volume of 100 μL containing 50 mM KCl, 10 mM Tris-HCl, pH 8.8, 2.5 mM $MgCl_2$, using 2 units/reaction of Taq DNA polymerase (DyNAzyme, Finnzymes, Espoo, Finland), 100 μM dNTPs, and 0.5 μM PCR primers on a GeneAmp PCR System 9600 (PE Applied Biosystems). The PCR conditions were as follows: denaturation, 30 s, 95 °C; annealing, 30 s, 65 °C; elongation, 15 s, 72 °C. The lengths of the Bt-F/Bt-R and ZM-F/ZM-R PCR products were 76 and 69 bp, respectively. To avoid reaching the plateau phase of the amplification reaction, PCRs were conducted for 10, 20, 30, 40, and 50 cycles and Southern blot assay of the products performed. This preliminary step is crucial for determining experimental conditions allowing quantitative determinations (*36*, *37*).

For immobilization onto SA sensor chips, PCR products were obtained using, in addition to Bt-R and ZM-R, the biotinylated biot-Bt-F and biot-ZM-F primers. PCR products were purified using Microcon-30 (Millipore Corp., Bedford, MA).

Surface Plasmon Resonance (SPR). BIAcore 1000 analytical system (BIAcore AB, Uppsala, Sweden) was used in all experiments. Sensor chips SA, precoated with streptavidin, were from BIAcore AB. Running buffer was HEPES-buffered saline-EP (HBS-EP), which contains 10 mM HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% (v/v) Surfactant P20 (BIAcore AB). The experiments were conducted at 25 °C. The flow rate was 5 μL/min. Sensorgrams were analyzed with BIAevaluation 2.1 software (*31*). Blank subtractions were performed in all of the experiments by subtracting, from the experimental sensorgrams, the sensorgrams obtained by performing the same protocol on an empty flow cell. To obtain an efficient capture of PCR products onto the sensor chip, the well-documented streptavidin−biotin interaction was employed (*38*). After a pretreatment with a 10 μL pulse of 50 mM NaOH, 80 pmol in HBS-EP of the biotinylated PCR products was injected over the sensor chip SA. This protocol was repeated three times to reach saturation of the sensor chip. Hybridization was carried out by injecting 20 μL of Bt-p and ZM-p probes (40 pmol in HBS-EP) at 25 °C and a 5 μL/min flow rate. The same flow cells were used for these experiments, which were sequentially injected with Bt-p and ZM-p probes. After each hybridization and washing with 10 μL of HBS-EP, the flow cells were regenerated by performing a 5 μL pulse of 50 mM NaOH. To verify reproducibility, different multiplex PCR products were immobilized onto different flow cells, and Bt-p and ZM-p probes were injected.

Sequencing of PCR Products. The zein and Bt-176 maize PCR products were purified with Microcon-30 (Millipore Corp.) and sequenced using the BigDye terminator cycle sequencing kit and the ABI Prism 377 DNA sequencer (PE Applied Biosystems).

Southern Blotting. Southern blotting was performed according to established procedures (*39*), which have been applied also to GMO detection (*40*) as well as for semiquantitative purposes in other experimental systems (*41*, *42*). DNA was isolated as previously

described and PCR was performed; electrophoretic separation of PCR product was carried out on a 2.5% agarose gel, and the PCR fragments were transferred onto a Hybond-XL filter (Amersham Biosciences, Buckinghamshire, U.K.). Hybridization was performed using 5× Denhardt's solution, 5× SSC, 0.5% (w/v) SDS, and 100 μg/mL salmon sperm DNA. Bt-176 and zein probes were ^{32}P-labeled Bt-p1 and ZM-p1 oligonucleotides (see **Table 1** for nucleotide sequences). After overnight hybridization, the filters were washed and autoradiography was performed.

Real-Time Quantitative PCR. Quantitative PCR was performed as recently described (*5*, *15*). PCR was performed using the Bt-176 and zein gene-specific forward and reverse primers shown in **Table 1**. Quantitative real-time PCR assay was carried out with the use of gene-specific double-fluorescently labeled probes using the ABI Prism 7700 Sequence Detector (Applied Biosystems). The following probe sequences were used for real-time PCR: Bt-176 probe, 5′-FAM-AAC AAC AAC GTG CCA CCT CGA CAG G-TAMRA-3′; zein gene probe, 5′-VIC-ATC ATC ACT GGC ATC GTC TGA AGC GG-TAMRA-3′, where the fluorescent reporter FAM and the quencher TAMRA are 6-carboxyfluorescein (FAM) and 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine (TAMRA), respectively. VIC is a trademark of PE Applied Biosystems.

RESULTS

Southern Blot Analysis of Multiplex PCR on Genomic DNA from Maize Powders Containing 0.1, 0.5, and 2% GM Bt-176 Maize. In **Table 1** the nucleotide sequences of maize zein and Bt-176 PCR primers and oligonucleotide probes are reported. We first verified whether multiplex PCR allows the production of different amounts of Bt-176 and zein PCR products when powders containing different amounts of GMO are employed. In this set of experiments, genomic DNA was extracted from conventional maize powder or powders containing 0.1, 0.5, and 2% Bt-176 GM maize. Multiplex PCRs were performed by employing different cycles (10, 20, 30, 40, and 50) using Bt-176 and zein primers; this was done to determine experimental conditions in which the multiplex PCRs are not at the completion of the amplification reactions, but still in the logarithmic phase. This is very important as it is well established that quantitation of the nonexponential plateau phase of a PCR is not possible (*36*, *37*). To verify that the PCR conditions employed were acceptable for quantitation purposes, PCR products were electrophoresed through a 2.5% agarose gel and Southern blotted in order to produce two identical nylon filters that were hybridized with ^{32}P-labeled Bt-176 and zein probes. The results demonstrate that PCR products obtained after 40 cycles are in the plateau level of the amplification reaction (data not shown). Because plateau levels were not reached after 30 cycles, we used these conditions for our experiments employing multiplex PCR. The results obtained in these experimental conditions are shown in **Figure 1** and demonstrate that increasing signals are detected using the Bt-176 probe (**Figure 1A**) when the multiplex PCR products are from maize powders containing a higher content of Bt-176 maize. On the contrary, the signals corresponding to zein sequences are relatively constant (**Figure 1B**).

SPR-Based BIA Format for GMO Detection and Quantification. Biotinylated zein and Bt-176 PCR products (see **Table 1** for nucleotide sequences) were stably immobilized onto an SA sensor chip by taking advantage of the streptavidin−biotin interaction as elsewhere described (*31*). In **Figure 2A** the employed SPR-based BIA approach is described. The approach is based on (a) immobilization on the same flow cell of two target PCR product (a representative example of immobilization is given in **Figure 2B**) obtained by multiplex PCR using biotinylated primers and (b) the following analysis

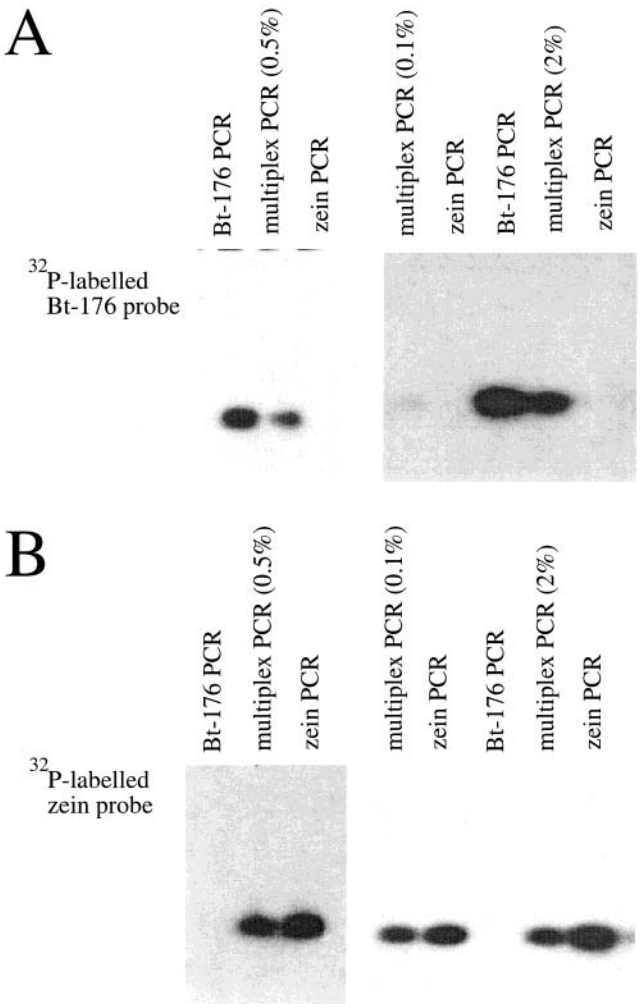

Figure 1. Southern blot analysis of Bt-176 (A) and zein (B) PCR products obtained by singleplex and multiplex PCR from maize containing 0.1, 0.5, and 2% Bt-176 sequences.

by sequential injection of suitable oligonucleotide probes. To produce double-stranded target gene sequences, multiplex PCR was performed using genomic DNA as template in the excess of the Bt-R and ZM-R primers with respect to biotinylated biot-Bt-F and biot-ZM-F. This was done to minimize the presence of biotinylated, unincorporated PCR primers in the mixture after PCR. The final multiplex zein and Bt-176 PCR products were in any case further purified with Microcon-30. Agarose gel electrophoretic analysis and direct sequencing of the PCR products confirmed the specificity of the PCR reaction (data not shown). To reach saturating levels of immobilized PCR products, three consecutive injections were performed using 40 μL of 2 μM PCR products in HBS-EP. Pulses with 50 mM NaOH were performed after each injection for the production of single-stranded PCR products able to hybridize with zein and Bt-176 probes. We expect that these two single-stranded PCR products will exhibit different secondary structures, as shown in **Figure 2A**. We routinely obtained ∼2000 RU of immobilized multiplex PCR products.

Hybridization of Bt-176 and Zein DNA Probes Following Injection to Flow Cells Carrying Multiplex PCR Products. Multiplex PCR reactions were performed using a primer mix constituted of biot-Bt-F, Bt-R, biot-ZM-F, and ZM-R PCR primers and using as target gene sequences genomic DNA from maize powders containing 0.5 and 2% Bt-176 GM maize. The two biotinylated multiplex PCR products were immobilized into

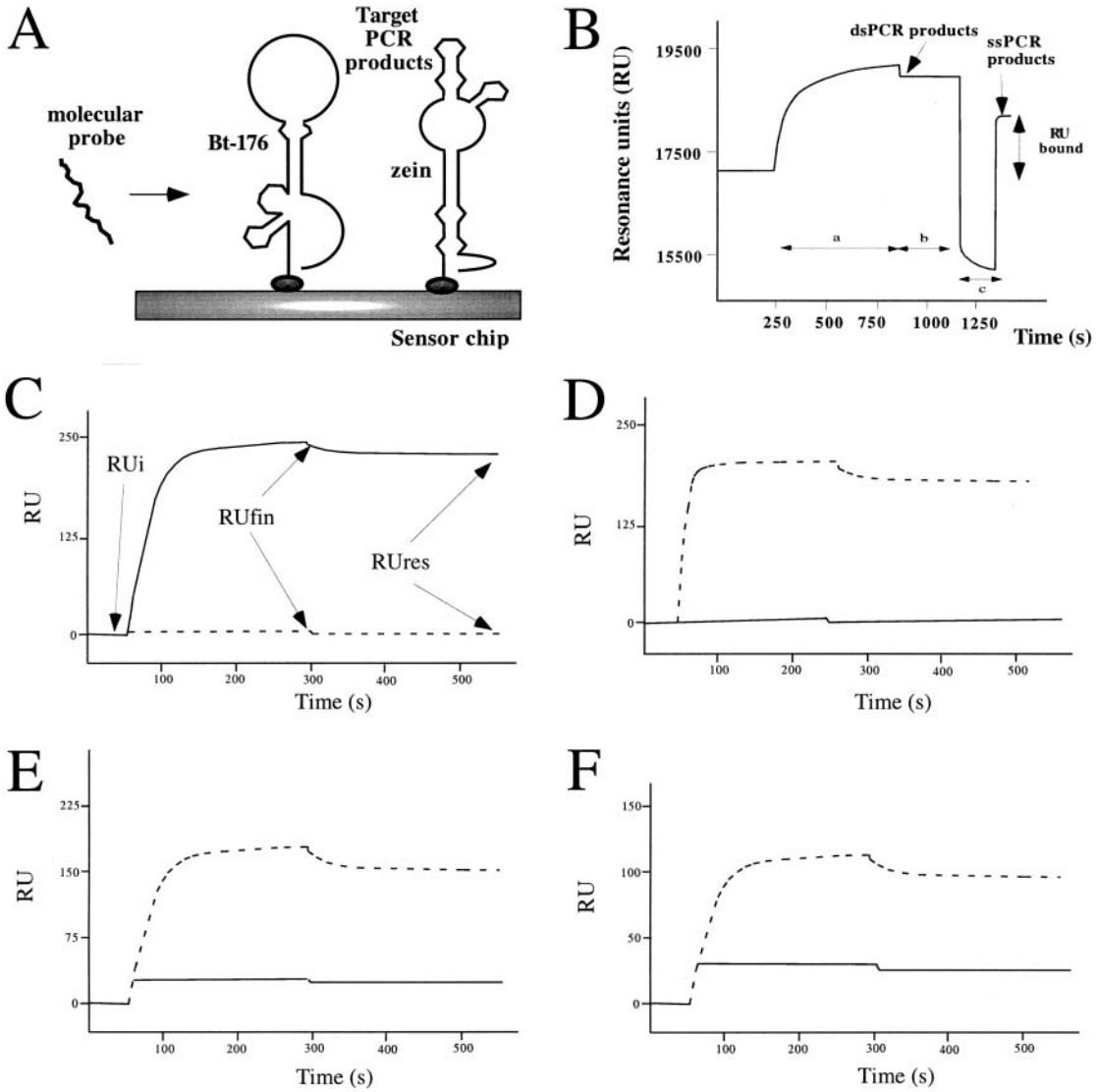

Figure 2. (A) Experimental strategy and format for GMO detection and quantification using SPR-based BIA and sensor chips carrying both zein and Bt-176 PCR products obtained by multiplex PCR. (B) Representative example of the increase of resonance units following injection of biotinylated Bt-176 and zein PCR products obtained by multiplex PCR. PCR products were injected in HBS-EP; after injection (segments "a" of the panel), injections of HBS-EP (segments "b" of the panel) and 50 mM NaOH (segments "c" of the panel) were performed. (C–F) Sensorgrams obtained after injection of 25 μL containing 40 pmol of Bt-176 (solid lines) and zein (dotted lines) probes on sensor chips carrying single Bt-F/Bt-R PCR products (C) or multiplex Bt-176 and zein PCR products obtained from maize containing 0% (D), 0.5% (E), and 2% (F) Bt-176 sequences.

two independent flow cells. In two additional flow cells (a) PCR products from a single PCR reaction performed using biot-Bt-F and Bt-R primers and (b) a multiplex PCR product obtained using target genomic DNA from conventional maize were immobilized. The results of injection of Bt-176 and zein probes are shown in **Figure 2C**−**F**. As expected, no hybridization was obtained following injection of the zein probe on the flow cell carrying a single Bt-176 PCR product (**Figure 2C**, dotted line); increase of RUfin − RUi values was on the contrary observed after the injection of the Bt-probe (**Figure 2C**, solid line). In the second control experiment, no hybridization was obtained following injection of the Bt-probe on the flow cell carrying multiplex PCR from conventional maize (see **Figure 2D**, solid line); increase of RUfin − RUi values was on the contrary observed after the injection of the zein probe (**Figure 2D**, dotted line).

When the zein and Bt-176 probes were injected on flow cells carrying the biotinylated multiplex PCR reactions performed using as target genomic DNA from maize powders containing 0.5 (**Figure 2E**) and 2% (**Figure 2F**) Bt-176 GM maize, the following results were obtained. When zein probes were injected, similar RUfin − RUi values were obtained (compare dotted lines of **Figure 2E**,**F**). When the Bt-176 probe was injected, different RUfin − RUi values were obtained, according to the different concentration of Bt-176 GMO (compare solid lines of **Figure 2E**,**F**).

Table 2 shows the comparison between Southern blotting and SPR-based BIA in determining the level of Bt-176 GMO. Comparison of the GMO_{Bt-176} indices (see **Table 2** for definitions) indicates that SPR-based BIA is a technique more efficient than Southern blotting in determining the concentration of Bt-176 sequences. The fold increase of the GMO_{Bt-176} indices from maize sample containing 0.5% Bt-176 sequences to maize sample containing 2% Bt-176 sequences was 3.14 ± 0.21 in the case of analysis employing SPR-based BIA but only 1.7 ± 0.15 in the case of Southern blotting.

Quantitative Real-Time PCR Analysis of Genomic DNA from Maize Powders Containing 0.5 and 2% GM Bt-176 Maize. To compare the results of SPR-based BIA with other well-established quantitative diagnostic approaches, real-time

Table 2. Quantification of Bt-176 Maize Sequences by Southern Blotting and SPR-Based BIA[a]

	GMO Bt-176 index		fold increase in
	0.5%	2%	2% Bt-176 maize[b]
Southern blotting[c]	0.74 ± 0.08	1.25 ± 0.13	1.7 ± 0.15
SPR-based BIA[d]	0.07 ± 0.01	0.21 ± 0.02	3.14 ± 0.21

[a] Results represent average ± SD of four independent experiments. [b] GMO Bt-176 index (2%)/GMO Bt-176 index (0.5%). [c] GMO Bt-176 index = [radioactivity (Bt-176)]/[radioactivity (zein)]. [d] GMO Bt-176 index = [(RUfin − RUi) (Bt-176)]/[(RUfin − RUi) (zein)].

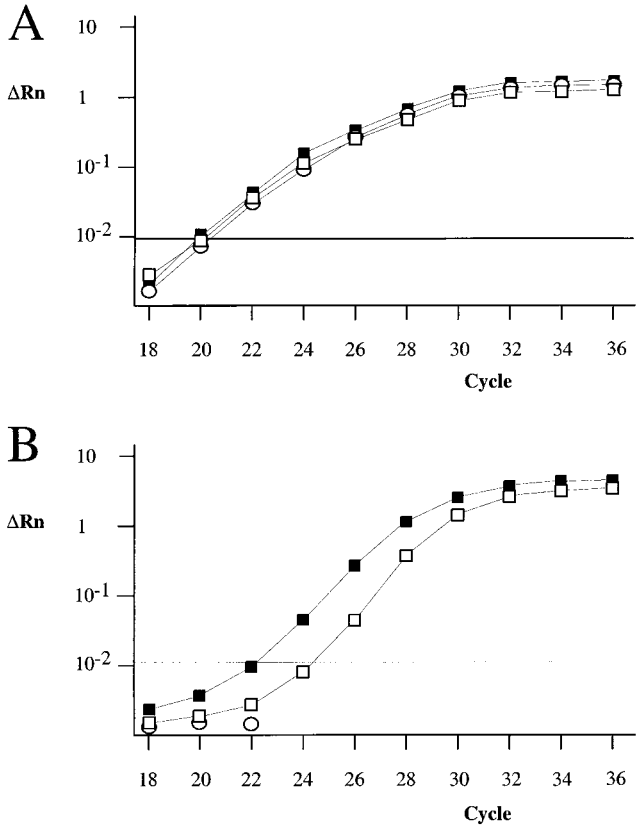

Figure 3. Quantitative real-time assay using as substrate genomic DNA from maize containing 0% (○), 0.5% (□), and 2% (■) Bt-176 sequences. Real-time PCR amplifications were conducted with primers/probes for zein (A) and Bt-176 (B) sequences (**Table 1**).

quantitative PCR was performed on the same samples used for SPR-based BIA. The results of real-time quantitative multiplex PCR using the ABI Prism 7700 Sequence Detector (Applied Biosystems) are shown in **Figure 3**. The kinetics of production of zein products is very similar when multiplex reactions are performed using genomic DNA samples from maize powders containing 0.5 and 2% Bt-176 GM maize (**Figure 3A**). On the contrary, as expected, the kinetics of production of Bt-176 PCR products is faster when DNA is used from a maize powder containing 2% Bt-176 GM maize (**Figure 3B**). **Table 3** shows the values of C_T and ΔC_T of the experiment. These data were analyzed using version 1.6.3 of the Sequence Detection Systems (Applied Biosystems) and give evidence for 4.53-fold content of Bt-176 maize sequences in samples from 2% Bt-176 as compared to those containing 0.5% Bt-176.

DISCUSSION

The detection and quantification of GMOs is becoming an issue of great interest (*1*−*6*). For the specific and sensitive detection of genetically modified material (for instance, Roundup-Ready soybeans and Bt-176 Maximizer maize) in foodstuffs, the PCR method has proved to be an invaluable tool (*7*−*12*). The detection of PCR products could be achieved by performing several techniques, including SPR-based BIA using biosensors (*28*−*31*). This technology has several important advantages, namely, the fact that no radioactive labeling is required, the procedure is performed in real time, and small amounts of ligand and analyte are required to obtain informative results (*21*−*23*).

Table 3. Quantification of Bt-176 Maize Sequences by Quantitative Real-Time PCR

	C_T		
	Bt-176	zein	ΔC_T
0.5% Bt-176	24.33	19.79	4.54
2% Bt-176	22.08	19.72	2.36

However, SPR-based BIA has not been employed to our knowledge using multiplex PCR products and, with respect to GMO analysis, GMOs have been identified by SPR-based BIA, but quantitative studies have not been reported (*31, 32*).

Several methods have been so far proposed to quantify PCR products, including Southern blot analysis of multiplex PCR performed employing few cycles (*40*−*42*), semiquantitative competitive PCR (*36*), and real-time quantitative PCR (*5*, *14*, *15*). Some of these methods are suitable to confirm the specificity of the obtained PCR products (for instance, all of the approaches employing a DNA specific probe).

The main issue of our study was to verify whether BIA, employing SPR and biosensor technologies, could be used to discriminate samples containing 0.5 and 2% Bt-176 maize.

The results obtained allow us to suggest that SPR-based BIA is an easy, speedy, and automated approach to quantify Bt-176 Maximizer sequences in maize. To our knowledge, this is the first example of quantitation of PCR products by SPR-based BIA.

The format used (**Figure 2A**) needs the immobilization of multiplex PCR products and the injection of zein and Bt-176 probes. This approach is based on the hypothesis that, when injection of products obtained by logarithmic phase multiplex PCR (the ligand) is carried out at saturating levels, the ratio Bt-176/zein PCR products bound to the chip is similar to that present in the PCR test tube. These amounts of PCR products are related to the initial copy number of Bt-176 and zein sequences if the multiplex PCR is carried on at the exponential phase. This appears to be the case, because sequential injections of Bt-176 and zein probes reproducibly allow the discrimination between multiplex PCR performed using maize genomic DNA containing 0.5% Bt-176 sequence and that performed using maize genomic DNA containing 2% Bt-176 sequence. The efficiency of SPR-based BIA in discriminating material containing 0.5% Bt-176 maize from that containing 2% Bt-176 maize is comparable to that of real-time quantitative PCR performed using the ABI Prism 7700 Sequence Detector (Applied Biosystems) (**Table 3**) and much more reliable than Southern blotting (**Table 2**).

We point out that the procedure described in the present paper is rapid, and informative results are obtained within ∼40 min. Furthermore, our approach allows several repetitions of the BIA assay on the same multiplex PCR product immobilized on the sensor chip. In fact, after washing with HBS-EP and regeneration of the flow cell with NaOH, the flow cell containing single-stranded multiplex Bt-176 and zein PCR products is ready for further hybridization steps. This is an interesting feature of the

protocol, allowing verification of the obtained results by the different teams involved in the application of the regulations that in different countries are the basis for the authorization of commercial foodstuffs containing GMO. At least in theory, the same chip could be maintained in HBS buffer, handed over from laboratory to laboratory, and reused several times.

Although this paper demonstrates that quantitation of Bt-176 sequences is feasible by SPR-based BIA, much work should be done (including the development of suitable internal standards) to apply this method to processed foodstuffs and other GMO products.

Finally, we emphasize that the approach described in this paper could be proposed for all of the SPR-based, commercially available biosensors (*43*−*46*). Some of these optical SPR-based biosensors use, instead of flow-based sensor chips, stirred microcuvettes, reducing the costs of the experimentation (*46*).

ABBREVIATIONS USED

GMO, genetically modified organisms; SPR, surface plasmon resonance; BIA, biospecific interaction analysis; RU, resonance units; SA, streptavidin; PCR, Polymerase Chain Reaction.

LITERATURE CITED

(1) Vollenhofer, S.; Burg, K.; Schmidt, J.; Kroath, H. Genetically modified organisms in food-screening and specific detection by polymerase chain reaction. *J. Agric. Food Chem.* **1999**, *47*, 5038−5043.

(2) Brodmann, P. D.; Ilg, E. C.; Berthoud, H.; Herrmann, A. Real-time quantitative polymerase chain reaction methods for four genetically modified maize varieties and maize DNA content in food. *J AOAC Int*. **2002**, *85*, 646−653.

(3) Vollenhofer, S.; Burg, K.; Schmidt, J.; Kroath, H. Short communication: Detection of Genetically Modified Organisms in Food. *Dtsch. Lebensm. Rundsch*. **1999**, *95*, 275−278.

(4) Permingeat, H. R.; Reggiardo, M. I.; Vallejos, R. H. Detection and quantification of transgenes in grains by multiplex and real-time PCR. *J. Agric. Food Chem*. **2002**, *50*, 4431−4436.

(5) Vaitilingom, M.; Pijnenburg, H.; Gendre, F.; Brignon, P. Real-time quantitative PCR detection of genetically modified Maximizer maize and Roundup Ready soybean in some representative foods. *J. Agric. Food Chem.* **1999**, *47*, 5261−5266.

(6) Tengel, C.; Schussler, P.; Setzke, E.; Balles, J.; Sprenger-Haussels, M. PCR-based detection of genetically modified soybean and maize in raw and highly processed foodstuffs. *Biotechniques* **2001**, *31*, 426−429.

(7) Lipp, M.; Anklam, E.; Stave, J. W. Validation of an immunoassay for detection and quantitation of a genetically modified mais in food and food fractions using reference materials: interlaboratory study. *J AOAC Int.* **2000**, *83*, 919−927.

(8) Meyer, R. Detection of genetically engineered plants by polymerase chain reaction (PCR) using the FLAVR SAVR tomato as an example. *Z. Lebensm. Unters. Forsch.* **1995**, *201*, 583−586.

(9) Meyer, R.; Chardonnens, F.; Hubner, P.; Luthy, J. Polymerase chain reaction (PCR) in the quality and safety assurance of food: detection of soya in processed meat products. *Z. Lebensm. Unters. Forsch*. **1996**, *203*, 339−344.

(10) Hupfer, C.; Hotzel, H.; Sachse, K.; Engel, K. H. Detection of the genetic modification in heat-treated products of Bt maize by polymerase chain reaction. *Z. Lebensm. Unters. Forsch*. **1998**, *206*, 203−207.

(11) Hassan-Hauser, C.; Mayer, W.; Hortner, H. Detection of the starch modifying gbss-antisense construct in transgenic potatoes. *Z. Lebensm. Unters. Forsch.* **1998**, *206*, 83−87.

(12) van Duijn, G. J.; van Biert, R.; Bleeker-Marcelis, H.; Van Boeijen, I.; Adan, A. J.; Jhakrie, S.; Hessing, M. Detection of genetically modified organisms in foods by protein- and DNA-based techniques: bridging the methods. *J. AOAC Int*. **2002**, *85*, 787−791.

(13) Vaitilingom, M.; Pijnenburg, H.; Gendre, F.; Brignon, P. Real-time quantitative PCR detection of genetically modified Maximizer maize and Bt-176 mais in some representative foods. *J. Agric. Food Chem.* **1998**, *47*, 5261−5266.

(14) Hoef, A. M.; Kok, E. J.; Bouw, E.; Kuiper, H. A.; Keijer, J. Development and application of a selective detection method for genetically modified soy and soy-derived products. *Food Addit. Contam.* **1998**, *15*, 767−774.

(15) Shindo, Y.; Kuribara, H.; Matsuoka, T.; Futo, S.; Sawada, C.; Shono, J.; Akiyama, H.; Goda, Y.; Toyoda, M.; Hino, A. Validation of real-time PCR analyses for line-specific quantitation of genetically modified maize and soybean using new reference molecules. *J. AOAC Int.* **2002**, *85*, 1119−1126.

(16) Hernandez, M.; Rio, A.; Esteve, T.; Prat, S.; Pla, M. A rapeseed-specific gene, acetyl-CoA carboxylase, can be used as a reference for qualitative and real-time quantitative PCR detection of transgenes from mixed food samples. *J. Agric. Food Chem*. **2001**, *49*, 3622−3627.

(17) Garcia-Canas, V.; Gonzalez, R.; Cifuentes, A. Detection of genetically modified maize by the polymerase chain reaction and capillary gel electrophoresis with UV detection and laser-induced fluorescence. *J. Agric. Food Chem*. **2002**, *50*, 1016−1021.

(18) Garcia-Canas, V.; Gonzalez, R.; Cifuentes, A. Ultrasensitive detection of genetically modified maize DNA by capillary gel electrophoresis with laser-induced fluorescence using different fluorescent intercalating dyes. *J. Agric. Food Chem.* **2002**, *50*, 4497−4502.

(19) Studer, E.; Rhyner, C.; Lüthy, J.; Hübner, P. Quantitative competitive PCR for the detection of genetically modified mais and maize. *Z. Lebensm. Unters. Forsch*. **1998**, *207*, 207−213.

(20) Ingham, D. J.; Beer, S.; Money, S.; Hansen, G. Quantitative real-time PCR assay for determining transgene copy number in transformed plants. *Biotechniques* **2001**, *31*, 132−141.

(21) Jonsson, U.; Fagerstam, L.; Ivarsson, B.; Johnsson, B.; Karlsson, R. Real-time biospecific interaction analysis using surface plasmon resonance and a sensor chip technology. *BioTechniques* **1991**, *11*, 620−627.

(22) Vadgama, P.; Crump, P. W. Biosensors: recent trends, a review. *Analyst.* **1992**, *117*, 1657−1670.

(23) Malmqvist, M. Biospecific interaction analysis using biosensor technology. *Nature* **1993**, *361*, 186−187.

(24) Wood, S. J. DNA-DNA hybridization in real time using BIAcore. *Microchem. J.* **1993**, *47*, 330−337.

(25) Nilsson, P.; Persson, B.; Uhlén, M.; Nygren, P. A. Real-time monitoring of DNA manipulations using biosensor technology. *Anal. Biochem.* **1995**, *224*, 400−408.

(26) Bianchi, N.; Rutigliano, C.; Tomassetti, M.; Feriotto, G.; Zorzato, F.; Gambari, R. Biosensor technology and surface plasmon resonance for real-time detection of HIV-1 genomic sequences amplified by polymerase chain reaction. *Clin. Diagn. Virol*. **1997**, *8*, 199−208.

(27) Nilsson, P.; Persson, B.; Larsson, A.; Uhlen, M.; Nygren, P. A. Detection of mutations in PCR products from clinical samples by surface plasmon resonance. *J. Mol. Recognit.* **1997**, *10*, 7−17.

(28) Feriotto, G.; Lucci, M.; Bianchi, N.; Mischiati, C.; Gambari, R. Detection of the ΔF508 (F508del) Mutation of the Cystic Fibrosis Gene by Surface Plasmon Resonance and Biosensor Technology. *Hum. Mutat.* **1999**, *13*, 390−400.

(29) Feriotto, G.; Ferlini, A.; Ravani, A.; Calzolari, E.; Mischiati, C.; Bianchi, N.; Gambari, R. Biosensor Technology for Real-Time Detection of the Cystic Fibrosis W1282X Mutation in CFTR. *Hum. Mutat.* **2001**, *18*, 70−81.

(30) Feriotto, G.; Corradini, R.; Sforza, S.; Bianchi, N.; Mischiati, C.; Marchelli, R.; Gambari, R. Peptide nucleic acids and biosensor technology for real-time detection of the cystic fibrosis W1282X mutation by surface plasmon resonance. *Lab. Invest*. **2001**, *81*, 1415−1427.

(31) Feriotto, G.; Borgatti, M.; Mischiati, C.; Bianchi, N.; Gambari, R. Biosensor technology and surface plasmon resonance for real-time detection of genetically modified Roundup Ready soybean gene sequences. *J. Agric. Food Chem.* **2002**, *50*, 955−962.

(32) Mariotti, E.; Minunni, M.; Mascini, M. Surface plasmon resonance biosensor for genetically modified organisms detection. *Anal. Chim. Acta* **2002**, *453*, 165−172.
(33) Matsuoka, T.; Kuribara, H.; Takubo, K.; Akiyama, H.; Miura, H.; Goda, Y.; Kusakabe, Y.; Isshiki, K.; Toyoda, M.; Hino, A. Detection of recombinant DNA segments introduced to genetically modified maize (*Zea mays*). *J. Agric. Food Chem*. **2002**, *50*, 2100−2109.
(34) Zuker, M.; Mathews, D. H.; Turner, D. H. Algorithms and thermodynamics for RNA secondary structure prediction: a practical guide. In *RNA Biochemistry and Biotechnology*; Barciszewski, J., Clark, B. F. C., Eds.; NATO ASI Series; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1999; pp 11−43.
(35) Mathews, D. H.; Sabina, J.; Zuker, M.; Turner, D. H. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J. Mol. Biol.* **1999**, *288*, 911−940.
(36) Hubner, P.; Studer, E.; Luthy, J. Quantitation of genetically modified organisms in food. *Nat. Biotechnol.* **1999**, *17*, 1137−1138.
(37) Heid, C. A.; Stevens, J.; Livak, K. J.; Williams, P. M. Real time quantitative PCR. *Genome Res.* **1996**, *6*, 986−994.
(38) Leblond-Francillard, M.; Dreyfus, M.; Rougeon, F. Isolation of DNA−protein complexes based on streptavidin and biotin interaction. *Eur. J. Biochem.* **1987**, *166*, 351−355.
(39) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning. A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989; Vol. 2, pp 9.31−9.58.
(40) McCabe, M. S.; Power, J. B.; de Laat, A. M.; Davey, M. R. Detection of single-copy genes in DNA from transgenic plants by nonradioactive Southern blot analysis. *Mol. Biotechnol*. **1997**, *7*, 79−84.
(41) Deng, G.; Kim, Y. S. Quantitation of erbB-2 gene copy number in breast cancer by an improved polymerase chain reaction (PCR) technique, competitively differential PCR. *Breast Cancer Res. Treat.* **1999**, *58*, 213-217.
(42) Hill, J. M.; Halford, W. P.; Wen, R.; Engel, L. S.; Green, L. C.; Gebhardt, B. M. Quantitative analysis of polymerase chain reaction products by dot blot. *Anal. Biochem.* **1996**, *235*, 44−48.
(43) de Mol, N. J.; Plomp, E.; Fischer, M. J.; Ruijtenbeek, R. Kinetic analysis of the mass transport limited interaction between the tyrosine kinase lck SH2 domain and a phosphorylated peptide studied by a new cuvette-based surface plasmon resonance instrument. *Anal. Biochem*. **2000**, *279*, 61−70.
(44) Edwards, P. R.; Maule, C. H.; Leatherbarrow, R. J.; Winzor, D. J. Second-order kinetic analysis of IAsys Biosensor data: its use and applicability. *Anal. Biochem.* **1998**, *263*, 1−12.
(45) Senzaki, K.; Ogawa, M.; Yagi, T. Proteins of the CNR family are multiple receptors for Reelin. *Cell* **1999**, *99*, 635−647.
(46) Myszka, D. G.; Rich, R. L. Implementating surface plasmon resonance biosensors in drug discovery. *Pharm. Sci. Technol. Today* **2000**, *9*, 3310−3317.

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