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# First Application of a Microsphere-Based Immunoassay to the Detection of Genetically Modified Organisms (GMOs): Quantification of Cry1Ab Protein in Genetically Modified Maize

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An innovative covalent microsphere immunoassay, based on the usage of fluorescent beads coupled to a specific antibody, was developed for the quantification of the endotoxin Cry1Ab present in MON810 and Bt11 genetically modified (GM) maize lines. In particular, a specific protocol was developed to assess the presence of Cry1Ab in a very broad range of GM maize concentrations, from 0.1 to 100% [weight of genetically modified organism (GMO)/weight[. Test linearity was achieved in the range of values from 0.1 to 3%, whereas fluorescence signal increased following a nonlinear model, reaching a plateau at 25%. The limits of detection and quantification were equal to 0.018 and 0.054%, respectively. The present study describes the first application of quantitative high-throughput immunoassays in GMO analysis.

**KEYWORDS: GMO; immunoassay; Cry1Ab; high-throughput analysis; quantitative detection assay; fluorescent beads**

## INTRODUCTION

Development of innovative analytical tools for the precise detection and quantification of genetically modified organism (GMO) presence in human food and animal feedstuffs is a legal prerequisite and technical challenge for official authorization and market access in the European Union (EU). With respect to genetically modified (GM) food and feed, the new European regulatory framework, that is, Regulations (EC) 1829/2003 and (EC) 1830/2003, aims at reinforcing the confidence of consumers by improving the traceability and control of food and feedstuffs with respect to legal and technical efforts to maintain identity preservation of GMO- and non-GMO-based product supply chains. They also identify a threshold value of 0.9% (*1*) for the presence of adventitious or technically unavoidable EU-approved GM content in otherwise GM-free product lines (*2*, *3*). The consequence of such a threshold setting, and the detailed traceability and labeling requirements, gives rise to the specific need for analytical methods for the reliable detection, identification, and quantification of a given authorized GM line, in particular, for general enforcement and control activities and in the event of any possible future risk management requirement. The most commonly applied methodology is based on the real time Polymerase Chain Reaction (RT-PCR) technique, which allows event-specific detection and quantification of a particular GM line according to the authorization requirements. However, RT-PCR is not suitable for high throughput, wide-scale screening approaches, particularly for routine control application at various critical control points along the food/feed chain where, for example, more immediate and fast assays can be usefully employed (*4*, *5*). In such circumstances the application of RT-PCR is unsustainable in terms of cost and management to be widely adopted; consequently, alternative platforms are needed. As potential complementary high-throughput approaches to such control screening requirements, developments in protein-based detection technologies could be harnessed that allow for trait-specific detection and quantification, as well as event-specific traits. Furthermore, protein-based methods have the potential to serve as cost-efficient, rapid screening tools, such as, the ELISA kits for GMO detection and quantification that have the potential of measuring the amount of Cry1Ab/Cry1Ac in IRMM certified reference materials (*4*). The present study outlines the application of a quantitative immunosystem based on fluorescent

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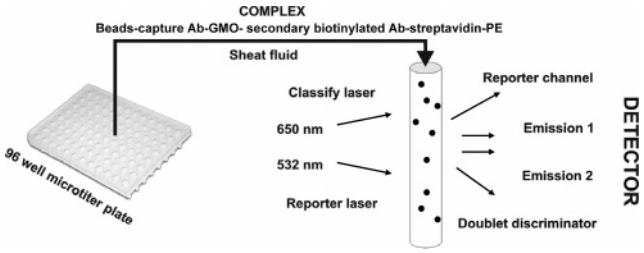


**Figure 1.** Description of the detection system. Single beads are transported by the fluid from each well of the 96-well microtiter plate to the laser chamber through a capillary. Inside the instrument the beads are struck by two different lasers, the classify channel (650 nm) and the reporter channel (532 nm). The first one excites the fluorescence identifying each bead and the second one excites the fluorescence of the streptavidin-conjugated phycoerythrin (PE). A combination of the two different emissions from the dye beads and the signal of the PE staining are detected by the instrument and plotted as mean fluorescent intensity (MFI). In addition, a side scatter detector allows double discrimination.


**Figure 2.** Capture antibody titration. The test was performed on 2% MON810, 0% MON810, and Bt176 included as negative controls. Fluorescent signal obtained was plotted as mean florescent intensity (MFI). The optimal concentration of capture antibody was obtained using 25 $\mu$g of capture antibody/5 $\times$ $10^6$ beads.

beads and liquid-array technology for high-throughput screening of a common GM trait (the insecticidal protein endotoxin Cry1Ab), representing the first experimental application of this technology for general GMO control purposes.

The assay is based on a specific monoclonal antibody covalently linked to color-coded fluorescent beads that specifically recognize target proteins present in the sample extracts. A secondary biotinylated antibody, directed against a different epitope of the same target protein, reacts with the samples, forming a complex that, in the presence of streptavidin−phycoerythrin (PE), can be detected. Detection and quantification of the immunocomplex is obtained via fluorescence emitted by the PE, and the specificity of the signal is assured by the color-coded beads to which it is coupled (**Figure 1**).

The advantage of this method would be the possibility of simultaneously detecting and quantifying up to 100 different target proteins within the same sample due to the color specificity of each bead combined to fluorescence intensity emitted by each complex after laser excitation.

The entire detection procedure takes less than an hour from the preparation of the lysate to sample analysis by the dedicated software. This method has been successfully applied in other fields of research such as multiple cytokine analysis, detection of cancer markers, apoptosis, gene expression, genotyping, and antibody screenings (*5*−*12*).

The present study describes the applicability of this method to the detection and quantification of Cry1Ab protein and sets the basis for the development of a high-throughput screening platform for this and other GM traits currently authorized in the EU and globally.

## MATERIALS AND METHODS

**Samples.** Certified reference materials (CRMs) for genetically modified MON810 maize (ERM-BF413) and CRMs for GM Bt-176 maize (ERM-BF411), both at six different mass fractions (<0.2, 0.1, 0.5, 1, 2, and 5%), as well as kernels of pure MON810 line were from IRMM (IRMM, Geel, Belgium). Cry1Ab purified protein was kindly provided by Monsanto (St. Louis, MO). A high-throughput device for Cry1Ab quantification [Luminex-100 (Luminex Corp., Austin, TX)] and carboxylated microspheres of a specific microsphere set (item L100-C154-01) were purchased from Luminex Corp. The pair of specific monoclonal antibodies recognizing Cry1Ab protein was kindly provided by Monsanto. Information on antibody-binding capacity is limited to data received from Monsanto (personal communication and unpublished data) from whom the antibodies were received. The antibodies were subsequently used according to the company's recommendation(s). However, we tested their specificity by ELISA and Western blot analysis, including ELISA reverse m&d as recently published (*5*).

Cross-linking of the fluorescent beads was performed according to the manufacturer's instructions. Briefly, 5 $\times$ $10^6$ beads were activated for 20 min in 80 $\mu$L of 100 mM monobasic sodium phosphate, pH 6.3, containing 500 $\mu$g of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) (Pierce,Rockford, IL; catalog no. 22980) and 500 $\mu$g of *N*-hydroxysulfosuccinimide (Sulfo-NHS, Pierce catalog no. 24500). Activated microspheres were washed twice with PBS, pH 7.4 (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4), suspended in 500 $\mu$L of PBS containing 125 $\mu$g of capture antibody, and incubated at room temperature for 2 h with mixing. Capture antibodies were dialyzed into PBS to avoid any primary amines at a final concentration of 1 mg/mL. Coupled microspheres were washed twice with PBS−TBN (PBS, pH 7.4, 0.02% Tween-20, 0.1% BSA, and 0.05% sodium azide) and stored in PBS−TBN at 4 °C in the dark.

Secondary antibody biotinylation was performed as follows: briefly, to remove any primary amines from the antibody solution, an overnight dialysis was performed at 4 °C in $NaH_3PO_4$ solution, pH 8.5. To each gram of antibody was conjugated 60 $\mu$g of $NH_2$−Zlink−biotin solution in DMSO by incubation at room temperature with shaking for 4 h. To remove unbound biotin the solution was dialyzed overnight in PBS, pH 7.4.

**Protein Extraction Procedure.** MON810 kernels were initially ground. Total proteins were extracted from the obtained flour and CRMs using a lysis buffer containing 10 mM sodium borate buffer, pH 7.5; 250 mg of each sample was incubated for 15 min in 1 mL of lysis buffer at room temperature. After centrifugation at 5300 rcf for 10 min, supernatants were recovered.

Serial dilutions (in PBS, 1% BSA, and 0.05% $NaN_3$) of the Cry1Ab purified protein were used as positive controls. Total protein concentration was assessed by conventional Bradford assay (Bio-Rad Laboratories, Hercules, CA; catalog no. 500-0001). For the Cry1Ab protein quantification assay, 50 $\mu$L of solution containing 5000 microspheres coupled with the capture antibody was added in each well of a filter bottom microtiter plate (Multiscreen$_{HTS}$ BV filter plates, Millipore Corp., Billerica, MA). Fifty microliters of samples was added to the microspheres, and the reactions were incubated for 20 min at room temperature. After incubation, the samples were washed twice by filtration and resuspended in 50 $\mu$L of PBS−BSA. Fifty microliters of secondary antibody (4 $\mu$g/mL) was added to each sample and incubated at room temperature for an additional 20 min. Samples were then washed twice by filtration and resuspended with 50 $\mu$L of PBS−BSA. Fifty microliters of streptavidin−*R*-phycoerythrin (4 $\mu$g/mL) was added, and the reactions were incubated at room temperature for 10 min. Samples were washed twice with PBS−BSA and finally resuspended in 100 $\mu$L before being analyzed by the Luminex-100 device.

**Statistical Tool.** Weighted ordinary least-squares regression was performed using SAS software (SAS Institute Belgium-Luxembourg Kasteel de Robiano, Tervuren, Belgium; SAS user base plus analyst

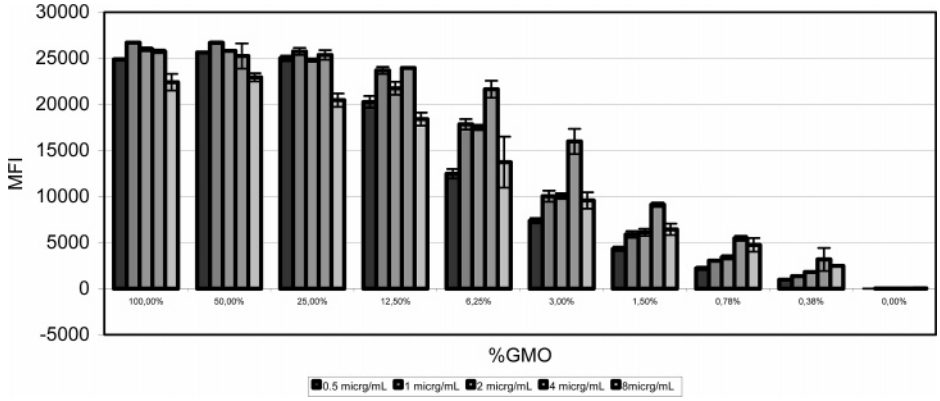


**Figure 3.** Titration of secondary antibody: Cry1Ab quantification assay performed on serial dilutions of pure MON810 maize line. The test was performed using five different concentrations of secondary antibody. Linearity was observed between 0 and 3% GMO content. The best secondary Ab concentration was 4 $\mu$g/mL, and MFI achieved saturation level at 25% of GMO content.

version 8.2). The squared standard deviation was used as a parameter for the SAS analysis.

The assay relies on the use of specific monoclonal antibodies selectively and covalently attached to color-coded beads that form a complex with the target protein when incubated with the given samples. A secondary biotinylated antibody binding to a different epitope of the same target protein forms a sandwich complex that is labeled in the presence of streptavidin−PE. Each sample is then collected and transported past the lasers via a capillary fluid sheet that allows single-bead suspension, essential for quantification reliability. Inside the instrument two different lasers strike the complexes: the 650 nm laser (classification channel) excites the fluorescence code of the bead, whereas the second 532 nm reporter channel excites the PE. The emissions of the two different wavelengths are collected by decoders, and the combinations of these two values first identify the target (specific antibody attached to each bead set) and then quantify the presence of each target (PE) (**Figure 1**). Simultaneous analysis of several samples was performed by using 96-wells microtiter plates. Multiple assays within each well can be performed for a maximum of 100 different targets. In our assay, the microspheres were efficiently coupled with specific monoclonal antibodies recognizing the protein Cry1Ab following the protocol described by Luminex (http://www.luminexcorp.com/support/protocols/protein.html). As part of the experimental design all experiments were repeated three times under independent conditions following the same experimental design. All samples were loaded in triplicate. Bt-176 GM maize was included in the experimental design in all experiments as a negative control.

## RESULTS

A quantitative immunoassay was established for the detection of the endotoxin protein Cry1Ab. Protein detection strictly depends on the availability of specific and functional antibodies that in the GMO field are not yet commercially available. The tests were performed on different types of GM maize matrices: flour of pure MON810 line and the conventional CRM, both sourced from the IRMM, Geel, Belgium, and pure Cry1Ab recombinant protein (Monsanto), using monoclonal antibodies recognizing the target protein kindly provided by the GMO producer (Monsanto). Following the procedure of in-house validation, assay reliability was tested for Cry1Ab protein quantification. To establish the optimal amount of antibody to be coupled to $5 \times 10^6$ beads, four different amounts (1, 5, 25, and 125 $\mu$g) of antibody were tested. Tests were performed on lysates of MON810 at 0 and 2% of GMO percentage using GM maize line Bt-176, which does not contain detectable Cry1Ab, as negative control. Coupling efficiency was evaluated by the Luminex-100 instrument measuring the sample's fluorescence intensity and using a standard concentration of secondary antibody (4 $\mu$g/mL). The maximum florescence intensity (MFI) was generated by samples of MON810 at 2% GMO level when 25 $\mu$g of antibody was coupled to $5 \times 10^6$ beads, and this value was not further increased when more antibody was used in coupling. No signal was detected by MON810 0% and by Bt-176 samples (**Figure 2**), indicating that no background signal was obtained as a result of unspecific binding of secondary antibody.

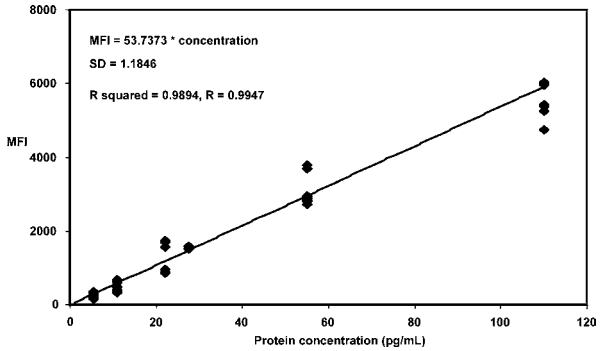


**Figure 4.** Weighted ordinary least-squares regression. The regression line was calculated using Cry1Ab purified protein within a range of 0–110 ng/mL.

Titration of the secondary antibody was also performed testing five different antibody concentrations (0.5, 1, 2, 4, and 8 ng/mL). The assay was performed on a serial dilution of protein extracted from the 100% GM MON810 in order to analyze a broad range of GMO percentages and repeated on CRM certified GMO standards at 0, 0.5, and 2%. In each analysis, the highest level of fluorescence intensity was achieved by using 4 mg/mL of secondary antibody (**Figure 3**).

The test was optimized to match standard requirements imposed for GMO analysis in compliance with EU legislation. Therefore, the GMO percent range of major interest was close to 0.9%. Subsequently, linear curves were obtained for each matrix based (Cry1Ab purified protein, IRMM CRMs, and dilution of 100% MON810 protein extracts) samples containing 0−3% GMO. Within the 0−3% range the assay shows linearity and optimal performance; calibration lines are shown in **Figures 4−6**. Coefficient of correlation values were 0.9947, 0.9842, and 0.9861, respectively (**Figure 4−6**). **Tables 1−4** show regression characteristics of the three curves, confirming that the best assay performance was achieved when Cry1Ab purified protein was used (data obtained by applying SAS software version 8.2 with a confidence level of 95%, **Tables 1−4**). For assay evaluation

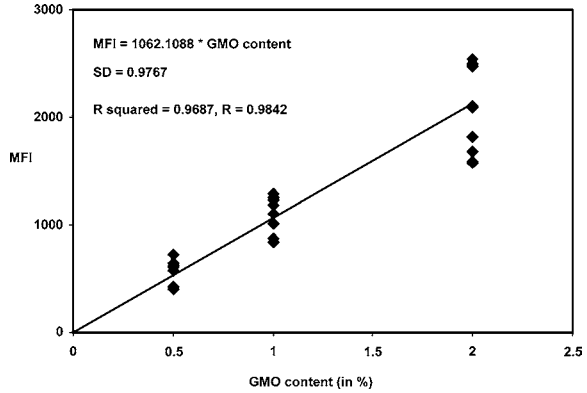


**Figure 5.** Weighted ordinary least-squares regression. The regression line was calculated using MON810 maize line at 0.5, 1, and 2%.

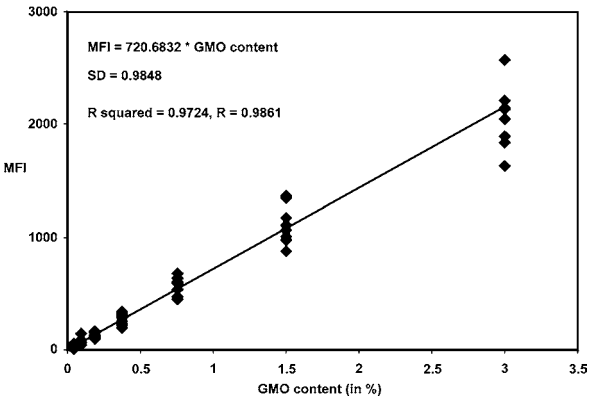


**Figure 6.** Weighted ordinary least-squares regression. The regression line was calculated using extracts of pure MON810 maize line within the range of 0–3%.

we took into consideration parameters such as recovery, reproducibility, and intermediate precision (*14*−*16*) (http://gmo-crl.jrc.it/doc/Method%20requirements.pdf).

Recovery is described as the closeness of agreement between the observed value and the expected value. It was calculated using the formula (observed value/expected value) × 100 and is expressed in percent. Assay recovery values were all in the acceptable range of 100 ± 25%. The repeatability standard deviation (RSD) expressed by the formula RSD = SD/$\bar{x}$ (in percent) exhibited values in the range of 1.9−10.8%, with only one exception at point D1 (**Table 3**). Repeatability RSD (percent) was calculated on three parallel measurements, whereas intermediate precision RSD (percent) was calculated on three values corresponding to the mean of three parallel measurements (**Tables 2**−**4**). Precision is the closeness of agreement between independent results obtained under stipulated conditions (e.g., repeatability, reproducibility).

Above values of 3% GMO, the signals increased following a nonlinear model, reaching a plateau at 25% (**Figure 3**). In particular, the assay conditions were optimized to reach values of limit of detection (LOD) and limit of quantification (LOQ) allowing the detection and quantification of contamination around the 0.1% level as with conventional ELISA tests (*4*). The LOD was calculated using the formula LOD = 3 × SD/slope (expressed in concentration units and in percent of GMO content). The LOD calculated using the slope value obtained from the purified protein measurements was 0.764 pg/mL. The LOD calculated starting from CRMs was equal to 0.018% (0.357* pg/mL), and the LOD calculated from 100% MON810 protein extract dilutions was 0.056% (∼0.751* pg/mL).

**Table 1.** Weighted Ordinary Least-Squares Regression Characteristics[a]

| | slope | standard error | *p* value | 95% probability bands lower | 95% probability bands upper |
|---|---|---|---|---|---|
| GMO content[b] | 53.7373 | 0.8118 | <0.0001 | 52.1041 | 55.3705 |
| GMO content[c] | 720.6832 | 15.4123 | <0.0001 | 689.8745 | 751.4919 |
| GMO content[d] | 1062.1088 | 37.4138 | <0.0001 | 985.2037 | 1139.0140 |

[a] Acquired by SAS software. [b] Regression characteristics are based on *purified protein*. [c] Regression characteristics are based on *IRMM standards*. [d] Regression characteristics are based on *IRMM dilutions*.

The LOQ is the lowest amount or concentration of target in a sample that can be reliably quantified with an acceptable level of precision and accuracy. It was calculated using the formula LOQ = 9 × SD/slope (expressed in concentration units and in percent of GMO content). The best value of LOQ equal to 0.054% (∼1.072** pg/mL) was achieved by using CRMs. The LOQ using purified protein and the LOQ of 100% MON810 protein extract dilutions were 2.292 pg/mL and 0.168% (∼2.253** pg/mL), respectively. Although not reported here, multiple quantitative analyses for both CP4EPSPS and Cry1Ab were also performed to verify system capability to detect and quantify different GMO targets simultaneously. However, results obtained on the CP4EPSPS quantification were not comparable, or consistent, as the single Cry1Ab data set, in particular, with reference to reproducibility. This may have been due to CP4EPSPS antibody instability (data not shown).

## DISCUSSION

This work highlights the use of an immunoassay as the basis of a high-throughput approach for applications in biotechnology, specifically to control enforcement actions related to genetically modified organisms. The high-throughput system we used allows for either 96- or 384-well plates with the option to analyze four plates simultaneously and is compatible with front-end plate-handling robotics. A 96-well plate assay is completed in less than 2 h and 50 min for the preparative steps and 1 h for instrument analysis. The estimation of the cost per assay is directly link to the number of targets included in the assay. For a single analysis the cost is close to that of the RT-PCR assay or ELISA (close to 1 and 2 euros per well, respectively), but in the case of multiple simultaneous detections the costs per sample would be proportionally reduced. Proteomic screening of potentially contaminated crops can in fact reduce uncertainty by providing more information about crop composition than targeted analysis alone or in combination with qualitative multiplex PCRs. In addition, multivariate statistical methods can be applied to analyze the results to obtain a clearer overall picture of how the given samples relate to each other, rather than the comparison of single compounds. These facts may make proteomics increasingly attractive, especially with the advent of second-generation GM crops containing multiple transgenes in the EU.

Consumer awareness on food safety is of considerable societal concern within the EU. Monitoring adventitious GM presence for identity preservation and traceability measures along the food/feed chain highlights the need of high-throughput analysis in the investigation of several contaminations in either raw samples or finished food products and feedstuffs. In this respect, the first application of the fluorescent bead based immunologic assay/Luminex platform to GMO protein for quantitative

**Table 2.** Regression Characteristics of the Curve Obtained Using CMRs at 0.5, 1, and 2% Mon810 Maize Line

| ID IRMM CRM | observed GMO content (%) | expected GMO content (%) | MFI | recovery (%) | repeatability RSD (%) | intermediate precision RSD (%) | predicted concentration (pg/mL) |
|---|---|---|---|---|---|---|---|
| ST1 | 0.52 | 0.5 | 553.4 | 104.2 | 8.1 | 26.8 | 10.3 |
| ST2 | 1.01 | 1 | 1069.9 | 100.8 | 1.9 | 18.9 | 19.9 |
| ST3 | 1.92 | 2 | 2037.1 | 95.9 | 5.2 | 21.9 | 37.9 |

**Table 3.** Regression Characteristics of the Curve Obtained Using Extracts of Pure MON810 GM Maize Line within the Range of 0.04–3%[a]

| ID of dilution | observed GMO content (%) | expected GMO content (%) | MFI | recovery (%) | repeatability RSD (%) | intermediate precision RSD (%) | predicted concentration (pg/mL) |
|---|---|---|---|---|---|---|---|
| D1 | 0.034 | 0.04 | 24.5 | 85.0 | 44.8 | 49.8 | 0.46 |
| D2 | 0.092 | 0.093 | 66.1 | 98.6 | 10.8 | 44.2 | 1.23 |
| D3 | 0.183 | 0.185 | 132.2 | 99.1 | 7.1 | 26.4 | 2.46 |
| D4 | 0.376 | 0.375 | 270.7 | 100.2 | 8.9 | 18.5 | 5.04 |
| D5 | 0.792 | 0.75 | 571 | 105.6 | 6.7 | 14 | 10.6 |
| D6 | 1.544 | 1.5 | 1112.6 | 102.9 | 6.4 | 21.6 | 20.7 |
| D7 | 2.858 | 3 | 2059.5 | 95.3 | 7.4 | 18 | 38.3 |

[a] D1 < LOD.

**Table 4.** Regression Characteristics of the Curve Obtained Using Cry1Ab Purified Protein within a Range of 5.5–550 ng/mL

| ID of purified protein | observed concentration (pg/mL) | expected concentration (pg/mL) | MFI | recovery (%) | repeatability RSD (%) | intermediate precision RSD (%) |
|---|---|---|---|---|---|---|
| PP1 | 4.5 | 5.5 | 244.1 | 82.6 | 7.3 | 33.9 |
| PP2 | 8.9 | 11 | 481.7 | 81.5 | 7.4 | 31.9 |
| PP3 (1)[a] | 23.9 | 22 | 1283.5 | 108.6 | 5.6 | 42.1 |
| PP4 (2)[b] | 28.7 | 27.5 | 1540.2 | 104.2 | 2.8 | |
| PP5 | 57.4 | 55 | 3086.8 | 104.4 | 2.5 | 16.4 |
| PP6 (1) | 102.5 | 110 | 5508.2 | 93.2 | 4.4 | 7.9 |
| PP7 (2)[b] | | 275 | 10002.2 | | 3.4 | |
| PP8* | | 440 | 8628.4 | | 4.8 | 14.4 |
| PP9 | | 550 | 11080.6 | | 3.5 | 22.6 |

[a] Outputs are based on six parallel measurements. [b] Outputs are based on three parallel measurements.

analysis can be foreseen as a complementary tool to the ELISA assays generally applied only for screening purposes, as we previously have shown (*4*). In particular, the assay conditions were optimized to reach LOD and LOQ values, allowing the detection and quantification of contamination around the 0.1% level as with conventional ELISA tests. Unfortunately, due to the lack of commercially retrievable antibodies against GMO related proteins, it was not possible for us to fully exploit the multiplex potentiality of the system. In the present paper, only the quantification of a single GMO target trait is reported. The data provided show that the system is linear and has its optimal performance within the range of 0−3% GMO, perfectly in line with the contaminations limits imposed by the legislation. This result is comparable to the performance of the commercial ELISA kits and of the ELISA reverse Cry1Ab quantitative assay in which, using the same antibodies, linearity was achieved within the range of 0−2% (*4*, *5*). Also, the evaluation of the other parameters, such as the assay recovery and RDS, confirms that the system is comparable with the ELISA and ELISA reverse assays. In particular, the assay recovery and RDS values were in the range of 81.5−108.6 and 1.9−10.8% (except point D1), respectively, in accordance with the values of the ELISA reverse assay, with which the accuracy was in the range of 86.52−113.47% and the RSD was from 2.05 to 14.17% (*5*). Commercial ELISA kits were, in general, less accurate, showing values of accuracy in the range of 52.9−266.4% and RDS values from 7.6 to 16.1% (*4*). The LOD and the LOQ values of the innovative assay were also comparable to the LOD and LOQ values of the ELISA reverse. Indeed, ELISA reverse LOD and LOQ were equal to 0.0056 and 0.0168%, respectively, whereas the microsphere-based immunoassay LODs, calculated using three different materials (the purified protein, CRMS, and 100% MON810 protein extract dilutions), were equal to 0.764 pg/mL, 0.018% (0.357* pg/mL), and 0.056% (∼0.751* pg/mL), respectively; meanwhile, the best value of LOQ was achieved by using CRMs and equal to 0.054% (∼1.072** pg/mL) (*5*). LOD and LOQ of commercial ELISA kits were in the range of 0.034−0.107 and 0.082−0.259%, respectively (*4*).

In conclusion, the potential of the system here described enables us to look forward to a multiple-target assay able to detect and quantify different GM traits simultaneously occurring within the same sample preparation. Here, a panel of different antigen-reactive capture antibodies could be cross-linked to individually distinguishable color-coded beads, allowing quantification of several targets as already demonstrated in other fields of application (*6*−*13*).

## LITERATURE CITED

(1) European Commission. Recommendation (EC) 787/2004 of 4 October 2004 on technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisms as or in products in the context

of Regulation (EC) 1830/2003. Text with EEA relevance. *Off. J. Eur. Communities* **2004**, *L348*, 0018−0026.
(2) European Commission. Regulation (EC) 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. *Off. J. Eur. Communities* **2003**, *L268*, 0001−0023.
(3) European Commission. Regulation (EC) 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC. *Off. J. Eur. Communities* **2003**, *L268*, 0024−0028.
(4) Ermolli, M.; Fantozzi, A.; Marini, M.; Scotti, D.; Balla, B.; Hoffmann, S.; Querci, M.; Paoletti, C.; Van den Eede G. Food safety: screening tests used to detect and quantify GMO proteins. *Accredit. Qual. Assur.* **2006**, *11*, 55−57.
(5) Ermolli, M.; Prospero, A.; Balla, B.; Querci, M.; Mazzeo, A.; Van den Eede, G. Development of an innovative immunoassay for CP4EPSPS and Cry1AB GM proteins detection and quantification. *Food Addit. Contam.* **2006**, *23*, 876−882.
(6) International Standard (ISO) 21572:2002. *Foodstuffs—Methods of Analysis for the Detection of Genetically Modified Organisms and Derived Products—Protein-Based Methods*; International Organization for Standardization: Genéva, Switzerland, 2002.
(7) International Standard (ISO) 5725:1994. *Accuracy (Trueness and Precision) of Measurement Methods and Results*; International Organization for Standardization: Genéva, Switzerland, 1994.
(8) International Union of Pure and Applied Chemistry (IUPAC). Gold Book. *Compendium of Chemical Terminology*, 2nd ed.; McNaught, A. D., Wilkinson, A., Eds.; Blackwell Scientific Publications, Royal Society of Chemistry: Cambridge, U.K. ,-1997.
(9) Carson, R. T., Vignali, D. A. Simultaneous quantification of 15 cytokines using a multiplex flow cytometer assay. *J. Immunol. Methods* **1999**, *227*, 41−52.
(10) Seideman, J.; Peritt, D. A novel monoclonal antibody screening method using the Luminex-100 micro-sphere system. *J. Immunol Methods* **2002**, *267*, 165.
(11) Martins, T. B. Development of internal controls for the Luminex instrument as part of a multiplex seven-analyte viral respiratory antibody profile. *Clin. Diagn. Lab. Immunol.* **2002**, *9*, 41−45.
(12) Yang, L.; Tran, D. K.; Wang, X. BADGE, Beads Array for the Detection of Gene Expression, a high-throughput diagnostic bioassay. *Genome Res.* **2001**, *11*, 1888−1898.
(13) Ye, F.; Li, M.; Taylor, J. D.; Nguyen, Q.; Colton, H. M.; Casey, W. M.; Wagner, M.; Weiner, M. P.; Chen, J. Fluorescent microsphere-based readout technology for multiplexed human single nucleotide polymorphism analysis and bacterial identification. *Hum Mutat.* **2001**, *17*, 305−316.
(14) Thompson, M.; Ellison, S. L. R.; Wood, R. Harmonized guidelines for single-laboratory validation of methods of analysis. IUPAC Technical Report. *Pure Appl. Chem.* **2002**, *74*, 835−855.
(15) Taylor, J. D.; Briley, D.; Nguyen, Q. Flow cytometric platform for high-throughput single nucleotide polymorphism analysis. *Biotechniques* **2001**, *3*, 661−666, 668−669.
(16) Janossy, G.; Jani, I. V.; Kahan, M.; Barnett, D.; Mandy, F.; Shapiro, H. Precise CD4 T-cell counting using red diode laser excitation: for richer, for poorer. *Cytometry* **2002**, *50*, 78−85.

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