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High Sensitive Detection of Cry1Ab Protein Using a Quantum Dot-Based Fluorescence-Linked Immunosorbent Assay

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ABSTRACT: Protein-based detection methods, enzyme-linked immunosorbent assay (ELISA) and lateral flow strip, have been widely used for rapid, spot, and sensitive detection of genetically modified organisms (GMOs). Herein, one novel quantum dot-based fluorescence-linked immunosorbent assay (QD-FLISA) was developed employing quantum dots (QDs) as the fluorescent marker for the detection of the Cry1Ab protein in MON810 maize. The end-point fluorescent detection system was carried out using QDs conjugated with goat anti-rabbit secondary antibody. The newly developed Cry1Ab QD-FLISA assay was highly specific to the Cry1Ab protein and had no cross-reactivity with other target proteins, such as Cry2Ab, Cry1F, and Cry3Bb. The quantified linearity was achieved in the value range of 0.05−5% (w/w). The limits of detection (LOD) and quantification (LOQ) of the QD-FLISA were 2.956 and 9.854 pg/mL, respectively, which were more sensitive than the conventional sandwich ELISA method. All of the results indicated that QD-FLISA was a highly specific and sensitive method for the monitoring of Cry1Ab in GMOs.

KEYWORDS: Cry1Ab, genetically modified maize, quantum dot, QD-FLISA

INTRODUCTION

More than 50 countries and areas have issued labeling regulations for genetically modified organisms (GMOs) since the GM tomato FLAVR SAVR was approved for commercialization. The common analytical methodologies for GMOs were mainly developed on the basis of nucleic acid and protein analyses. Particularly, the protein-based methods, enzyme-linked immunosorbent assay (ELISA) and lateral flow strip, have been used in quick and spot field tests because of time savings and low costs.[1] Recently, one immunoassay was validated for the quantification of GM maize MON810 by international collaborative studies.[2−4] However, the conventional immunoassays for food sample analysis are hampered by the different levels of protein expression in various tissues and growing conditions, as well as the reduced sensitivity because of protein denaturation during food processing.[5] Therefore, the development of innovative and rapid analytical methods with high sensitivity is necessary and important.

With the development of nanotechnology, one novel fluorescent homogeneous semiconductor nanocrystal named quantum dot (QD) has been developed.[6] QDs, as a brand new class of luminescent inorganic fluorophores, have the advantage of narrow emission, broad excitation spectra, long-term photostability, and high-quantum yield. The QDs with different sizes can be excited using only a single excitation laser wavelength.[7] Moreover, the emission color of QDs is tunable by changing the nanocrystal size and the type of core material used.[8] Therefore, simultaneous multi-analyte detection can be realized using multicolor QDs.[9−11] QDs have been successfully used for a variety of analysis purposes, such as fluorescence resonance energy transfer (FRET),[12] cellular labeling,[13] and immunoassays.[14]

In this study, we developed one novel quantum dot-based fluorescence-linked immunosorbent assay (QD-FLISA) employing red QDs (emission at 655 nm) as a fluorescent marker for the detection of the Cry1Ab protein in GM maize MON810. Furthermore, the analytical performance of Cry1Ab QD-FLISA was evaluated and compared to that of the conventional ELISA with colorimetric detection.

MATERIALS AND METHODS

Materials and Chemicals. The seeds of GM maize event MON810 were supplied by the Monsanto Company (St. Louis, MO). Non-GM maize was purchased from the local market in Shanghai, China, and verified with non-GM maize events in the GMO Detection Laboratory of Shanghai Jiaotong University, Shanghai, China. The maize seeds were immersed in liquid nitrogen and pulverized with a magnetically driven impactor using the model 6870 freezer/mill. The practical

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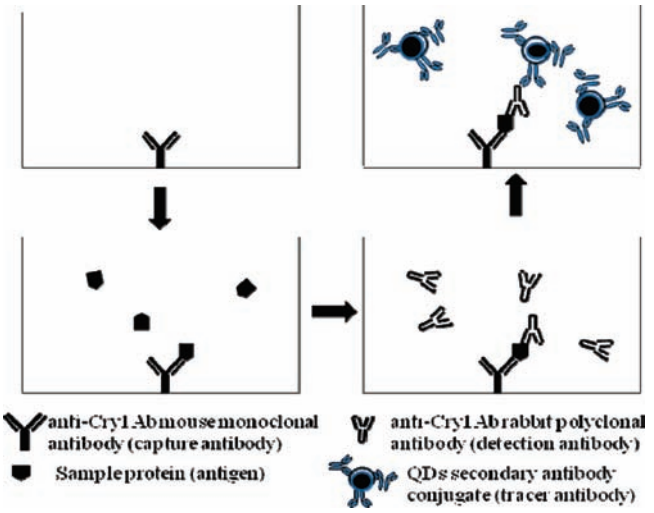

Figure 1. Schematic diagram of the QD-FLISA procedure.

GM maize samples with different GM contents (0.05, 0.1, 0.15, 0.3, 0.6, 1.25, 2.5, 5, 10, and 20%) were artificially prepared by mixing the ground MON810 maize with non-GM maize powder based on the weight ratio. Purified Cry1Ab protein and the monoclonal antibody against Cry1Ab were kindly provided by Shanghai Jieme Baol Inmal Biological Engineer Co., Ltd. (Shanghai, China). The 3-mercaptopropionic acid (MPA)-coated CdTe QDs were synthesized by the College of Chemistry, Shanghai Jiaotong University, Shanghai, China.[15] 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, >98%) and *N*-hydroxysuccinimide (NHS, >98%) were purchased from the Sigma Chemical Co. (St. Louis, MO). Goat anti-rabbit IgG was purchased from GenScript Corporation (Piscataway, NJ).

Synthesis and Purification of QD−Ab Conjugates. The QD−Ab conjugates were synthesized according to the following protocol: First, mix 25 μL of solution of MPA-coated CdTe QDs (1 mg/mL) with 20 μL of EDC (10 mg/mL) and 10 μL of NHS (1.5 mg/mL) and incubate at room temperature for 5 min. Then, add 0.2 mg of goat anti-rabbit antibodies (Abs) in phosphate-buffered saline (PBS) to the above reaction solution and incubate for 4 h with gentle agitation.[16,17] Finally, the synthesized QD−Ab conjugates were purified using an ultrafiltration membrane (Micoron YM-50-50 000NMWL, Millipore, Billerica, MA) according to the instructions of the manufacturer.[18]

Production and Purification of Polyclonal Antibody. The polyclonal antibody against the Cry1Ab protein was obtained by immunizing the New Zealand white rabbit with purified Cry1Ab protein. In brief, the Cry1Ab protein dissolved in 0.9% NaCl was emulsified with an equal volume of Freund's complete adjuvant to give a final concentration of 1 mg/mL. This mixture was given to the animal in five intradermic injections. Boosting injections of Freund's incomplete adjuvant were made at 2 week intervals. Blood samples were collected 1 week after the third booster injection and tested for titer concentration. The blood samples from the rabbits were first allowed to stand overnight at 4 °C and then centrifuged at 2000*g* for 15 min. A total of 20 mL of antiserum was purified according to the adsorption method.[19] The titer of purified antiserum (least detectable dose) for indirect ELISA was estimated by known an amount of purified Cry1Ab protein.

Development of QD-FLISA. The schematic diagram of the QD-FLISA procedure was shown in Figure 1. Each well of the opaque white microtiter plate (Bio Basic, Inc., Ontario, Canada) was coated overnight (4 °C) with 100 μL of anti-Cry1Ab mouse monoclonal antibody (capture antibody) conjugate dissolved in coating buffer (0.05 M carbonate buffer at pH 9.6) at the optimal dilution. After the excess antibody was removed, the plate was triple-washed with washing buffer (0.05 M phosphate buffer at pH 7.4 and containing 0.1% Tween-20). The excess binding sites were blocked with 200 μL of blocking buffer (coating buffer with 0.5% casein) at 37 °C for 1 h. Subsequently, 100 μL samples (tissue extract or standard serial dilutions of Cry1Ab in PBS) were added to the wells (in triplicate), along with the same volume of buffer containing no antigen to control for non-specific binding. Then, the plate was incubated at 37 °C for 30 min and washed 3 times with washing buffer. After washing, 100 μL of anti-Cry1Ab rabbit polyclonal antibody (detection antibody) was added to the wells and incubated for 30 min at 37 °C. Then, the plate was washed again before adding 100 μL of QD−Ab (1:1000 dilutions in PBS) into each well and incubating at 37 °C for 30 min. After incubation, unbound QD conjugates were washed 3 times and padded dry. The fluorescent signal of each well was measured in Fluoroskan Ascent (Thermo Fisher Scientific, Inc., Fremont, CA). Standard curves were plotted as fluorescent intensity against the reference analyte concentration. The relative fluorescence intensity was calculated using the value of analyte minus the blank controls. All of the data were statistically analyzed using SPSS 13.0 software (SPSS, Inc., Chicago, IL).

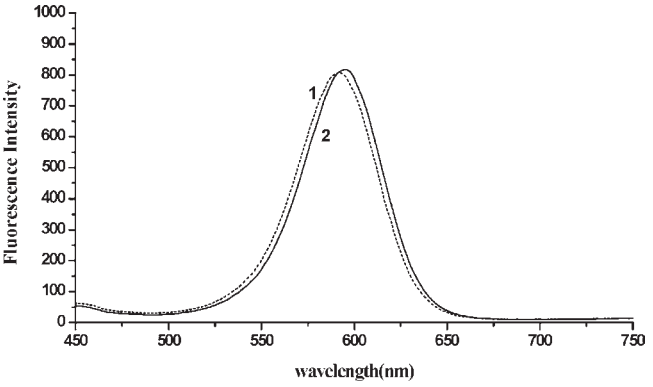

Figure 2. Fluorescence spectra of (1) CdTe nanoparticle solution and (2) CdTe−IgG solution. The emission peak is at 605 nm, with full width at half-maximum of 50 nm.

Protein Extraction and Practical Sample Analysis. The dried and homogenized GM maize seed powder samples were subjected to the protein extraction prior to analysis. An aliquot of 0.5 g of powder sample was added to 2 mL of extraction buffer [PBST with 1% polyvinyl pyrrolidone (PVP)].[20] The suspension was incubated for 20 min at room temperature with gentle shaking and centrifuged for 5 min at 3000*g*. The supernatant with the extracted protein was also confirmed by a conventional ELISA method. The total protein concentration was determined by a conventional Bradford assay.[21] For quantitative analysis of the practical GM samples, serial dilutions of the Cry1Ab protein with the concentrations of 3, 6, 12.5, 25, 50, 100, and 200 pg/mL were used to construct the calibration curve. In the sample analysis, a total of 100 μL of sample extract was used in each QD-FLISA reaction and each sample was analyzed with three replicates and repeated 3 times.

RESULTS AND DISCUSSION

Fluorescent Features of the QD−Ab Conjugate. The emission peaks of CdTe and CdTe−IgG were measured using luminescence spectrometer LS-55 (Perkin-Elmer, Inc., Waltham, MA) with 589 and 597 nm, respectively. The emission spectra of CdTe−IgG shifted 8 nm toward the red spectrum, which was caused by the cross-linking agent EDC, the carboxyl group on the surface of CdTe nanoparticles, and the amino of IgG that were conjugated by covalent bonding (Figure 2). QDs are one kind of large molecule that can be connected with a number of IgG, which made the distance among QDs closer. The interactions of the dipole between particles become much stronger, resulting in

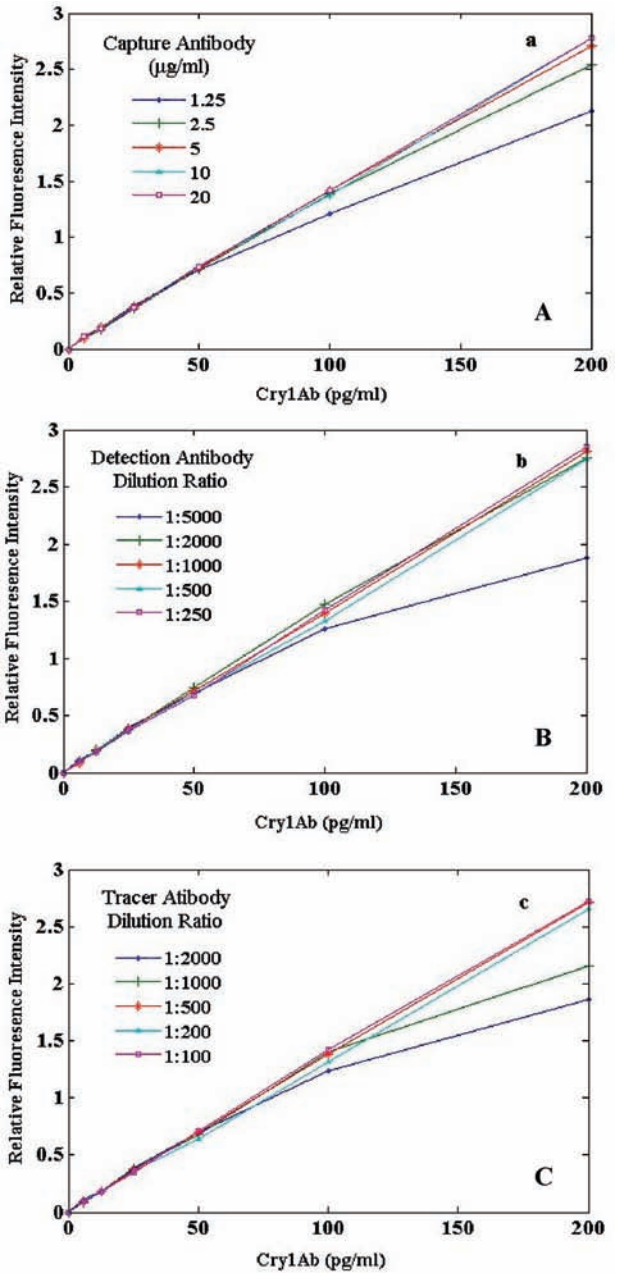

Figure 3. Optimization of the immunoassay: (A) calibration curves obtained for the 96-well QD-FLISA immunoassay using different concentrations of capture antibody, (B) detection antibody, and (C) tracer antibody. Fixed antibody concentrations were (a) detection antibody, 1:250, and tracer antibody, 1:100; (b) capture antibody, 10 μg/mL, and tracer antibody, 1:100; and (c) capture antibody, 10 μg/mL, and detection antibody, 1:1000.

the red shift of the emission spectrum of CdTe nanoparticles. The full width at half-maximum of CdTe and CdTe−IgG did not change, indicating that the reunions did not happen in the course of QDs conjugating to IgG. Therefore, the spectra demonstrated that QD conjugates to the antibody were effective.

Optimization and Development of QD-FLISA. One quantitative QD-FLISA assay was established for the detection of the endotoxin protein Cry1Ab. To determine the optimal dilution ratio, the titer of purified antiserum for indirect ELISA was estimated by decouple dilutions of the purified Cry1Ab protein

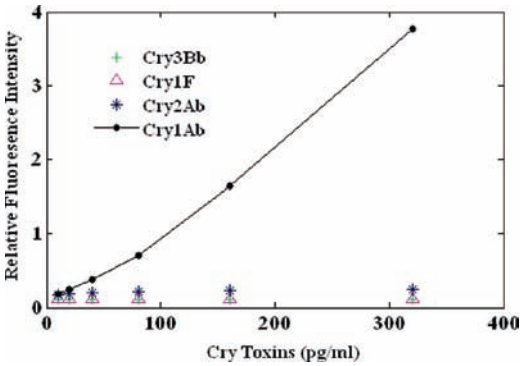

Figure 4. Cross-reaction test of other Bt toxins with Cry1Ab antibody in developed QD-FLISA.

in carbonate buffer from 1 to 1 × 10^{-10} μg/mL. The optimal dilution ratio of titer was 1:5000. The optimal concentrations of the anti-Cry1Ab capture antibody, anti-Cry1Ab detection antibody, and QD-labeled anti-rabbit tracer antibody were determined (Figure 3). For the immobilization of the anti-Cry1Ab capture antibody, physical adsorption on polystyrene microtiter plates was chosen because of its simplicity, reproducibility, and stability. The concentration of the capture antibody was determined by coating 96-well microtiter plates with different dilutions of the capture antibody solutions within the range of 1.25−20 μg/mL. The standard curve for the QD-FLISA assay was constructed using pure Cry1Ab protein samples with the concentration of 3, 6, 12.5, 25, 50, 100, 200, and 400 pg/mL. In the established QD-FLISA, the capture antibody with the concentration of 10 μg/mL was selected according to the quantified dynamic range (6−200 pg/mL) and linearity coefficient of determination (R^2) of the standard curve. A similar procedure was employed to optimize the concentrations of the anti-Cry1Ab detection antibody and the QD-labeled goat anti-rabbit tracer antibody, and the optimal diluted ratio of the anti-Cry1Ab detection antibody and the QD-labeled goat anti-rabbit tracer antibody were 1:1000 and 1:500, respectively.

Evaluation of the Cry1Ab QD-FLISA. *Cross-reactivity.* Three different *Bacillus thuringiensis* (Bt) proteins (Cry2Ab, Cry1F, and Cry3Bb) were used to validate the specificity of the QD-FLISA assay, and the cross-reactivities with these three Bt endotoxins were verified by producing dose−response curves for them. The results showed that Cry2Ab, Cry1F, and Cry3Bb did not interfere with the immunoassay (Figure 4), indicating that the QD-FLISA has low cross-reactivity against these Bt endotoxins, and this assay can be used for unique quantification of the Cry1Ab toxin.

Limits of Detection (LOD) and Quantification (LOQ). The LOD and LOQ are key parameters for each protein detection method, and the LOD and LOQ can be expressed in two different formats, relative and absolute LOD and LOQ. Following the established guidelines (http://gmo-crl.jrc.ec.europa.eu/), the LOD was calculated as 3 times the ratio of the standard deviation (SD) to the slope of the low-concentration calibration plot (LOD = 3SD/slope) and LOQ was calculated as 10 times the same ratio (LOQ = 10SD/slope).

To estimate the absolute LOD and LOQ, the pure Cry1Ab protein samples with different concentrations (3, 6, 12.5, 25, 50, 100, 200, and 400 pg/mL) were used. A good linear correlation was obtained from these samples, with the concentrations ranging from 6 to 200 pg/mL (Figure 5), and the absolute LOD and LOQ were 2.956 and 9.854 pg/mL, respectively. The

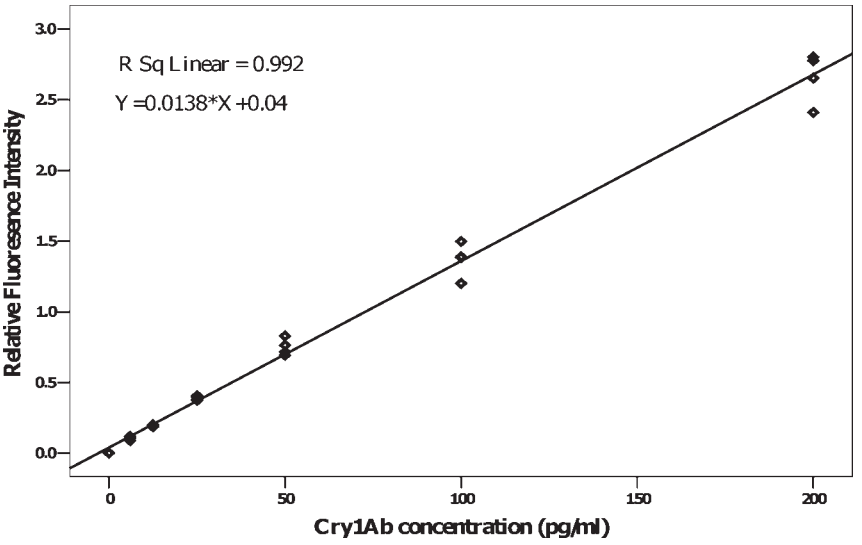

Figure 5. Regression line was calculated using purified Cry1Ab protein within the range of 0−200 pg/mL, where *y* represents the value of relative fluorescence intensity and *x* represents the value of concentration.

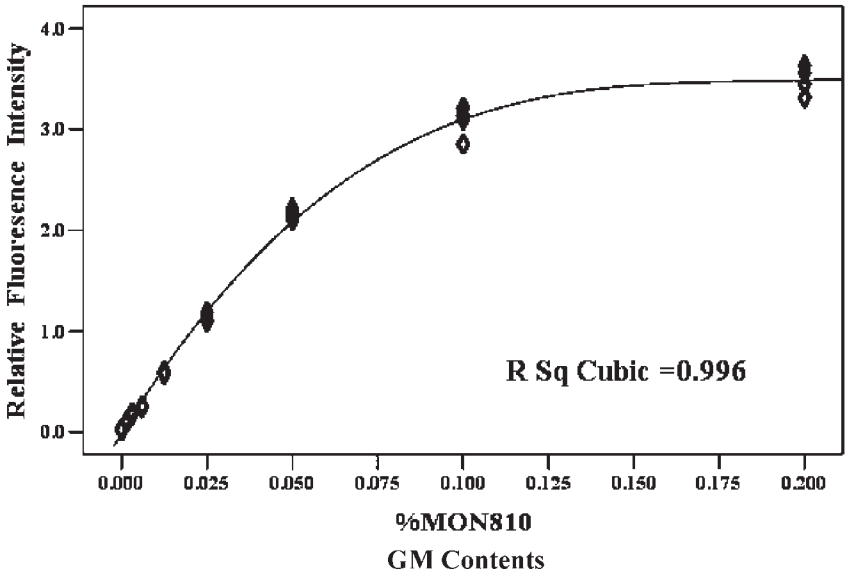

Figure 6. Cry1Ab quantification assay performed using serial dilutions of mixed MON810 maize samples with different GM contents of 0.15, 0.3, 0.6, 1.25, 2.5, 5, 10, and 20%.

values of LOD and LOQ allowed for the detection and quantification of contamination around the 0.1% level, as well as conventional ELISA tests.[20]

The relative LOD and LOQ were tested employing the mixed maize powder materials with different GM contents of 0.15, 0.3, 0.6, 1.25, 2.5, 5, 10, and 20%. The results showed that the good linear correlation existed in the range of 0.15−5% in the optimized QD-FLISA assay (Figure 6), with the calculated LOD value of 0.019% and LOQ value of 0.066%. In comparison to LOD (from 0.034 to 0.107%) and LOQ (from 0.082 to 0.259%) of conventional ELISA assays, the developed QD-FLISA assay was more sensitive because the tracers (QDs) are more sensitive than the enzymes.

Practical Sample Quantification Using QD-FLISA. *Accuracy.* In brief, the accuracy of the method was determined by comparing the measured value to the true value, which was expressed as percent recovery. Recovery is described as the closeness of the agreement between the observed value and the theoretical value. It is calculated using the formula (observed value/expected value) × 100 and is expressed in percent. Good recovery was achieved for each fortified concentration at or above the LOQ. To determine the method accuracy, different amounts of pure Cry1Ab protein and extracted protein of MON810 were analyzed by QD-FLISA. The recoveries of the pure and extracted protein samples were shown in Tables 1 and 2, respectively. The recoveries for pure Cry1Ab protein and protein extracts of MON810 ranged from 91.6 to 105.9% (Table 1) and from 86.5 to 110% (Table 2), indicating that the satisfied accuracy was obtained using the developed QD-FLISA compared to the accuracy (52.9−266.4%) of commercial ELISA kits.[22]

Precision. Precision is the closeness of agreement between independent results obtained under stipulated conditions. The

Table 1. Regression Characteristics of the Obtained Curve Using Protein Extractions of Mixed MON810 Maize Samples within the Range of 0.05−5%

sample name	experimental GM content (%)	theoretical GM content (%)	recovery (%)	SD	CV (%)
D1	0.043	0.05	86.5	0.0053	12.3
D2	0.097	0.1	96.8	0.0185	19.1
D3	0.144	0.15	95.8	0.0106	7.3
D4	0.327	0.3	108.9	0.0299	9.1
D5	0.66	0.6	110	0.025	3.8
D6	1.229	1.2	102.4	0.0609	5
D7	2.346	2.5	93.8	0.1814	7.7
D8	4.909	5	98.1	0.0704	1.4

Table 2. Regression Characteristics of the Obtained Curve Using Purified Cry1Ab Protein within the Range of 3−200 pg/mL

sample name	experimental value (pg/mL)	therortical value (pg/mL)	recovery (%)	SD	CV (%)
P1	2.5	3	83.3	0.3568	14.6
P2	5.6	6	93.3	0.3219	5.8
P3	12.3	12.5	98.8	0.3681	3
P4	26.3	25	105	1.142	4.4
P5	52.3	50	104.5	2.34	4.5
P6	105.9	100	105.9	3.275	3.1
P7	182.3	200	91.2	6.605	3.7

precision was evaluated on the basis of the results of experiments with fortified samples or positive-control samples by multiple analyses on different days. Each level of fortified extract and pure Cry1Ab protein were run in duplicate on each QD-FLISA plate. The average of measured concentrations, SD, and percent coefficient of variation (CV, %) were calculated for each sample on each day. The precision data were shown in Tables 1 and 2. The CV values were both lower than 10% in pure Cry1Ab protein samples, except for P1, and practical GM MON810 maize samples, except for D1 and D2, suggesting the good precision of the developed QD-FLISA method.

In conclusion, in this work, one QD-FLISA was developed targeting the Cry1Ab protein. The specificity and cross-reactivity were well-verified using different exogenous Bt proteins. Also, the good precision and accuracy were evaluated, employing different Cry1Ab protein samples. In addition, the highlights of the developed QD-FLISA method are the high sensitivity and time savings compared to those of conventional sandwich enzyme immunoassay techniques. As concluded from the results, we believed that the developed QD-FLISA method might do a great favor to the practical GM sample analysis. Furthermore, the developed FLISA method based on the QDs supplied one possible strategy for the development of multiplex protein analysis methods employing different QD conjugates.

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