LWT - Food Science and Technology 125 (2020) 109176

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

# A universal analytical approach for screening and monitoring of authorized and unauthorized GMOs

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ARTICLE INFO

*Keywords:*
Screening approach
Genetically modified organisms
Unauthorized GMOs
Microfluidic chip
Digital PCR

ABSTRACT

Release of various genetically modified organisms (GMOs) into market during the past decade contributes to concerns regarding the traceability of food and feed products. In this study, a comprehensive list of inserted elements of commercialized GMOs has been collected, which contained information of 191 singular genetically modified (GM) varieties. Several elements were selected to develop a universal analytical approach for screening GM presence in food and feed products. This approach achieved coverage of those singular GM events as well as their hybrid crosses. The developed screening approach showed great specificity and sensitivity of less than 25 copies that meet the labeling demands for all countries. Moreover, microfluidic chip and digital PCR were combined in this approach to detect GM varieties and estimate GM content, both of which achieved high-throughput and accurate identification of unauthorized GMOs. Overall, this new analytical approach will serve as a functional tool for accurate control of authorized and unauthorized GMOs.

## 1. Introduction

Genetically modified (GM) crops achieved an unprecedented 100-fold increase in planting area during the past 20 years, reaching to 189.8 million hectares in 2017 (James, 2018). Moreover, over 500 GM varieties (or called events) have been developed by institutes and agricultural biotech corporations, most of which are not well assessed and are undergoing additional, large-scale field trials in preparation for moving towards the commercialization (Moses, Abdallah, & Prakash, 2012). Reports from scientific laboratories and official academies evaluated effects of GM crops on human health, environment, agronomy, and economy and found no adverse effects attributed to genetic engineering in human population (EFSA, 2008; Fu et al., 2019; National Academies of Sciences & Medicine, 2017). Genetically modified organisms (GMOs) are now in strict regulation among countries and regions to track GM presence, to identify the estimated content and to cover unauthorized GMOs (Gruère & Rao, 2007; Marmiroli et al., 2008).

Analytical methods that have been successfully applied in the field of nucleic acids detection are developed for GMO identification and manipulation, such as polymerase chain reaction (PCR), enzyme-linked immunosorbent assay and next-generation sequencing (NGS) (Arulandhu et al., 2018; Fraiture, Herman, Papazova, et al., 2017; Fraiture et al., 2018; Querci, Van den Bulcke, Žel, Van den Eede, & Broll, 2010; Wei et al., 2018). A universal high-throughput method for screening is ideally the best solution to identify GM presence and possible unauthorized GMOs, which need comprehensive summaries of inserted genetic elements of commercialized GM events (Fraiture, Herman, De Loose, Debode, & Roosens, 2017). Numerous efforts are designed to detect as many targets as possible in one tube based on the conventional multiplex PCR (Cottenet, Blancpain, Sonnard, & Chuah, 2013; 2019; Köppel, Bucher, Bär, van Velsen, & Ganeshan, 2018; Querci et al., 2009). Hundreds of GM events made this method cost- and labor-consuming, despite a multiplex real-time PCR method that can simultaneously detect up to 47 targets was validated (Li et al., 2015). Another option for GMOs screening is to select several inserted elements to cover as many GM events as possible (Angers-Loustau et al., 2014; Huber et al., 2013; Park, Kim, & Kim, 2015; Randhawa, Morisset, Singh, & Žel, 2014; Rosa et al., 2016). This screening method is also applied for unauthorized GMOs identification. For example, five elements have been used as a universal screening approach for at least 81 GM events in the German market (Waiblinger, Grohmann, Mankertz, Engelbert, & Pietsch, 2010). Nine screening targets were also selected in three individual triplex PCR systems to identify 24 GM soybean events

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https://doi.org/10.1016/j.lwt.2020.109176
Received 18 October 2019; Received in revised form 14 January 2020; Accepted 14 February 2020
Available online 17 February 2020

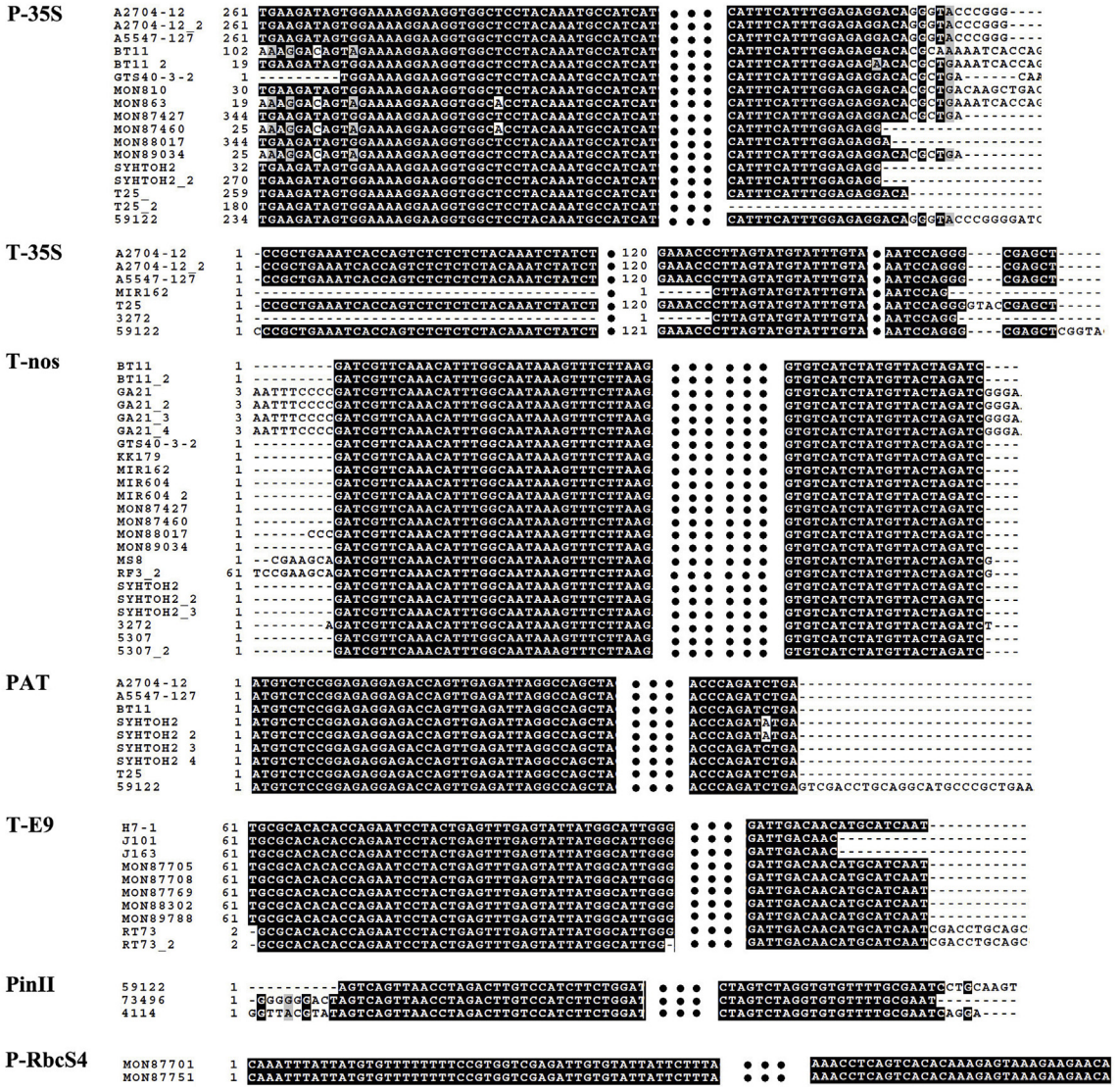


**Fig. 1.** Multiple sequence alignment of conserved sequence. Result of P–35S was shown as an example. Other alignment result can be seen from Fig. S1. Red letters mean conserved sequence, which was omitted by black dots. Yellow and green case indicated the location of primer and probe, respectively.

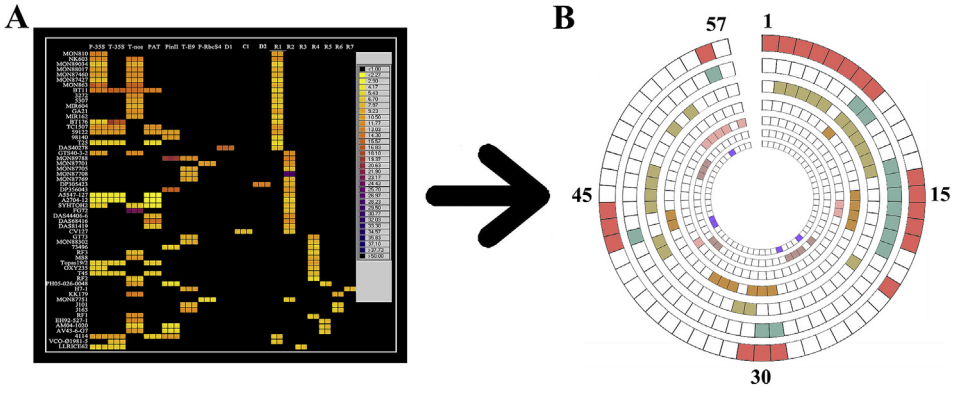


**Fig. 2.** Specificity validation for selected target. (A) Experimental result in the Biomark 48 × 48 chip. Colored squares indicated positive chambers. Yellow color means low Ct value, while blue color means high Ct value. D1, D2, C1 demonstrates DAS40278, DP305423 and CV127, respectively. R1-R7 means plant reference gene for maize, soybean, rice, canola, potato, alfalfa and sugarbeet. (B) The Seven rings with different colors demonstrate seven elements. Each pane in the radial direction represents the screening results of a single GM event by real-time qualitative PCR. Positive screening elements are demonstrated as red (P–35S), green (T-35S), khaki (T-nos), orange (PAT), pink (PinII), purple (T-E9) and rosy brown (P-RbcS4). Blank pane represents negative results. GM events numbered from 1 to 57 were MON810, NK603, MON89034, MON88017, MON87460, MON87427, MON863, Bt11, 3272, 5307, MIR604, GA21, MIR162, Bt176, TC1507, 59122, 4114, T25, VCO-Ø1981-5, DAS40278, GTS40-3-2, MON89788, MON87701, MON87705, MON87708, MON87769, MON87751, DP305423, A5547–127, A2704-12, SYHTØH2, FG72, DAS44406-6, DAS68416-4, DAS81419, CV127, RT73, MON88302, 73496, RF3, MS8, Topas19/2, T45, OXY-235, MS1, RF1, RF2, H7-1, J101, J163, KK179, AM04-1020, EH92-527-1, PH05-026-0048, AV43-6-G7, LLRICE62 and DP356043, respectively.

**Table 1**
Sensitivity of elements in universal analytical approach for screening.

| target | cp[a] | 1 | 2 | 3 | 4 | 5 | 6 | Average | STDEV[b] | RSD(%)[c] |
|---|---|---|---|---|---|---|---|---|---|---|
| T-nos | 10 | 37.4 | 36.9 | 38.21 | 35.81 | 35.44 | 37.1 | 36.81 | 1.03 | 2.79 |
| P–35S | 5 | 36.83 | 35.7 | 37.03 | 35.9 | 36.75 | 36.1 | 36.39 | 0.55 | 1.52 |
| T-35S | 5 | 38.54 | 38.52 | 38.72 | 38.2 | 38.82 | 38.52 | 38.55 | 0.21 | 0.55 |
| PAT | 4 | 36.97 | 37.85 | 37.51 | 37.78 | 37.75 | 36.78 | 37.44 | 0.46 | 1.22 |
| T-E9 | 2 | 36.87 | 36.57 | 36.72 | 36.91 | 36.33 | 37.02 | 36.74 | 0.25 | 0.69 |
| PinII | 10 | 37.87 | 38.17 | 38.52 | 38.21 | 38.01 | 37.64 | 38.07 | 0.30 | 0.80 |
| P-RbcS4 | 5 | 37.21 | 37.51 | 37.49 | 37.51 | 37.74 | 37.25 | 37.45 | 0.20 | 0.52 |

[a] cp means detected lowest copy numbers.
[b] STDEV indicates standard deviation.
[c] RSD indicates relative standard deviation, which equals to the ratio of STDEV to average number.

**Table 2**
GM identification of food and feed samples from market.

| Sample description | Taxon | Detected target | events need to be detected[c] | Number of commercialized events[d] | GM events |
|---|---|---|---|---|---|
| Soybean powder, imported[a], contained GM soybean[b] | soybean | P–35S, T-nos | 5 | 27 | GTS40-3-2 |
| Cornflakes, imported, contained GM maize[b] | maize | P–35S, T-nos, PAT | 22 | 45 | MON89034, TC1507 |
| Wafers, imported, contained GM canola[b] | maize, canola | T-E9 | 2 | 15 | RT73 |
| Animal feed, contained GM content[b] | alfalfa, sugarbeet | T-E9 | 4 | 4 | J101, H7-1 |

[a] Imported means that samples were imported from countries outside China.
[b] Samples are labeled as GMO positive in the package.
[c] number of GM events need to be detected based on Table S1.
[d] Number of GM events need to be detected based on ISAAA.

(Park et al., 2015). European Union Reference laboratory for GM food and feed (EURL-GMFF) developed the GMO matrix containing the sequence information of known GM-events and the other validated PCR-based detection and identification methods, which providing a useful tool for method validation and evaluation (Angers-Loustau et al., 2014). However, these established screening methods showed limited coverage of GM events, which cannot satisfy routine screening for over 500 GM events, especially for those crops in food and feed chain.

Recent progress seemed to combine the element screening and high-throughput analysis together. Microfluidic chip has been used in a number of high-throughput analysis such as gene expression (Monks et al., 2018; Wang et al., 2014), and thereby have been applied in GM events identification (Fu et al., 2015; Li et al., 2015; Shao et al., 2014). Otherwise, droplet digital PCR (ddPCR) is a newly developed absolute quantitative PCR-based method that is dependent on the limited separation of the reaction volume and independent on PCR efficiency for DNA quantification, which has been applied for GMO screening and quantitation (Dobnik, Spilsberg, Bogožalec Košir, Holst-Jensen, & Ž;el, 2015; Košir, Demšar, Štebih, Žel, & Milavec, 2019; Zhu et al., 2016). NGS seems to be the promising technology for high-throughput screening and identification of GM contamination and has been applied in Europe to track the unauthorized GMOs (Liang et al., 2014; Pauwels et al., 2015; Wahler, Schauser, Bendiek, & Grohmann, 2013; Yang et al., 2013).

Considering labor and cost in the certified laboratories across the world, PCR-based screening approach seems to be applicable and viable method to screen GMO presence and identify unauthorized GMOs for routine manipulation. In this study, inserted information of 191 singular GM events (21 varieties) for food and feed has been collected and investigated to search for the proper elements for wide-range screening. A universal analytical approach for GMO screening was then developed to serve manipulation of these GMOs and their hybrid crosses. Two strategies combined with this approach were then developed to identify and control unauthorized GMOs.

## 2. Material and methods

### 2.1. Plant and food materials

A list of 164 GM varieties was collected in this study. The insertion element summary of them was obtained from the GM Approval Database of the International Service for the Acquisition of Agri-biotech Applications (ISAAA) and shown in Table S1 (updated in 2019.9). Two events, MON87751 soybean and KK179 alfalfa, were kindly provided by Monsanto Co. Ltd. TT51-1(Bt63) rice was from archived samples stored at laboratory of Institute of Plant Quarantine in Chinese Academy of Inspection and Quarantine. Other GMOs and non-GM potato and sugarbeet samples were purchased from Reference Materials and Measurements (IRMM, Geel, Belgium) and from the American Oil Chemists' Society (AOCS, Urbana, USA). The non-GM soybean, maize, canola, alfalfa and rice samples were from archived samples stored at the laboratory described above. All food samples were collected from local market, including cornflakes, wafers, soybean powders, soybean oils, chips and Chinese tofu (ingredients are showed in Table 2).

### 2.2. DNA extraction

The seeds/grains were firstly grounded by a Retsch® MM400 mixer (Verder Shanghai Instruments and Equipment Co., Ltd., Shanghai, China). The mixed samples were then placed in a Dynamic CM-200 mixer and were shaken overnight to achieve equal distribution. Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Shanghai, China) from 50 μg of powdered samples. The extraction procedure was conducted according to the manufacturer's instruction. The concentration and purity of each sample were determined using NanoDrop N2000 (ThermoFisher Scientific Inc., Shanghai, China) according to the OD260 (optical density on 260 nm) and OD280 (optical density on 280 nm) values. Samples were then diluted to a final concentration of 50 ng/μL for this study.

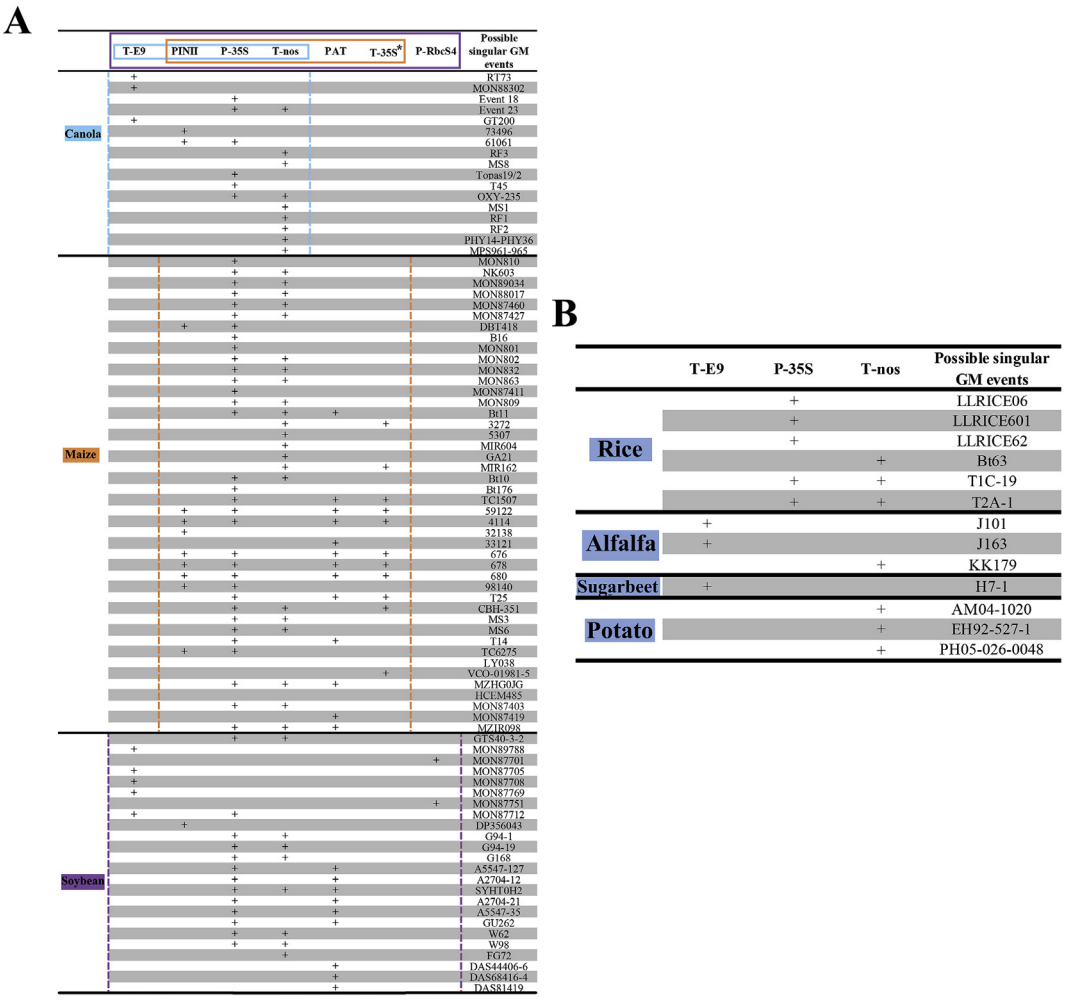


**Fig. 3.** Combinations assisted in choosing the most appropriate and cost-effective screening element to screen maximum number of GM events. Maize, soybean, canola, potato, sugarbeet, alfalfa and rice are listed to show the necessary elements used for singular GM events identification. Blue, orange and purple pane shows the concrete screening elements. T-35S was labeled with * to show that this element was not included in the screening of soybean.

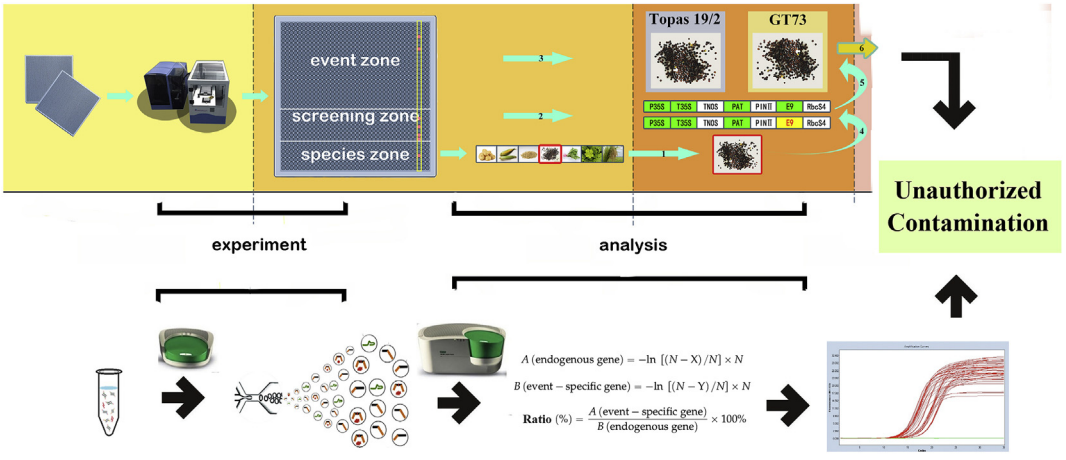


**Fig. 4.** Detection strategy to track unauthorized GM events. The process was performed on the microfluidic chip was divided into three pieces: Species zone, Screening zone and Events zone. Analyzing process was marked as Arrows 1 to 6. Positive results were marked as red in the chip (Arrow 1, 2 and 3). Species were firstly determined. Then, the screening results were compared to *in silico* results. The practical results and results *in silico* were listed (Arrow 4). unpredicted results from Screening zone and Events zone indicate unauthorized contamination (Arrow 5 and 6). Digital strategy was performed following digital PCR experiment, ratio calculation for indication of unauthorized contamination and qPCR validation, which showed on the bottom of this figure.

### 2.3. Oligonucleotides primers

All of the primers and probes used in this study were designed by PrimerQuest Tool (Integrated DNA Technologies, Coralville, USA) and were synthesized by Invitrogen (Thermofisher Scientific Inc., Shanghai, China). Probes were labeled by 6-carboxy-fluorescein (FAM) at the 5′ end and carboxytetramethylrhodamine (TAMRA) at the 3' end region, except for that of endogenous gene, which were labeled by VIC at the 5′ end region and Black Hole Quencher 1 (BHQ-1) at the 3′ end region. Detailed primer and probe information was listed in Table S2 and Table S3. Primers from Chinese standard (standard number: SN/T 1204–2016) were also used for verification.

### 2.4. Experimental specificity analysis

All DNA samples were tested by BioMark system (Fluidigm, South San Francisco, USA) equipped with a 48 × 48 digital array (Fluidigm) based on the manufacturer's instructions. All samples were tested in duplicate. The experimental conditions contained 1 × TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, USA), 2 × sample loading reagent (Fluidigm, South San Francisco, USA), 0.2 μM of each primer, 0.1 μM of each probe, and 100 ng DNA samples to meet the globally accepted requirement (ENGL, 2015). The cycling programs was based on the previous study (Fu et al., 2015), which including 50 °C for 5 min, 95 °C for 5 min, and then 50 cycles of 95 °C for 1 min and 60 °C for 1 min. Fluorescence was captured at the annealing step of each cycle. Baseline and thresholds were set manually.

The specificity was also validated by real-time PCR. The experimental conditions contained 1 × TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, USA), 0.2 μM of each primer, 0.1 μM of each probe, and 100 ng DNA samples. The cycling programs and fluorescence capture were the same as that in Biomark system. All samples were tested in triplicate.

### 2.5. Sensitivity analysis

DNA samples were prepared to contain 10, 5, 4, 3, 2 and 1 copies/μL, respectively. To accomplish this, the initial copy number of the individual DNA samples was estimated by running a droplet digital PCR (ddPCR) in quadruplicate. Sensitivity was tested by adding a 1 μL DNA template to the real-time PCR in triplicate. The limit of detection (LOD) was determined as the last dilution level at which no negative result was observed. 95% of replicates providing a positive response validate the data.

### 2.6. Chip and dPCR assay

Chip assay were evaluated through the Wafergen SmartChip (Microanaly Inc., Hefei, China) Real-time PCR system. PCR mixtures (100 nl for each well) consisted of 1 × TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, USA), 0.2 μM of each primer, 0.1 μM of each probe, and 100 ng DNA samples. PCR mixtures were dispensed into a 5184-well chip. After the initial enzyme activation at 95 °C for 10 min, 40 cycles of the following program were used for amplification: denaturation at 95 °C for 30 s ad annealing at 60 °C for 30 s. Fluorescence was captured at the annealing step of each cycle.

dPCR assay were evaluated through the Bio-rad QX200™ (Bio-rad, Hercules, USA). DNA were quantified in a 20 μL reaction mix containing 2X QX200™ ddPCR SuperMix for probes (no dUTP) (Bio-rad, Hercules, USA), 400 nM of each primer and 200 nM of the fluorescently labeled probe. Droplet generation and reading were performed on the Biorad QX200™ Droplet Digital PCR System (Bio-rad, Hercules, USA) and PCRs were run on the Biorad C1000 Touch™ Thermal Cycler (Bio-rad, Hercules, USA) according to the manufacturer's instructions. Data were analyzed using the QuantaSoft Software version 1.6.6 (Bio-rad, Hercules, USA).

## 3. Results and discussion

### 3.1. Collection of GM events and the selected targets

Over 500 GM events have been developed for food and feed use in 2018. A list of singular GM crops and their major inserted elements were shown in Table S1. GM crops mainly for food and feed were under consideration for this research. Some GM crops were not released into the market or have been withdrawn from the market, although they are approved in at least one country (Casacuberta, Nogué, & Du Jardin, 2017, p. 297; Lisowska, 2011).

Several elements were selected to cover these GM events for food and feed. As stacked GM events are usually hybrid crosses between singular GM events or retransformed singular GM events, these elements can also be available for stacked GMOs identification. A great diversity of genetic elements was observed both for crops and vegetables. Most frequently present genetic elements were 35S promoter of cauliflower mosaic virus (P–35S), the terminator of cauliflower mosaic virus (T-35S) and the terminator the nopaline synthase (T-nos), the 3' UTR terminator of Rubisco small subunit E9 gene from *Pisum sativum* (T-E9), phosphinothricin acetyltransferase gene (PAT), proteinase inhibitor II of *Solanum tuberosum* (PinII) and Terminator of ribulose 1, 5-bisphosphate carboxylase small subunit promoter of *Arabidopsis thaliana* (P-RbcS4). Rest of GM crops, including DAS40278, LY038, DP305423, CV127 and other uncommercialized events, which contained none of these seven elements and were detected directly through event-specific method. The analytical approach was then developed to contain these seven elements.

The screening approach for the presence or absence of candidate elements can facilitate rapid and cost-efficient discrimination of samples (Querci et al., 2010). This idea of the screening approach in this study is similar to the GMO matrix from EURL-GMFF that referred to the relationships between the two variables: GMOs and the detected element contained in samples (Angers-Loustau et al., 2014). Innovatively, coverage provided in the study is more comprehensive than any other published methods, which allows fast screening of 191 singular and their derived stacked events.

### 3.2. Multiple sequence alignment for all selected targets

The inserted DNA sequences of major GM crops were obtained from APHIS (Animal and Plant Health Inspection Service of United States of America) and developing corporations with official transfer agreement. Sequences were extracted and aligned by ClustalW sequence alignment algorithm (Tamura, Dudley, Nei, & Kumar, 2007; Thompson, Gibson, & Higgins, 2002), as shown in Fig. 1 and Fig. S1. The longest sequence of inserted element was considered if GM events contained more than one copy. Some truncated sequence, such as T-35S sequence in MIR162, was omitted to guarantee the compatible 'conserved' region. The length of target sequence was 257bp (P–35S), 203bp (T-35S), 253bp (T-nos), 548bp (PAT), 325bp (PinII), 572bp (T-E9) and 1723bp (P-RbcS4), respectively. All the sequences were validated to show the specificity *in silico* by blasting conserved region to full-length insertion sequence and corresponding reference genome, and were then used for primer and probe design.

### 3.3. Specificity, sensitivity and application

It is difficult to obtain all 191 commercialized GM crops for specificity validation. A list of 48 events, some of which were obtained from developing corporations were collected and used to assess the specificity of the designed primers and probes through quantitative PCR (Fig. 2). Ct value that less than 35 of each target was identified as positive, and was colored in Fig. 2B. No cross-reactivity was found between the experimental and *in silico* results, and amplification was observed for all expected positive targets. The results of quantitative

PCR confirmed the specificity of designed primers and probes.

Estimation of the limit of detection (LOD) is shown in the form of lowest detected copy numbers in all parallel experiments (Table 1). LOD determination was performed and calculated by six parallel experiments. The tested LOD was consistent with the minimum performance requirements for analytical GMO detection methods as defined by the EURL GMFF guidance document (ENGL, 2015), specifying that the LOD should be less than 25 copies. As the sensitivity requirement in EU is the strictest standard all over the world, our screening method could be accepted globally by countries and regions.

100 food products from local market were also collected for detection in order to test the validity of this screening approach (Table 2). These food products consist of flour, cookies, chips, cornflakes, as well as homemade tofu. Six of them were tested as positive GM derived products, which was also verified with standard method (Fig. S2). This comparison showed the great applicability of this approach for detection of field samples.

### 3.4. Chip strategy to identify unauthorized GMOs

The most common condition of unauthorized GMOs is when GMOs are authorized in one jurisdiction but not in another (Ruttink et al., 2010). DNA samples that consist of two or more GM events may be an unauthorized mixture to some extent (Holst-Jensen et al., 2012), such as the illegal contaminant of GM maize 4114 in GM maize MON810 that is exported to China. The concrete GM events should be identified, although screening results can be indicative of the presence of unauthorized GMOs through results of element screening. A series of reduced screening approaches were proposed by choosing several elements to screen GM events in one variety. Maize, one of the main crops all over the world, can only be screened with P–35S, T-35S, T-nos, PAT and PinII (Fig. 3A). Alfalfa only need to be screened with T-nos and T-E9 (Fig. 3B).

The strategy to identify unauthorized GM events can be performed on a microfluidic chip (Fig. 4), which were separated into several zones. Species zone was designed for species determination through taxon-specific detection. Screening zone was aimed to identify the GMO content from the previous developed screening approach. Templates in Event zone were detected through 48 event-specific methods. All the results were compared with *in silico* information under comprehensive analysis after the template DNA were amplified in these three zones simultaneously. For example, one food sample were detected through this chip-based strategy and identified canola content. However, the screening results (P–35S, T-35S, PAT and T-E9) is not matched with any events in Table 1. Results in event zone identified Topas 19/2 contamination, which is not authorized in European Commission. This detection strategy is satisfactory to decrease the number of necessary event-specific detections and provide a clue for identification of GM events (Fig. 4).

### 3.5. Content estimation to identify unauthorized GMOs

It is known that GM events and the corresponding screening elements already provides a corresponding relation, which means that any wrong indication between positive detected elements and GM events may indicate the presence of unauthorized GM events. Taking advantage of absolute quantitation characteristics of digital PCR, screening elements can also be used of GM content estimation, which provide another clue to identify the concrete GM events combined with event-specific detections. Droplet digital PCR (ddPCR) provide the concrete copy numbers of detected elements for calculation of content of elements. This estimated content was then used for comparison with theoretical content in Table 1. This comparison serves as a base for prediction of GM events and unauthorized contamination, which need fewer number of event-specific detections than chip strategy (Table S4). We also used 10%, 1% and 0.1% CRMs as positive calibrator to determine the LOD of this method. Generally, 2, 3 and 5 droplets are usually determined as positive replicate and a threshold of 2 droplets was applied in this study (Niu et al., 2018). The sensitivity was determined to be 0.13–0.14% content that is satisfied for EU labeling threshold (Table S5). Compared to developed method, this strategy get rid of large amount of detection of concrete GM events and was sufficient to determine unauthorized GMOs in complex matrixes, which is free from large amounts of events-specific detection (Košir et al., 2019).

## 4. Conclusions

The universal screening approach consists of ten elements/species, which cover the majority of commercialized GM events. Although the screening approach is designed for GMOs for food and feed, it can also apply to screen other GMOs (Table S1). The microfluidic chip strategy and ddPCR strategy were combined in this approach to enable an efficient and comprehensive identification of authorized and unauthorized GMOs. The developed approach is also able to detect GM events in raw materials and derived products for unauthorized GMOs control. This approach is not suitable for detection of absolutely unknown GMOs that contained unfamiliar inserts and unknown inserted site (Kovalic et al., 2012).

## CRediT authorship contribution statement

**Wei Fu:** Methodology, Resources, Writing - original draft, Writing - review & editing, Conceptualization. **Chenguang Wang:** Methodology, Resources, Writing - original draft, Writing - review & editing, Investigation, Formal analysis, Validation. **Pengyu Zhu:** Methodology, Validation. **Wenjie Xu:** Validation, Writing - original draft. **Xinshi Li:** Methodology, Supervision, Project administration, Supervision. **Shuifang Zhu:** Conceptualization, Methodology, Supervision, Writing - review & editing, Project administration, Funding acquisition.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

This work was funded by the National Science and Technology Major Project (2018ZX08012001-001) and the basic scientific research foundation in the Chinese Academy of Inspection and Quarantine (2017JK006). We herein thank Monsanto Far East Co. Ltd, Pioneer Hi-Bred International, Inc., Dow AgroSciences LLC, Syngenta Biotechnology (China) Co. Ltd, Bayer Cropscience NV, BASF Plant Science L.P and CropLife China for their kindly help in providing inserted information of GM events and their comments on the manuscript.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.lwt.2020.109176.

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