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MPIC: A High-Throughput Analytical Method for Multiple DNA Targets

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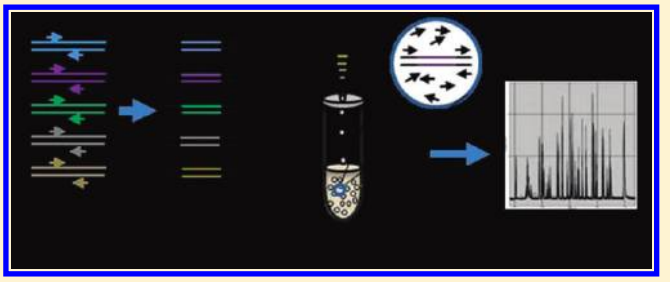
Supporting Information

ABSTRACT: We describe the development of a novel combined approach for high-throughput analysis of multiple DNA targets based on multiplex Microdroplet PCR Implemented Capillary gel electrophoresis (MPIC), a two-step PCR amplification strategy. In the first step, the multiple target DNAs are preamplified using bipartite primers attached with universal tail sequences on their 5′-ends. Then, the preamplified templates are compartmentalized individually in the microdroplet of the PCR system, and multiple targets can be amplified in parallel, employing primers targeting their universal sequences. Subsequently, the resulting multiple products are analyzed by capillary gel electrophoresis (CGE). Using genetically modified organism (GMO) analysis as a model, 24 DNA targets can be simultaneously detected with a relative limit of detection of 0.1% (w/w) and absolute limit of detection of 39 target DNA copies. The described system provides a promising alternative for high-throughput analysis of multiple DNA targets.



The advent of modern analytical technologies provides researchers and laboratories involved in nucleic acid diagnostics with new opportunities for analyzing multiple target DNAs in the fields of medical diagnostics,[1−3] food/feed identification,[4−6] environmental monitoring,[7−10] etc. Owing to the increase in the number of molecular targets to be analyzed, assay miniaturization and cost-efficiency become necessary.

The DNA microarray is a frequently used technology for analyzing complex nucleic acid samples. It presents the advantages of high-throughput, miniaturized, and automatic biological assays in one single experiment.[2,4,7−10] High density microarrays allow up to approximately 10^6 tests sites in a 1−2-cm^2 area, giving the opportunity for simultaneous analysis of thousands of different targets.[7,10] Nevertheless, the microarray analytical approach is relatively expensive and complicated, limiting its extensive availability. Additionally, for the analysis of minimal amounts of initial targets, direct detection by DNA hybridization onto microarrays is impracticable, and therefore additional target enrichment steps are required, such as the polymerase chain reaction (PCR).[4,6]

Currently, PCR, and especially multiplex PCR, is routinely used for simultaneous amplification and identification of several DNA targets and offers several advantages, e.g., increased throughput and reduced turnaround times of detection. These methods become even more attractive if they can be combined with other technologies capable of automation. A PCR-CGE method combining multiplex PCR amplification with capillary gel electrophoresis (CGE) has been recently shown to allow simultaneous detection of multiple DNA targets.[11] However, the level of multiple PCR amplification is usually limited to a maximum of 10 targets simultaneously amplified in a single reaction.[12] The development of conventional multiplex PCR for high-throughput target analysis suffers from several limitations including the preferential amplification of partial target molecules within the whole set of analytical targets, and the nonspecific amplification due to the interference of multiple primer pairs in a single reaction.[13,14] To overcome such limitations, measures including the increase of template amount and/or reduction of the number of PCR amplification cycles have been reported.[4,6,15] The reported high-throughput DNA detection methods such as

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Table 1. Composition of the Simulated DNA Samples Used To Test the 24-plex MPIC Test in This Study[a]

target	sample A, %	sample B, %	sample C, %	sample D, %	sample E, %	sample F, %	sample G, %	sample H, %	sample I, %	sample J, %
Bt176	0.15	0.15	0.5	0.25	0.5	0.1	0.15	0.1	10.0	1.0
RT73	0.5	0.5	0.5	0.5	0.25	0.05	0.1	0.63	10.0	1.0
Bt11	0.25	0.25	0.3	0.1	0.75	0.15	0.38	0.38	5.0	0.5
TC1507	0.25	0.25	0.5	0.38	1.25	0.25	0.25	0.1	7.5	0.75
HN-1	0.5	0.5	0.2	0.1	0.0	0.0	0.1	0.15	5.0	0.5
MON531	0.75	0.75	0.5	0.0	0.5	0.1	0.25	0.25	10.0	1.0
OXY235	0.25	0.25	1.0	0.63	0.5	0.1	0.25	0.63	20.0	2.0
RRS	0.25	0.25	0.25	0.1	0.25	0.05	0.0	0.0	5.0	0.5
NK603	0.38	0.38	0.1	0.38	1.0	0.2	0.38	0.38	0.0	0.0
T25	0.2	0.2	0.2	0.2	0.5	0.1	0.05	0.05	5.0	0.5
MON88913	0.13	0.13	0.1	0.13	0.0	0.0	0.1	0.1	5.0	0.5
MIR604	0.1	0.1	0.1	0.0	1.0	0.2	0.0	0.0	0.0	0.0
GA21	0.75	0.75	0.1	0.75	0.75	0.15	0.5	0.15	0.0	0.0
MON1445	0.38	0.38	1.0	0.1	0.0	0.0	0.38	0.25	17.5	1.75

[a] Samples A, D, E, F, and G were prepared by mixing the different GM events with non-GM maize. Sample B, C, H, and J were prepared by mixing the different GM events with non-GM soybeans. Sample I was prepared by mixing the different GM events together, without other non-GM DNA solutions. The percentages of GM events were calculated by dividing the amount of DNA from corresponding GM events by the total DNA amounts in the sample.

NASBA implemented microarray analysis (NAIMA) and multiplex quantitative DNA array-based PCR (MQDA-PCR) used one or several initial preamplification steps in multiplex PCR followed by other amplification approaches.[4,6]

Recently, the microdroplet-based PCR technology has been successfully used for many applications including single-molecule PCR amplification,[16,17] high-throughput screening,[18] large scale genome sequencing,[19,20] etc. In microdroplet PCR, the template DNA molecules are segregated in millions of microdroplets of about 0.5 fl to 0.5 pl in volume in a water-in-oil (w/o) emulsion.[20] Each template molecule can be amplified individually in the microdroplet, alleviating the above-described drawbacks of conventional multiplex PCR and allowing uniform and parallel amplification of as many as 10^{10} DNA targets in 1 mL of w/o emulsion.[21,22] Microdroplet PCR provides a powerful high-throughput format for biological analysis.[23−25] To date, multiple DNA targets cannot be simultaneously and effectively amplified for target-specific analysis using various primer pairs in microdroplet PCR; the random combination of a large number of different primers arithmetically reduces the number of microdroplets with correct primer pair combination.

In this study, we develop a robust high-throughput analytical approach named multiplex Microdroplet PCR Implemented Capillary gel electrophoresis (MPIC) combining the advantages of bipartite primers, microdroplet PCR and CGE for multiple target DNA analysis. Genetically modified organism (GMO) diagnostics present several challenges worldwide, one of them being the great number of targets that should be detected to screen for all possible GMOs on the market.[26,27] For this reason, we have selected a total of 24 target sequences used in GMO diagnostics to demonstrate the high throughput, sensitivity, great flexibility, and applicability that are the advantages of the established MPIC. The developed MPIC system provides an alternative approach to current diagnostics tools for high-throughput detection and/or identification of various DNA targets and can be applied in different domains where such multiplex nucleic acid detection is needed.

EXPERIMENTAL SECTION

Plant Materials. GM maize (MON810, NK603, and GA21), GM soybean (Roundup Ready soybean, hereafter termed RRS), GM canola (RT73), and GM cotton events (MON88913, MON1445, and MON531) were kindly supplied by Monsanto Company (St. Louis, MO). GM maize events Bt176 and Bt11 were kindly supplied by Syngenta Seeds, Inc. (Minnetonka, MN). The GM canola OXY235 and maize MIR604 and T25 events were kindly supplied by Bayer CropScience Co. (Monheim am Rhein, Germany). GM maize TC1507 was kindly supplied by Dow Agrosciences LLC (Indianapolis, IN). Leaves from the GM Huanong No. 1 (HN-1) papaya and non-GM papaya were supplied by South China Agriculture University, China. Seeds from nontransgenic crops (maize, rice, wheat, barley, soybean, cotton, and canola) were purchased from local markets in Shanghai, China, and checked for the absence of any GM events before use in this study.

Sample Preparation and DNA Extraction. The genomic DNAs of all plant materials were extracted and purified using a mini-plant genomic DNA extraction kit (Shanghai Ruifeng Agrotech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The concentration and quality of purified DNA samples were determined using a NanoDrop 1000 UV/vis spectrophotometer (NanoDrop Technologies, LLC, Wilmington, DE). All extracted DNA samples were adjusted to a final concentration of 20 ng/μL using 1×TE (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA).

To evaluate the developed MPIC system, a series of GM DNA solutions were prepared by mixing the genomic DNA of each 14 GM events with non-GM wheat genomic DNA. The different mixed GM DNA solutions were prepared in order that the final relative GMO content of each event was 5.0%, 1.6%, 0.8%, 0.16%, 0.08%, 0.032%, and 0.0% (w/w), respectively. Additionally, 10 GM DNA mixtures (hereafter termed "simulated samples") containing various GMO contents were also prepared to test the applicability of MPIC. The details about GM contents in each of these simulated samples are given in Table 1.

Oligonucleotide Primers. All bipartite primer pairs (or so-called "tailed" target-specific primer pairs) used in this study were

designed using the Primer Express software version 2.0 (Applied Biosystems, Foster City, CA) and are listed in Table S1 (Supporting Information). The locations of primers annealing on the genomic DNA are indicated in the Figure S1 (Supporting Information) (http://gmdd.shgmo.org/).[28] Each forward bipartite primer was modified by the addition of a universal sequence (Uni-F, 5′-GCCTTGTTCTACCATTACGC-3′) at its 5′-end, and each reverse bipartite primer was modified by the addition of another universal sequence (Uni-R, 5′-TCAGCCACCTCTT-TGTCCTT-3′) at its 5′-end. The universal Uni-F/R primers were designed according to the principle described in previous reports.[29,30] All primers were synthesized by Invitrogen Co., Ltd. (Shanghai, China)

Singleplex PCR. Singleplex PCR assays were carried out in a final volume of 25 μL on a PTC-100 thermocycler machine (MJ Research, Waltham, MA) for evaluating the specificity of the bipartite primers and universal primers. Each reaction mixture contained the following reagents: 1×buffer for *KOD-Plus*, 1 mM $MgSO_4$, 200 μM dNTP, 200 nM each primers, 0.5 unit of *KOD-Plus* DNA polymerase (Toyobo Biotech Co., Ltd., Shanghai, China), and 10 ng of genomic DNA. Singleplex PCR programs were as follows: 5 min of predenaturation at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 35 s at 68 °C, and a final extension of 7 min at 68 °C.

Set-up of MPIC Reaction. *Preamplification of Multiple Target DNA Fragments.* The reactions were performed in 25 μL PCR reaction mixtures containing the following reagents: 1×buffer for *KOD-Plus*, 1.5 mM $MgSO_4$, 200 μM dNTP, appropriate bipartite primer mix as shown in Table S2 (Supporting Information), and 0.5 unit of *KOD-Plus* DNA polymerase (Toyobo Biotech Co., Ltd., Shanghai, China). Unless specially stated, 100 ng of genomic template DNA was used in each reaction. The 8-plex PCR preamplification reactions were performed using the following program: 5 min of predenaturation at 94 °C, followed by 11 cycles of 30 s at 94 °C, 30 s at 60 °C, and 35 s at 68 °C, and a final extension of 7 min at 68 °C.

Cleanup of the Multiplex Preamplification PCR. To remove the remaining free bipartite primers after the preamplification reaction, the multiplex preamplified products were purified using the AxyPrep PCR Cleanup Kit (Axygen Scientific, Inc., Union City, CA) according to the manufacturer's instructions and eluted in 25 μL of elution buffer supplied in the kit.

Microdroplet Universal PCR Amplification. Microdroplet universal PCR was performed in microdroplet compartments based on a previously published method with slight modifications.[22] The oil phase for water-in oil (w/o) emulsion contained 4.5% (v/v) Span 80, 0.4% (v/v) Tween 80, and 0.05% (v/v) Triton X-100 in mineral oil. The aqueous phase consisted of 1×buffer for *KOD-Plus*, 10 g/L BSA, 1.5 mM $MgSO_4$, 200 μM dNTP, 400 nM each Uni-F/R, and 5.2 units of *KOD-Plus* DNA polymerase (Toyobo Biotech Co., Ltd., Shanghai, China) or 2.6 units of Hotstar *Taq* DNA polymerase (TaKaRa Biotech Co., Ltd., Dalian, China) and 5 μL of purified preamplification products in a total volume of 260 μL.

The w/o emulsion was generated by adding 200 μL of the aqueous phase to the 400 μL oil−surfactant mixture in a dropwise manner over 1.5 min while vortexing the oil−surfactant mixture at 25 °C. After the addition of the aqueous phase was completed, the vortex mixing step was continued for 5 min at 25 °C. The obtained w/o emulsion was dispensed into PCR tubes as 10 aliquots of 50 μL. As a control of the microdroplet universal PCR, conventional universal PCR (i.e., nonemulsified reaction) was performed using 50 μL of the aqueous phase. PCR amplification programs were as follows: 5 min of predenaturation at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 35 s at 68 °C (or 72 °C for *Taq* system), and a final extension of 7 min at 68 °C (or 72 °C for *Taq* system).

After completion of the microdroplet universal PCR, the w/o emulsion was centrifuged (16 000 g, 5 min at room temperature) to gather the microdroplets. The products of microdroplet universal PCR reaction were purified using a AxyPrep PCR Cleanup Kit (Axygen Scientific, Inc.) according to the manufacturer's instructions and eluted in 25 μL of elution buffer supplied in the kit before analysis on agarose gel electrophoresis or CGE.

CGE Conditions. QIAxcel System (Qiagen Shanghai Co., Ltd., China) and the QIAxcel DNA High Resolution kit were used to separate PCR amplicons according to the manufacturer's instructions. Amplicon samples were run using the separation method OH400 (QIAxcel System) at room temperature. Sample injection at 2000 V for 20 s using N_2 pressure (0.5 psi), and sample separation at 6000 V for 520 s. The amplicons with expected size obtained from the 24 individual singleplex PCR were mixed and used as positive controls for all reactions. The data were analyzed by QIAxcel BioCalculator Analysis software (cat. no. 9018391, Qiagen Shanghai Co., Ltd.). As a threshold signal value, a level corresponding to 5% of the highest peak value was chosen as acceptance criterion for a positive signal.

RESULTS AND DISCUSSION

The Design and Principle of MPIC. In the MPIC reaction (Figure 1), each target DNA was preamplified by multiplex PCR over a few cycles (in this study, the optimized preamplification was performed with 11 cycles), employing bipartite primers with the same set of universal sequences tails. The obtained preamplified DNA fragments harbored two different universal sequences at their 3′- and 5′-ends, respectively, and were cleaned to remove the residual bipartite primers. The cleaned preamplification DNA products were used as templates in the next universal microdroplet PCR amplification. In this second step, only one universal primer pair targeting both universal tail sequences was used in the microdroplet PCR system containing millions of independent emulsified microdroplets. The relatively smaller number of template DNA molecules could be randomly compartmentalized in the microdroplets (that were in large excess compared to the target DNA molecules), allowing each droplet to contain no more than a single template DNA molecule. In other words, each single template DNA molecule was theoretically incorporated in an individual emulsion droplet, and therefore single molecule PCR amplifications were performed in parallel in individual droplets. In this way, the multiple DNA target molecules could be coamplified with high efficiency and throughput in microdroplet PCR. Finally, the microdroplets were pooled, and the resulting products were purified and analyzed by CGE based on the different DNA samples sizes.

Specificity Test of Bipartite Primers and Universal Primers. In the MPIC system, the universal and bipartite primers should be carefully selected and designed by bioinformatics analysis before experimental verification (Table S3, Supporting Information). In this study, all the singleplex PCR assays based on the use of the bipartite and universal primers were experimentally tested on several GM plant genomic DNAs. This verification showed that the universal primers did not prime any nonspecific amplification, and the bipartite primers could

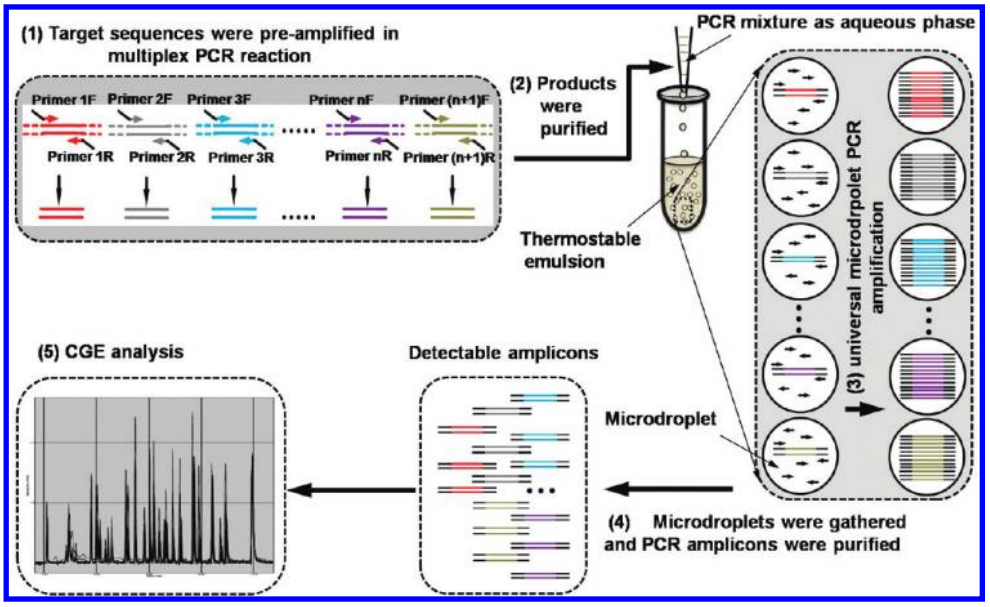

Figure 1. Schematic diagram of the MPIC assay. (1) Target-specific DNA products with universal tails on each side were generated in multiplex PCR preamplification, using bipartite primers. (2) The products of the multiplex preamplification were purified and used as templates in the second step, universal microdroplet PCR amplification, using universal primer pairs. (3) The microdroplet PCR was performed, employing the universal primer pair Uni-F/R in the emulsion droplets. (4) The w/o emulsion was centrifuged to gather all microdroplets, and DNA fragments were purified for subsequent CGE-based detection. (5) Detectable amplicons were analyzed by CGE.

only amplify the expected target DNA sequences (data not shown).

Optimization of the MPIC System. For convenience and ease of the development, the MPIC amplification optimization was performed based on a 8-plex microdroplet PCR platform designed for the detection of eight GM events (Bt176, Bt11, HN-1, RRS, T25, MON88913, MIR604, and MON1445), hereafter referred to as combination A (Table S2, Supporting Information). Various parameters known to significantly influence the generation of nonspecific fragments were assessed: the type of DNA polymerase, the number of PCR cycles, and the concentrations of Mg^{2+} and primers. During this optimization phase and unless specifically specified, the 8-plex microdroplet PCR was tested on a DNA mixture containing all 14 GM events (each at a final concentration of 1.6% (w/w)). All reactions were performed at least in triplicate.

Selection of DNA Polymerase. The production of amplified products was shown to be probably affected by the type of DNA polymerase.[31] In this study, we compared two thermostable DNA polymerases, *Hotstar Taq* (TaKaRa Biotech. Co., Ltd., Dalian, China) and *KOD-Plus* (Toyobo Biotech Co., Ltd., Shanghai, China), by amplifying the same template using the same primer combination with varying Mg^{2+} concentrations. The *KOD-Plus* DNA polymerase system showed the better performance for the development of the MPIC assay in this study (Figure S2, Supporting Information).

Primer Concentration. Primer concentration/ratio is a critical parameter for successful multiplex PCR. Initially, bipartite primers were used with an equal final concentration (50 nM) in the multiplex PCR, and the amplicons were detected by agarose gel electrophoresis. On the basis of the electrophoresis results, we adjusted the ratios of several bipartite primers in the reaction. The final optimized concentration (37.5−125 nM) for each primer is listed in Table S2 (Supporting Information).

Cleanup of the Preamplified Amplicons. To investigate the influence of the residual presence on the MPIC performance, we tested whether cleaning the preamplified amplicons before the microdroplet universal PCR could improve the procedure. To this end, two DNA solution mixtures containing all 14 GM lines (at 1.6% and 0.8% for each GM line, respectively) were used for multiplex preamplification. Purified and nonpurified preamplification products were then used as templates in the following universal microdroplet PCR step. As a result, all expected amplicons were amplified in universal microdroplet PCR, but weaker amplicons were obtained from preamplification products that were not cleaned prior to the universal microdroplet PCR (Figure S3a, Supporting Information). Similar results were also reported in a previous study using a two-step PCR procedure.[4] This is probably due to the presence of bipartite primers and multiplex PCR reagents throughout the MPIC, causing a high number of possible primer combinations (primer dimer and cross dimer) and therefore reducing amplification efficiency of the microdroplet PCR. Therefore, it is necessary to remove the residual bipartite primers and PCR reagents to improve the efficiency and sensitivity of MPIC and reduce the nonspecific interactions.

Mg^{2+} Concentration. For a successful multiplex PCR assay, the balance between magnesium chloride (Mg^{2+}) and nucleotides (dNTPs) is an important parameter.[32] As other DNA polymerases, the *KOD-Plus* DNA polymerase requires free magnesium to work properly. During the MPIC assay optimization, both PCR amplification steps (multiplex preamplification and microdroplet PCR) were tested with various Mg^{2+} concentrations (0.5 mM, 1 mM, 1.2 mM, 1.5 mM, 2 mM, 2.5 mM, 3 mM, and 4 mM) while keeping the dNTP concentration constant (200 μM), suggesting that the universal microdroplet PCR efficiency was not obviously influenced by Mg^{2+} concentration. However, Mg^{2+} significantly influenced the multiplex preamplification step: some target products were barely visible or even

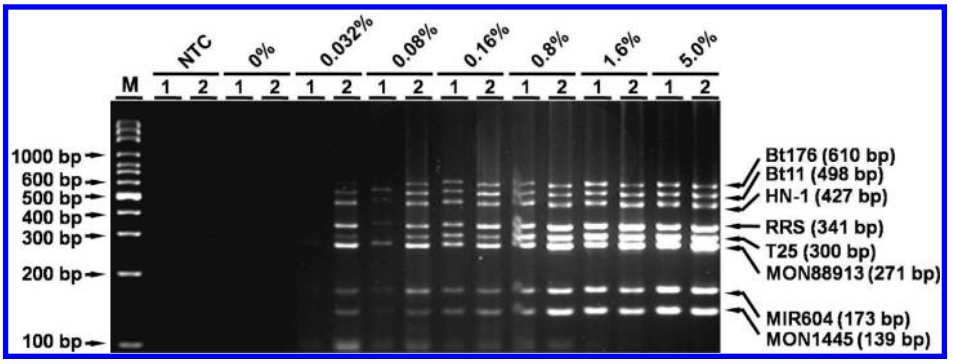

Figure 2. Sensitivity test of 8-plex microdroplet PCR employing primer combination A. Lane 1: nonemulsification control. Lane 2: amplification result of 8-plex microdroplet PCR in different dilutions of GM DNA mixture templates. Lane M: 100 bp DNA Ladder (Axygen Scientific, Inc.).

disappeared with the Mg^{2+} concentration below 1.5 mM or above 2.0 mM in multiplex PCR (the Mg^{2+} concentration being 1.5 mM in the following universal microdroplet PCR) (Figure S2, Supporting Information). In conclusion, the ideal results were obtained when both multiplex PCR and microdroplet PCR were performed with a Mg^{2+} concentration of 1.5 mM.

The Number of Preamplification Cycles. The influence of the number of cycles (5, 8, 11, 14, 17, and 20 cycles) during the multiplex preamplification PCR was investigated using a mixture of the 14 GM event DNA solutions (0.8% for each GM event) as template. Increasing the number of cycles in the first preamplification PCR step improved the sensitivity of the MPIC assay by increasing the amounts of target products. We also observed that 11 amplification cycles were sufficient for the multiplex preamplification PCR step to generate enough target templates to be used in the following microdroplet PCR (Figure S3b, Supporting Information).

Formation of Emulsion Droplets for Microdroplet PCR. The emulsified droplets were produced based on previously published methods with slight modification.[22] The microdroplets generated by the emulsion were observed using a Leica DM2500 microscope with a 40× objective lens, and their average diameter was estimated to be about 4−6 μm (Figure S4a, Supporting Information). The diameter of microdroplets is close to previous reports.[33] Following this observation, we calculated that a 200 μL PCR reaction volume contains approximately 1.8×10^9 to 6.0×10^9 microdroplets in this study. We also observed that the size distribution of microdroplets had less change before and after microdroplet PCR amplification (Figure S4b, Supporting Information), suggesting the high thermostability of the generated microdroplets.

Compartmentalization of the Template Molecules in Droplets. Target DNA molecules should be distributed in microdroplets based on the Poisson distribution.[23] According to Poisson statistics, many empty microdroplets are required in order to make sure that only one single DNA target molecule is contained in a given microdroplet. For this reason, we calculated the theoretical enrichment in terms of target molecules after the multiplex PCR preamplification step (Method S1, Supporting Information). The theoretical maximal final amount of multiplexed template DNA molecules in the 200 μL w/o emulsion is about 3.9×10^8 molecules. As mentioned above in Formation of Emulsion Droplets for Microdroplet PCR, a 200 μL microdroplet PCR mixture reaction volume contains approximately 1.8×10^9 to 6.0×10^9 microdroplets. So, it is reasonable to suggest that most of droplets contain only a single-copy template DNA molecule.

Specificity of the 8-plex Microdroplet PCR Assay. For evaluating the specificity of the 8-plex microdroplet PCR assay (combination A), a mixed GM DNA solution containing the 14 GM events (with a content of 1.6% for each GM event), genomic DNA solutions containing eight single GM events (Bt176, Bt11, HN-1, RRS, T25, MON88913, MIR604, and MON1445, 100% (w/w) content for the events), and non-GM crops were used. As shown in Figure S5 of Supporting Information, all of the eight event-specific targets were correctly amplified in a single reaction using the GM DNA mixture as template. Similarly, specificity of the 8-plex was confirmed on the DNA materials containing a single GM event: only the expected DNA target was amplified. Finally, no amplification product was observed in the no-template control (NTC), as well as in reaction using non-GM crops DNA solutions as templates. These results demonstrated the specificity of the bipartite primers used in the preamplification step as well as the specificity of the microdroplet PCR amplification using universal primers pair. Therefore, the 8-plex (combination A) microdroplet PCR assay showed high accuracy and specificity for detection of multiple targets in complex mixture.

Sensitivity of the 8-plex Microdroplet PCR Assay. For testing the sensitivity of the 8-plex microdroplet PCR assay (combination A), a series of mixed GM DNA solutions containing the 14 GM events (fortified at 5.0%, 1.6%, 0.8%, 0.16%, 0.08%, 0.032%, and 0% for each GM event) were used as templates. A 100 ng amount of total genomic DNA was used in each PCR preamplification reaction. As shown in Figure 2, all eight event-specific amplicons could be simultaneously detected from all levels except for the DNA mixture with 0.0% GMO content. The smallest level of GM content that could be detected using the 8-plex microdroplet PCR assay (combination A) was as low as 0.032%, which corresponds to 12−83 copies according to the genome sizes of plant species.[34] In the conventional universal PCR reaction (i.e., the universal PCR amplification in nonemulsified reaction), several amplicons were not detected in template solution where the GM content was below 0.16%, even when the PCR amplification was increased to 50 cycles (Figure S6, Supporting Information). These results showed that the developed 8-plex microdroplet PCR technique can be used for practical GM samples detection where high sensitivity is required.

The Flexibility of the MPIC Setup. The above results showed that the optimized multiplex microdroplet PCR assay using the 8-plex combination A is stable, accurate, and presents good specificity and high sensitivity. To show the applicability of

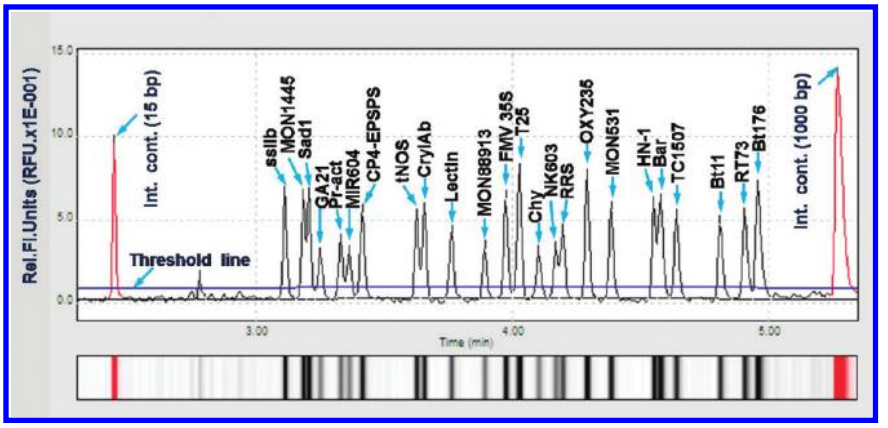

Figure 3. Capillary gel electrophoresis analysis of the 24-plex MPIC assay for detection of 14 different GM events using 24 bipartite primer pairs. Amplicons were produced from a GM DNA solution mixture containing 1.6% of each of the 14 different GM events. The peaks of the amplicon are indicated by blue arrows. The profile shown under the electrophoregram is the simulated gel image corresponding to the results of the capillary gel electrophoresis analysis.

MPIC for analysis of a higher number of target DNAs, we performed simultaneous analysis of 24 different GMO targets in a single MPIC reaction by adding another 16 targets to our platform. Two additional 8-plex microdroplet PCR assays were developed according to the above optimized procedure except for primer concentration (Table S2, Supporting Information). These two additional 8-plex assays showed high specificity and high sensitivity (as low as 0.032% w/w, data not shown). Subsequently, we performed the combined 24-plex MPIC assay to simultaneously analyze these 24 different targets (Bt176, Bt11, TC1507, NK603, T25, MIR604, GA21, MON531, MON1445, MON88913, RT73, OXY235, RRS, HN-1, *ssIIb*, *Lectin*, *Sad1*, *Chy*, tNOS, FMV35S, *CP4-EPSPS*, *CryIAb*, *Bar*, and Pr-act) with the use of 24 bipartite primer pairs distributed in three primers combinations, respectively.

The 24-plex MPIC assay was tested using the GM DNA solution mixture containing the 14 GM events (fortified at 1.6% for each GM event) as template. For this, the 24 targets were first preamplified in three different 8-plex PCR reactions (combinations A, B, and C). In a second step, all the preamplified products were amplified in a single universal microdroplet PCR reaction using a single pair of universal primers. Eventually, the 24 amplicons with differences in size as small as 5 bp were unambiguously separated by CGE. As shown in Figure 3, all the expected 24 amplicons were clearly identified by CGE when the GM DNA dilution solution mixture containing the 14 GM events (100 ng) was used. Also, when testing simulated DNA samples containing various amounts of the 14 GM events in maize or soybean background, all GMO-specific target amplicons could also be generated and clearly identified with little signal strength variation (Figure 4a,b), further showing the flexibility of MPIC.

Evaluation of the Applicability of the 24-plex MPIC for GMOs. The applicability of the 24-plex MPIC assay was evaluated employing ten simulated DNA sample sets (samples A−J). The detailed composition of each simulated DNA sample is given in Table 1. DNA samples A, B, and C contained all 14 GM events at different concentration levels. The samples D−J were similarly prepared but with at least one GM event being absent in the samples. GMO contents in all these simulated samples were ranging from 0.05 to 20.0%. To assess the robustness of the multiplex assay, the simulated DNA samples were prepared such that the different target contents are unbalanced, especially when comparing the taxon target content vs transgenic element (event-specific or screening elements) contents. The MPIC assay was performed in triplicates for each sample set, and 100 ng of DNA was used as templates.

As shown in Figure 4, the different 24-plex MPIC reactions were specific and accurate: only the expected targets (taxon-specific sequences, event-specific sequences, and screening elements) were detected by CGE, and no false-positive or false-negative signal was observed. However, for RT73 event-specific target in sample F, the signal was not significantly above the background threshold, according to our criteria. The reason for this is probably from the low level of RT73 content (0.05%) in the sample F (close to the lowest detection level observed with the 8-plex combination A). Additionally, all the 14 GM events in the simulated DNA samples with contents as low as 0.1% (0.05% for RRS) could be accurately detected and identified. This observation means that the lowest sensitivity level of the 24-plex MPIC assay is at least 0.1% (or approximately 39 maize haploid genome copies) for each of the 14 different GM events. Together with these results, it is shown that all 24 primer pairs can specifically anneal to their corresponding target sequences and prime specific amplification, and that our MPIC approach can be successfully used for high-throughput analysis of multiple targets DNA in practical samples.

CONCLUSIONS

In this work, one novel multiplex, high-throughput, and sensitive nucleic acid detection method, termed MPIC, was developed by dexterously combining the universal tailed primers and microdroplet PCR approaches with the CGE analysis technique. GMO diagnostics, a domain of great importance for food and feed safety and control, were selected as a model to show the applicability of this method. MPIC was successfully employed to qualitatively analyze 24 different target DNA fragments from 14 GM events. The generally good performance of the MPIC method (good specificity and high observed sensitivity of at least 0.1% for all the targets or 39 maize genome copies) is associated with flexibility, high throughput, and low cost (due to high multiplexing). In addition to its above-mentioned qualities, this new approach offers identification of multiple DNA targets with automation capabilities. In particular, the predominant

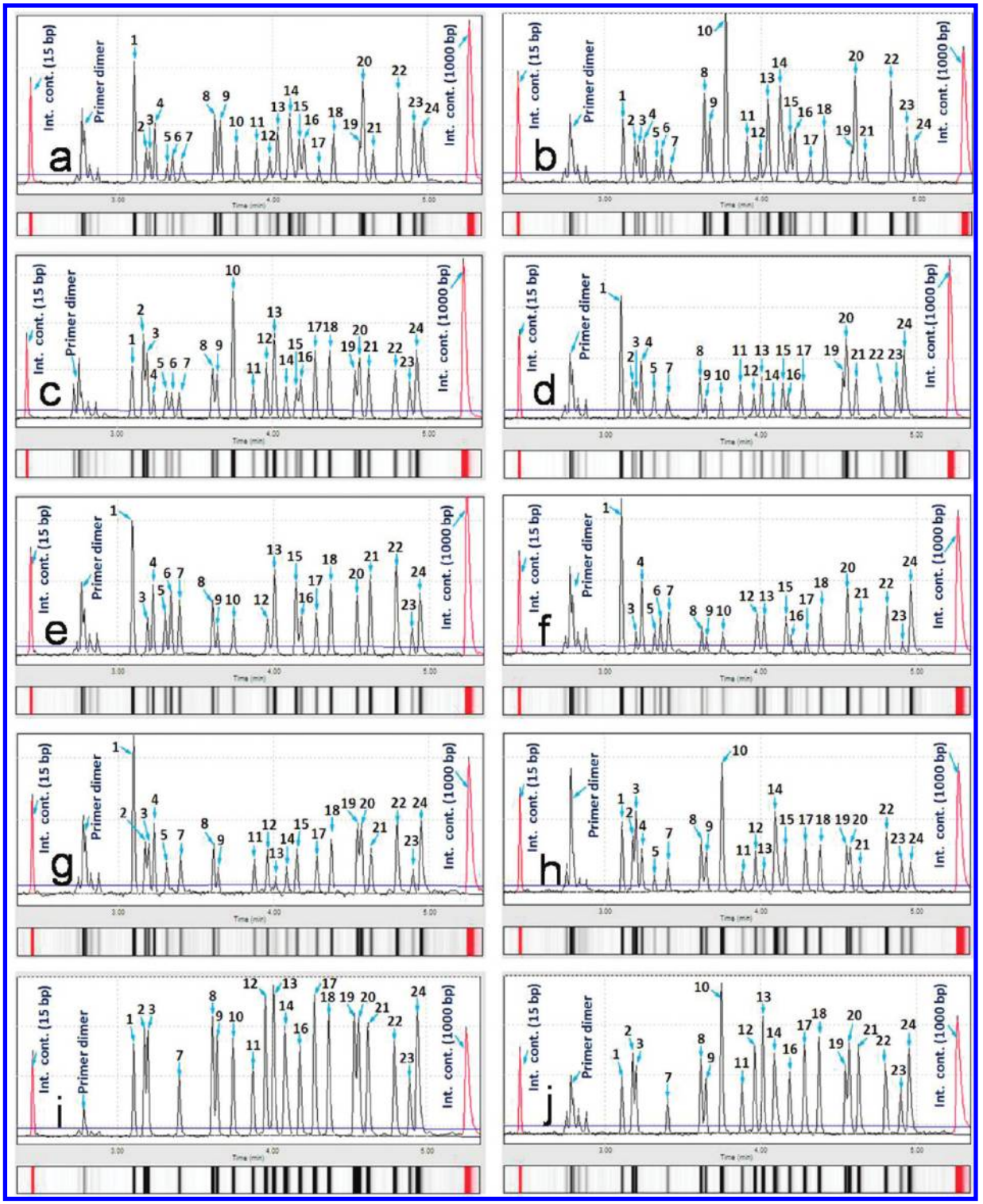

Figure 4. Capillary gel electrophoresis analysis of the 24-plex MPIC assay for detection of 14 different GM events using 24 bipartite primer pairs. (a−j) Amplicons were produced from simulated DNA samples A to J. The peak numbers correspond to the respective amplification targets: (1) *ssIIb*, (2) MON1445, (3) *Sad1*, (4) GA21, (5) Pr-act, (6) MIR604, (7) *CP4-EPSPS*, (8) tNOS, (9) *CryIAb*, (10) *Lectin*, (11) MON88913, (12) FMV35S, (13) T25, (14) *Chy*, (15) NK603, (16) RRS, (17) OXY235, (18) MON531, (19) HN-1, (20) *Bar*, (21) TC1507, (22) Bt11, (23) RT73, and (24) Bt176.

advantages of MPIC compared with the actual multiplex methods (such as multiplex PCR and PCR-CGE) are as follows: (i) one universal primer pair for all different target DNA simultaneous amplification avoiding the numerous primer competition and interference; (ii) each target DNA molecule can be amplified in separate single microdroplet with high efficiency avoiding amplicon competition; (iii) the properties of the MPIC demonstrated on GMO samples are not limited to this domain of diagnostics and should allow the approach to be applicable in various DNA-based diagnostics fields.

■ ASSOCIATED CONTENT

Supporting Information. Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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