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MACRO: A Combined Microchip-PCR and Microarray System for High-Throughput Monitoring of Genetically Modified Organisms

Ning Shao,[†,§,∥] Shi-Meng Jiang,[‡] Miao Zhang,[‡] Jing Wang,[‡] Shu-Juan Guo,[†,§,∥] Yang Li,[†,§,∥] He-Wei Jiang,[†,§,∥] Cheng-Xi Liu,[†,§,∥] Da-Bing Zhang,[‡] Li-Tao Yang,*[,‡] and Sheng-Ce Tao*[,†,§,∥]

[†]Shanghai Center for Systems Biomedicine, Key Laboratory of Systems Biomedicine (Ministry of Education), Shanghai Jiao Tong University, Shanghai 200240, China

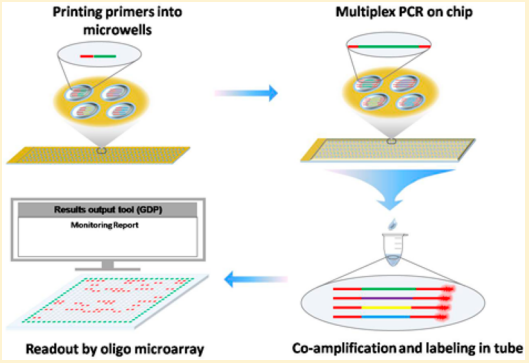
[‡]Collaborative Innovation Center for Biosafety of GMOs, National Center for the Molecular Characterization of Genetically Modified Organisms, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China

[§]State Key Laboratory of Oncogenes and Related Genes, Shanghai 200240, China

[∥]School of Biomedical Engineering, Shanghai Jiao Tong University, Shanghai 200240, China

Supporting Information

ABSTRACT: The monitoring of genetically modified organisms (GMOs) is a primary step of GMO regulation. However, there is presently a lack of effective and high-throughput methodologies for specifically and sensitively monitoring most of the commercialized GMOs. Herein, we developed a multiplex amplification on a chip with readout on an oligo microarray (MACRO) system specifically for convenient GMO monitoring. This system is composed of a microchip for multiplex amplification and an oligo microarray for the readout of multiple amplicons, containing a total of 91 targets (18 universal elements, 20 exogenous genes, 45 events, and 8 endogenous reference genes) that covers 97.1% of all GM events that have been commercialized up to 2012. We demonstrate that the specificity of MACRO is ~100%, with a limit of detection (LOD) that is suitable for real-world applications. Moreover, the results obtained of simulated complex samples and blind samples with MACRO were 100% consistent with expectations and the results of independently performed real-time PCRs, respectively. Thus, we believe MACRO is the first system that can be applied for effectively monitoring the majority of the commercialized GMOs in a single test.



In the past decade, we have witnessed an explosive growth in the planting and consuming of genetically modified organisms (GMOs). By the end of 2012, a total of 170 million hectares of genetically modified (GM) crops were planted in 28 countries, 319 GM events of 25 species were approved, and 2 497 regulatory approvals were issued in 59 countries.[1] Meanwhile, the concerns and debates about the safety of GMOs, specifically about their unknown impact on the environment and human health, remain widely prevalent issues.[2,3] Additionally, despite strict regulations,[4−7] unauthorized GMOs (UGMs) have been occasionally released into the market. There is thus an urgent need for high capacity monitoring of GMOs.[8,9]

An ideal solution for GMO monitoring would be a single test that is specific, sensitive, cost-effective, and capable of covering the majority of the GM events,[5,7] To date, a wide variety of methodologies have been developed for the monitoring of GM contents,[7] but these fall short of the ideal goal as, generally, they are effective for only a limited number of samples. Of these methodologies though, polymerase chain reaction (PCR) is currently the most widely applied technique owing to its high sensitivity, low cost, and easy handling.[10]

A PCR-based GMO monitoring methodology is usually composed of three key steps: namely, DNA extraction, target DNA fragment amplification, and qualitative or quantitative readout of the amplified target DNA fragment. There are a variety of PCR-based methodologies for high-throughput monitoring of GM contents, such as multiplex PCR,[11−13] ready-to-use 96/384 well PCR plates,[14] DNA microarray,[15−17] liquid beads array,[18] and droplet PCR.[19] Multiplex PCR, which can simultaneously amplify multiple DNA targets in one reaction and thus enable simultaneous monitoring of multiple GM contents, is the basic strategy for multiple target DNA fragment amplification. DNA microarrays, which include up to several millions of different oligo probes for identifying many targets on a single microarray, are usually the first choice for readout of the enriched target DNAs. As such, many of the high-throughput methods were established based on multiplex PCR coupled with different DNA microarrays,[11,15,16] although multiplex PCR has also been combined with capillary gel

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electrophoresis (CGE),[20−22] liquid beads array,[18] or HPLC.[23] However, because of the intrinsic interference and competition between primers, the number of target DNA fragments that can be simultaneously amplified in a traditional multiplex PCR is very limited.[13] For this reason, some have modified or substituted the target amplification step with other nucleic acid amplification techniques, including multiplex quantitative DNA array-based PCR (MQDA-PCR),[24] NASBA implemented microarray analysis (NAIMA),[25] padlock probe ligation in combination with microarray detection (PPLMD),[26] and SNPlex assay.[27] Droplet PCR, which has been developed and successfully employed for randomly enriching millions of different sequences in next-generation sequencing, might be a potential solution for high-throughput target DNA enrichment.[19,28,29] The disadvantage of droplet PCR though lies in the sophistication of generating designated droplets. Nonetheless, although showing high-throughput potential, none of these methodologies is capable of efficient monitoring of more than 50 GM contents simultaneously in a single test, not to mention being capable of readily adapting to the introduction of new GM events. To date, the maximum number of enriched DNA targets that has been successfully decoded simultaneously is 47.[14]

To achieve the goal of full coverage monitoring of commercialized GMOs, herein we developed a combined microarray system, taking advantage of a highly efficient multiplex chip-PCR strategy (MPH&HPM)[30] that we have previously developed. This MACRO system is composed of a PCR chip for the multiplex amplification of 91 target DNA fragments and an oligo microarray for the readout of the amplified DNA fragments. With this system, we show a GMO event coverage of 97% and a limit of detection (LOD) of MACRO that is suitable for practical applications. The MACRO results of simulated complex samples and blind samples were 100% consistent with expectations and the results of independently performed real-time PCRs, respectively. To our knowledge, MACRO is the first system that could be applied to effectively monitor the majority of the commercialized GMOs in a single test.

EXPERIMENTAL SECTION

Plant Materials and DNA Isolation. All the plant materials of the GM events were genially offered by several organizations and companies, the details are listed in Supplementary Table 1 in the Supporting Information. Samples of nontransgenic plant materials were obtained from local markets in Shanghai, China, and analyzed for the absence of any GMO ingredients. The blind samples were kindly supplied by Shanghai Entry-Exit Inspection and Quarantine Bureau (SHCIQ). Genomic DNA of all aforementioned plant materials were extracted and purified using a commercial DNA extraction kit (Shanghai Ruifeng Agrotech Co., Ltd., Shanghai, China). The concentration and quality of the DNA samples were evaluated by a NanoDrop 1000 UV−vis spectrophotometer (NanoDrop Technologies, LLC, Wilmington, DE) and 1% agarose gel electrophoresis.

Chemicals and Reagents. Unless otherwise stated, all the chemicals were purchased from Sigma-Aldrich, (Missouri). EasyTaq DNA polymerase and dNTPs were purchased from TransGen Biotech (Beijing, China); 50× Denhardt's solution was purchased from Sangon Biotech (Shanghai, China). Piranha solution (H_2SO_4/H_2O_2 = 2: 1, v/v); hydrofluoric acid etching solution ($HF/HNO_3/H_2O$ = 20:14:66, v/v).

Primers and Probes. All the specific primer pairs and probes used in this study were designed using Primer Premier 6.0 software (PREMIER Biosoft International, CA). The qualified primers of 105 targets and the final 91 optimal primers and probes were listed in Supplementary Table 2 in the Supporting Information. All the primers had similar TM value allowing a 3 °C difference and similar length of 22−28 nt. Sizes of the corresponding amplicons were between 150 and 350 bp. Each forward primer was modified by adding a 5′ universal sequence (5′-TCACTTGCTTCCGTTGTCC-3′), and each reverse primer was modified by adding another 5′ universal sequence (5′-TGATGCAGGTCCCAATTAGG-3′). The universal primer sequences were artificially generated and BLAST checked to avoid cross-reaction with any of the plant genomic DNA that may involved in this studied. . Similar melting temperature (65 °C ± 5 °C) and probe length (26 nt ±4 nt) were also set for designing the probes. All the primers and probes were synthesized and purchased from Invitrogen Co., Ltd. (Shanghai, China).

Microwell Chip-PCR Amplification. The chip fabrication steps were performed as described by Li et al.[30] The 5 μM specific primer pairs were dissolved in 0.65% (w/v) chitosan (MW = 750 K_d) solution with 0.15 μM Hex-labeled oligos, and then the specific primer pairs were printed into microwells individually by a SmartArray48 Microarray Spotter (CapitalBio, Beijing, China) at a humidity of 60−70%. Chip PCR was performed on an in situ thermal cycler (Eastwin Biotech, China) with 50 μL of reaction mixture, including 1× EasyTaq PCR buffer, 250 nM dNTP, 8 unit of EasyTaq DNA polymerase, 100 μg of BSA, and 20 ng of genomic DNA. The PCR program was set as follows: 94 °C for 5 min; 5 cycles of 94 °C for 30 s, 53 °C for 30 s and 72 °C for 1 min; 20 cycles of 94 °C for 30 s, 68 °C for 90 s; 72 °C for 5 min. After the PCR amplification, the chip-PCR products were collected from microwells by centrifuging the chip (1200*g*, 5 min) in a 15 mL tube filled with mineral oil. The recovered chip PCR products were then diluted in 50 μL of ddH_2O, 2 μL of which was used as template for second round universal PCR.

Second Round Universal PCR. The second round PCR was performed in a 200 μL tube in a regular thermal cycler. Each reaction mixture contained the following reagents: 1 × EasyTaq PCR buffer, 250 nM dNTP, 200 nM each universal primers, 4 unit of EasyTaq DNA polymerase, 50 μg of BSA, and 2 μL of diluted chip PCR products. The PCR program was set as follows: 94 °C for 10 min; 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min; 72 °C for 5 min.

Readout on Oligo Microarray. Oligo probes were dissolved in 50% DMSO with a final concentration of 10 μM and printed on aldehyde-modified glass slides (CapitalBio, Beijing, China) by a SmartArray48 Microarray Spotter. The microarrays for probe screening were printed with two spots per probe, and the final optimal microarrays for practical sample test were printed with four spots per probe. Hex-labeled oligos were spotted around the probe area as a landmarker. A total if 12 identical subarrays were printed on each microarray. The slides were incubated at 37 °C in a humid chamber overnight, then soaked in a freshly prepared 0.3% $NaBH_4$ solution (ethanol/PBS = 15:45, v/v, as solvent) for 5 min to block the free aldehyde groups, followed by washing with 0.2% SDS for 5 min. The dried slides were preserved and desiccated at room temperature in dark.

The PCR products were hybridized with the oligo microarray as follows. A volume of 25 μL of hybridization mixture was

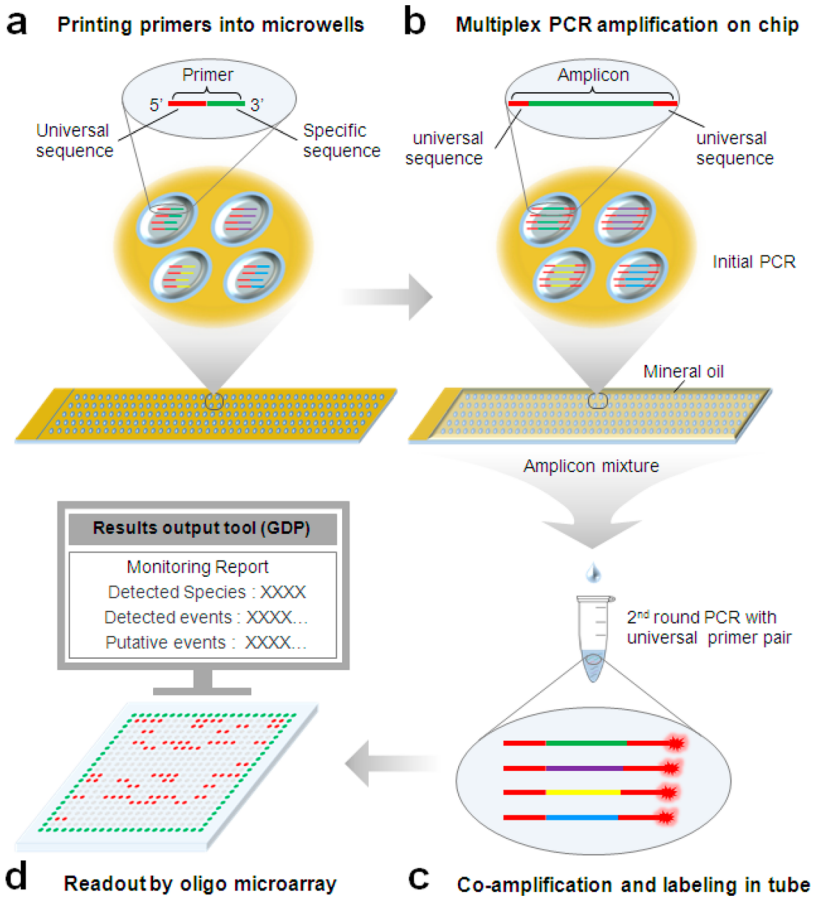

Figure 1. Schematic diagram of the MACRO system. (a) Specific primer pairs with universal 5′-tags were loaded to individual microwells by microarray printing. (b) PCR mixture was loaded into the microwells and covered with mineral oil immediately. The initial on-chip PCR was performed on an *in situ* thermal cycler. (c) Initial PCR products were collected and diluted. The diluted PCR products were used as templates for the second round PCR. The second round PCR was performed in tube with a pair of 5′ Cy5 tagged universal primers. (d) The fluorescent labeled PCR products were decoded by an oligo microarray, and the microarray results were processed by a GM contents Determined Program (GDP) for generating the final report.

prepared for each reaction, which contained 4 μL of Cy5-labeled PCR products, 5× Denhardt's reagent, 2× SSC, and 0.2% (w/v) SDS. The hybridization mixture was denaturated for 5 min at 99 °C and chilled on ice immediately for 3 min. The hybridization was performed at 55 °C in a humidity chamber for 2 h followed by washing at 42 °C by 0.2× SSC/0.2% SDS and 0.2× SDS. After drying, the microarray results were recorded by a GenePix 4200A scanner (Molecular Devices, CA). The signal to noise ratio (SNR) was defined as the ratio of the median value of the foreground signal to median value of the background signal. The cutoff to determine the positive spots was set as SNR > 3.5. All the SNR values of the positive spots were shown in Supplementary Table 3a–d in the Supporting Information.

GM Events Determination Program (GDP). To accurately transfer the microarray hybrid signal to the final results, an automatic GM events determination program (GDP) was developed. GDP was developed based on the knowledge of known GM events, the contained GM contents (elements, transgenes, event-specific sequences), and species endogenous reference genes (Supplementary Table 4 in the Supporting Information). In GDP, the results of the event level were deduced according to the detected signals of the species endogenous reference gene, event-specific sequence, transgenes, and universal elements. For example, the signal of the species endogenous reference gene was first used to confirm the origin of a sample. If the signal of soybean endogenous reference gene was negative, all the GM soybean events will be excluded for further analysis. The signal of event-specific sequence was then used to determine the specific GM event. At last, the signals of transgenes and universal elements were used to speculate the potential GM events besides the confirmed GM events, and the probability of the speculated GM events was also included according to the known information of GM contents. The GDP is publically available (http://202.120.45.92/GMO_DETECT/index.php).

RESULTS

Principle of the MACRO System. The MACRO system is modified from the MPH&HPM strategy that we have recently developed.[30] There are four major steps of the MACRO system (Figure 1): the preparation of the microchip for PCR, initial PCR on the microchip, amplification and labeling of the PCR products using universal primers, and finally decoding the PCR products on an oligo microarray. Briefly, in the first step, a hydrophobically and hydrophilically patterned microwell chip with 200 microwells and a volume of ~50 nL/well is manufactured and the specific primer pairs with universal 5′-tags are printed into individual microwells (Figure 1a). The PCR mix containing the DNA template is then loaded into the

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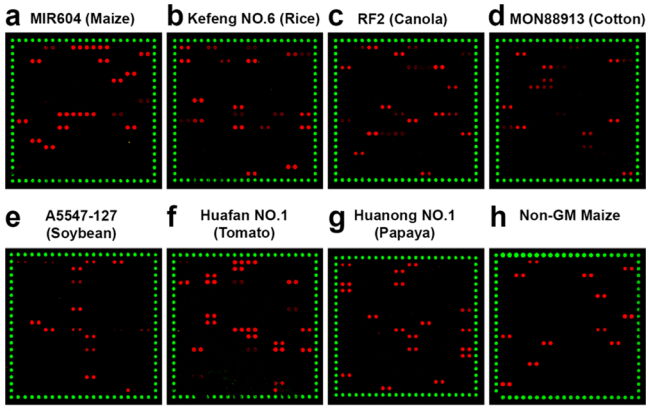
microwells making use of the hydrophobic and hydrophilic pattern on the microchip. The initial chip-PCR amplification is carried out on an *in situ* thermal cycler (Figure 1b). By employing the diluted product of the initial PCR as a template, the second round of PCR amplification is carried out in a tube, using a 5′ Cy5-conjugated downstream universal primer and a nonlabeled upstream universal primer (Figure 1c). The fluorescently labeled PCR products are then hybridized onto a GMO-specific DNA microarray and decoded using an appropriate computer software (Figure 1d).

Preparation of the Primer Pairs and Probes. To obtain the maximum coverage for monitoring GMOs, we focused on the top eight most frequently planted GM crops (maize, soybean, canola, cotton, rice, tomato, papaya, and sugar beet), which account for >99.5% of the planted area with GM crops.[1] We collected 51 GM elements (i.e., 23 universal elements and 28 exogenous genes), 57 GM events, and 8 endogenous reference genes of those eight species (*zSSIIb* for maize, *lectin* for soybean, *HMG 1/Y* for canola, *Sad1* for cotton, *SPS* for rice, *Lat52* for tomato, *CHY* for papaya, and *GluA* for sugar beet), for a total of116 potential targets from the GMO Detection Method Database (GMDD)[31] and NCBI database (as shown in Supplementary Figure 1 in the Supporting Information). Specific primers were then designed for these 116 potential targets.

To ensure the efficient amplification of all the potential targets on a single microchip, all of the primer pairs were designed with the following parameters: similar melting temperature (60 ± 3 °C), length of primers (25 ± 3 nt), and length of the PCR products (250 ± 100 bp). The primers were then subjected to BLAST analysis as a first *in-silico* specificity check. To experimentally verify the specificity and sensitivity, genomic DNAs of different GM events which contain these potential targets were employed as templates. All of the primer pairs were tested using identical thermal cycling conditions by both conventional PCRs and microchip PCRs (Supplementary Figure 2 in the Supporting Information). A set of 105 primer pairs targeting 42 GM elements (i.e., 22 universal elements, 20 exogenous genes), 55 GM events, and 8 endogenous reference genes were experimentally verified (Supplementary Table 2 in the Supporting Information).

To efficiently decode the products of the microchip PCR, a DNA microarray was designed according to the sequences of the amplicons from 105 primer pairs. To ensure efficient hybridization of all the targets on the DNA microarray, probes were designed with similar melting temperature (65 ± 5 °C) and length (26 ± 4 nt). Two to four probes were designed for each universal element, exogenous gene, and endogenous reference gene, while only one probe was designed for each GM event because of the high specificity of the event-specific junction between the transgene and the neighboring host DNA (Supplementary Figure 1 in the Supporting Information). The probes were synthesized and printed in duplicate on an aldehyde-derivatized glass slide to fabricate the DNA microarray. The DNA microarray was then individually hybridized with fluorescent labeled PCR products of the 105 targets (Supplementary Figure 3 in the Supporting Information). To determine the positive spots with high confidence, the signal-to-noise ratio (SNR) was set as >3.5. After the removal of the probes with a weak and/or cross hybridization signal, a final set of 91 probes, corresponding to 38 GM elements (i.e., 18 universal elements -9 promoters, 6 terminators, and 3 marker genes-and 20 exogenous genes), 45 event-specific sequences from eight types of crop (maize, soybean, canola, cotton, rice, tomato, papaya, and sugar beet), and 8 endogenous reference genes of the above 8 species, were determined to further establish the MACRO system (Supplementary Table 2 in the Supporting Information). The 91 verified primer pairs were then used to construct the microchip for multiplex PCR, and the 91 probes were printed along with a Hex labeled landmark to produce the DNA microarray.

High Specificity of the MACRO System. To evaluate the specificity of the MACRO system, powder samples of 8 seeds (maize MIR604, cotton MON88913, canola RF2, soybean A5547, rice Kefeng No.6, tomato Huafan No.1, papaya Huanong No.1, and a non-GM maize) were tested in duplicates (Figure 2 and Supplementary Table 3a in the Supporting Information). The results of the 8 samples obtained from the MACRO system were then compared with the GM content records from the GMDD and GM Crop Database (http://cera-gmc.org/index.php?action=gm_crop_database). For example, for the GM maize MIR604, positive signals were obtained for probes 5, 6, 7, 20, 22, 27, 49, and 90, which corresponded to GM contents: *pmi*, *T-nos*, *mcry3A*, *P-Ubi*, *P-LEG1*, *T-LEG1*, event MIR604, and endogenous reference gene *zSSIIb*, respectively (Figure 2a). With GM rice Kefeng No. 6, the target DNAs of *P-CaMV35S*, *bla*, *T-nos*, *P-ract1*, *P-Ubi*, *P-Orbcs*, *cry1Ab/c*, *CpT1*, event Kefeng No. 6, and endogenous reference gene *SPS* were detected (Figure 2b). Similarly, with GM canola RF2, *bar*, *T-nos*, *nptII*, *T-E9*, *P-nos*, *T-g7*, *P-TA29*, *barstar*, eventRF2, and endogenous reference gene *HMG I/Y* were



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Samples	Detected contents (Red: false positives)	FPR	FNR
MIR604	pmi, T-nos, mcry3A, P-Ubi, P-LEG1, T-LEG1, MIR604, zSSIIb	0	0
Kefeng NO. 6	P-CaMV35S, bla, T-nos, P-ract1, P-Ubi, P-Orbcs, cry1Ab/c, CpT1, Kefeng No.6, Sps	0	0
RF2	T-nos, nptII, P-nos, T-g7, P-TA29, barstar, RF2, Hmg1/y	0	0
MON88913	CP4 epsps, P-CMoVb, T-E9, P-FMV/TSF1, MON89034, MON88913, Sad1	0.55%	0
A5547-127	P-CaMV35S, bla, T-nos, Pat, A5547-127, Lectin	0.55%	0
Huafan NO. 1	P-CaMV35S, T-nos, nptII, Anti-EFE, P-nos, GUS, Huafan NO.1, Lat52	0	0
Huanong NO.1	P-CaMV35S, T-nos, nptII, Nib, Huanong NO.1, CHY	0	0
Non-GM Maize	P-Ubi, P-LEG1, T-LEG1, zSSIIb	0	0

Figure 2. The specificity of the MACRO system. (a–h) Microarray testing results of MIR604 (a), MON88913 (b), RF2 (c), A5547 (d), Kefeng No. 6 (e), Huafan No. 1 (f), Huanong No. 1, (g), and non-GM maize (h). (i) Summary of the specificity tests. FPR, false positive rate; FNR, false negative rate.

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detected (Figure 2c), and with the non-GM maize sample, only the *P-Ubi*, *P-LEG1*, *T-LEG1,* and the *zSSIIb* gene of the maize genome were detected (Figure 2h). In summary, all of the results from two replicates showed that all of the expected GM contents were successfully detected in the eight tested samples (Figure 2i), except for two unexpected signals of *T-nos* in soybean A5547-127 and event MON89034 in the cotton MON88913sample, respectively (Figure 2f).

Sensitivity of the MACRO System Is Suitable for Routine GMO Monitoring. To measure the sensitivity of the MACRO system, serially diluted genomic DNAs (i.e., 10, 5, and 1 copy haploid genome/microwell) were tested. Five GM event samples were studied (i.e., maize MIR604, cotton MON88913, canola RF2, soybean A5547-127, and rice KeFeng No. 6). All of the experiments were performed with two duplicates. As shown in Figure 3 and Supplementary Table 3b in the Supporting Information, all of the expected GM contents were successfully detected at a sample concentration of 10 copies/microwell and a sensitivity of 5 copies/microwell was achieved for the samples MON88913 and A5547-127. Moreover, expected signals were partially detected in each test with sample dilution of 1 copy/microwell, such as elements *CP4 epsps* and event MON 88913 that were observed in the test of cotton MON 88913 and elements *T-nos*, *mcry3A*, *P-LEG1*, *T-LEG1*, and event MIR604 that were observed in the test of maize MIR604. These results suggest that the LOD of MACRO is ≤10 copies of target GM DNA per microwell. Because the volume of the microwell on the PCR microchip is ∼50 nL, the sensitivity of the MACRO system can thus be estimated as ∼200 copies/μL, which corresponds to ∼0.56 ng/μL of maize genomic DNA, ∼0.23 ng/μL of soybean genomic DNA, and ∼0.09 ng/μL of rice genomic DNA.[32] When handling GMO samples, this concentration (∼200 copies/μL) of genomic DNA can be easily achieved by conventional DNA extraction methods.[33] These results suggested that the sensitivity of the MACRO system is suitable for routine GMO monitoring[33,34] after further validation of the MACRO system with processed samples.

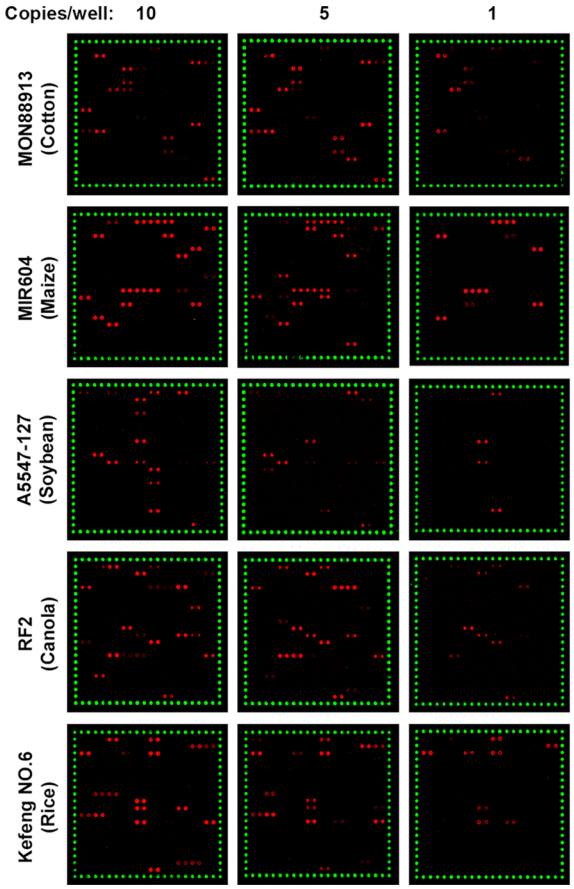

Figure 3. Sensitivity of the MACRO system. Five samples with simple GM contents were applied, i.e., MIR604, MON88913, RF2, A5547-127, and KeFeng No. 6. Serial diluted GM DNAs, i.e., 10, 5, and 1 copies of haploid genome/microwell were tested.

High Accuracy of MACRO in the Analysis of Simulated and Blind Samples. To test the performance of the MACRO system for GMO monitoring, two types of practical samples were prepared and analyzed. The first type was a simulated sample prepared by mixing multiple GM events with known concentrations. The six simulated samples were S1 (BT176, BT11, 59122, 3272, GA21, MIR604, and MON810), S2 (MON863, MON88017, MON89034, NK603, TC1507, and BVLA430101), S3 (15985, 531, 88913, and LLCotton25), S4 (RT73, OXY235, RF2, MS8, T45, and Topas 19-2), S5 (A2704-12, A5547-127, GTS40-3-2, and MON89788) and S6(TT51-1, Kefeng No. 6, T2A-1, KMD1, Bar68-1, t1c-19, and RJ5). To prepare these samples, all the required components were mixed with equal quantity. The second type of consisted of real samples collected by the Shanghai Entry-Exit Inspection and Quarantine Bureau (SHCIQ) from the Shanghai port, including five samples of soybean seeds (2527, 6727, 2958, 4527, 8179) and five samples of maize seeds (2002, 5216, 5215, 51624, 1-79227). There was no prior knowledge of the GMO contents of SHCIQ samples. All the tests were performed in duplicates.

The MACRO results of the simulated samples are shown in Figure 4 and Supplementary Table 3c in the Supporting Information. Positive signals of probes 1, 2, 3, 5, 6, 7, 8, 9, 10, 15, 19, 20, 22, 27, 40, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, and 90 were observed in sample S1, which correspond to elements *P-CaMV35S*, *bar*, *bla*, *pmi*, *T-nos*, *mcry3A*, *pat*, *cry34Ab1*, *cry35Ab1*, *nptII*, *P-ract1*, *P-Ubi*, *P-LEG1*, *T-LEG1*, *amy797E*, events BT176, BT11, 59122, 3272, GA21, MIR604, MON810 and endogenous reference gene *zSSIIb*, respectively. Similarly, GM canola events RT73, OXY235, RF2, MS8, T45, and Topas19-2 were detected in sample S4, and the presence of GM rice events TT51-1, Kefeng No. 6, T2A-1, KMD1, Bar68-1, t1c-19, and RJ5 were observed in sample S6. According to the GMDD and GM Crop database, the MACRO results of the simulated samples show nearly 100% of the expected results, with the exceptions of one element *T-pinII* (probe 4) absent in the results of sample S1 and two other false positive signals (*P-Orbcs* and MON89034) obtained in S2 and S3, respectively. For many DNA microarray applications, the false positive/negative rate is usually defined as the ratio of the number of false positive/negative probes in one test to the number of total probes on the microarray.[35] Using this definition, the false negative rate of S1 is 1.1%, and the false positive rate of samples S2 and S3 is each 0.55% (Figure 4).

Double blind experiments using the SHCIQ samples were performed to further test the practicability of the MACRO system. As shown in Table 1, different numbers of GM events were detected in individual SHCIQ samples (Supplementary Table 3d in the Supporting Information). For example, the GM events 59122, GA21, MON 810, MON 88017, MON 89034,

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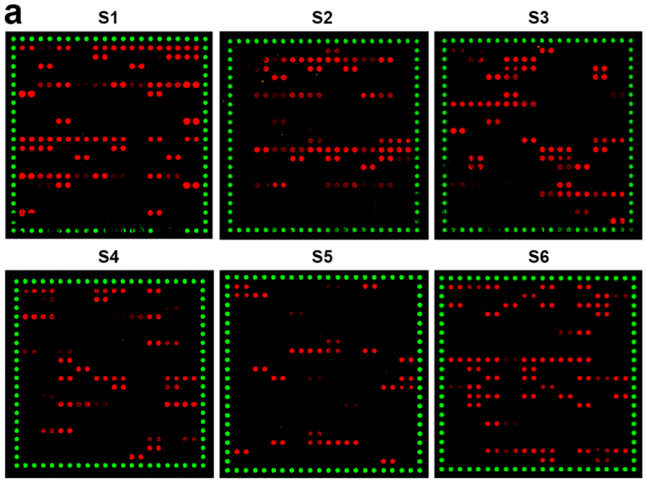

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Samples	Detected Events	Detected / Contained	FPR	FNR
S1	BT176, BT11, 59122, 3272, GA21, MIR604, MON810	7 / 7	0	1.1%
S2	MON863, MON88017, MON89034, NK603, TC1507, BVLA430101	6 / 6	0.55%	0
S3	15985, 531, MON88913, LLCotton25, MON89034	5 / 4	0.55%	0
S4	RT73, OXY235, RF2, MS8, T45, Topas 19-2	6 / 6	0	0
S5	A2704-12, A5547-127, GTS40-3-2, MON89788	4 / 4	0	0
S6	TT51-1, Kefeng NO.6, T2A-1, KMD1, Bar68-1, t1c-19, RJ5	7 / 7	0	0

Figure 4. Monitoring results of the simulated samples. (a) Microarray results of the six simulated samples, i.e., S1−S6. (b) Summary of the results of the simulated samples. Detected/Contained, the number of the detected events to the number of contained events. Red, false positives. FPR/FNR, false positive/negative rate.

NK603, and TC1507 were detected in sample 2002, and only one GM event GTS 40-3-2 was detected in sample 2527. To verify these results, independent tests were performed in SHCIQ on these samples using real-time PCR (Supplementary Tables 5 and 6 in the Supporting Information). The detected GM events were completely consistent between the two independent tests (Table 1). Besides the GM events, GM elements, exogenous genes, and endogenous reference genes were also correctly detected by the MACRO system, based on expectations from the GMDD and GM Crop Database.

DISCUSSION

There are presently more than 300 approved GM events, and the number of new approvals is rapidly increasing every year. Thus, an effective methodology that can comprehensively monitor many GM contents at the same time is urgently needed. To this end, we have developed the MACRO system by integrating a microchip-based multiplex PCR for GMO target amplification and a GMO specific DNA microarray for GMO event decoding. To our knowledge, the MACRO system is the most comprehensive GMO monitoring system presently designed, with a capacity of monitoring 91 GM contents simultaneously in a single test (except for processed materials samples), providing coverage of more than 97% of all the commercialized GM events.

Table 1. Summary of the Double-Blinded Testing Results of SHCIQ Samples[a]

ID	results from our method		results from real-time PCR	
	true positive signals	detected events	detected events	
2002	1,6,8,9,10,11,12,13,14,15,16,17,19,20,22,27,29,44,45,48,50,51,53,54,55,57	59122, GA21, MON810, MON88017, MON89034, NK603, TC1507	59122, GA21, MON810, MON88017, MON89034, NK603, TC1507	
5216	1,4,6,8,9,10,11,12,13,16,19,20,22,27,29,45,48,50,51,53,57,90	59122, GA21, MON810, MON88017, TC1507	59122, GA21, MON810, MON88017, TC1507	
5215	1,4,6,8,9,10,11,12,13,14,16,17,19,20,22,27,29,45,50,51,53,54, 55,90	MON 810, 59122, MON88017, MON89034, NK603	MON810, 59122, MON88017, MON89034, NK603	
51624	1,4,5,6,7,8,9,10,11,12,13,14,16,17,19,20,22,24,27,29,43,44,45,48,49,50,51,53,54,55,57,90	BT11,59122,GA21, MIR604,MON810, MON88017,MON89034,NK603, TC1507	BT11, 59122, GA21, MIR604, MON810, MON88017, MON89034, NK603, TC1507	
1-79227	1,6,8,9,10,11,12,13,14,16,17,19,20,22,27,29,44,45,48,50,51,53, 54, 55,57,90	59122,GA21,MON810, MON88017,MON89034, NK603,TC1507	59122, GA21, MON810, MON88017, MON89034, NK603, TC1507	
2527	1,6,11,15,39,76,77,92	GTS 40-3-2	GTS 40-3-2	
6727	1,6,11,15,76,77,92	GTS 40-3-2	GTS 40-3-2	
2958	1,6,11,12,15,29,34,76,77,78,92	GTS 40-3-2, MON89788	GTS 40-3-2, MON89788	
4527	1,6,8,11,12,15,29,34,74,76,77,78, 92	A2704−12, GTS 40-3-2, MON89788	A2704-12, GTS 40-3-2, MON89788	
8179	1,6,11,12,15,29,34,76,77,78,92	GTS 40-3-2, MON89788	GTS 40-3-2, MON89788	

[a]The true signals were signals of elements, events, and endogenous reference genes, which were detected by the MACRO system and real-time PCR independently.

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Besides its high specificity and sufficient sensitivity, the MACRO system possesses several significant advantages over other GMO monitoring methodologies. First, in its current version, of all present methodologies, the MACRO system covers the highest number of GMO related DNA targets. There are several high-throughput methodologies for GMO monitoring, such as MQDA-PCR,[24] multiplex PCR,[11,12] microarray,[15,16] MPIC,[19] and ready-to-use 96/384 well PCR plates.[14] Among these methodologies, Cottenet et al. have developed a ready-to-use 384 well PCR plates, which can monitor 47 GMO targets, represents the highest number of DNA targets prior to MACRO.[14] However, strictly speaking, this method is not a real multiplex PCR method because PCR mix still needs to be added to each well individually, which may greatly increase labor burden. On the contrary, the MACRO system can simultaneously enrich and detect 91 DNA targets in a single test.

Second, GM contents can be monitored on different levels (screen, gene specific, event specific, and endogenous reference gene) in a single MACRO test, and the combination of the results from different levels could enhance the accuracy of the GMO monitoring. Generally, there are several GMO monitoring strategies, such as species identification, screening of universal elements and screening for GMO constructs, exogenous gene-specific detection, and event-specific detection.[36] Usually, traditional PCR methods could be applied to analyze one level a time or detect several levels at a time by a combined strategy; however, the targets that could be analyzed simultaneously in a combined strategy is usually limited. While the 91 targets that MACRO covers could be divided into four types, such as universal elements, exogenous transgenes, event-specific sequence, and endogenous reference genes. Thus the final results of a MACRO test can cover most of above levels of information at once. Because the results of these levels must be consistent, as in an ordinary bar-coding system, the information from different levels could serve as error correcting codes for each other and thereby enhance the accuracy and specificity of the MACRO system.

Third, the MACRO system is well suited for the monitoring of future GMOs. The current commercialized GM events still belong to second generation GMO types, which are characterized by the introduction of insect-resistant and herbicide-tolerant exogenous genes with the help of universal exogenous elements into the host plant genome. The 18 universal elements and the 20 exogenous genes that the MACRO system covers are frequently used in the development of many GMOs. Initially, the MACRO system was designed based on the 2011 GMDD and GM crop databases, and 96.9% of the known GMOs by 2011 could be directly or indirectly monitored. However, the number of GMO events increased in the two main databases from 195 by 2011 to 243 by 2012. Surprisingly, even though designed according to the 2011 GMO info, the MACRO system was still compatible with the 2012 events, exhibiting an even slightly higher coverage of 97.1% in 2012 (Table 2). This result strongly suggests that the MACRO system in its current version would sustain a high coverage in global GMOs monitoring in the next few years, unless new generation GM events are introduced with totally new GM elements or cisgenes.

Fourthly, the MACRO system may be suitable for UGM monitoring. The MACRO system has the most comprehensive coverage of GMO events and is also capable of monitoring GMOs at several different levels in a single test (except for processed materials samples). Thus, detailed GM contents of a sample could be easily obtained even if it is an UGM sample. For example, if the unexpected elements, such as *CaMV35s* promoter, NOS terminator, and NPTII marker genes, etc., were detected in the known GM events sample, the sample must contain but is not limited to the GM contents of known events and the unexpected GM contents might be come from the UGM.

Table 2. Coverage of the MACRO Platform for the Known GMOs[a]

	events number in 2011			events number in 2012		
species	cover	total	coverage (%)	cover	total	coverage (%)
maize	55	57	96.5	80	82	97.6
rice	12	12	100	12	12	100
canola	24	24	100	26	26	100
soybean	17	21	81.0	20	24	83.3
cotton	26	26	100	34	34	100
tomato	11	11	100	14	14	100
sugar beet	7	7	100	7	7	100
papaya	3	3	100	3	3	100
others	34	34	100	40	41	97.6
total	189	195	96.9	236	243	97.1

[a]For each species, "cover" and "total" represent the number of events covered by MACRO system and the total number of commercialized events, respectively. "Coverage" was calculated by the ratio of "cover" to "total".

Fifthly, the MACRO system is an open system with good flexibility and expandability. The MACRO system is composed of two chips/microarrays: the microwell chip for PCR and the DNA microarray for decoding. Because both the primer pairs printed on the microwell chip and the probes immobilized on the DNA microarray are physically isolated, there is little interference among the primer pairs during PCR amplification and among the probes during hybridization. Thus a primer pair or a probe could be flexibly removed or added and enable the upgrade of the monitoring scope to include newly introduced targets on both of the two microarrays. Another level of flexibility lies in the independence of the PCR microchip and the DNA microarray, both of which could be readily integrated with other techniques for efficient detection of GMOs.

To make the MACRO system more applicable for routine GMO monitoring, there is still room for improvements. Though the sensitivity of the current MACRO system is sufficient for routine GMO monitoring, it could be challenging for monitoring samples with low concentration of GM contents, such as less than 200 copies/μL. However, this challenge can be overcome by a simple DNA preconcentration procedure, which can easily increase the template DNA concentration by 10−100 times and thus increase the sensitivity of MACRO to as low as ∼20 copies/μL. The current MACRO system is an open system, and the different stages are handled manually to avoid cross contamination and to ensure a better reproducibility. Yet it could be sealed and integrated with a miniaturized thermal cycler and an automatic sampling and detection system. To take advantage of the high coverage of the MACRO system, an ideal strategy would be to analyze a sample first by the MACRO system to screening the possible GM content and then focus on the GM content by real-time PCR for confirmation and quantitative information. In the future, without the DNA microarray for decoding, the PCR microchip could also be integrated with an automatic fluorescence

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detector for direct or even quantitative GMO monitoring. To facilitate the final readout of the MACRO system, a computational tool which can directly transfer the microarray hybrid signal to GM contents is necessary. Bearing this in mind, we have developed an online tool, GDP (GM events Determination Program, see the Experimental Section) for rapidly reporting the MACRO results. In the GDP program, the final detection results at the event level can be analyzed by a computer, and the potential GM events can be identified according to a complex combination of different GM elements, transgenes, and species endogenous reference genes. With the help of GDP, it is possible to easily obtain the final GM events or potential GM events in a mixture of samples with high accuracy.

In summary, we have developed the MACRO system by effectively integrating a microchip-based PCR for multiplex GMO target amplification and a GMO specific DNA microarray for GMO event decoding. Our MACRO system can monitor 91 DNA targets in a single test, and it is so far the most comprehensive GMO monitoring system presently constructed with a high sensitivity and sufficient specificity. This system is capable of covering 97% of all 319 known GMO events,[1] and it could be flexibly updated whenever necessary. Because of its superior advantages over the existing GMO monitoring methodologies, we strongly believe that the MACRO system will be widely applied for future routine GMO monitoring and UGM inspection.

■ ASSOCIATED CONTENT

Supporting Information

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

■ AUTHOR INFORMATION

Corresponding Authors

*Phone: +86 21 34207174. E-mail: yylltt@sjtu.edu.cn.
*Phone: +86 21 34207069. E-mail: taosc@sjtu.edu.cn.

Author Contributions

The first two authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

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