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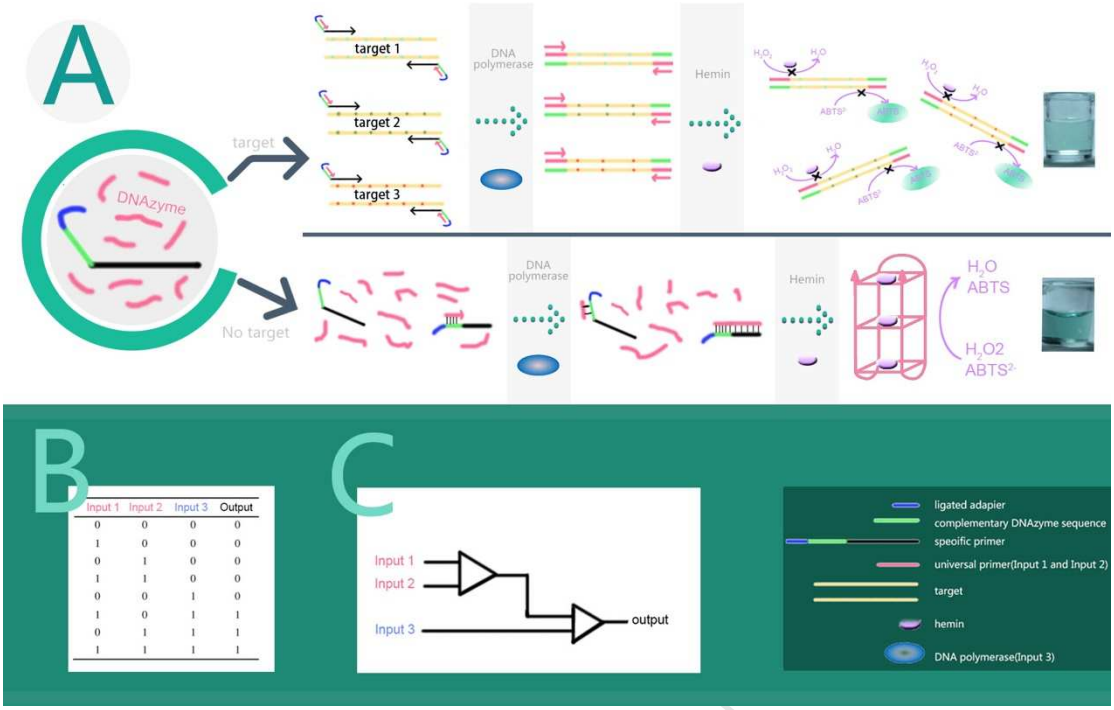
Graphical Abstracts



Colorimetric biosensor based on a DNAzyme primer and its application in logic gate operations for DNA screening

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ABSTRACT

A colorimetric biosensor for DNA screening was designed based on the conformational changes of the horseradish peroxidase (HRP)-mimicking DNAzyme. The scheme of DNA biosensing was designed based on the base pairing of DNAzyme sequence to inhibit the formation of HRP-mimicking hemin/G-quadruplex structures in the process of amplification. DNA could be amplified via the universal primer multiplex polymerase chain reaction (UP-M-PCR) and innovatively detected as color disappear in the reaction visible to the naked eye. The input of key factors and the output of optical characteristics in the reaction inspired the development of an OR logic gate operation for DNA detection. This biosensor overcomes self-inhibition and amplification disparity with the help of UP-M-PCR, thereby exhibiting high specificity and high-throughput without the requirement of gel analysis work. This biosensing system also presented 1% sensitivity and approximately 180 copy numbers in triplicate. The biosensor was used to screen elements from genetically modified organisms (GMOs) and covered more than 90% of all globally authorized events in the world. The designed colorimetric biosensor is a rapid, portable and versatile tool for nucleic acids detection and diagnosis in the field.

Key words: biosensors, colorimetric, nucleic acids, logic gate, genetically modified organisms

1. Introduction

Researchers have aimed to develop a universal molecular diagnostic method that is inspired by the replication and proliferation of living organisms. Molecular biologists followed the principle of self-reproduction to develop polymerase chain reaction (PCR). Over the past 20 years, the specificity and sensitivity of PCR has been improved through substantial research efforts. Various types of PCR technology, such as the multiple PCR, nested PCR, isothermal amplification, as well as the introduction of quantitative PCR (qPCR) and digital PCR (dPCR), have therefore been applied to detect, screen, and quantify targets [1-6]. However, PCR results have to be analyzed based on amplicon length, or the monitoring of fluorescent change, thus complicating analysis for point-of-care diagnosis [7-12].

Recent biotechnological developments, such as optical, electronic, and microgravimetric platforms, to offer a variety of alternative choices for interpreting result [13-15]. Visual detection, in which the presence of a target analyte is directly observed with the naked eye, has received increasing interest due to its simplicity and low cost [16, 17]. Of all these detection methods, DNAzyme provide flexibility, functional information and specific binding properties that are highly desirable for bioanalytical applications [18, 19]. DNAzyme are peroxidase-like complexes of nucleic acids, which are usually in the form of single-stranded DNA/RNA [20]. DNAzyme can fold into a stable and catalytically active G-quadruplex in the presence upon hemin binding. DNAzyme can catalyze some chemical and colorimetric reactions, such as the H_2O_2-mediated oxidation of 2,2'-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid ($ABTS^{2-}$) to $ABTS^{\bullet-}$. The reaction product, $ABTS^{\bullet-}$, had a maximal absorption of 420 nm and a green color that is visible to the naked eye [21]. The assembly of DNAzymes with nucleic acid sequences has allowed the development of systems for different targets, multiplexed sensing of analytes, and amplified sensing [22-24]. With the main advantages of

unmodified functional nucleic acid sequences and versatile sensing media, DNAzymes have shown to be very useful in target screening [25-27].

Molecular logic gate, a concept derived from conventional computer microprocessors, is a series of molecular computation that process chemical or physical "inputs" to generate "outputs" based on a set of molecular computation [28-31]. Recent development employed fluorescent, colorimetric, electrochemical, or electrochemiluminescent signals as outputs of logic gates, which benefit from its, sensitive readout, time-saving and somewhat optical convenience. Coupled with DNAzyme as inputs, logic gates could operate sequence hybridization or amplification based on G-quadruplex formation to facilitate fast and efficient target detection [28, 32, 33].

The rapid development of genetically modified organisms (GMOs) makes GMO detection technology a prerequisite for genetic management and manipulation [34, 35]. However, GMO detection usually depended on PCR-based methods that requires tedious gel electrophoresis or costly and complicated machines [36-40]. In the present study, we have successfully developed a DNAzyme-mediated colorimetric biosensor with logic gate applications for GMO screening. Three concrete DNA sequence motifs (CaMV 35S promoter, Nos terminator and EPSPS gene) present in transgenes were selected as screening targets for the presence of positive GMOs. The presence of target can be viewed as color disappearance from the reaction because of structural changes in DNAzyme and catalytic activity. The OR logic gate operates a simple demonstration of process of GMOs identification.

2. Experimental

2.1. Chemicals and materials

The multiplex PCR Assay Kit Ver. 2 used for UP-M-PCR was purchased from Clontech (Takara Co. Ltd., Dalian, China). Hemin, ABTS, H_2O_2 and citric acid were purchased from Aladdin Reagents (Shanghai, China). All primers were synthesized

and purified by Invitrogen Co. Ltd. (Shanghai, China). The primer sequences are shown in Table 1. All other reagents were of analytical grade and were used without further purification. Other primers used in this study are listed in Table S1.

Phosphate buffer (10 mM NaH_2PO_4/Na_2HPO_4, 100 mM KCl, 2 mM $MgCl_2$, and 0.003% (v) Triton X-100, pH = 7.0) was used as a colorimetric working buffer solution. Stock buffer (1.843 g $Na_2HPO_4.12H_2O$, 0.933 g citric acid, 100 mL, pH 4.0) was used as the enzymatic working buffer solution.

Materials used in our research included the GMO events MON87460, MON810, MON87701, MON87705, GT73, MON888302, H7-1, MON89788, J101 and TT51-1, which were kindly supplied by Monsanto Co. and Huazhong Agricultural University.

2.2. Extraction and evaluation of genomic DNA

Genomic DNA was extracted from 50 ng of a powdered sample using a DNA genome extraction kit (Food Safety Tech. Co, Ltd., Beijing, China). Isolated genomic DNA was diluted with 60 μL of ddH_2O. The concentration and purity were determined using a NanoDrop N2000 (Thermo Fisher Scientific Inc., Wilmington, USA) at both OD260 and OD280. Genomic DNA samples were diluted from 100% to 0.1% for further use in the experiments.

2.3. Biosening systems

Universal primer multiplex PCR (UP-M-PCR) was performed using the Multiplex PCR Assay Kit Ver. 2 in a 50 μL reaction volume containing 1×multiplex PCR buffer, 1× multiplex PCR enzyme mix, various amounts of universal primer, 0.04 μM each of CaMV 35S-F/R, NOS-F/R and CP4-EPSPS-F/R and various amounts of templates. ddH_2O was added to the reaction mixture to a final volume of 50 μL. The procedure was performed at 95 °C for 1 min, 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min for 40 cycles, and a holding step at 72 °C for 10 min. All the reactions were performed in a screw type of PCR-tube that was designed by our laboratory (China

Patent Application No. 201510293982.2) and produced by Beijing Food Safety Tech. Co., Ltd. (Beijing, China).

For optimization, a mixture of the universal primer and its derived primer with base deletion (1:1-1:5) was evaluated in the existing system. The total concentration of universal primer in the system was 0.4 μM.

After amplification, 80 μL of phosphate buffer and 10 μL of hemin (12 μM) were added to a 10 μL of post-PCR samples. The mixtures were incubated at 37 °C for 20 min. Subsequently, 30 μL stock buffer was added to the mixture, and photographs of the color development of the solutions were taken using a canon EOS 550D camera after 5 min of color development. Absorption was measured at 412 nm at room temperature with a Thermo Scientific Varioskan Flash (ThermoFisher Scientific Inc., DE, USA) and DCN Fluorescent Assay Visualizer (DCN Diagnostics, CA, USA).

2.4. Specificity and sensitivity

GMO events used for the specificity test were mixed as presented in Table 2. Sensitivity was defined as the lowest copy numbers of target in the reaction. A gradient dilution of GMO event mixture (100%, 50% 20%, 10%, 1%, and 0.1%) was prepared for the sensitivity test. The corresponding copy numbers were then calculated.

3. Results and Discussion

3.1. Principle of colorimetric detection

The main principle of the DNA biosensing process is illustrated in Fig. 1. This system is based on UP-M-PCR and the colorimetric property of DNAzymes. Two pairs of primers, namely, specific and universal primers, exist in the UP-M-PCR mixture. All the specific primers contain a common sequence, which is also the sequence of universal primer, in the 5’ end. Two types of universal primers were synthesized and tested (Table 1). One type is the exact DNAzyme sequence (ue-primer), and the other type is the complementary strand of the DNAzyme sequence (se-primer). Although

no color change is visible to the naked eye when these two primers are used, the absorption value at 412 nm slightly decreases when ue-primers are used (Fig. 2). We selected the ue-primers for optimization given their low absorption value and extensive usage in the reaction. When target exists in the reaction (upper section of Fig. 1A), specific primers are functional in identifying and amplifying in the first ten cycles of amplification, and universal primers become a major activator in the lasting cycles (pink lines in Fig. 1A). Double-stranded amplicons exponentially accumulate per cycle of PCR. The DNAzyme is also amplified to form double-stranded DNAzyme sequence (pink and green lines in Fig. 1A). The double-stranded DNAzyme sequence does not catalyze the conversion of a colorless $ABTS^{2-}$ to green $ABTS^{-}$ in the presence of hemin and H_2O_2. Nevertheless, single-stranded DNAzyme sequences in the negative control are not amplified and remained in the reaction, except sporadic complementary structure between universal and specific primers (lower section of Fig. 1A). Dissociative DNAzyme sequences catalyze the oxidation to produce the colored product $ABTS^{\bullet-}$ in the presence of hemin and H_2O_2 (pink G-quadruplex structure), which make the color to green and is visible from naked eye.

3.2. System optimization for colorimetric detection

The colorimetric reaction in the PCR-DNAzyme biosensor system was optimized. We devised a series of derived ue-primers with several base deletions in the 5’ and 3’ regions to investigate the effect of primer length on the colorimetric reaction (Table S1, ue-d-primer). According to the previous reports [41, 42], ue-d-primers inhibit the catalysis of oxidization, which changes optical density. Moreover, the primers may also lower the background signal if they remain in the system until the amplification is finished. As shown in Fig. 2A, a mixture of ue-primers and ue-d-primers allowed for visual discrimination between positive and negative samples. Moreover, Fig.2A indicated that further base deletion improved the visual effect. Therefore, the ue-d-primer 3’-3 was selected for the following research.

In addition to primer structure, primer ratio also influences the visual effect. The ue-primer and ue-d-primer ratio of 1:1 (0.2: 0.2 μM) was tested and selected for the evaluation of visual performance (Fig. 2A). DNAzyme with base deletions (ue-d-primer) functioned to lower the background noise. However, the addition of more ue-d-primer failed to decrease background noise and may disturb the visual evaluation. After the optimization (Fig. 2B), a criterion based on the absorption value was established to prevent false-positives identification, as follows: First, the system is visually evaluated and a color change can be seen. Then, when visually identifying color change is difficult, samples are considered positive if their ODs are at least 1.5 times less than that of the negative control. When ODs are 1.5–0.5 times less than the negative control, they are evaluated by using gel electrophoresis or qPCR.

3.3. Specificity, fitness and sensitivity

The applicability of the colorimetric biosensor was investigated by using GMO samples. For GMO detection, the screening work has attracted considerable research attention because it serves as the preliminary and efficient way to identify GMO presence [43-45]. Some common sequence motifs were selected for target screening, which often locates on promoters, terminators and some common interest genes. Only positive samples need to be tested using event-specific method to determine the concrete GMO event. In this study, three targets were selected for validation: cauliflower mosaic virus 35S promoter (P-35S), nopaline synthase terminator (T-NOS) and herbicide-resistant 5-enolpyruvulshikimate-3-phosphate synthase (EPSPS) gene. Three pairs of primers were all added to the UP-M-PCR reaction system. After amplification, the target was detected through the color change induced by catalytic reaction. Any existing target could result in the oxidation of $ABTS^{2-}$ and force to produce the colored product. This screening may limit the list of candidate GMO events and provide evidence for the identity of presented GMOs. Event detection is the highest specific detection for GMOs, and is usually applied in commercial trade and plant quarantine.

The specificity and fitness of the biosensor were tested by detecting different GMO materials and determining whether they contained GMOs. The detection results for TT51-1 (containing one element), MON87460 (containing two elements), and MON87701 (no element contained) all accurately confirmed our predicted results (Fig. 3A). The fitness of the biosensor was validated using GMOs mixture containing maize, soybean, canola and rice that randomly contained five GMO mixtures (Fig. 3 and Fig. S1). Samples from local supermarkets were also validated using this method. Therefore, the proposed detection could be used to determine whether they have contained GMO content (Table S2).

Sensitivity was tested using MON87460 and MON88302 combined at different ratios. The mixed sample presented 1% sensitivity, which is approximately equal to 180 haploid maize genomic copies because maize genomic DNA is 2.725 pg per haploid genome [46]. Although this sensitivity is not superior to those of other quantitative methods [29, 38, 39], our system is more versatile, could be feasibly used in high-throughput screening, and has a detection limit within the allowable threshold for GMO content in most countries.

3.4. Logic gate operation of the colorimetric biosensor

The target of this biosensor was also transformed to a logic value through logic gate computation. Three target genes were selected as the inputs: P-35S, T-NOS and EPSPS. We defined “0” as the absence of each element in the PCR cycles. Other conditions were defined as “1”. When at least one of the target gene exists in the system, the output is defined as “TRUE” with a hierarchical color indicator (Fig. 1B). Others are identified as “FALSE”. The UP-M-PCR biosensor worked well in the presence of either CaMV 35S, T-NOS or EPSPS (Fig. 1B). The indication of positive GMO in the detected samples can be decided from the truth table (Fig. 1C). The final results of the logic gate operations, as indicated through the output volume, are presented in Table 2. The selected targets, P-35S, T-NOS and EPSPS, covered more than 90% of all the commercialized GM events reported by the reports of

International Service for the Acquisition and Agri-biotech Applications (ISAAA). Thus, most of GM products can be identified if it is GMO positive by this colorimetric biosensor. It is meaningful for scientific worker to control overall GM positive content in practical samples rather than to control single GMO event content [47], which indicating the principal and necessary status of screening. This readout platform of this colorimetric biosensor exactly exhibits convenient and time-saving properties that is suitable for preliminary screening. This colorimetric biosensor is also feasible for overall target control and manipulation of highly polluted samples, such as quality control of foodborne pathogens, ecological microbial diversity and adulteration identification [48-50]. Therefore, although our system shows no improvements in sensitivity (1% GM content), it is much more applicable in practical GM screening work.

4. Conclusion

We have developed a colorimetric biosensor for GMO identification. A DNAzyme sequence was embedded in the primer sequence. The target in the biosensor was firstly amplified, and then can be evaluated through color change, identified by OR logic gate operations. This biosensor was combined with PCR, DNAzyme and logic gate to facilitate a convenient target screening, especially GMO screening. This system will be more profitable for field diagnosis when utilized with a hand-held UV spectrometer, portable PCR instrument and even isothermal technology [51-53].

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References

[1] C. Zhang, W. Xu, Z. Zhai, Y. Luo, X. Yan, N. Zhang, et al., Universal primer-multiplex-polymerase chain reaction (UP-M-PCR) and capillary electrophoresis–laser-induced fluorescence analysis for the simultaneous detection of six genetically modified maize lines, Journal of agricultural and food chemistry, 59(2011) 5188-94.

[2] W. Xu, Basic Rules in Nucleic Acid-Mediated Amplification and Hybridization Methods in Food Safety Detection: A Review, Functional Nucleic Acids Detection in Food Safety, Springer2016, pp. 37-61.

[3] W. Xu, Detecting Targets Without Thermal Cycling in Food: Isothermal Amplification and Hybridization, Functional Nucleic Acids Detection in Food Safety, Springer2016, pp. 185-218.

[4] M. Baker, Digital PCR hits its stride, nature methods, 9(2012) 541.

[5] C.A. Heid, J. Stevens, K.J. Livak, P.M. Williams, Real time quantitative PCR, Genome research, 6(1996) 986-94.

[6] C. Mengoli, M. Cruciani, R.A. Barnes, J. Loeffler, J.P. Donnelly, Use of PCR for diagnosis of invasive aspergillosis: systematic review and meta-analysis, The Lancet infectious diseases, 9(2009) 89-96.

[7] S. Huang, Y. Xu, X. Yan, Y. Shang, P. Zhu, W. Tian, et al., Development and application of a quantitative loop-mediated isothermal amplification method for detecting genetically modified maize MON863, Journal of the Science of Food and Agriculture, 95(2015) 253-9.

[8] W. Bai, W. Xu, K. Huang, Y. Yuan, S. Cao, Y. Luo, A novel common primer multiplex PCR (CP-M-PCR) method for the simultaneous detection of meat species, Food Control, 20(2009) 366-70.

[9] L.H. Bachmann, R.E. Johnson, H. Cheng, L.E. Markowitz, J.R. Papp, E.W. Hook, Nucleic acid amplification tests for diagnosis of Neisseria gonorrhoeae oropharyngeal infections, Journal of clinical microbiology, 47(2009) 902-7.

[10] K. Hsieh, A.S. Patterson, B.S. Ferguson, K.W. Plaxco, H.T. Soh, Rapid, Sensitive, and Quantitative Detection of Pathogenic DNA at the Point of Care through Microfluidic Electrochemical Quantitative Loop-Mediated Isothermal Amplification, Angewandte Chemie, 124(2012) 4980-4.

[11] Y. Shang, P. Zhu, W. Xu, T. Guo, W. Tian, Y. Luo, et al., Single universal primer multiplex ligation-dependent probe amplification with sequencing gel electrophoresis analysis, Analytical biochemistry, 443(2013) 243-8.

[12] T.L. Hawkins, J.C. Detter, P.M. Richardson, Whole genome amplification—applications and advances, Current opinion in biotechnology, 13(2002) 65-7.

[13] R. de la Rica, M.M. Stevens, Plasmonic ELISA for the ultrasensitive detection of disease biomarkers with the naked eye, Nat Nanotechnol, 7(2012) 821-4.

[14] J.L. MacLean, K. Morishita, J. Liu, DNA stabilized silver nanoclusters for ratiometric and visual detection of Hg(2)(+) and its immobilization in hydrogels, Biosens Bioelectron, 48(2013) 82-6.

296 [15] P. Gopinath, V. Anitha, S.A. Mastani, Microcantilever based biosensor for
297 disease detection applications, science, 24(2015) 34.
298 [16] W. Zhou, X. Gong, Y. Xiang, R. Yuan, Y. Chai, Target-triggered quadratic
299 amplification for label-free and sensitive visual detection of cytokines based on
300 hairpin aptamer DNAzyme probes, Analytical chemistry, 86(2013) 953-8.
301 [17] L.Z. N. Cheng, Y. Xu, W. Xu, A Review of Test Strips in Rapid Detection of
302 Food Safety, Austin Journal of Nutrition and Food Sciences, 2(2014) 6.
303 [18] J. Kosman, B. Juskowiak, Peroxidase-mimicking DNAzymes for biosensing
304 applications: a review, Analytica chimica acta, 707(2011) 7-17.
305 [19] C. Yang, V. Lates, B. Prieto-Simón, J.-L. Marty, X. Yang, Aptamer-DNAzyme
306 hairpins for biosensing of Ochratoxin A, Biosensors and Bioelectronics, 32(2012)
307 208-12.
308 [20] H.-J. Xiao, H.C. Hak, D.-M. Kong, H.-X. Shen, Sequence-specific detection of
309 nucleic acids utilizing isothermal enrichment of G-quadruplex DNAzymes, Analytica
310 chimica acta, 729(2012) 67-72.
311 [21] L. Yang, F. Du, G. Chen, A. Yasmeen, Z. Tang, A novel colorimetric PCR-based
312 biosensor for detection and quantification of hepatitis B virus, Analytica chimica acta,
313 840(2014) 75-81.
314 [22] R. Orbach, B. Willner, I. Willner, Catalytic nucleic acids (DNAzymes) as
315 functional units for logic gates and computing circuits: from basic principles to
316 practical applications, Chemical Communications, 51(2015) 4144-60.

317 [23] C. Zhang, J. Yang, S. Jiang, Y. Liu, H. Yan, DNAzyme-based logic gate-mediated
318 DNA self-assembly, Nano letters, 16(2015) 736-41.
319 [24] M. Kahan-Hanum, Y. Douek, R. Adar, E. Shapiro, A library of programmable
320 DNAzymes that operate in a cellular environment, Scientific reports, 3(2013) 1535.
321 [25] N. Kaneko, K. Horii, S. Kato, J. Akitomi, I. Waga, High-throughput quantitative
322 screening of peroxidase-mimicking DNAzymes on a microarray by using
323 electrochemical detection, Analytical chemistry, 85(2013) 5430-5.
324 [26] J. Zhuang, W. Lai, G. Chen, D. Tang, A rolling circle amplification-based DNA
325 machine for miRNA screening coupling catalytic hairpin assembly with DNAzyme
326 formation, Chemical communications, 50(2014) 2935-8.
327 [27] Y. Wen, Y. Xu, X. Mao, Y. Wei, H. Song, N. Chen, et al., DNAzyme-based
328 rolling-circle amplification DNA machine for ultrasensitive analysis of microRNA in
329 Drosophila larva, Analytical chemistry, 84(2012) 7664-9.
330 [28] J. Chen, Z. Fang, P. Lie, L. Zeng, Computational lateral flow biosensor for
331 proteins and small molecules: a new class of strip logic gates, Analytical chemistry,
332 84(2012) 6321-5.
333 [29] Y. Huang, W. Wen, D. Du, X. Zhang, S. Wang, Y. Lin, A universal lateral flow
334 biosensor for proteins and DNAs based on the conformational change of hairpin
335 oligonucleotide and its use for logic gate operations, Biosensors and Bioelectronics,
336 61(2014) 598-604.
337 [30] J. Andréasson, U. Pischel, Smart molecules at work—mimicking advanced logic

338 operations, Chemical Society Reviews, 39(2010) 174-88.

339 [31] J. Elbaz, O. Lioubashevski, F. Wang, F. Remacle, R.D. Levine, I. Willner, DNA
340 computing circuits using libraries of DNAzyme subunits, Nature nanotechnology,
341 5(2010) 417-22.

342 [32] X. Liu, R. Aizen, R. Freeman, O. Yehezkeli, I. Willner, Multiplexed aptasensors
343 and amplified DNA sensors using functionalized graphene oxide: application for logic
344 gate operations, ACS nano, 6(2012) 3553-63.

345 [33] N. Cheng, P. Zhu, Y. Xu, K. Huang, Y. Luo, Z. Yang, et al., High-sensitivity assay
346 for Hg (II) and Ag (I) ion detection: a new class of droplet digital PCR logic gates for
347 an intelligent DNA calculator, Biosensors and Bioelectronics, 84(2016) 1-6.

348 [34] C. James, Global Status of Commercialized Biotech/GM Crops: 2016, ISAAA
349 Brief, (2017).

350 [35] G. Brookes, P. Barfoot, Environmental impacts of genetically modified (GM)
351 crop use 1996–2013: Impacts on pesticide use and carbon emissions, GM crops &
352 food, 6(2015) 103-33.

353 [36] X. Han, H. Wang, H. Chen, L. Mei, S. Wu, G. Jia, et al., Development and
354 primary application of a fluorescent liquid bead array for the simultaneous
355 identification of multiple genetically modified maize, Biosensors and Bioelectronics,
356 49(2013) 360-6.

357 [37] X. Li, Y. Wu, J. Li, Y. Li, L. Long, F. Li, et al., Development and validation of a
358 48-target analytical method for high-throughput monitoring of genetically modified

organisms, Scientific reports, 5(2015) 7616.

[38] W. Fu, P. Zhu, C. Wang, K. Huang, Z. Du, W. Tian, et al., A highly sensitive and specific method for the screening detection of genetically modified organisms based on digital PCR without pretreatment, Scientific reports, 5(2015).

[39] W. Xu, Z. Zhai, K. Huang, N. Zhang, Y. Yuan, Y. Shang, et al., A novel universal primer-multiplex-PCR method with sequencing gel electrophoresis analysis, PLoS One, 7(2012) e22900.

[40] F. Zhang, R. Wang, L. Wang, J. Wu, Y. Ying, Tracing phosphate ions generated during DNA amplification and its simple use for visual detection of isothermal amplified products, Chemical Communications, 50(2014) 14382-5.

[41] A. Trifonov, E. Sharon, R. Tel-Vered, J.S. Kahn, I. Willner, Application of the Hybridization Chain Reaction on Electrodes for the Amplified and Parallel Electrochemical Analysis of DNA, The Journal of Physical Chemistry C, 120(2016) 15743-52.

[42] S. Shimron, F. Wang, R. Orbach, I. Willner, Amplified detection of DNA through the enzyme-free autonomous assembly of hemin/G-quadruplex DNAzyme nanowires, Analytical chemistry, 84(2011) 1042-8.

[43] S.F. Rosa, F. Gatto, A. Angers-Loustau, M. Petrillo, J. Kreysa, M. Querci, Development and applicability of a ready-to-use PCR system for GMO screening, Food Chem, 201(2016) 110-9.

[44] S.-B. Park, H.-Y. Kim, J.-H. Kim, Development of a screening method for the

monitoring of 38 genetically modified maize events in food and feed in South Korea, Food Control, 73(2017) 1459-65.

[45] M. Querci, M. Van den Bulcke, J. Žel, G. Van den Eede, H. Broll, New approaches in GMO detection, Analytical and Bioanalytical Chemistry, 396(2010) 1991-2002.

[46] K. Arumuganathan, E. Earle, Nuclear DNA content of some important plant species, Plant molecular biology reporter, 9(1991) 208-18.

[47] Y. Li, Y. Peng, E.M. Hallerman, K. Wu, Biosafety management and commercial use of genetically modified crops in China, Plant cell reports, 33(2014) 565-73.

[48] H. Dong, C. Wang, Y. Xiong, H. Lu, H. Ju, X. Zhang, Highly sensitive and selective chemiluminescent imaging for DNA detection by ligation-mediated rolling circle amplified synthesis of DNAzyme, Biosensors and Bioelectronics, 41(2013) 348-53.

[49] Q. Wang, X. Yang, X. Yang, F. Liu, K. Wang, Visual detection of myoglobin via G-quadruplex DNAzyme functionalized gold nanoparticles-based colorimetric biosensor, Sensors and Actuators B: Chemical, 212(2015) 440-5.

[50] J. Song, F. Wu, Y. Wan, L.-H. Ma, Visual test for melamine using silver nanoparticles modified with chromotropic acid, Microchimica Acta, 181(2014) 1267-74.

[51] J.R. Askim, K.S. Suslick, Hand-Held Reader for Colorimetric Sensor Arrays, Analytical chemistry, 87(2015) 7810-6.

401 [52] C.D. Ahrberg, B.R. Ilic, A. Manz, P. Neužil, Handheld real-time PCR device,
402 Lab on a Chip, 16(2016) 586-92.
403 [53] Y. Chen, N. Cheng, Y. Xu, K. Huang, Y. Luo, W. Xu, Point-of-care and visual
404 detection of P. aeruginosa and its toxin genes by multiple LAMP and lateral flow
405 nucleic acid biosensor, Biosensors and Bioelectronics, 81(2016) 317-23.
406

407 Table 1 Primers used in this study

Primer name	Sequence (5'-3')[a]	Amplicon	Reference
se-CaMV 35S-F	**AGGGTTGGGCGGGATGGG**CATCATTGCGATA AAGGAAAGGC	177	Present study
se-CaMV 35S-R	**AGGGTTGGGCGGGATGGG**TGCTTTGAAGACG TGGTTGGA		
se-NOS-F	**AGGGTTGGGCGGGATGGG**GAATCCTGTTGCC GGTCTTG	232	
se-NOS-R	**AGGGTTGGGCGGGATGGG**TTATCCTAGTTTG CGCGCTA		
se-CP4-EPSPS-F	**AGGGTTGGGCGGGATGGG**ACGGTGACCGTCT TCCAGTTAC		
se-CP4-EPSPS-R	**AGGGTTGGGCGGGATGGG**GAACAAGCAAGG CAGCAACCA	385	
ue-CaMV 35S-F	**TCCCAACCCGCCCTACCC**CATCATTGCGATA AAGGAAAGGC	177	
ue-CaMV 35S-R	**TCCCAACCCGCCCTACCC**TGCTTTGAAGACG TGGTTGGA		
ue-NOS-F	**TCCCAACCCGCCCTACCC**GAATCCTGTTGCC	232	

	GGTCTTG		
ue-NOS-R	**TCCCAACCCGCCCTACCC**TTATCCTAGTTTGC GCGCTA		
ue-CP4-EPSPS-F	**TCCCAACCCGCCCTACCC**ACGGTGACCGTCT TCCAGTTAC	385	
ue-CP4-EPSPS-R	**TCCCAACCCGCCCTACCC**GAACAAGCAAGGC AGCAACCA		
se-universal primer	TCCCAACCCGCCCTAC	-	
ue-universal primer	AGGGTTGGGCGGGATGGG	-	[19]

[a]sequences in bold letter demonstrate DNAzyme sequence in specific embedded primer and complementary sequence of DNAzyme in the universal embedded primer.

410 Table 2 Specificity and fitness of the screening system

Sample number	GM events	Target	Input	Output
1	MON810	CaMV 35S	(1,0,0)	TRUE
2	TT51-1	NOS	(0,1,0)	TRUE
3	MON87460	CaMV 35S, NOS	(1,1,0)	TRUE
4	MON88302	CP4-EPSPS	(0,0,1)	TRUE
5	H7-1	CP4-EPSPS	(0,0,1)	TRUE
6	MON87705	CaMV 35S, CP4-EPSPS	(1,0,1)	TRUE
7	J101	CP4-EPSPS	(0,0,1)	TRUE
8	MON89788	CP4-EPSPS	(0,0,1)	TRUE
9	MON810+TT51-1	CaMV 35S, NOS	(1,1,0)	TRUE
10	MON87460+TT51-1	CaMV 35S, NOS	(1,1,0)	TRUE
11	MON87460+MON88302	CaMV 35S, NOS CP4-EPSPS	(1,1,1)	TRUE
12	MON87701	none	(0,0,0)	FALSE
N	Negative control	none	(0,0,0)	FALSE

Figure captions

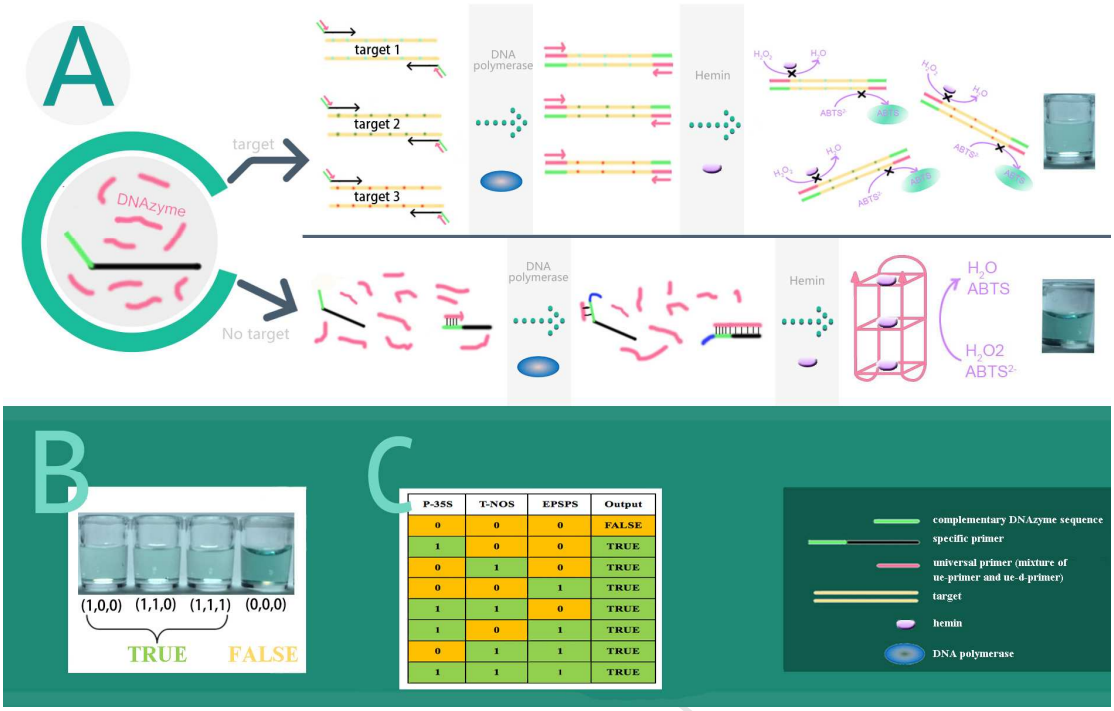
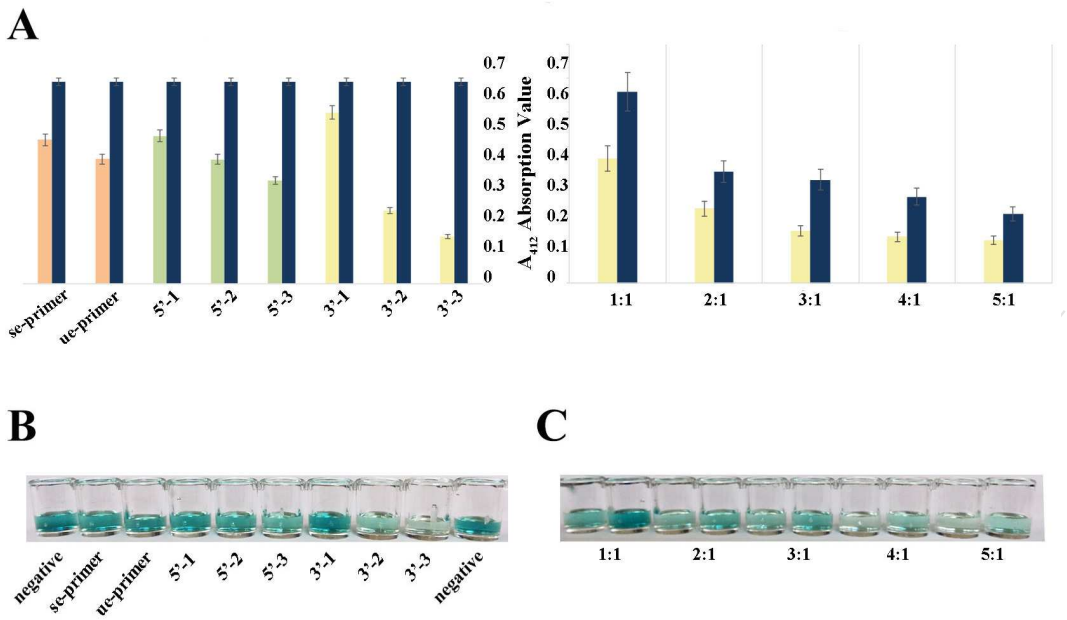


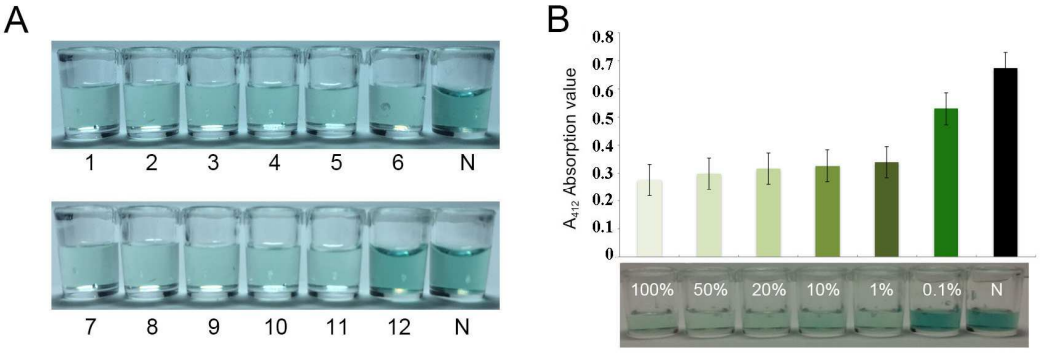
Fig. 1 The scheme of colorimetric biosensor for DNA screening. (A) Target in the system will be amplified by universal primer-multiplex-PCR (UP-M-PCR). At the beginning of PCR, specific primer is mainly responsible for amplification (Black part indicates the specific region). When the specific primer is used up, the universal primer (pink) begins to play a leading role to take the amplicons as templates. Double-stranded DNAzyme sequence will not undergo the catalytic colorimetric reaction (upper section in Fig. 1A). Negative control has an abundance of dissociative universal primers, transforming the reaction color from bright to green (lower section in Fig. 1A). These two processes exhibit different colors, which are shown in the right side of the figure. Three targets (yellow line with red, green and blue dots) make an OR logic gate operation. The corresponding indication and truth table are shown in (B) and (C), respectively.



425

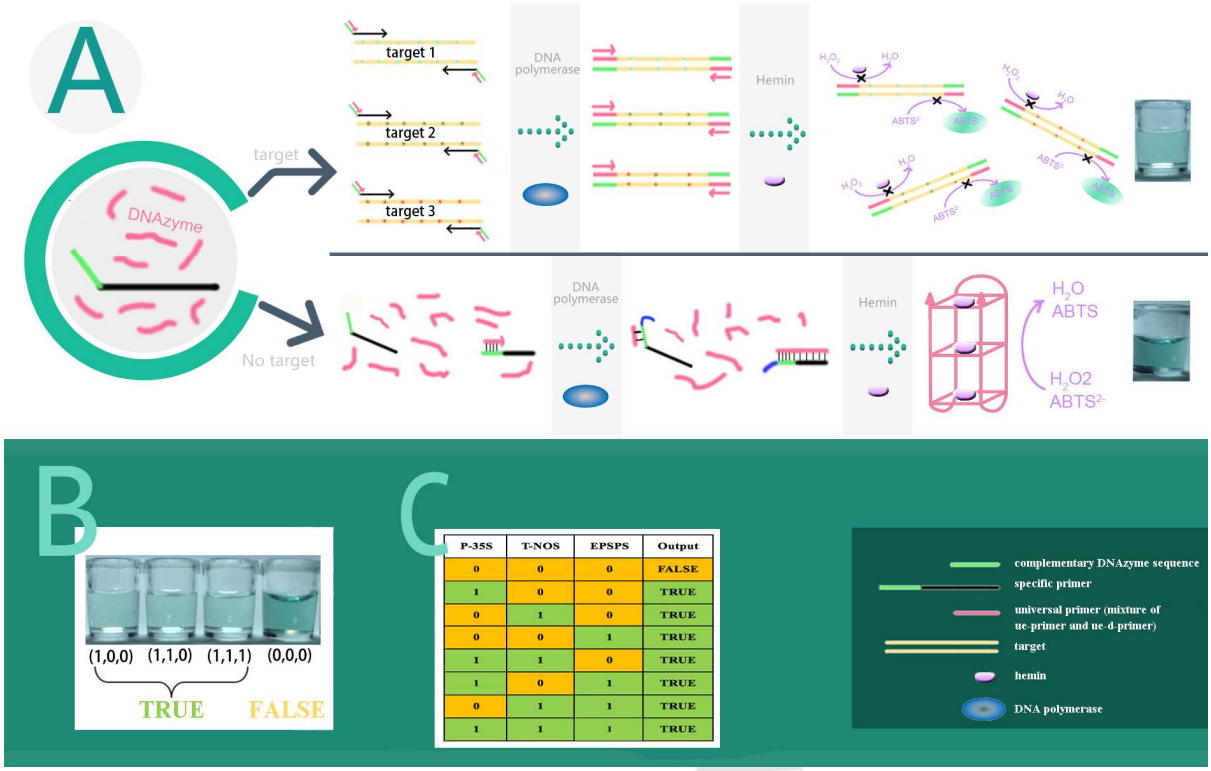
426 Fig. 2 (A) Primer selection for light-off evaluation. The Y-axis represents absorption
427 value at 412nm. The dark columns represent absorption value of negative control.
428 Columns from bottom to top represent UP-M-PCR evaluation with se-primers,
429 ue-primers (pink), ue-d-primers with 5' deletion (green) and 3' deletion (yellow). 5'-1,
430 5'-2 and 5'-3 represent one, two and three bases deletion on the 5' region of
431 ue-primers, respectively. 3'-1, 3'-2 and 3'-3 represent one, two and three bases
432 deletion on the 3' region of ue-primers, respectively. 3'-3 primer was chosen to be
433 mixed with ue-primer and the ratio was also evaluated (right in Fig.2A). 1:1, 2:1, 3:1,
434 4:1 and 5:1 represents the ratio of ue-d-primers (3'-3) and ue-primers. (B) (C) Visual
435 readouts of UP-M-PCR optimization. Negative means negative control, and other
436 captions are in accordance to Fig.2A.

437

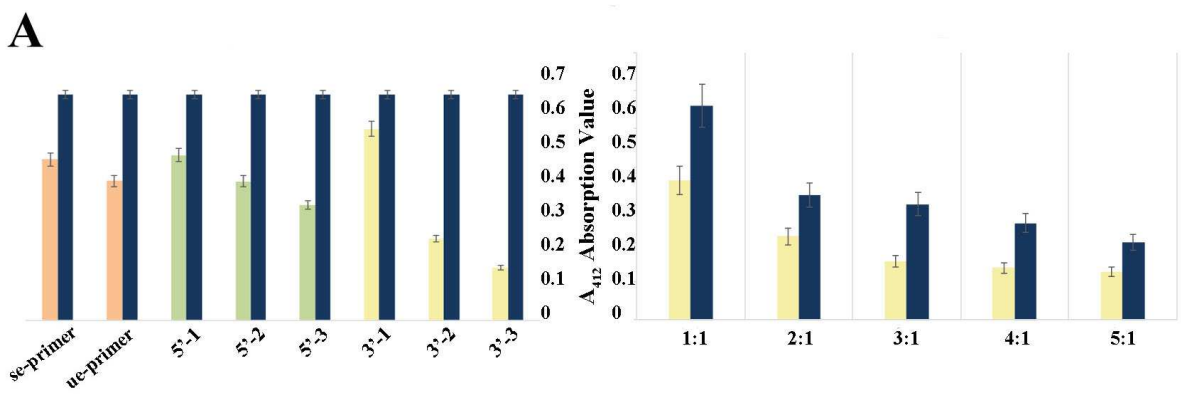


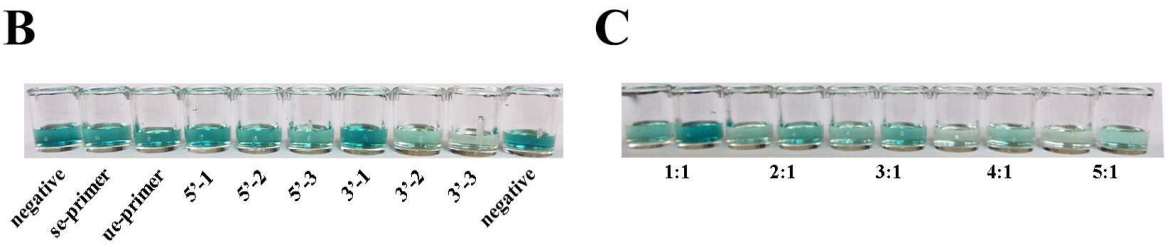
438

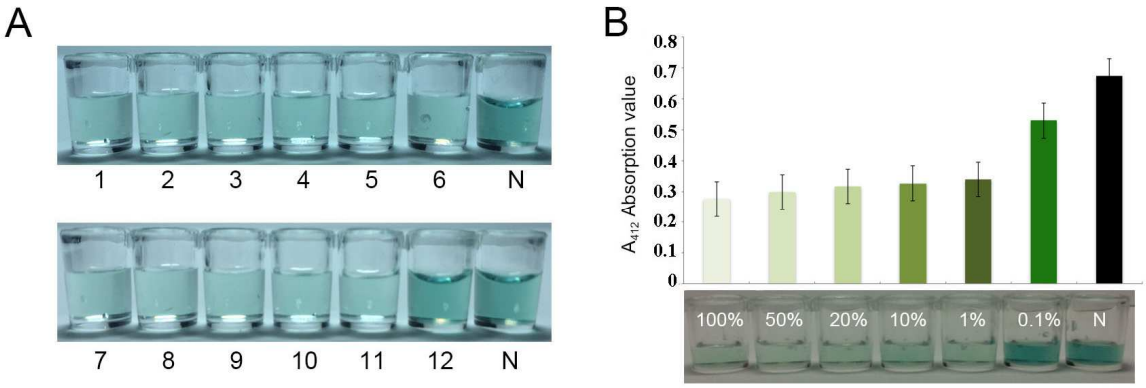
439 Fig. 3 Specificity (A) and sensitivity (B) of the visual light-off logic computation for
440 GMO screening. Sample components in (A) are shown in Table 2, and N represents
441 negative control.



P-35S	T-NOS	EPSPS	Output
0	0	0	FALSE
1	0	0	TRUE
0	1	0	TRUE
0	0	1	TRUE
1	1	0	TRUE
1	0	1	TRUE
0	1	1	TRUE
1	1	1	TRUE







Highlights

- HRP-mimicking DNAzyme was added in the primer.
- Biosensing reaction is obtained with naked eye.
- Logic gate operations were designed and applied.
- The system was applied for the high-throughput GMO screening.