Journal of Visualized Experiments
Visual Detection of Multiple Nucleic Acids in a Capillary Array
--Manuscript Draft--

Abstract:	Multi-target, short time, and resource-affordable methodologies for the detection of multiple nucleic acids in a single easy-to-operate test are urgently needed in disease diagnosis, microbial monitoring, as well as genetically modified organism (GMO) detection and forensic analysis. we have described the platform called CALM (Capillary Array-based Loop-mediated isothermal amplification for Multiplex visual detection of nucleic acids).Herein, we described the improved fabrication and performance process. In this platform, we apply a small ready-to-use cassette assembled by capillary array for multiplex visual detection of nucleic acids. In this method, the capillary array is pre-treated into hydrophobic and hydrophilic pattern before fixing loop-mediated isothermal amplification(LAMP) primer sets in capillaries. After assembly of the loading adaptor, LAMP reaction mixture is loaded and isolated into each capillary due to capillary force by a single pipetting. The LAMP reactions are

	performed in parallel in the capillaries. The results are visually read out by illumination with a hand-held UV flashlight. Using this platform, we demonstrated how to monitor 8 frequently appeared elements and genes in GMO samples with high specificity and sensitivity. In summary, the platform described here is intended to facilitate detection of multiple nucleic acids. We believe it will be widely applicable in fields where high-throughput nucleic acid analysis is required.
Author Comments:	
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter.	

SHANGHAI JIAOTONG
U N I V E R S I T Y

Shanghai Center for Systems Biomedicine

800 Dongchuan Road, Shanghai, China 200240

Dear Nandita Singh,

We are very excited to submit our manuscript entitled "Visual Detection of Multiple Nucleic Acids in a Capillary Array" by Chen *et al.*, for publication in JoVE (Journal of Visualized Experiments).

Fast and low-cost methodologies for monitoring of multiple nucleic acids in a single test with simple operation is urgently desired for Genetically modified organisms (GMO) monitoring, infectious disease detection and even anti-terrorism. For example, in genetically modified organisms (GMO) monitoring, there are hundreds of known genetic modifications to be tested for a single sample. Similarly, in infectious disease diagnosis, such as pneumonia, which could be caused by a variety of pathogens. To achieve rapid diagnosis and give the patients proper treatments, doctors need to identify the causative pathogen/s or subtype/s from many possibilities as soon as possible. Importantly, the requirement of simplicity and multiplicity is even higher in point-of-care test circumstance, which is usually resource-limited.

However, traditional methodologies can hardly meet these requirements. PCR-based methodologies are usually limited by its low multiplicity, high dependency to professional equipment. Isothermal amplification methodologies also short of multiplicity.

To this end, we have described the platform called CALM (Capillary Array-based Loop-mediated isothermal amplification for Multiplex visual detection of nucleic acids) (Shao et. al., Lab chip, 2017). Herein, we described the improved fabrication and performance process. In this platform, we apply a small ready-to-use cassette assembled by capillary array for multiplex visual detection of nucleic acids. In this method, the capillary array is pre-treated into hydrophobic and hydrophilic pattern before fixing loop-mediated isothermal amplification(LAMP) primer sets in capillaries. After assembly of the loading adaptor, LAMP reaction mixture is loaded and isolated into each capillary due to capillary force by a single pipetting. The LAMP reactions are performed in parallel in the capillaries. The results are visually read out by illumination with a hand-held UV flashlight. Using this platform, we demonstrated how to monitor 8 frequently appeared elements and genes in GMO samples with high specificity and sensitivity.

To our knowledge, CALM is the most high-throughput nucleic acids detection system based on LAMP. Meanwhile, it is easy-to-use. Due to the simple operation,

small size and naked eye-readout, CALM has great potential in POCT. The inherent flexibility of the capillary array format gives CALM great flexibility and expandability to be adapted to broad applications.

Reviewers who are especially well qualified to review the manuscript, along with their affiliation and contact information, are listed below:
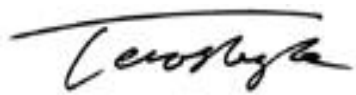
Name	Affiliation	Email
Jianhua Qin	Dalian Institute of Chemical Physics, Chinese Academy of Sciences	jhqin@dicp.ac.cn
Xian-en Zhang	Chinese Academy of Sciences	zhangxe@ibp.ac.cn
Arne Holst-Jensen	National Veterinary Institute，Food and Feed Hygiene	arne.holst-jensen@vetinst.no
Kristina Gruden	Department of Biotechnology and Systems Biology, National Institute of Biology	Kristina.Gruden@nib.si
Keyue Shen	University of Southern California	keyue.shen@usc.edu
Tony Jun Huang	The Pennsylvania State University	junhuang@psu.edu

We claim that none of the material in this manuscript has been published or is under consideration elsewhere, including the Internet.

Below, please find the information of authors' names, telephone\ fax numbers, and e-mail addresses:

Jian-Wei Chen, +86 21 34207314, naughtygene@sjtu.edu.cn
Ning Shao, +86 21 34207314, shaoning064@163.com
Jia-Ying Hu, +86 21 34207174, doubleluyao@163.com
Rong Li, +86 21 34207174, lirong190805020@163.com
Yuanshou Zhu, +86 34207314, 115415910008@sjtu.edu.cn
Da-Bing Zhang, +86 21 34207174, zhangdb@sjtu.edu.cn
Shu-Juan Guo, +86 21 34207314, gsj0215@126.com
Zhi Geng, +86-10-62798732, gengzthu@163.com
Jun-Hou Hui, +86-10-62798732, junhou_0816@hotmail.com
Peng Liu, +86-10-62798732, pliu@mail.tsinghua.edu.cn
Li-Tao Yang, +86 21 34207174, yylltt@sjtu.edu.cn
Sheng-Ce Tao, +86 21 34207069, taosc@sjtu.edu.cn

Sincerely,

Sheng-CeTao   Litao Yang

TITLE:
Visual Detection of Multiple Nucleic Acids in a Capillary Array

AUTHORS & AFFILIATIONS:
Jianwei Chen[a,c,d,†], Ning Shao[a,c,d,†], Jiaying Hu[b,†], Rong Li[b], Yuanshou Zhu[a,c,d], Dabing Zhang[b,f], Shujuan Guo[a,c,d], Junhou Hui[e], Peng Liu[e], Litao Yang[b], Sheng-ce Tao[a,c,d]

[a]Key Laboratory of Systems Biomedicine (Ministry of Education), Shanghai Center for Systems Biomedicine, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, China.
[b]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. E-mail: yylltt@sjtu.edu.cn
[c]State Key Laboratory of Oncogenes and Related Genes, Shanghai 200240, China
[d]School of Biomedical Engineering, Shanghai Jiao Tong University, Shanghai 200240, China
[e]Department of Biomedical Engineering, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, School of Medicine, Tsinghua University, Beijing 100084, China
[f]Key Laboratory of Crop Marker-Assisted Breeding of Huaian Municipality, Jiangsu Collaborative Innovation Center of Regional Modern Agriculture and Environmental Protection, Huaian 223300, China

† These authors contributed equally to this work.

EMAIL ADDRESSES:
Sheng-Ce Tao (taosc@sjtu.edu.cn)
Jianwei Chen (shaoning064@163.com)
Ning Shao (shaoning064@163.com)
Jiaying Hu (doubleluyao@163.com)
Rong Li (lirong190805020@163.com)
Yuanshou Zhu (115415910008@sjtu.edu.cn)
Dabing Zhang (zhangdb@sjtu.edu.cn)
Shujuan Guo (gsj0215@126.com)
Junhou Hui (junhou_0816@hotmail.com)
Peng Liu (pliu@mail.tsinghua.edu.cn)
Litao Yang (yylltt@sjtu.edu.cn)

CORRESPONDING AUTHORS:
Litao Yang (yylltt@sjtu.edu.cn)
Sheng-ce Tao (taosc@sjtu.edu.cn)

KEYWORDS:
Capillary array, LAMP, multiplex nucleic acid detection, genetically modified organisms (GMO), easy-to-operate, visual detection.

SHORT ABSTRACT:
This protocol describes the fabrication of a small, ready-to-use cassette that can be applied for visual detection of multiple nucleic acids in a single, test that is easy to operate. In this approach, a capillary array was used for multiplex and highly efficient detection of GMO targets.

LONG ABSTRACT:
Multi-target, short time, and resource-affordable methodologies for the detection of multiple nucleic acids in a single, easy to operate test are urgently needed in disease diagnosis, microbial monitoring, genetically modified organism (GMO) detection, and forensic analysis. We have previously described the platform called CALM (**C**apillary **A**rray-based **L**oop-mediated isothermal amplification for **M**ultiplex visual detection of nucleic acids). Herein, we describe improved fabrication and performance processes for this platform. Here, we apply a small, ready-to-use cassette assembled by capillary array for multiplex visual detection of nucleic acids. The capillary array is pre-treated into a hydrophobic and hydrophilic pattern before fixing loop-mediated isothermal amplification (LAMP) primer sets in capillaries. After assembly of the loading adaptor, LAMP reaction mixture is loaded and isolated into each capillary, due to capillary force by a single pipetting step. The LAMP reactions are performed in parallel in the capillaries. The results are visually read out by illumination with a hand-held UV flashlight. Using this platform, we demonstrate monitoring of 8 frequently appearing elements and genes in GMO samples with high specificity and sensitivity. In summary, the platform described herein is intended to facilitate the detection of multiple nucleic acids. We believe it will be widely applicable in fields where high-throughput nucleic acid analysis is required.

INTRODUCTION:
Low-cost, quick, and easy to use systems for the simultaneous detection of multiple nucleic acids are urgently needed in a wide range of fields, such as clinical diagnostics[1-3], GMO detection[4-6], microbial monitoring[7-9], forensic analysis[10,11], and especially point-of-care tests (POCTs), where resources are usually limited[12-14].

Polymerase chain reaction (PCR), including its derivative methods real-time PCR and multiplex PCR, is the most widely applied technique for detection in these fields. However, these methods typically only detect one target in one test[15] and they require electricity and sophisticated professional equipment.

Another promising technology for detecting nucleic acids is Loop-mediated isothermal amplification (LAMP), which was first described in 2000[16]. LAMP is a high efficiency DNA detection method. Theoretically, it can amplify from 1 copy to 10^9 copies of amplicons within one hour, all performed at a constant temperature, (*i.e.*, between 60–65 °C). Successful amplification will produce a large amount of the insoluble byproduct pyrophosphate and cause a change in turbidity[17], which could be directly observed by the naked eye. A color change can also be observed by the addition of metal ions or fluorescent dyes such as Calcein[18], Nucleic acid dye[19], and hydroxyl naphthol blue[20]. Because of the advantages of high sensitivity and convenience of operation, LAMP is being widely applied in nucleic acid detection.

Currently, there are mainly two strategies for multiplex LAMP assays. One is to perform multiple LAMP assays by having multiple LAMP primer sets in one tube[21-23]. However, the multiplicity and the amplification efficiency would be limited by the intrinsic interference and competition among different primer sets. Furthermore, it can be difficult to identify different LAMP products in the same reaction. Another strategy is based on physical isolation. Different primer sets were isolated into individual miniaturized compartments, and multiple LAMP reactions are then performed simultaneously[24,25]. These approaches, which are generally based on microfluidic chips, provide a potential solution for high-throughput LAMP reactions. However, the manufacture of the chips and the multiplex pre-coating of primer sets is complicated, which may increase costs and decrease reproducibility.

Recently, a few studies have described performing LAMP reactions in capillaries to bypass the complicated fabrication of microfluidic chips and have achieved low-cost detection[26,27]. However, with regards to high-throughput analysis, these capillaries are similar to miniature versions of PCR strip tubes, because the samples and reaction reagents (including the different primer sets) must be individually prepared and delivered to different reaction units within capillaries. To achieve parallel and multiplex analysis, additional equipment, for example a multichannel syringe pump, is required for parallel loading of samples or reagents.

To overcome the limitations associated with the current methods for multiplex detection of nucleic acids, we have developed a miniaturized platform which combines visual LAMP technology with a capillary array. This platform is multi-target, compact in size, low cost, and easy to operate[28]. Herein, we describe the details of how to fabricate the capillary array and perform the LAMP reactions in the array. The protocol described here has been standardized using genetically modified organism (GMO) detection as a model. Importantly, this protocol can also be used in high-throughput detection of other nucleic acid targets.

PROTOCOL:

Note: This protocol assumes that the stainless steel mold bearing the shape for the desired micro-channels and the loading adaptor have already been made (3D files are provided as Supplemental Files 1 and 2). This protocol also assumes that plant DNA isolation has already been carried out.

1. Fabrication of the Capillary Array-based Ready-To-Use Cassette

1.1. Clean the stainless-steel mold.

1.1.1. Wash the stainless-steel mold by detergent, ethanol, and deionized water to remove the potential contaminants. Dry the mold by nitrogen.

1.2. Clean the capillaries

1.2.1. Wear safety goggles, acid and alkali-resistant rubber uniforms, and chemical protective gloves.

1.2.2. Place capillaries into an acid cylinder. Clean the capillaries with 30 mL acetone for 5 min to remove organic matter, and then wash the capillaries with deionized water.

Caution: Handle acetone in a fume hood.

1.2.3. Pour 10 mL of H_2O_2 into the acid cylinder and then add 30 mL of H_2SO_4 into the H_2O_2 with slow shaking to prevent overheating. Make sure that the solution (piranha solution) completely covers the capillaries for at least 30 min.

Caution: Be careful while handling piranha solution (H_2SO_4/H_2O_2 = 3: 1, v/v). If the piranha solution is spilled, wash away the solution quickly with a large amount of water and wipe the surface with paper towels.

Note: Thorough cleaning of capillaries is one of the key points to ensure the success of the LAMP reaction. It is necessary to shake the acid cylinder during the cleaning process to remove the bubbles in capillaries, and the clean time can also be extended when required.

1.2.4. Wash away the piranha solution with a large amount of water. Wash capillaries with ethanol and deionized water for 5 min each. Dry the capillaries in a drying oven.

1.3. Pour polydimethylsiloxane (PDMS)

1.3.1. Weight out PDMS base and curing reagents with a ratio of 1:1 in a beaker. Typically, mix 5 g of elastomer base to 5 g of elastomer curing agent.

1.3.2. Stir the mixture thoroughly for about 5 min with a glass rod. Place the beaker with PDMS in a vacuum bell jar for 30 min for de-gassing.

1.3.3. Pour the PDMS slowly into the cylinder of the stainless-steel mold. Allow the PDMS to cure for 3 h in a drying oven at 60 °C

Note: Be careful to avoid air bubbles when pouring PDMS. After pouring PDMS, allow it stand for 5 min for natural removal of bubbles out of PDMS.

1.4. Remove the mold from PDMS

1.4.1. After curing the PDMS, remove the mold by pulling out the mold with cylinder. Remove PDMS from the cylinder by cutting the margin of mold with a scalpel.

1.4.2. Wash PDMS three times with ethanol and deionized water. Dry the PDMS support with nitrogen.

1.5. Treat the lower surface of the PDMS support to be hydrophobic. Soak the PDMS support in a super-hydrophobic coat for 1 s. Dry it for 10 min at room temperature.

1.6. Insert the capillary into the PDMS support

1.6.1. Insert the cleaned 4 mm capillaries into the holes of the PDMS support and leave 0.5 mm of the capillaries outside the top surface of the PDMS support. Make sure the ends of capillaries are on the same level.

1.7. Treat the outer surface of the capillaries and the top surface of the PDMS support to be hydrophobic.

1.7.1. Add 15 µL super-hydrophobic coat onto the top surface of the PDMS support. Note that the coating immediately spreads all over the top surface (including the top surface of the PDMS support and the outer surfaces of the exposed part of the capillaries) by capillary force.

1.7.2. Air dry the super-hydrophobic modified capillary array.

1.8. Fix primer into capillary array

1.8.1. Prepare primer solution. Weight out 0.65 g chitosan (molecular formula: $(C_6H_{11}NO_4)_n$) and dissolve it into 50 mL deionized water by adjusting the pH to 4.5-5.5 with acetic acid to achieve a chitosan concentration of 1.3%. Prepare primer components mix as per **Table 1**. See **Supplementary Table 1** for primers

[Place Table 1 here]

1.8.2. Add 1.6 µL of the mixture of one set of primers to fill one corresponding capillary of the capillary array according to a pre-designed order.

Note: The remaining two blank capillaries were set as negative controls.

1.8.3. Anchor the array in a transparent well of a standard flat bottom 96-well plate and dry the array at 60 °C for at least 2 h.

1.9. Assemble the cassette as follows. Put a loading adaptor onto the top of anchored array. Make sure the dish of the adaptor just covers the exposed parts of all ten capillaries (See **Figure 1**).

[Place Figure 1 here]

2. Performance of LAMP Reaction in Capillary Array

2.1. Prepare the reaction mix

2.1.1. Prepare the LAMP reaction mix as per **Table 2**.

[Place Table 2 here]

2.1.2. Add reagents to mixture according to the chosen order, as in **Table 2**, and vortex the reaction mixture for 5 s after adding calcein. Gently invert the tube 20 times after *Bst* polymerase is added.

2.2. Load the reagents and seal the cassette

2.2.1. Pipette 20 μL LAMP reaction mixture with a standard 100-μL tip. Insert the tip into the inlet of the loading adaptor to lock it. Gently inject the reaction mixture into the dish of adaptor; the reaction mixture will quickly fill the dish and then load the capillaries automatically through capillary force.

2.2.2. Remove the adaptor with the locked tip and seal the well by a PCR-compatible transparent sealing film.

Note: Only the capillaries touching the reaction mixture in the hydrophilic dish could be filled. So make sure the top side of all the capillaries are of the same height (See **Figure 2**).

[Place Figure 2 here]

2.3. Incubate the capillary array in an incubator at 63 °C for 1 h.

3. Results Readout and Data Analysis

3.1. Acquire images of the fluorescence emission.

3.1.1. Fix a UV filter on the top surface of a small hand-held UV LED flashlight to filter the visible light.

3.1.2. Excite the dissociated calcein to emit fluorescence with the UV flashlight. Capture images from the top of the capillary array by either a digital camera or a smartphone.

3.1.3. When taking an image, ensure that the camera is zoomed in on the area of the capillaries as much as possible to get high quality, clear images.

3.2. Analyze the results

3.2.1. Open the image by image analysis software and then select "Image>Mold>Grayscale>Yes" and "Image>Mold>16bit>Yes". Select "File>Save as>format>TIFF" to convert the image to 16-bit TIFF format.

Calculate the SNR to define whether LAMP is successfully performed in the capillaries.

Note: Signals of the capillaries were obtained by recording the gray values of the spots and signal to noise ratios (SNRs) were defined as ratios of mean values of foreground signals of the targets to average mean values of foreground signals of the two negative controls. The cutoff to determine positive signals was set as SNR >2.

REPRESENTATIVE RESULTS:
In this method, it is important to prevent cross-contamination among different capillaries during the sample loading. For this purpose, chitosan was introduced, which could retain the primers in individual capillaries. To test whether it worked or not, we pre-fixed the ADH1 (endogenous reference gene of maize) primer set in the capillary cassette with the pattern of "T" and "U", as illustrated in **Figure 3a.** As expected, only the capillaries contained primer sets showing positive signals (**Figure 3b**).

[Place Figure 3 here]

To further evaluate the ability to monitor GMO, we chose seven frequently-used transgenic elements which cover ~75% of the commercialized GMO events (*i.e.*, P-CaMV35S, bar, cp4 epsps, P-FMV35S, pat, T-nos and nptII) (**Figure 4, layout**) which correspond to the capillaries number 1-7, and one endogenous reference gene for maize (ADH1). To show the specificity of this method, three GM events (MON863, MON89034, and 59122) were selected and applied to CALM. To analyze the results, fluorescence images were taken by camera and analyzed as described in the protocol steps. Expected results were obtained for all the tests (**Figure 4**). For example, for the GM maize MON863, positive signals were obtained for capillaries 1, 6, 7, and 8, which correspond to targets P-CaMV35S, T-nos, nptII, and the endogenous reference gene ADH1, respectively.

[Place Figure 4 here]

To test the performance of our method in a real world application, two practical maize samples were selected for analysis by CALM. Then the results were compared with that of real-time PCR and results were consistent (**Figure 5, Supplementary Table 2**)

[Place Figure 5 here]

FIGURE LEGENDS:

Figure 1: The cassette fabrication and assembly. (a) Stainless mold and the PDMS support. The mold consists of 3 parts: cylinder, dam board, and pillar plate. **(b)** The schematic of PDMS support fabrication and assembly of the capillary cassette. The whole process contains 5 steps: 1. PDMS pouring, 2. Mold removing, 3. Capillary inserting and surface coating, 4. primer fixing and 5. cassette anchoring. 1. Pour PDMS into cylinder of the mold; 2. Push out the dam board to remove the mold from PDMS support; 3. Coat the down surface of PDMS support and then insert the capillaries into the PDMS support, lastly coat the upper surface of PDMS support and exposed capillaries. The thick blue line indicates super-hydrophobic coat; 4. Load primer set into individual capillaries; 5. Anchor the capillary array in a single 96-well plate and install a sample loading adaptor onto it. The details have been described in protocol steps 1.1-1.9.

Figure 2: Diagram of sample-loading using the loading adaptor. The picture shows the loading process employing blue solution as an example. Insert the tip into the inlet and inject the sample into the adaptor slowly, and then remove the adaptor with locked tip.

Figure 3: Examples of capillary result. **(a)** The layout of the capillary array. The green spots indicate that ADH-1 primer sets are pre-fixed in capillaries. **(b)** Fluorescent photographs of the two capillary arrays after LAMP. The green color presented the positive LAMP amplification. The test was performed in duplicate. Successful amplification only presented in primer-fixed capillaries and without contamination among blank capillaries.

Figure 4: The specificity for monitoring GMOs. The detection of different DNA samples, *i.e.*, MON863, MON89034, 59122, mix of all the above three GM events (GMO mix), Non-GM maize and pure water (no template control, NTC). 1–8: Capillaries pre-fixed with LAMP primer sets of *P-CaMV35S, bar, cp4 epsps, P-FMV35S, pat, T-nos, nptII,* and *ADH-1*, individually. 9–10, two no-primer controls. The test was performed in duplicate and we found that all the results were consistent with expectations. See **Supplementary Table 2** for data analysis

Figure 5: The results of testing two maize samples. 1–8: Capillaries pre-fixed with the LAMP primer set of *P-CaMV35S, bar, cp4 epsps, P-FMV35S, pat, T-nos, nptII,* and *ADH-1*, individually. 9–10, two no-primer controls. The test was performed in duplicate and then the results were compared with that of real-time PCR and the results were consistent. See **Supplementary Table 3** for comparison results. See **Supplementary Table 2** for data analysis.

Table 1: The components of LAMP primer fixing reagents. The components of LAMP primer fixing mix are listed in the left column of the table, and the volume of each component is listed in the right column.

Table 2: The reaction system of the capillary array-LAMP. The components of the reaction system of capillary array LAMP components are listed in left column, and the volume of each component is listed in the right column.

Supplementary Table 1: List of LAMP primer sequences.

Supplementary Table 2: Data analysis of the LAMP array experiments. The green color coded cells in the table indicate the positive result

Supplementary Table 3: The real-time PCR results reporting form.

DISCUSSION:
The CALM platform demonstrated here, which combines the LAMP technology with a capillary array, enables the simultaneous detection of multiple GMO-related gene targets in a single, highly effective and easy to operate test.

To successfully perform the multiplex LAMP reactions in the cassette, three critical points need to be noticed. Firstly, achieving the same height for the upper side of the capillaries and the hydrophilic and hydrophobic pattern of the capillary array are critical for simultaneously loading reagents into all the capillaries. The capillaries should be aligned by a plate after initial inserting into the PDMS support to ensure all of them can touch the loaded reaction mixture in the dish of the loading adaptor. When treating the capillary array with the super-hydrophobic coat, do not allow the coat to soak into the inner surface of the capillaries. Treat the outer surface of the capillaries by just loading coat on the top surface of the top PDMS support, allowing the coating to spread to the outer surfaces of the upper parts of the capillaries. If it occasionally happens that not all the capillaries are filled with the reagents, it may be prudent to load reagent directly into a specific capillary.

Secondly, be careful with the preparation of LAMP reagents. The whole process should be gently and quickly carried out on ice, due to the relatively low reaction temperature of LAMP and the fragility of the *Bst* polymerase. It's advisable to shake the reaction tube with the LAMP mixture gently instead of vortexing the tube violently after adding *Bst* polymerase. An inappropriate handling may cause failure of the reaction. In this experiment, ADH1(endogenous reference gene of Maize) is set as the positive control and two capillaries without primers are set as blank controls. So, if LAMP mixture is correctly handled, the positive control would show green and the blank control would remain unchanged after the test is performed.

Thirdly, due to the high efficiency of the LAMP reaction, false positive or carryover contamination may happen if there is a trace amount of DNA amplicon in the environment. Therefore, always bear in mind that the operating processes of pre-reaction and post-reaction should be strictly

separated in different areas and the LAMP products should be tightly sealed and discarded after analysis. Furthermore, a blank control should be set for indicating an adequate performance of the LAMP.

Intrinsically, CALM is a universal multi-target nucleic acid detection method with good flexibility and expandability. LAMP reactions performed in capillaries can be easily substituted by other types of isothermal amplification methods, such as rolling circle amplification (RCA)[29], and recombinase polymerase amplification (RPA)[30]. It can also be applied in other detection fields, such as pathogen detection[27] and disease diagnosis[31].

In the current form of this method, although primer sets are pre-fixed with chitosan, the process of LAMP mixture preparation is still needed when the test is performed, which lowers the simplicity of the method. To address this, we would try to preload all the reagents of LAMP in the capillary and then pipette the sample into the cassette. Another limitation for our method may be the lack of nucleic acid extraction, but we are now developing a mobile-based machine which would combine the extraction of nucleic acids, automatic image taking, and data analysis to achieve a real "sample-in and results-out" method.

In summary, we have developed the CALM platform by integrating multiplex LAMP with a capillary array. As a general nucleic acid detection method, CALM may also have potential in a wide range of other nucleic acid analysis applications.

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:

1   Urdea, M. *et al.* Requirements for high impact diagnostics in the developing world. *Nature.* **444 Suppl 1** 73-79, doi:10.1038/nature05448, (2006).
2   Yager, P., Domingo, G. J. & Gerdes, J. Point-of-care diagnostics for global health. *Annu Rev Biomed Eng.* **10** 107-144, doi:10.1146/annurev.bioeng.10.061807.160524, (2008).
3   Opota, O., Jaton, K. & Greub, G. Microbial diagnosis of bloodstream infection: towards molecular diagnosis directly from blood. *Clin Microbiol Infec.* **21** (4), 323-331, doi:10.1016/j.cmi.2015.02.005, (2015).
4   Guo, J. *et al.* MPIC: A High-Throughput Analytical Method for Multiple DNA Targets. *Anal Chem.* **83** (5), 1579-1586, doi:10.1021/ac103266w, (2011).
5   Shao, N. *et al.* MACRO: a combined microchip-PCR and microarray system for high-

throughput monitoring of genetically modified organisms. *Anal Chem.* **86** (2), 1269-1276, doi:10.1021/ac403630a, (2014).

6   Kamle, S. & Ali, S. Genetically modified crops: detection strategies and biosafety issues. *Gene.* **522** (2), 123-132, doi:10.1016/j.gene.2013.03.107, (2013).

7   Galvin, S., Dolan, A., Cahill, O., Daniels, S. & Humphreys, H. Microbial monitoring of the hospital environment: why and how? *J Hosp Infect.* **82** (3), 143-151, doi:10.1016/j.jhin.2012.06.015, (2012).

8   Sciancalepore, A. G. *et al.* Microdroplet-based multiplex PCR on chip to detect foodborne bacteria producing biogenic amines. *Food Microbiol.* **35** (1), 10-14, doi:10.1016/j.fm.2013.02.010, (2013).

9   Saxena, G., Bharagava, R. N., Kaithwas, G. & Raj, A. Microbial indicators, pathogens and methods for their monitoring in water environment. *J Water Health.* **13** (2), 319-339, doi:10.2166/wh.2014.275, (2015).

10   Hopwood, A. J. *et al.* Integrated Microfluidic System for Rapid Forensic DNA Analysis: Sample Collection to DNA Profile. *Anal Chem.* **82** (16), 6991-6999, doi:10.1021/ac101355r, (2010).

11   Estes, M. D. *et al.* Optimization of multiplexed PCR on an integrated microfluidic forensic platform for rapid DNA analysis. *Analyst.* **137** (23), 5510-5519, doi:10.1039/c2an35768b, (2012).

12   Niemz, A., Ferguson, T. M. & Boyle, D. S. Point-of-care nucleic acid testing for infectious diseases. *Trends Biotechnol.* **29** (5), 240-250, doi:10.1016/j.tibtech.2011.01.007, (2011).

13   Peeling, R. W. & Mabey, D. Point-of-care tests for diagnosing infections in the developing world. *Clin Microbiol Infec.* **16** (8), 1062-1069, doi:10.1111/j.1469-0691.2010.03279.x, (2010).

14   Perkins, M. D. & Kessel, M. What Ebola tells us about outbreak diagnostic readiness. *Nat Biotechnol.* **33** (5), 464-469 (2015).

15   Li, Y. *et al.* A universal multiplex PCR strategy for 100-plex amplification using a hydrophobically patterned microarray. *Lab Chip.* **11** (21), 3609-3618, doi:10.1039/c1lc20526a, (2011).

16   Notomi, T. *et al.* Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res.* **28** (12), doi:10.1093/nar/28.12.e63, (2000).

17   Mori, Y., Nagamine, K., Tomita, N. & Notomi, T. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochem Biophys Res Commun.* **289** (1), 150-154, doi:10.1006/bbrc.2001.5921, (2001).

18   Tomita, N., Mori, Y., Kanda, H. & Notomi, T. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nat Protoc.* **3** (5), 877-882, doi:10.1038/nprot.2008.57, (2008).

19   Iwamoto, T., Sonobe, T. & Hayashi, K. Loop-mediated isothermal amplification for direct detection of Mycobacterium tuberculosis complex, M-avium, and M-intracellulare in sputum samples. *J Clin Microbiol.* **41** (6), 2616-2622, doi:10.1128/Jcm.41.6.2616-2622.2003, (2003).

20   Goto, M., Honda, E., Ogura, A., Nomoto, A. & Hanaki, K. Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. *Biotechniques.* **46** (3), 167-172, doi:10.2144/000113072, (2009).

21   Iseki, H. *et al.* Development of a multiplex loop-mediated isothermal amplification (mLAMP) method for the simultaneous detection of bovine Babesia parasites. *J Microbiol Methods.* **71** (3), 281-287, doi:10.1016/j.mimet.2007.09.019, (2007).

22   Liang, C. *et al.* Multiplex loop-mediated isothermal amplification detection by sequence-

based barcodes coupled with nicking endonuclease-mediated pyrosequencing. *Anal Chem.* **84** (8), 3758-3763, doi:10.1021/ac3003825, (2012).

23   Shao, Y., Zhu, S., Jin, C. & Chen, F. Development of multiplex loop-mediated isothermal amplification-RFLP (mLAMP-RFLP) to detect Salmonella spp. and Shigella spp. in milk. *Int J Food Microbiol.* **148** (2), 75-79, doi:10.1016/j.ijfoodmicro.2011.05.004, (2011).

24   Fang, X., Chen, H., Yu, S., Jiang, X. & Kong, J. Predicting viruses accurately by a multiplex microfluidic loop-mediated isothermal amplification chip. *Anal Chem.* **83** (3), 690-695, doi:10.1021/ac102858j, (2011).

25   Stedtfeld, R. D. *et al.* Gene-Z: a device for point of care genetic testing using a smartphone. *Lab Chip.* **12** (8), 1454-1462, doi:10.1039/c2lc21226a, (2012).

26   Liu, D., Liang, G., Zhang, Q. & Chen, B. Detection of Mycobacterium tuberculosis using a capillary-array microsystem with integrated DNA extraction, loop-mediated isothermal amplification, and fluorescence detection. *Anal Chem.* **85** (9), 4698-4704, doi:10.1021/ac400412m, (2013).
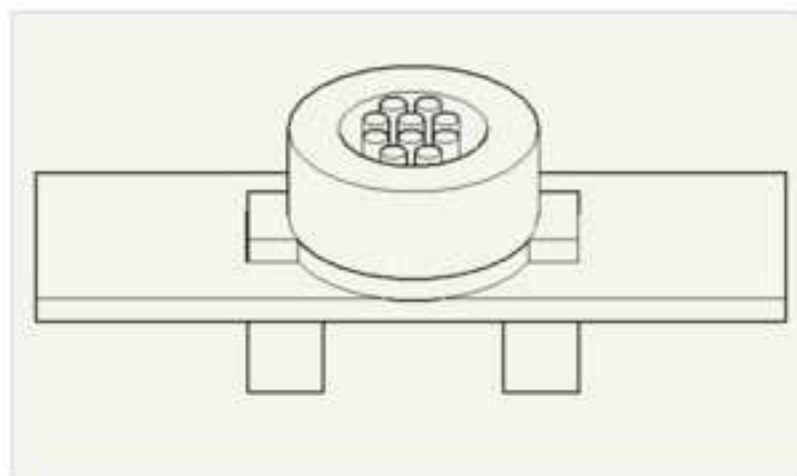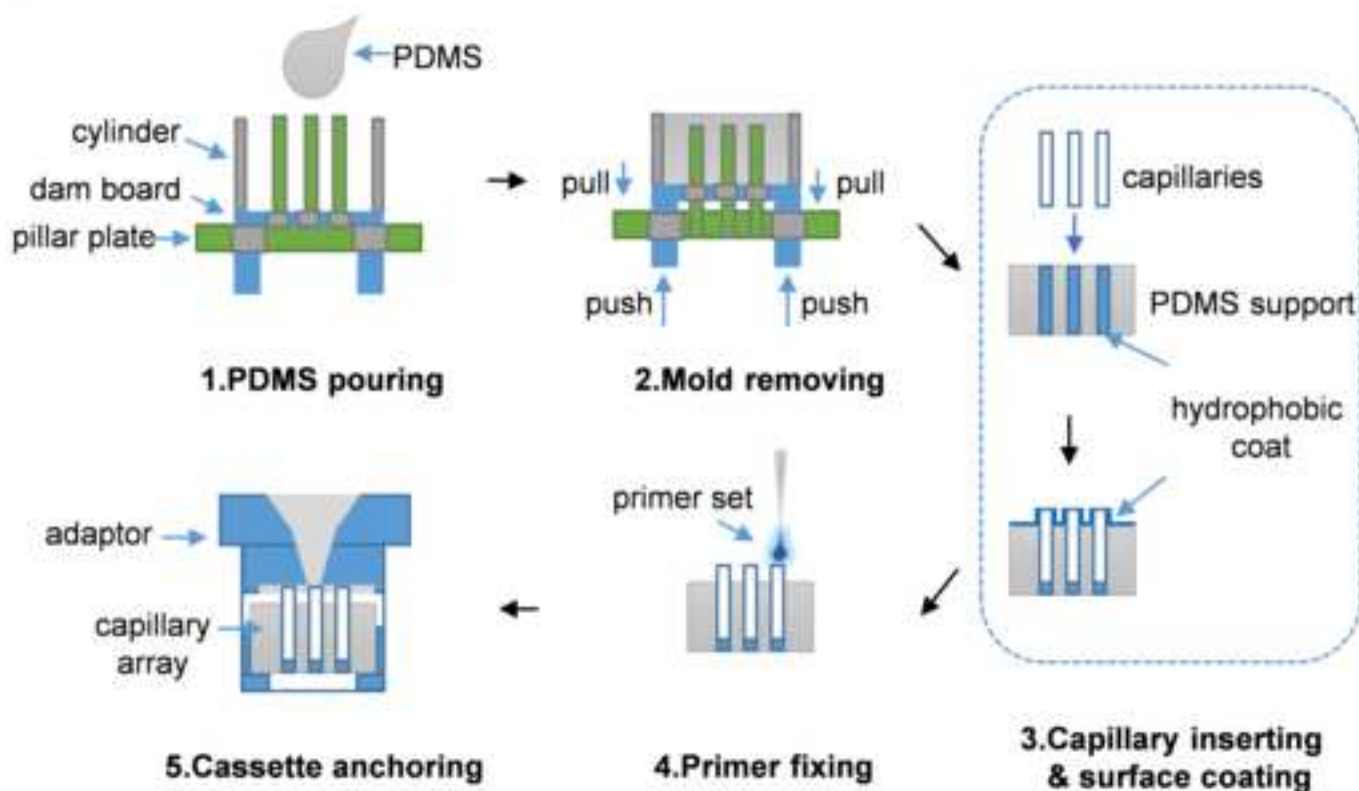
27   Zhang, Y. *et al.* Point-of-Care Multiplexed Assays of Nucleic Acids Using Microcapillary-based Loop-Mediated Isothermal Amplification. *Anal Chem.* **86** (14), 7057-7062, doi:10.1021/ac5014332, (2014).

28   Shao, N. *et al.* Visual detection of multiple genetically modified organisms in a capillary array. *Lab Chip.* **17** (3), 521-529, doi:10.1039/c6lc01330a, (2017).

29   Lizardi., P. M. *et al.* Mutation detection and single-molecule counting using isothermal rolling-circle amplification. *Nat Genet.* (1998).

30   Piepenburg, O., Williams, C. H., Stemple, D. L. & Armes, N. A. DNA detection using recombination proteins. *Plos Biol.* **4** (7), 1115-1121, doi:10.1371/journal.pbio.0040204, (2006).

31   Opota, O., Jaton, K. & Greub, G. Microbial diagnosis of bloodstream infection: towards molecular diagnosis directly from blood. *Clin Microbiol Infect.* **21** (4), 323-331, doi:10.1016/j.cmi.2015.02.005, (2015).

a

b

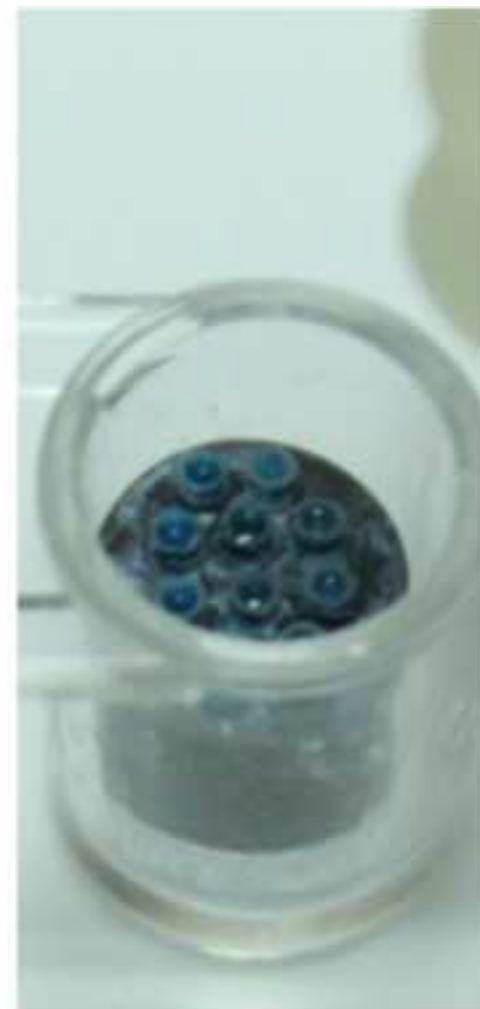1.PDMS pouring

2.Mold removing

3.Capillary inserting & surface coating

4.Primer fixing

5.Cassette anchoring

20 mm

Layout

Expected signals

MON863
1,6,7,8

MON89034
1,4,6,8

MON59122
1,5,8

GMO mix
1,4,5,6,7,8

Non-GM maize
8

NTC
None

20 mm
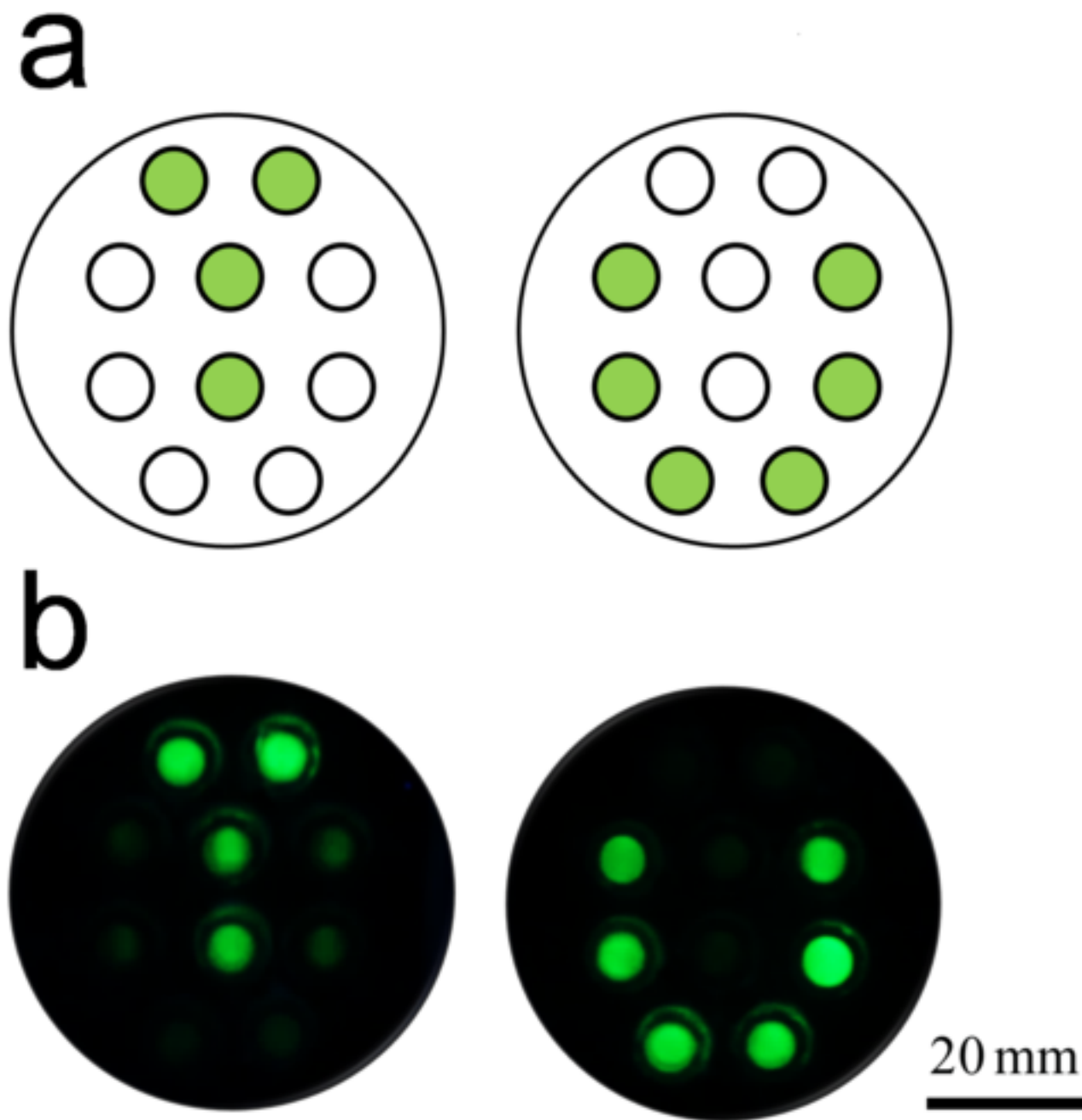
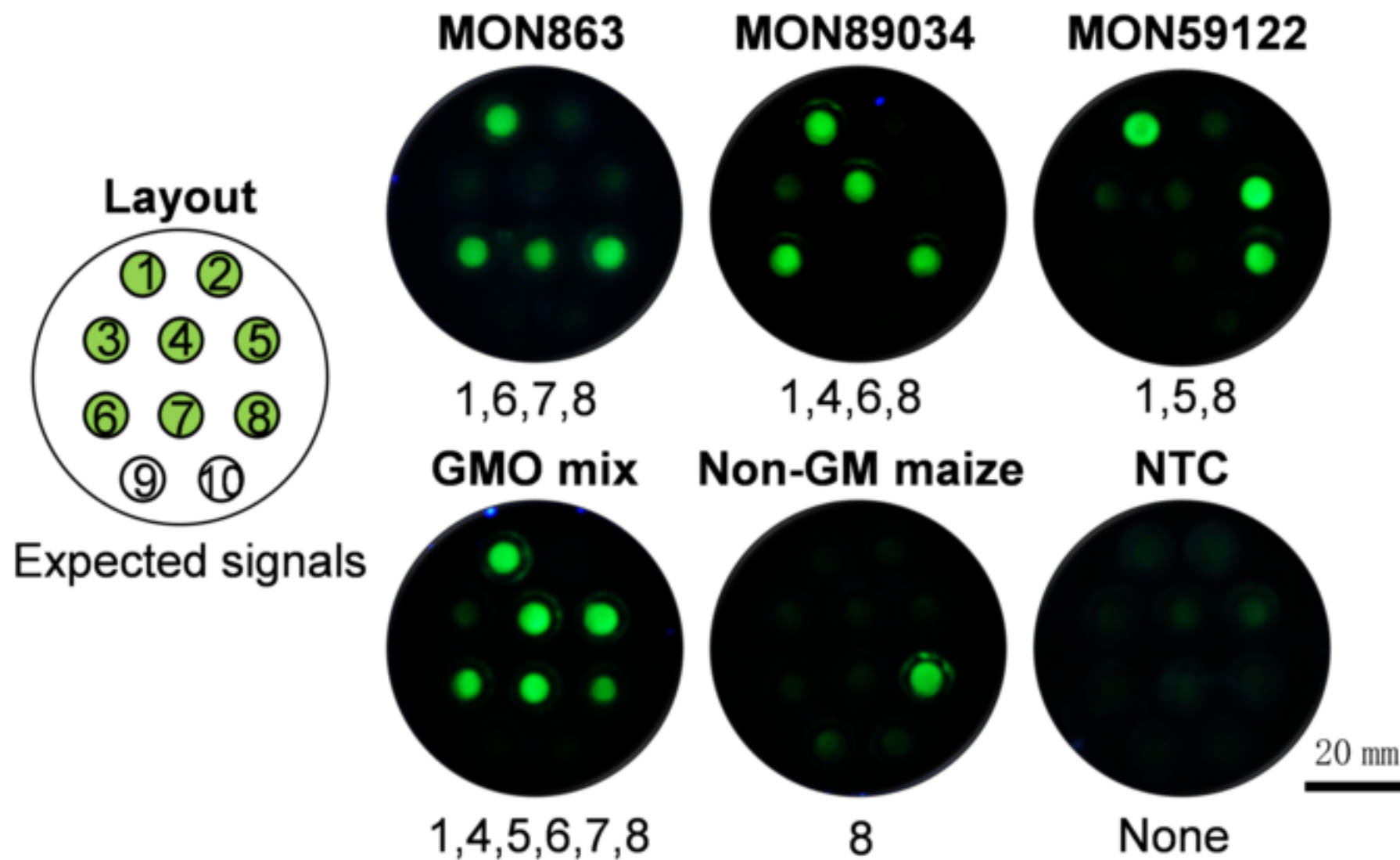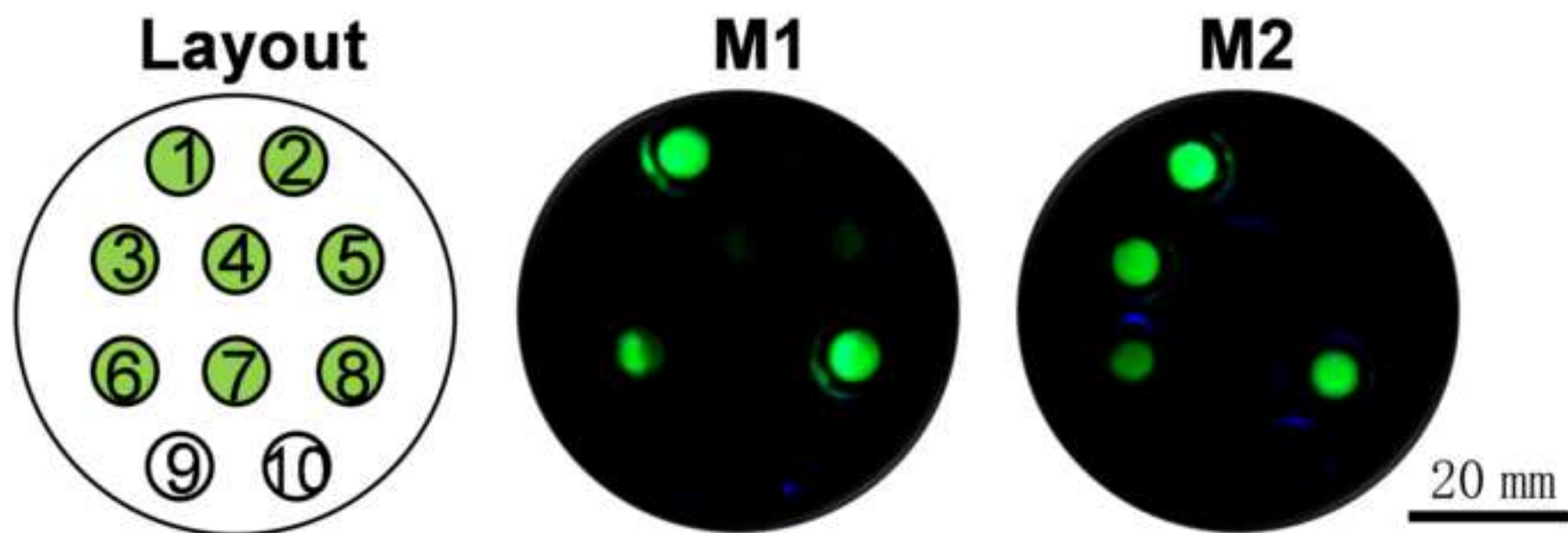Table 1

Table 1. The components of LAMP primer fixing reagents

LAMP primer fixing mix components（initial concentration）	Volume (μL)
ddH$_2$O	17.0
Chitosan（1.3%）	1.0
FIP/BIP primer（20 μM）	2.0/2.0
LoopF/LoopB primer（20 μM）	1.0/1.0
F3/B3 primer（20 μM）	0.5/0.5
Total volume	25.0

Table 2

Table 2. The reaction system of the capillary array-LAMP

LAMP components （initial concentration）	Volume (µL)
ddH$_2$O	11.6
MgSO$_4$ (100 Mm)	2.0
dNTPs (25 mM)	1.4
Betaine (5 M)	4.0
Buffer(10x)	2.5
Calcein (1.25 mM)	0.5
MnCl$_2$ (25 mM)	0.5
Bst polymerase(8U/µL)	1.5
plant DNA(10ng/µL)	1.0
Total volume	25.0

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
UltraEverDry(super-hydrophobic coat)	UltraTech	4001	supplier:Exiron chemistry(CHINA) CO.,LTD.
PDMS	Dow Corning	8332557	
Bst polymerase	New England BioLabs	M0275L	
betain	Sigma-Aldrich	B0300-1VL	
calcein	Sigma-Aldrich	C0875-5G	
MnCl$_2$	Sigma-Aldrich	MKBP0495V	
MgSO$_4$	New England BioLabs	B1003S	
dNTPs	Shanghai Sangon	B804BA0022	
chitosan	Shanghai Sangon	LJ0805S309J	
Photoshop 7.0 software	Adobe Systems Inc., CA, USA		Image analysis
GenePix Pro 6.1	Molecular Devices, CA, USA		microarray analysis software
AutoCAD	Adobe Systems Inc.		3D construction software

UV filter（ZWB2）　　　　　　YXSensing　　　　　　supplier：taobao

jove
JOURNAL OF
VISUALIZED EXPERIMENTS

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Visual Detection of Multiple Nucleic Acids in a Capillary Array

Author(s): Jianwei Chen, Ning Shao, Jiaying Hu, Rong Li, Yuanshou zhu, Dabing Zhang, Shujuan Guo, Junhou Hui, Peng Liu, Litao Yang, SHENG-CE, TAO

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: ✓ Standard Access ☐ Open Access

Item 2 (check one box):

✓ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

1

612542.6

4.   Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5.   Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6.   Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7.   Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8.   Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9.   Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party.   The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10.   JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE.   JoVE and its employees, agents and independent contractors shall have

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. <u>Indemnification</u>. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's
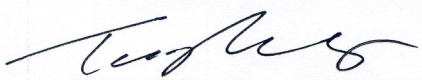
expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. <u>Fees</u>. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. <u>Transfer, Governing Law</u>. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name: Shence Tao

Department: Shanghai Center for Systems Biomedicine

Institution: Shanghai Jiao Tong University

Article Title: Visual Detection of Multiple Nucleic Acids in a Capillary Array

Signature: *[signature]*

Date: 2017-5-10

Please submit a <u>signed</u> and <u>dated</u> copy of this license by one of the following three methods:
1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

612542.6

Dear Dr. Alisha DSouza:

Thank you and the referees for helping us to improve our work. We have carefully checked the comments and suggestions. Please see below for the point-to-point response. We have also modified the manuscript accordingly. We wish our manuscript is now acceptable for publication in *JoVE*. Again, thank you very much for your help.

Sincerely yours,
Sheng-ce Tao

Changes recommended by the JoVE Scientific Review Editor: **(Dark green mark in the manuscript)**

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

We have checked it.

• Please include at least six keywords/phrases.

We have checked it.

• Abstracts: Please remove the superscript citations from the long abstract, and reorder the references accordingly.

We have omitted the citations and reorder the references.

• Introduction: Please expand your Introduction to include the following: The advantages over alternative techniques with applicable references to previous studies; Description of the context of the technique in the wider body of literature; Information that can help readers to determine if the method is appropriate for their application.

We have expanded the introduction. Line 87-92

• Protocol Language: Please ensure that all text in the protocol section is written in the imperative tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

We have checked it.

• Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, for steps that will not be filmed, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. For example:

1) 3.2.1: Please mention what button is clicked on in the software to do this, or which menu items need to be selected.

We have mentioned it in line 255-269.

• Protocol Numbering: Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.

We have checked it.

• Protocol Highlight: After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

We have highlighted the protocol.

• Results: Please add at least one paragraph of results text that explains your representative results in the context of the technique you describe; i.e. how do these results show the technique, suggestions about how to analyze the outcome etc. This text should be written in paragraph form under a "Representative Results" heading and should refer to all of the results figures. You may include the figure captions under this heading but the captions and figure text must be separate entities.

We have added the representative results. line279-296.

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We have checked and revised it.

• Figures::

1) Please provide each figure as an individual PDF, TIFF, JPEG or PNG files.

2) Fig 3b, 4,5: Please provide scale bars, and define them in the figure legend.

We have provided the scale bars.

3)All figures and tables need to be referenced in your manuscript text. Please include call outs at appropriate locations.

We have checked it.

• Tables:: Please remove the embedded Tables from the manuscript. All tables should be uploaded to the Editorial Manager site in the form of Excel files. A description of the table should be included with the Figure legends.

We have removed the embedded tables and included the description of the table. line 300-306

• Figure/Table Legends:: Please expand the legends to adequately describe the figures/tables. Each figure or table must have an accompanying legend including a short title, followed by a short description of each panel and/or a general description.

We have expanded the legends.

• References: Please abbreviate all journal titles.

We have checked and revised it.

• Commercial Language:JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are SYBR Green, AutoCAD (Adobe), Ultra-Ever Dry Top Coat, ThermoPol, ZWB2, photoshop 7.0 software (Adobe Systems Inc., GenePix Pro 6.1, etc.

1) Please use MS Word's find function (Ctrl+F or Command+F (on Mac)), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

We have revised it in the manuscript. line 168, 179, 246,255,261 359.

• Please define all abbreviations at first use.

We have checked it.

• Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.

We have checked it.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

We have checked it.

Reply to Reviewer #1: (**green mark in the manuscript**):

Manuscript Summary:

This paper present a capillary array device designed for visual detection of multiple nucleic acids. The main contribution of this work is reagents preloading and multiplexed detection to enhance the analysis throughput. However, the nucleic acid detection assay depends on manual operation; this may hinder acceptation of this methodology. The absence of nucleic acids extraction /purification is another limitation, which denies the device in dealing with large volume or complex samples.

Major Concerns:

It is advised that the author should add a figure showing the hydrophobic and hydrophlic area within the capillary, and the process illustration of primer immobilization, reagent preloading and sample injection.

Answers：We thank the reviewer for pointing out this. In fact, we have showed the whole process of making capillary cassette in figure 1, including PDMS pouring, mold removing, capillary inserting & surface coating, primer fixing and cassette anchoring. And we have showed the process of sample injection in figure 2. line 308-323

Reviewer #2: (**pink mark in the manuscript**)

Manuscript Summary:

This manuscript describes the fabrication and use of a cassette containing an array of capillaries where primers can be placed for the detection of target DNA sequences. The detection is carried out by first amplifying the DNA by LAMP and then detecting the UV absorbance of the product. As the authors state, the method appears promising for a number of environmental, industrial and health-related applications.

Major Concerns:

1) I think the English of the article should be reviewed. In its current form, I had difficulty understanding some parts, including parts of the protocol, which is key in this article.

Answers：We thank the reviewer for pointing out this. We have carefully checked and revised the manuscript.

2) The authors indicate that the protocol assumes that the mold with the shape for the micro-channels and the loading adapter has already been made. Although it is true that it may be too much for this protocol to explain the actual manufacturing of these parts, I think that it hinders the usefulness of the protocol. A possible solution would be for the authors to provide a working AutoCAD file of the design of the mold and the corresponding files necessary for 3D printing the adapter. They could have these files available in a repository owned by their institution or offer to provide these files to requesting researchers/developers. [Editor's Note: CAD files can be added as supplemental files in your submission]

Answers：We thank the reviewer for pointing out this. We have added the working Auto CAD file of the design of the mold and the adapter as supplemental files. We have also revised the manuscript accordingly, line 105-108.
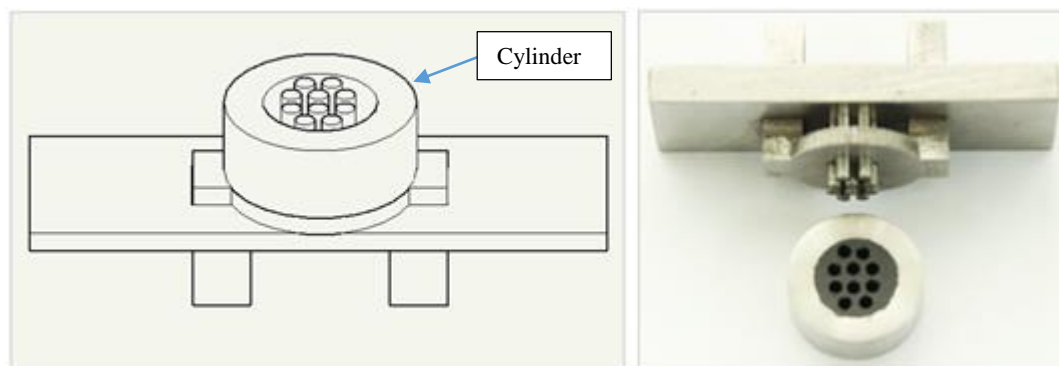
Minor Concerns:

- Step 1.2.3.) The authors indicate that the capillaries need to be shaken during the cleaning process to remove bubbles. It would be interesting here if they state whether sonication may be used for this purpose and how it compares in time and efficiency with just shaking it. They state that cleaning time may be extended when required. In this regard, it would be interesting if they state how long it may take for this cleaning process (and then, they could compare it with the time required using sonication)

Answers：Although the capillaries need to be shaken during the cleaning process to remove bubbles, it do not need to shake all the time. It's enough to shake the capillaries every 10 minutes. Bubble is usually produced at the beginning of adding H_2O_2 and as the time goes the amount of bubbles is reduced. So we haven't try sonication. Usually 30 min is enough for cleaning of the capillaries and we haven't observed obvious effect when extend the cleaning time.

- Step 1.3.) Although it may be rather evident and eventually the video will show it, it would be good to state explicitly where exactly the PDMS is poured onto.

Answers：We thank the reviewer for pointing out this. There is a cylinder of mold that will contain PDMS, when fabricating the reaction unit, we just directly pour PDMS into it. It is also showed in Figure 1. We have revised the manuscript accordingly. line 150.

- The authors mention in the Discussion section that the LAMP mixture should be shaken gently instead of vortexing it because it may cause failure of the reaction. Given this consideration, the authors should discuss the convenience of having a positive control (and what they would use for that) together with the blank control they propose as a negative control.

Answers：The positive and blank control would help us for better interpretation of the results. In this experiment, ADH1(endogenous reference gene of Maize) is set as the positive control and two capillaries without primers are set as blank controls. So, if LAMP mixture is correctly handled, the positive control would show green and blank control would keep unchanged after the test is performed. We have now discussed this in the manuscript line 368-371.

- The authors should indicate in the table of materials and equipment a suitable supplier for the Ultra-Ever Dry Top Coat for other researchers/developers to be able to obtain it

Answers：we have provided the supplier for the Ultra-Ever Dry Top Coat. Researchers can obtain it from Exiron chemistry (CHINA) CO., LTD.
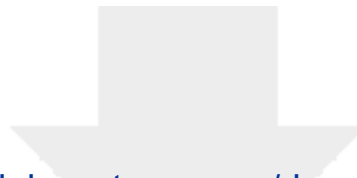
Click here to access/download
Supplemental File
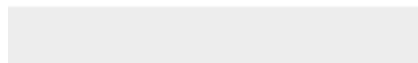Supplemental File 1-Sample Loading Adaptor.dwg

Click here to access/download
Supplemental File
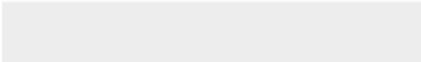Supplemental File 2-Steel Mold for PDMS Support.dwg

Supplemental Table 1

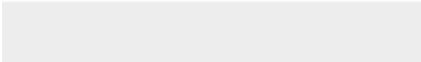Click here to access/download
Supplemental File
Supplementary Table 1_rev.xlsx

Supplemental Table 2

Click here to access/download
Supplemental File
Supplementary Table 2_rev.xlsx

Click here to access/download
Supplemental File
Supplementary Table 3_rev.xlsx