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Colorimetric detection of *Maize chlorotic mottle virus* by reverse transcription loop-mediated isothermal amplification (RT-LAMP) with hydroxynapthol blue dye

Zhanmin Liu[a*], Xueying Xia[a], Cuiyun Yang[b], Junyi Huang[a]

a School of Life Sciences, Shanghai University, Shanghai, P.R.C, 200444, China;

b Shanghai Entry-Exit Inspection and Quarantine Bureau, Shanghai 200135, China.

Abstract

Maize chlorotic mottle virus causes corn lethal necrosis disease, and can transmit via infected maize seeds. It remains a challenge to detect this virus to prevent its introduction, infection and wide transmission fields. For this purpose, colorimetric assay for detection of *Maize chlorotic mottle virus* was developed in cooperation with RT-LAMP and hydroxynapthol blue dye(HNB). The reaction was performed to amplification in one step in a single tube at the optimum condition (64°C for 60 min, 150 mM HNB and 2 mM $MgSO_4$). Samples infected with MCMV developed a characteristic sky blue color after the reaction but those uninfected with MCMV or infected with other plant pathogenic virus did not. Results of HNB staining method were reconfirmed through LAMP products gel electrophoresis. The sensitivity of this assay was 4.8 pg/μl of RNA of *Maize chlorotic mottle virus* per reaction, which was approximately 10-fold higher sensitivity over conventional RT-PCR test. The results indicate that this assay is highly species-specific, simple, low-cost, and visual for easy detection of *Maize chlorotic mottle virus* in plant tissues. Therefore, colorimetric detection of *Maize chlorotic movirus ittle* s a potentially useful tool for middle or small-scales corporations and entry-exit inspection and quarantine bureau to detect maize seeds or plant tissues infected with *Maize chlorotic mottle virus*.

Keywords: Colorimetric detection; *Maize chlorotic mottle virus*; Loop-mediated isothermal amplification; Hydroxynapthol blue dye

Introduction

Maize chlorotic mottle virus (MCMV) is the only species in the genus *Machlomo virus* (family Tombusviridae), and it is the single strand RNA virus. As an important plant pathogenic virus,

*Corresponding author. Zhanmin Liu,School of Life sciences, Shanghai University, 333 Nanchen Road, Shanghai, 200444, China. Tel.: +86-21-66135166; Fax: +86-21-66135166.
E-mail: zhmliu@shu.edu.cn

MCMV was first reported infecting *Zea mays* in Peru[1] where it caused losses of 10-15% in floury and sweet corn cultivars. The combination of MCMV with the *Maize dwarf mosaic virus*, *Sugarcane mosaic virus*, or *Wheat streak mosaic virus* may also cause a severe symptom known as maize lethal necrosis[2]. In addition, this virus can be introduced readily into other countries by seeds or vectors[2, 3]. Because of the potential threat to the production of maize crops, it was listed as a quarantine pest by the Chinese government in 2007, and was identified in maize seeds imported from the United States, Germany, and Mexico, indicating a high risk of MCMV introduction with the increasing international exchange of maize seeds. In order to prevent introduction of MCMV through international exchange of maize seeds and wide spread in the fields, many kinds of assays have been used for detection of MCMV, including biological indexing[4], ELISA[5], electron microscopy[6], a real-time RT-PCR[7], and surface plasmon resonance[5]. However, biological indexing is time-consuming, and labor-intensive; the results of ELISA are dependent on the quality and availability of expensive antibodies; electron microscopy, real-time RT-PCR and surface plasmon resonance require very expensive equipment; gold nanoparticles is not extremely stable; RT-PCR is the requirement of running gels increases the risk of contamination during post-PCR manipulations.

More and more assays have been developed for detection of biomolecular, such as DNA/RNA[8-11], protein[12], Dopamine[13], Interleukin-6[14] and cysteine[15]. Furthermore, Loop-mediated isothermal amplification (LAMP), a novel constant temperature nucleic acid amplification technique, has demonstrated a rapid, low-cost, easy operating, highly sensitive and specific detection method applied in several fields[16, 17]. Under theisothermal conditions, the target sequences are amplified with high efficiency, rapidity and specificity according to 5 primers (two internal and two external primers, and one loop primers) designed by several regions of target gene and *Bst* DNA polymerase with strain displacement activities. In addition, accelerated amplification via loop primers reduces the building of artefacts to a minimum[18]. Furthermore, LAMP also offers the possibility of analysis without gel electrophoresis, for instance by measuring the turbidity of magnesium pyrophosphate that is formed as aside product in positive reactions by using dyes like SYBR-Green[19] or hydroxynapthol blue (HNB)[20]. The simplicity of the LAMP method, which does not require special equipment including a thermal cycler, makes it suitable for field testing. At present, this method has been successfully used for detections of *Mycobacterium tuberculosis*, and *Listeria monocytogenes*[19, 21]. In the paper, we developed a rapid LAMP method for diagnosis of MCMV and explored the optimal reaction conditions to determine the detection

system of MCMV and verify the reliability through detecting the contaminated samples. Moreover HNB is used in our assays, which enables an easier, cheaper and more sensitive discrimination of positive (blue) and negative (violet) reactions with the naked eye compared to other methods for LAMP product detection.

Materials and methods

Materials and reagents

MCMV-Agdia2219 was purchased from Agdia, USA; MCMV-ZJ was provided by Beijing Entry-Exit Inspection and Quarantine Bureau, Beijing, China; MCMV-BJ was obtained from China Agricultural University; MCMV-field was obtained from field; MCMV-1087, MCMV-2094, MCMV-1907 isolates were supplied by Shanghai Entry-Exit Inspection and Quarantine Bureau, Shanghai, China. *Carnation ringspot virus* (CRSV), *Odontoglossum ringspot virus* (ORSV),*Cucumber green mottle mosaic virus* (CGMMV), *Lily symptomless virus* (LSV),*Cymbidium mosaicvirus* (CymMV),*Southern bean mosaic virus* (SBMV) isolates were kept in Shanghai Entry-Exit Inspection and Quarantine Bureau, Shanghai, China. MCMV was inoculated in *Zea mays*, and the infected tissues were harvested. *Arabis mosaic virus* (ArMV), *Carnation ringspot virus* (CRSV), and *Tobacco rattle virus* (TRV) were inoculated in *Chenopodium quino*, CGMMV were inoculated in *Cucumissativus*, and the infected tissues were harvested after 14 days. The leaf of *Phalaenopsis Aphrodite* with ORSV, and the lily bulbs with LSV were intercepted in entry plants on port. DNA Marker I was purchased from TIANGEN Biotech (Beijing) Co.,Ltd (Beijing. China), *Bst* DNA polymerase was purchased from NEB, Betaine and HNB was purchased from Sigama Co.,Ltd. AMV reverse transcriptase was purchased from Takara BiotechCo.,Ltd (Dalian, China).

Viral RNA extraction

Viral genomic RNA was extracted from100 mg of leaves infected with the virus, respectively using TIANamp Virus RNA Kit (Beijing. China) according to the manufacturer's instructions. The RNA was elutedin70 μl of RNase-free water and stored at -80°C.

Primers design

A sequence alignment of the 400-bp sequences located at nucleotide position 4001-4400of nine MCMV genomes (GenBank accession no. KP851970.1, NC_003627.1, KJ782300.1, KF010583.1, JQ982470.1, JQ982469.1, GU138674.1, EU358605.1 and X14736.2) was aligned using Vector

NTI Advance version 11 (Invitrogen, Auckland,New Zealand). A reference template was generated according to conserve region of the alignment. Primer explorer v3 software (http://primerexplorer.jp/e/index.html) was used to design the MCMV specific primers. The primers consisted of two outer primers (F3 and B3), two inner primers(FIP and BIP), and one loop primers (LF). The specificity of the primer and probe sequences were analyzed using the Basic Local Alignment Search Tool (BLAST). The sequences of the oligonucleotide primers were synthesized by Sangon Biotech (Shanghai, China) shown in Table 1, and Fig. 1 described primers for RT-LAMP method.

Table 1 The RT-LAMP primers used in this study

Primers	sequences
F3	5′-AGACCGGAATAACCAGTCCT- 3′
B3	5′-TGCCCCAGGGTTAAGTGTA- 3′
LF	5′-CGATTTAGGCTCCCAAACA C-3′
FIP	5′-CTCCAGTCATGGTCATCACGCATTTTGGCAGAGTCCTGCCAATC- 3′
BIP	5′-CAACCGCAGACTGGGCGTATTTTTGCACCGTTCGTAAGTACGT- 3′

Optimization of RT-LAMP reaction conditions

Optimization of concentrations of the $MgSO_4$ in the reaction system was performed as followed. the RT-LAMP reaction was carried out in 25 μl reaction mixture containing 20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.2% TritonX-100, 1.2 mM dNTPs, 1.6 mM each FIP and BIP primers, 0.2 μM each F3 and B3 primers, 0.8 μM LF primers, 1 μl template RNA, 10 U *Bst* DNA polymerase , 5 U AMV reverse transcriptase, dNTPs (2.5mM) , 0, 2 mM, 3 mM, 4 mM, 6 mM, 8 mM $MgSO_4$, respectively and adding ultrapure water to 25 μl. RT-LAMP reaction was performed at 64 °C for 60 min and then heated at 80 °C for 5 min to terminate the reaction. The amplified products were analyzed by 2% agarose gel electrophoresis and the results were documented by a gel imaging system.

Optimization of reaction temperature of reaction system was carried out according to the procedure below, the RT-LAMP reaction was carried out in 25 μl reaction mixture containing 20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.2% TritonX-100, 1.2 mM dNTPs, 1.6 μM of each FIP and BIP primers, 0.2 μM of each F3 and B3 primers, 0.8 μM of LF primers, 1 μl template RNA, 10 U *Bst* DNA polymerase , 5 U AMV reverse transcriptase, dNTPs (2.5mM) , 2

mM $MgSO_4$ and add ultrapure water to 25 µl. RT-LAMP reaction was performed at 60, 62, 64, 66 °C for 60 min respectively and then heated for 5 min at 80 °C to terminate the reaction. The amplified products were analyzed by 2% agarose gel electrophoresis and the results were documented by a gel imaging system.

Optimization of reaction time of reaction system was followed as the protocol below, the RT-LAMP reaction was carried out in 25 µl reaction mixture containing 20mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.2% TritonX-100, 1.2 mM dNTPs, 1.6 µM of each FIP and BIP primers, 0.2 µM of each F3 and B3 primers, 0.8 µM of LF primers, 1 µl template RNA, 10U *Bst* DNA polymerase , 5 U AMV reverse transcriptase, dNTPs (2.5mM), 2 mM $MgSO_4$ and add ultrapure water to 25 µl. The LAMP reaction was performed at 64 °C for 30, 45, 60, 75 and 90 min, respectively and then heated at 80 °C for 5 min to terminate the reaction. The amplified products were analyzed by 2% agarose gel electrophoresis and the results were documented by a gel imaging system.

Optimization of concentrations of the HNB in the reaction system was performed as followed, the RT-LAMP reaction was carried out in 25 µl reaction mixture containing 20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.2% TritonX-100, 1.2 mM dNTPs, 1.6 µM each FIP and BIP primers, 0.2 µM each F3 and B3 primers, 0.8 µM LF primers, 1 µl template RNA, 10 U *Bst* DNA polymerase , 5 U AMV reverse transcriptase, dNTPs (2.5mM), 2 mM $MgSO_4$, 100, 120, 150, 200 µM HNB respectively and adding ultrapure water to 25 µl. RT-LAMP reaction was performed at 64 °Cfor 60 min and then heated at 80 °C for 5 min to terminate the reaction. The amplified products were analyzed by 2% agarose gel electrophoresis and the results were documented by a gel imaging system. In addition, the amplification product could also be visually inspected according to the color change from violet to sky blue, while the negative control remained violet. The tubes were observed by naked eyes and photographed under the natural light.

Specificity of colorimetric detection

The specificity of RT-LAMP was verified by performing the assay of RNA of MCMV-Agdia2219 and other viral, including CRSV, ORSV, CGMMV, LSV, CymMV and SBMV. The RT-LAMP assay was done as described earlier. As before, the assays were assessed based on gel electrophoresis and HNB-visualized color change.

Sensitivity of colorimetric detection

The sensitivity of RT-LAMP was estimated through detecting the seven serial dilutions

containing serial 10-fold dilutions of RNA (48 ng/µl to 4.8 fg/µl). The RT-LAMP assay was done as described earlier. As before, the assays were assessed based on gel electrophoresis and HNB-visualized color change.

Colorimetric detection of MCMV from artificially contaminated maize leaves

In order to evaluate the capability of colorimetric detection of MCMV, twelve maize leaves contaminated with viruses analyzed using conventional RT-PCR were tested as described above.

Results

Determination of the optimal reaction condition of RT-LAMP for MCMV

The RT-LAMP was performed using MCMV genomic RNA as the template to determine the optimal temperature, reaction time and concentration of $MgSO_4$ in the study. The assay was further optimized the concentration of HNB for an optimal hydroxynaphthol blue color change during the reaction. A successful RT-LAMP with specific primers at 60–66 °C for 60 min produced many bands of different sizes upon agarose electrophoresis because the RT-LAMP products consisted of several inverted-repeat structures (Fig. 2). It was evident that distinct bands at 64 °C were brighter than those of other reaction temperatures (Fig. 2), so the optimal temperature for this RT-LAMP is 64 °C. The optimum concentration of $MgSO_4$ required for amplification by RT-LAMP was studied in the study, and the results suggested that efficient amplification of template RNA could be obtained at 2 mM $MgSO_4$ during the concentration of $MgSO_4$ from 0 to 8 mM (Fig. 3). Although positive reactions were detected using different reaction time among 30 and 90 min (Fig. 4). No difference was observed when the RT-LAMP assays were performed in 60, 75 and 90 min, respectively. No amplification was observed for 30 min and amplifications of 45 min were less than others. Hence, a reaction time of 60 min was selected as the optimum reaction time for a RT-LAMP assay. Optimization of RT-LAMP reaction conditions (temperature, time and concentration of $MgSO_4$) revealed that the ideal settings for the primer set were 64 °C for 60 min and concentration of $MgSO_4$ was 2 mM. Therefore, the RT-LAMP assays were subsequently performed under these conditions. According to the optimal reaction condition of RT-LAMP for MCMV, HNB was tested and optimized within the range of 100 to 200 mM and had an optimum at 150 mM. RT-LAMP products were detected both by agarose gel electrophoresis and by visual inspection (Fig. 5A, 5B).

Specificity test results

The specificity of RT-LAMP was verified by performing the assay of RNA of MCMV-Agdia2219 and other viral, including CRSV, ORSV, CGMMV, LSV, CymMV and SBMV. RT-LAMP products were detected both by agarose gel electrophoresis and by visual inspection (Fig. 6), the results described that only maize leaves infected with MCMV could amplify the bright, specific band in lan1 in Fig.6A, and correspondingly, there appeared the blue in Fig6B, other viruses and ddH_20 could not amplify the products in Fig.6A and showed the violet in Fig.6B. RT-LAMP amplified only MCMV genome with no cross-reactivity with other viruses tested in the colorimetric assay. Colorimetric assay can observe visually the change of color there by no electrophoresis instrument is needed. The results suggested the colorimetric detection of MCMV had highly specific to MCMV.

Sensitivity of colorimetric detection of MCMV

In order to determine the sensitivity of the colorimetric detection of MCMV, RT-LAMP was performed using 10-fold serial dilutions of MCMV-Agdia2219 genomic RNA. RT-LAMP products were detected both by agarose gel electrophoresis and by visual inspection (Fig. 7). RT-LAMP was successfully amplified when the concentrations of template were no less than 4.8 pg/μl (Fig. 7A). Simultaneously, the reaction products were blue (Fig.7B). Therefore, the sensitivity of the colorimetric detection was 4.8 pg/μl MCMV genomic RNA.

Detection MCMV in crude extractions from artificially contaminated maize leaves

The utility of the colorimetric assay of MCMV was examined using five artificially contaminated maize leaves, one field leaf infected with MCMV and six other viruses (CRSV, ORSV, CGMMV, LSV, CymMV and SBMV). The results indicated that in this test, six samples were MCMV positive tested by RT-LAMP and colorimetric assay (Fig. 8A, B) which had been analyzed using conventional RT-PCR (data unpublished). The detection accuracy of colorimetric assay was 100% when compared with the RT-PCR. The results showed that it is reliable to use a colorimetric assay to detect MCMV in samples.

Discussion

Recently, moving protein gene has been used as markers for *Cucumber green mottle mosaic virus*[22, 23], the signal transduction gene, *vicK* has been identified as a molecular marker for detection of *Staphylococcus aureus* and lmo0460 sequence has been identified as a molecular marker for detection of *Listeria monocytogenes* in our previous research based on comparative genome and sequences alignment analysis[21, 24, 25], which implies some sequences in genome are

possible to be molecular markers for virus except for coat protein gene[21, 24, 26]. Based on the similar strategy, 400-bp sequence (X14736.2) has been identified as a novel molecular marker for detection of MCMV by means of bioinformatics. Simultaneously, RT-LAMP also confirmed the result in our study.

Huang et al reported that the sensitivity of quartz crystal microbalance based sensor for detection of MCMV is 250 pg/μl, which is similar to that of the existing ELISA method[27]. However, in this study, colorimetric assay was developed to detect MCMV with the sensitivity to detect as low as 4.8 pg of total RNA in the reaction system, which was more sensitive than a quartz crystal microbalance based sensor for MCMV diagnosis (Table 2). Compared with conventional RT-PCR (30 pg of total RNA), colorimetric detection of MCMV had significantly higher sensitivity levels. This result was concordant with previous reports of the LAMP method for detecting *Listeria monocytogenes*[28].

Table 2 Several assays for detection of MCMV

Methods	Materials	Linear range	Detection limit	References
Biological indexing	Maize leaf	*****	*****	4
ELISA	Maize leaf	1--1000ppb	100 ppb	5
Electron microscopy	Maize leaf	*****	*****	6
Real-time RT-PCR	Maize seeds	≥4 fg	4 fg	7
SPR	Maize leaf	1--1000ppb	100 ppb	5
RT-LAMP	Maize leaf	≥4.8 pg	4.8 pg	In this study

Note: ***** show no data.

The reliability of performance was evaluated using eleven maize leaves artificially contaminated with viruses, and one field sample had been determined using conventional RT-PCR. Of all samples, the rate of coincidence was 12 of 12 (100%), which is consistent with the results of RT-PCR, and the result indicated the colorimetric assay is valid. This is because that China has strengthened the prevention and control of introduction of maize seed through international exchange of maize seeds, there is seldom a report MCMV transmission, and it is very difficult to gain field isolates. Therefore, MCMV transmission in the corn field was simulated in our lab, artificially infected samples were prepared to evaluate the novel assay for MCMV detection. The results showed that the positive results of infected samples were same, which was due to the specificity and reliability of the RT-LAMP. Since we did not have access to more field isolates, the current method has only been tested on the limited number of known MCMV isolate. Therefore, we hope to be able to obtain more trials with field isolated viruses to tamp fundamentals for assay applications.

In recent year, severe chlorotic mottle symptoms found in sweat corns or sugarcans have been observed at the base of infected leaves with MCMV in many countries and regions[26, 29, 30]. Sugarcan and corn in field were also found to be infected with MCMV in Yunnan province, China in 2013. Because this colorimetric assay only required simple sample preparation and the results were obtained less than two hours, which make it rapid, sensitive, simple detection of MCMV. Colorimetric assay for MCMV detection is very suitable for the detection field leaves samples without than other assays according to only our sample preparation and RT-LAMP.

Conclusion

In this work, we took full advantage of RT-LAMP with HNB-based color change and provided a low-cost, easy to operate, and sensitive assay for visual detection of MCMV. The assay described is easily read with the naked eye. In comparison with other methods for detection of MCMV, the method is more attractive because of its high sensitivity, low cost, ready availability and simple manipulation. This is the first application of RT-LAMP with HNB-based color change for detection of MCMV by the naked eye without the need of expensive detection instruments.

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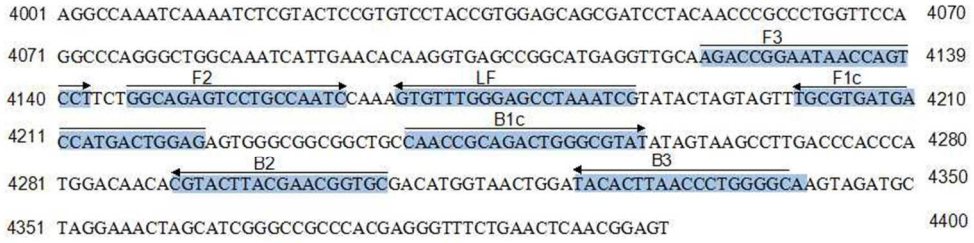
Figure legends:

Fig.1. Position and orientation of MCMV RT-LAMP primers within the reverse nucleotide sequence of MCMV (GenBank accession no. EU358605.1). Nucleotide positions from 40001 to 4400 are shown.

Fig. 6 Specificity analyses of colorimetric detection of MCMV. (A) RT-LAMP detection of MCMV by electrophoresis; (B) Color reaction with HNB. Lane M, 600 bp DNA marker; Lane 1: MCMV-Agdia2219; lane 2: CRSV; lane 3: ORSV; lane 4: CGMMV; lane 5: LSV; lane 6: CymMV; lane 7: SBMV; lane 8: ddH_20.

Fig.7 Sensitivity analysis of colorimetric detection of MCMV. (A) RT-LAMP detection of MCMVby Electrophoresis; (B) Color reaction with HNB. Lane M, DNA marker 600; line 1-8: diluted samples of MCMV with RNA crude solution, 48 ng/µl, 4.8 ng/µl, 480 pg/µl, 48 pg/µl, 4.8 pg/µl, 480 fg/µl, 48 fg/µl, 4.8 fg/µl, respectively. Lane 9: negative reaction.



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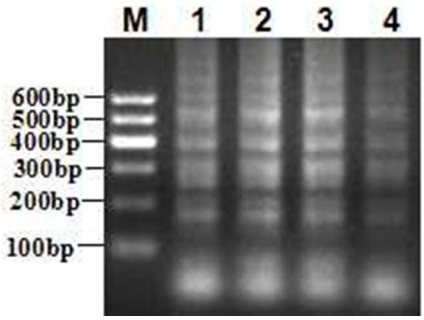


Fig. 2 Temperature optimization results of RT-LAMP amplified MCMV Lane M, the DNA marker 600; Lane 1-4: 60, 62, 64, 66℃.
58x42mm (96 x 96 DPI)

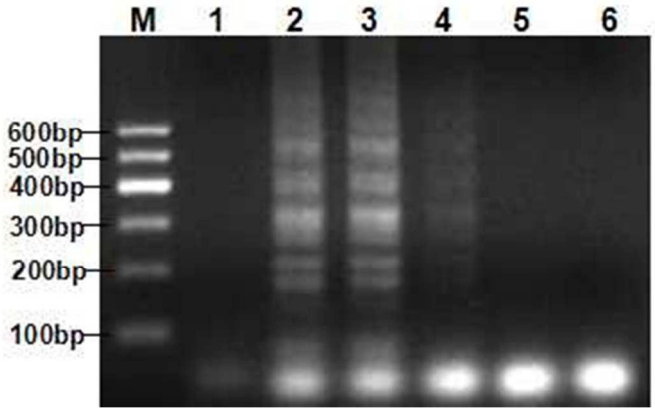


Fig. 3 Mg2+ optimization results of RT-LAMP amplified MCMV. Lane M, DNA marker 600; lane 1-6: 0, 2, 3, 4, 6, 8 mM.
88x55mm (96 x 96 DPI)

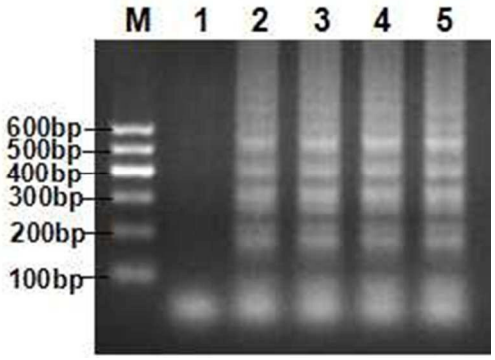


Fig. 4 Time optimization results of RT-LAMP amplified MCMV. Lane M, DNA marker 600; lane 1-5: 30, 45, 60, 75, 90 min.
66x48mm (96 x 96 DPI)

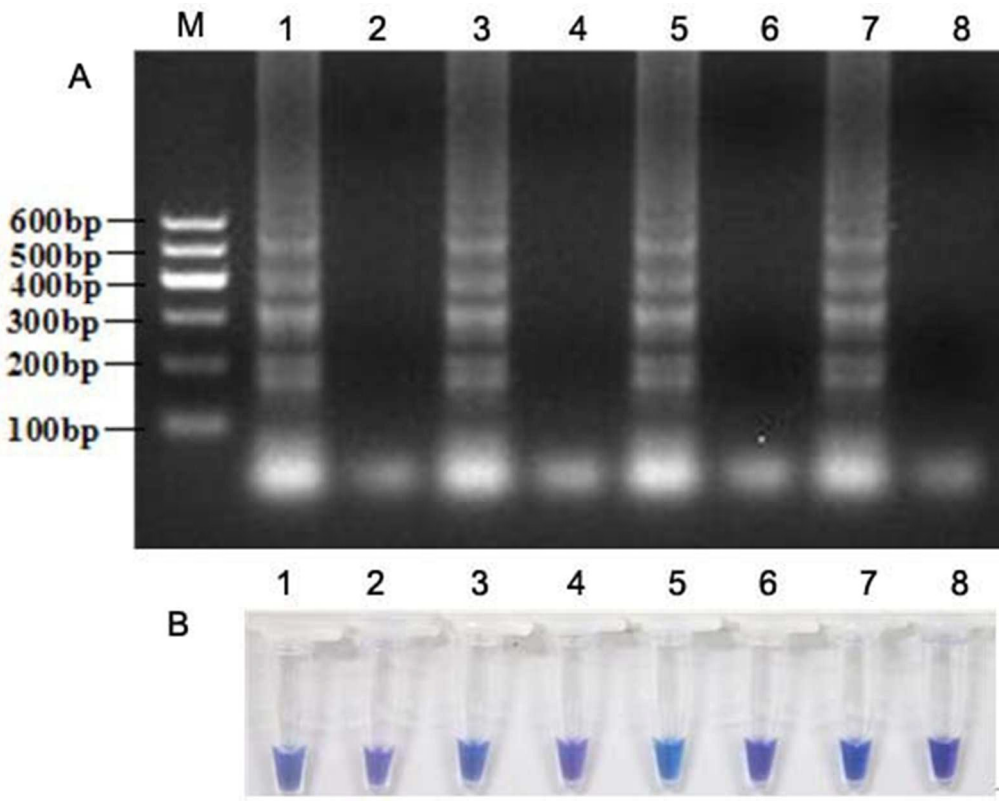
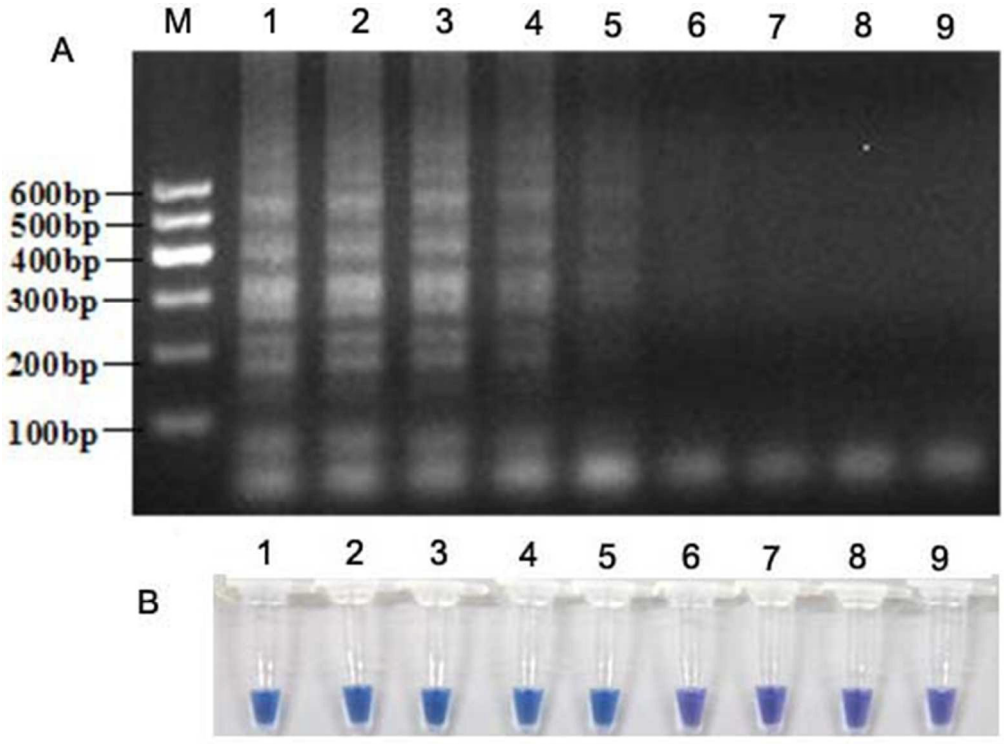
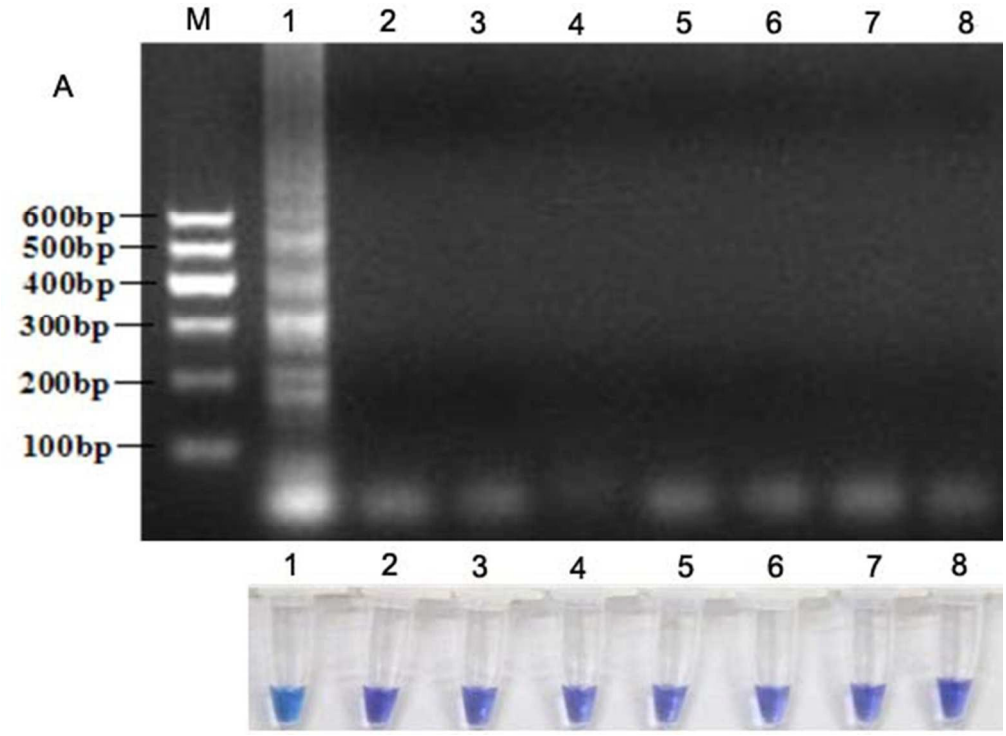


Fig. 5 HNB optimization results of RT-LAMP detection for MCMV. (A) RT-LAMP detection of MCMV by Electrophoresis; (B) Color reaction with HNB. Lane M, DNA marker 600; line 1, 3,5,7: positive reaction with HNB; line 2, 4, 6, 8:negative reaction with HNB; line 1, 2: 100 mM; line 3, 4: 120 mM; line 5, 6: 150 mM; line 7, 8: 200 mM;
186x148mm (72 x 72 DPI)



184x147mm (72 x 72 DPI)



207x152mm (72 x 72 DPI)

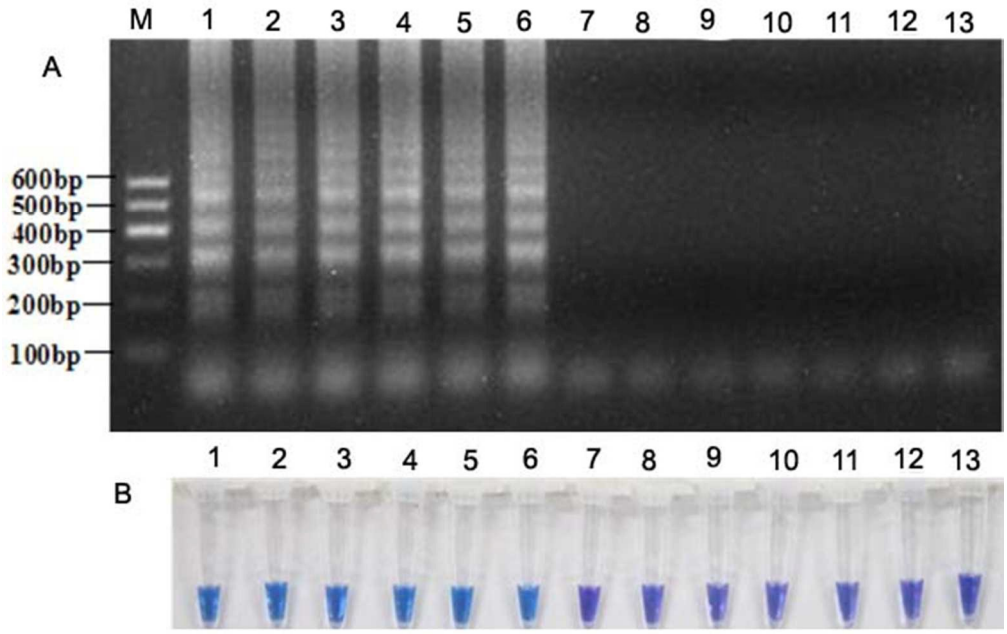


Fig. 8 Results of colorimetric assay for MCMV detection. (A) RT-LAMP detection of MCMV by electrophoresis; (B) Color reaction with HNB. Lane M, DNA marker 600; lane 1: MCMV-ZJ; lane 2: MCM-V1087; lane 3: MCMV-C2094; lane 4: MCMV-C1907; lane 5: MCMV-BJ; lane 6: MCMV-field; lane 7: CRSV; lane 8: ORSV; lane 9: CGMMV; lane 10:LSV; lane 11: CymMV; lane 12:SBMV; lane 13: ddH2O.
223x143mm (72 x 72 DPI)