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Rapid Screening of Barley Stripe Mosaic Virus Via Microfluidic Chip Method

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Abstract: *Barley stripe mosaic virus*, the type member of *Hordeivirus*, constitutes an important seed-transmitted pathogen distributed in almost every barley growing area worldwide. Researches on efficient detection of BSMV suitable in wild application are in urgent need. New platforms like microfluidic technology keep progressing rapidly in past decades and gains increasing influence on life sciences. Thus in this study, one rapid screening method for BSMV via microfluidic chip was established. Through method validity, we succeeded in demonstrating the amplification of BSMV within 30 min at one constant temperature using a microfluidic chip assay. Sensitivity analysis conformed that this method obtained a detection limit at 1.00×10^2 copies/μL, which was even more sensitive than real-time RT-PCR. *Maize chlorotic mottle virus*, *Maize dwarf mosaic virus*, *Oat mosaic virus*, and *Wheat streak mosaic virus* were chosen in specificity test along with BSMV, but only BSMV expressed typical amplification curves and Ct. Microfluidic analytic systems realize miniaturization by reducing the reaction volume to just 5 μL, which has several superiorities such as saving expensive reagents and reducing inspection costs. Moreover this microfluidic chip assay can simultaneously detect a variety of viruses, and the whole process takes just 1 hour, which greatly speeds up the detection and improves efficiency. All in all, this microfluidic chip method has the potential to be further implemented by phytosanitary services for routine diagnose as well as rapid screening in places like ports.

Keywords: Barley Stripe Mosaic Virus, Microfluidic Chip, Routine Diagnose, Rapid Screening, Disease Control

1. Introduction

Barley stripe mosaic virus (BSMV), the type member of genus *Hordeivirus*, constitutes an important seed-transmitted pathogen distributed in almost every barley planting area around the world [1]. Virion of BSMV is non-enveloped, rod-shaped, about 20×110-150 nm in size, helically symmetrical with a pitch of 2.5 nm [2]. BSMV features three positive sense ssRNAs designated as α (RNA 1), β (RNA 2) and γ (RNA 3), respectively. All three RNAs are essential for systemic infection of hosts [3]. Thermal inactivation of viral infectivity ranges from 63 to 70°C. BSMV chooses barley (*Hordeum vulgare*) as native host, yet it infects other grasses (Gramineae) [4] as well as dicotyledonous plants [5]. Long distance spread occurs mainly via seed transportation while field transmission happens efficiently by direct leaf contact [6]. So far, there have been no known vectors for BSMV. BSMV causes enormous damages in cereal production resulting in significant economic losses. As symptoms are not obvious at low temperature (<12°C), and some wheat varieties have cryptic phenomenon in the late growth period [7], it is difficult to diagnose the exact disease. Thus research on efficient detection of BSMV suitable in wild application is in urgent need.

Currently there are several methods for the detection and identification of BSMV, i.e., biological, serological and molecular ones. Biological assays include electron microscope observation [8] and host inoculation, which are both cumbersome in operation and time consuming. Besides, biological assays require quarantine greenhouse, electron microscope or other facilities and instruments, which makes it less efficient for routine inspection. Enzyme-linked

immuno-assay (ELISA), the most widely used serological method, has some shortcomings such as low sensitivity and easy contamination [9]. Molecular methods, like reverse transcription polymerase chain reaction (RT-PCR) and real-time fluorescence RT-PCR technology [10], are the most popular, as they do not have to prepare antibodies, are obviously more sensitive and efficient compared with the former two. However, regular molecular methods are still not suitable in field application because they need complex nucleic acid amplifier.

Microfluidic technology keeps progressing rapidly in past decades and gains increasing influence on life sciences. Since early 1990s, microfluidic technology has been applied in multiple research areas including chemical synthesis [11], high-throughput screening, environmental analysis and medical diagnostics [12]. Microfluidic chips combined with specific detection technique are supposed to be suitable for high-throughput screening of plant pathogens.

In this study, we introduce the application of microfluidic chips in rapid detection of BSMV. Through analysis, this method was proved to be sensitive, specific and efficient, which has the potential for field survey.

2. Materials & Methods

2.1. Sample Preparation

Five leaf samples (Table 1) infected by BSMV, *Maize chlorotic mottle virus* (MCMV), *Maize dwarf mosaic virus* (MDMV), *Oat mosaic virus* (OMV), and *Wheat streak mosaic virus* (WSMV), respectively, were used in specificity analysis. Based on the conserved sequence of BSMV from GenBank (accession number: X03854.1) [13], position 2380-2719 was selected, synthesized and inserted into plasmid puc57 vector as positive control used in sensitivity test (carried out by NingBo iGene Technology Co., Ltd, China).

2.2. RNA Extraction

Nearly 30-60 mg of ground leaf tissue from each sample was prepared for total RNA extraction. Rneasy Plant Mini Kit 50 (QIAGEN, Germany) was used according to the Kit User Manual. RNA purity and concentration were conformed by Nanodrop 2000 (Thermo, USA). RNA solutions (in RNase free ddH_2O) were stored at -20°C for further use. TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TRANS, Beijing) was used for cDNA synthesis.

2.3. Primer Design

Primer design and further assay development were based on the conserved sequence (position 2380-2719, 340 bp in length) of BSMV type strain (accession number: X03854.1). On-line tool Primer Explorer v4.0 (http://primerexplorer.jp/elamp4.0.0/index.html) was employed. Primer synthesis and purification were performed by BGI Co., Ltd (Guangdong).

2.4. Establishment of Microfluidic Chip Method

Fluorescent isothermal amplification premix (Ningbo iGene Technology Co., Ltd, China) was adopted according to the manufacturer's instruction. The primer concentration was set at 100 μM. Reaction mixture (50 μL, for 8 reactions, 5 μL per reaction, the rest for error volume) consisted of 18 μL premix, 0.1 μL F3, 0.1 μL B3, 0.8 μL FIP, 0.8 μL BIP, 0.4 μL LP and 29.8 μL RNA template. Reaction was performed via heating at 63.5°C for 30 min using Microfluidic fluorescence detector MA2000 (Ningbo iGene Technology Co., Ltd, China). Results were represented in both Ct and amplification curve.

2.5. Reaction Validity, Specificity and Sensitivity

Reaction validity was carried out using cDNA synthesized and extracted from leaf sample infected by BSMV. To analyze the sensitivity of microfluidic chip method, a recombinant puc57 plasmid [14] containing target BSMV sequence was used as the parent solution (1.00×10^6 copies/μL). It was then serially diluted at a 10-fold gradient with RNase free ddH_2O, resulting in 7 concentrations from 1.00×10^6 to 1.00×10^0 copies/μL. The assay specificity was assessed via 5 leaf samples infected by BSMV, MCMV, MDMV, OMV, and WSMV (Table 1), respectively. RNase free ddH_2O was treated as the negative control.

Table 1. *Plant samples used in method analysis.*

No.	Samples	Sources
1	Leaf powder infected by BSMV	Agdia Inc., USA
2	Leaf powder infected by *Maize chlorotic mottle virus*, MCMV	Agdia Inc., USA
3	Leaf powder infected by *Maize dwarf mosaic virus*, MDMV	Agdia Inc., USA
4	Leaves infected by *Oat mosaic virus*, OMV	Reserved positive samples
5	Leaf powder infected by *Wheat streak mosaic virus*, WSMV	Agdia Inc., USA

3. Result and Analysis

3.1. Primer Design and Method Validity

Primers were designed and chosen accordingly via Primer Explorer v4.0 (Table 2), resulting in an amplicon of 340 bp in length. Through method validity test, it was showed that all 8 repetitions of BSMV treatment had a Ct ranging from 11 to 16 (Table 3) and typical amplification curves (Figure 1), which suggested those primer pairs and reaction sets worked efficiently and were able to provide an accurate result.

3.2. Specificity Tests

Total RNA was extracted from 5 leaf samples listed in table

1 and cDNA was synthesized accordingly. The concentrations of cDNA were adjusted varying from 1.00×10^5 to 1.00×10^6 copies/μL. Each treatment had 4 repetitions. Positive results were observed in only BSMV treatment with Ct ranging from 11 to 15, and typical amplification curves, yet not in other 4 virus treatments or negative control (Table 4, Figure 2), ensuring enough specificity of this method for BSMV detection.

Table 2. *Primers designed for BSMV.*

Name	Sequence (from 5' to 3')
BSMV-F3-2	GGATTGTTTGCGTATTTGATCT
BSMV-B3-2	CCGATGATGGTAAGCATTG
BSMV-FIP-2	GTAACTACCTCCGTTGGCGACAAAAACATTCTACGGAATCCG
BSMV-BIP-2	AGAGACGGGTCAAAGAGTATAAGTGGGCAACAACATTCCAGG
BSMV-LP-2	TTGCCCGCAATGCTTACC

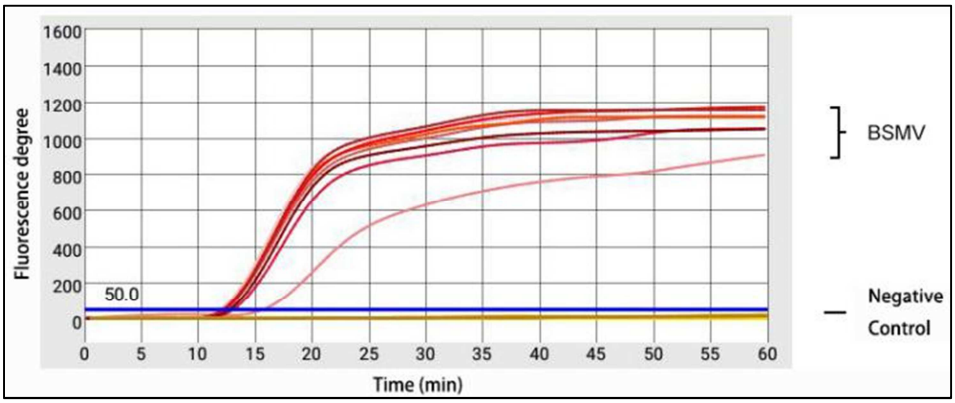

Figure 1. *Amplification curves for method validity.*

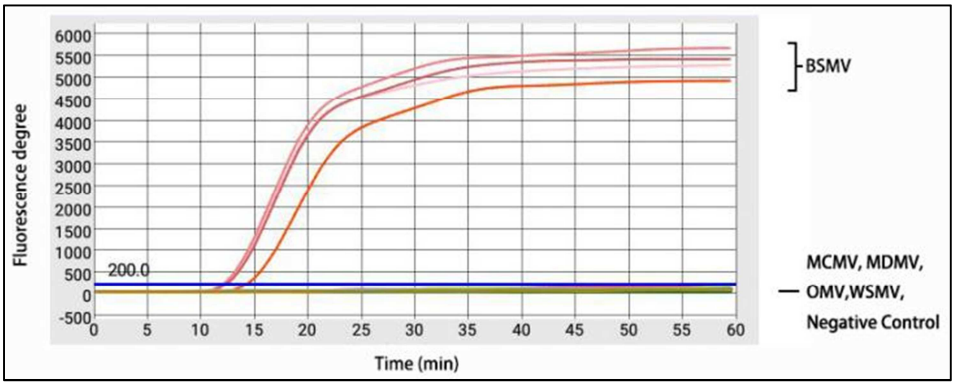

Figure 2. *Amplification curves for specificity test.*

Table 3. *Method validity result.*

Treatment	No.	Ct	Judgment
BSMV	1	11.78	+
	2	15.61	+
	3	12.2	+
	4	12.09	+
	5	12.11	+
	6	12.95	+
	7	12.29	+
	8	12.64	+
Negative Control	9	0	-
	10	0	-
	11	0	-
	12	0	-
	13	0	-
	14	0	-
	15	0	-
	16	0	-

Table 4. *Specificity test result.*

Treatment	No.	Ct	Judgment
BSMV	1	12.06	+
	2	11.94	+
	3	12.27	+
	4	14.17	+
MCMV	5	0	-
	6	0	-
	7	0	-
	8	0	-
MDMV	9	0	-
	10	0	-
	11	0	-
	12	0	-
OMV	13	0	-
	14	0	-
	15	0	-
	16	0	-
WSMV	17	0	-
	18	0	-
	19	0	-
	20	0	-
Negative Control	21	0	-
	22	0	-
	23	0	-
	24	0	-

Table 5. *Sensitivity analysis result.*

Treatment	No.	Ct	Judgment
1.00×10^6	1	11.67	+
	2	12.16	+
	3	11.93	+
	4	12.18	+
1.00×10^5	5	14.26	+
	6	14.14	+
	7	14.15	+
	8	14.04	+
1.00×10^4	9	16.02	+
	10	16.08	+
	11	16.01	+
	12	16.21	+
1.00×10^3	13	18.11	+
	14	21.41	+
	15	19.79	+
	16	18.73	+
1.00×10^2	17	23.54	+
	18	28.82	+
	19	28.04	+
	20	20.03	+
1.00×10^1	21	0	-
	22	0	-
	23	0	-
	24	0	-
1.00×10^0	25	0	-
	26	0	-
	27	0	-
	28	0	-
Negative control	29	0	-
	30	0	-
	31	0	-
	32	0	-

3.3. Sensitivity Analysis

Seven concentrations of the recombinant puc57 plasmid solutions ranging from 1.00×10^0 to 1.00×10^6 copies/μL were amplified. Each treatment had 4 repetitions. According to the Ct and amplification curves, concentration of 1.00×10^2 copies/μL was regarded as the detection limit of this microfluidic chip method (Table 5, Figures 3-6).

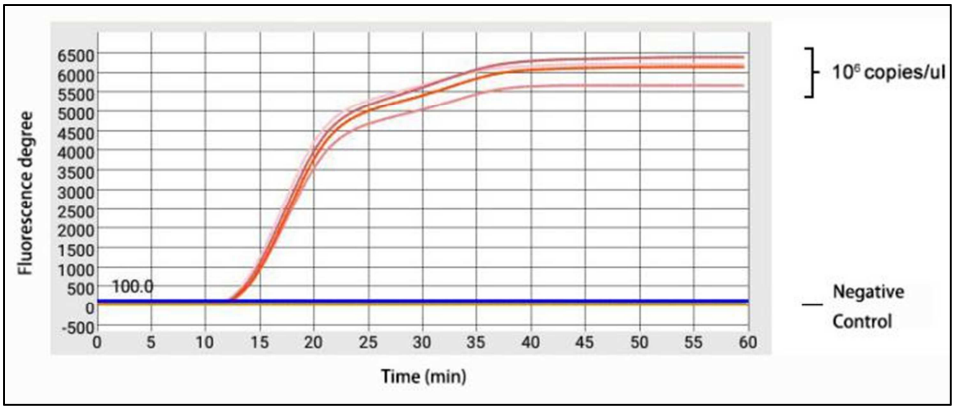

Figure 3. *Amplification curves for concentration of 1.00×10^{6} copies/μL.*

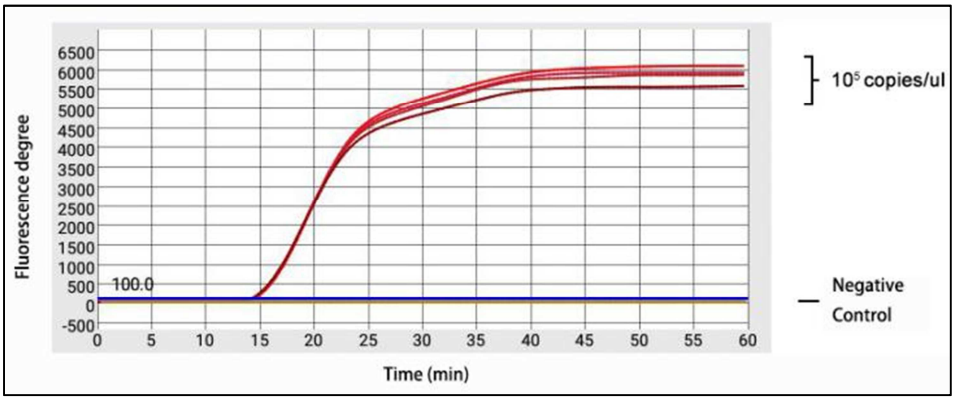

Figure 4. *Amplification curves for concentration of 1.00×10^{5} copies/μL.*

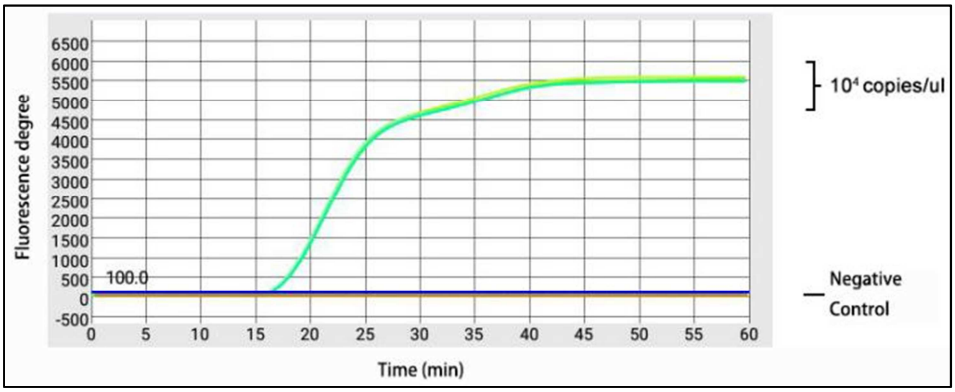

Figure 5. *Amplification curves for concentration of 1.00×10^{4} copies/μL.*

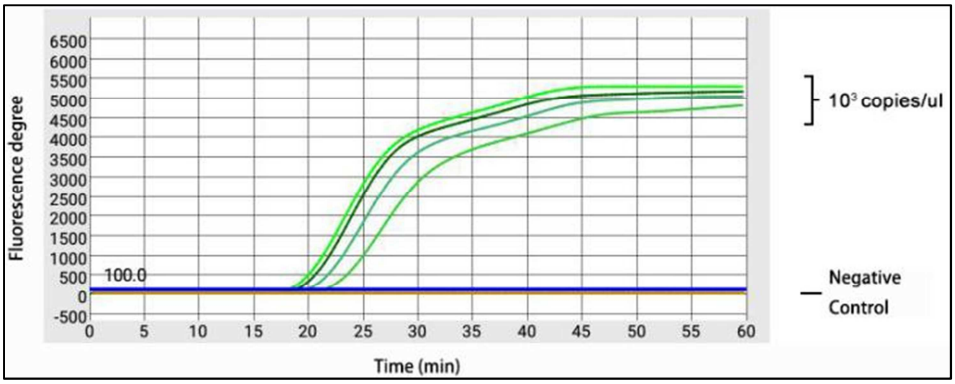

Figure 6. *Amplification curves for concentration of 1.00×10^{3} copies/μL.*

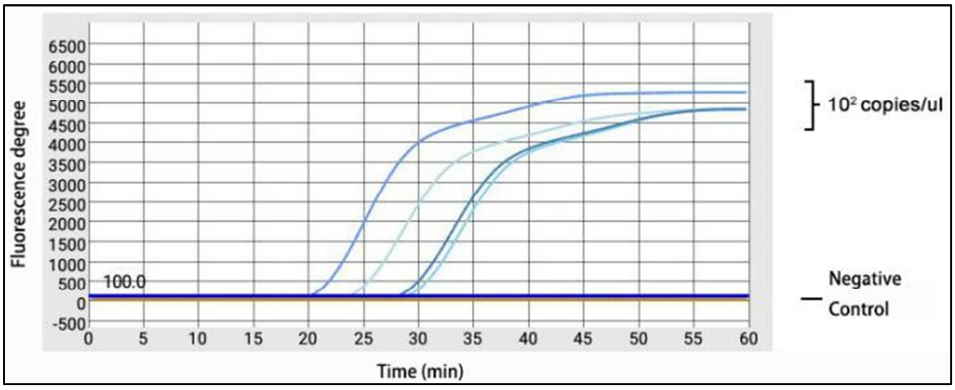

Figure 7. *Amplification curves for concentration of 1.00×10^2 copies/μL.*

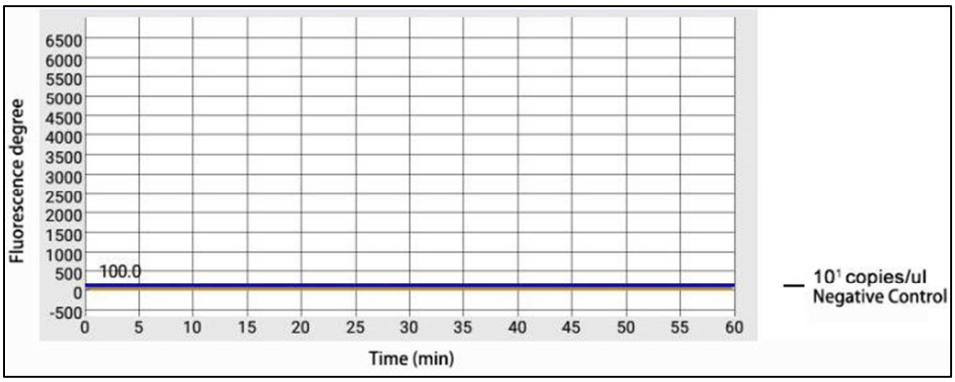

Figure 8. *Amplification curves for concentration of 1.00×10^1 copies/μL.*

4. Discussion

Pathogen diagnostic methods play a vital role in both disease control and economic loss prevention resulting from yield and quality decline. In this study, we succeeded in demonstrating the amplification of BSMV within 30 min at one constant temperature using a microfluidic chip assay. It has been reported that conventional RT-PCR features a detection limit at 4.65×10^5 copies/μL, whereas real-time RT-PCR is able to amplify viral RNA at the concentration of 4.60×10^3 copies/μL. Immunocapture-real-time RT-PCR further improves the sensitivity basing on real-time PCR, which at the same time does not require RNA isolation [15]. RT loop-mediated isothermal amplification (RT-LAMP) assay for detection of BSMV has been established before with the sensitivity of 1.10×10^4 copies/μL, ranging between conventional and real-time RT-PCR [16]. Result of our sensitivity test revealed that this microfluidic chip method was the most sensitive one, which can detect target sequence at the concentration as low as 1.00×10^2 copies/μL. To date, there have been several methods for BSMV detection in seeds and other plant materials. Electron microscopy and host plant inoculation, depending on experimental conditions, generally take weeks or even months, whereas ELISA assay needs 5-6 hours. Conventional RT-PCR normally costs 2-3 hours per detection, and real-time RT-PCR needs 1.5 hours. Microfluidic chip method can simultaneously screen a variety of viruses, and the whole process takes just 1 hour (including the preparation time), which greatly speeds up the detection and improves efficiency.

Platform like microfluidic technology offers new insights into biological processes and enables the efficient and rapid screening of plant pathogens. Microfluidic analytic systems realize miniaturization which has several superiorities such as saving expensive reagents and reducing inspection costs. It is worth mentioning that reaction volume of microfluidic chip was just 5 μL, while that of conventional RT-PCR or real-time RT-PCR was at least 20 μL [17]. RT-LAMP published in 2017 performed its amplification in a final volume of 25 μL [16]. Thus, this method reduces reagent consumption and makes the whole procedure cost-effective.

In specificity test, MCMV, MDMV, OMV and WSMV were chosen as they shared some characters with BSMV. MCMV, one member of the genus *Machlomovirus* (Tombusviridae), induces maize (*Zea mays*) lethal necrosis disease [18]. MDMV, belonging to the genus *Potyvirus* (Potyviridae), epidemic worldwide, is regarded as one of the most important pathogens for monocotyledonous hosts. MDMV was reported to cause nearly 70% loss in corn production all over the world since 1960 [19]. The load of MCMV was 5.4 times higher in plants infected by both MCMV and MDMV than in those infected only by MCMV. Besides, hosts infected by both had less chlorophyll and a lower ratio of chloroplast to cytoplasmic rRNA [20]. OMV, a soil-borne virus with rod-shaped particles, can be transmitted through soil by fungus. As one member in *Bymovirus* (Potyviridae), its host range is limited in *Avena* species

[21-23]. WSMV, the type member of *Tritimovirus* (Potyviridae), is a monopartite, positive-sense, ssRNA virus threatening wheat production worldwide [24].

This rapid screening method for BSMV via microfluidic chip has been demonstrated in this paper to be highly specific and sensitive, which has the potential to be further implemented by phytosanitary services for routine diagnose. Moreover, this method could be a useful tool for BSMV screening in places like ports, where high reliability and efficiency are also required.

5. Conclusion

In this study, one rapid screening method for BSMV via microfluidic chip was established. Through method validity, we succeeded in demonstrating the amplification of BSMV within 30 min at one constant temperature using a microfluidic chip assay. Sensitivity analysis conformed that this method obtained a detection limit at 1.00×10^2 copies/μL, which was even more sensitive than real-time RT-PCR. *Maize chlorotic mottle virus*, *Maize dwarf mosaic virus*, *Oat mosaic virus*, and *Wheat streak mosaic virus* were chosen in specificity test along with BSMV, but only BSMV expressed typical amplification curves and Ct. Microfluidic analytic systems realize miniaturization by reducing the reaction volume to just 5 μL, which has several superiorities such as saving expensive reagents and reducing inspection costs. Moreover this microfluidic chip assay can simultaneously detect a variety of viruses, and the whole process takes just 1 hour, which greatly speeds up the detection and improves efficiency. All in all, this microfluidic chip method has the potential to be further implemented by phytosanitary services for routine diagnose as well as rapid screening in places like ports. Future research is suggested to focus on high throughput screening system development and stability improvement.

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