Chapter 4

Rapid Extraction of Plant Nucleic Acids by Microneedle Patch for In-Field Detection of Plant Pathogens

Rajesh Paul, Emily Ostermann, and Qingshan Wei

Abstract

Plant diseases pose a significant threat to global food security. Molecular diagnosis currently plays a crucial role in mitigating the negative impacts of plant diseases by accurately identifying the disease-causing pathogens and revealing their genotypes. However, current molecular assays are constrained to the laboratory because of the cumbersome protocols involved in plant nucleic acid extraction. To streamline this, we have developed a polymeric microneedle (MN) patch-based nucleic acid extraction method, which can be applied to various plant tissues and easily performed in field settings without using bulky laboratory equipment. The MN patch instantly isolates both host and pathogen's DNA and RNA from plant leaves by two simple steps: press and rinse with a buffer solution or nuclease-free water. The MN-extracted DNA and RNA are purification-free and directly applicable to downstream molecular assays such as polymerase chain reaction (PCR), reverse transcription-polymerase chain reaction (RT-PCR), loop-mediated isothermal amplification (LAMP), and reverse transcription loop-mediated isothermal amplification (RT-LAMP). Here, we describe the fabrication procedures of the MN patch and demonstrate the application of the MN method by extracting *Phytophthora infestans* DNA and tomato spotted wilt virus (TSWV) RNA from infected tomato leaves. After MN extraction, we directly utilize the MN-extracted nucleic acid samples to run PCR, RT-PCR, LAMP, or RT-LAMP reactions to amplify various biomarker genes, such as the ribulose-bisphosphate carboxylase (*rbcL*) gene of host tomato DNA, internal transcribed spacer (ITS) region of *P. infestans* DNA, and nucleocapsid (N) gene of TSWV RNA. Furthermore, this simple and rapid nucleic acid method can be integrated with portable nucleic acid amplification platforms such as smartphone-based microscopy devices to achieve "sample-to-answer" detection of plant pathogens directly in the field.

Key words Microneedle (MN) patch, Nucleic acid extraction, Plant pathogen, Polymerase chain reaction (PCR), Loop-mediated isothermal amplification (LAMP)

1 Introduction

In-field detection of plant pathogens is crucial for protecting crops from disease. Currently, growers rely mostly on visible symptoms to identify the plant pathogens in their field. However, diseased plants often show similar signs for different pathogens. Therefore, visual symptoms may fail to recognize the causal agent in the field. To

Nicola Luchi (ed.), *Plant Pathology: Methods and Protocols*, Methods in Molecular Biology, vol. 2536,
https://doi.org/10.1007/978-1-0716-2517-0_4,

overcome this issue, several immunoassay methods (e.g., ELISA and LFA) have been developed for in-field detection of plant pathogens [1, 2]. However, antibody-based assays usually suffer from low sensitivity and specificity. Moreover, many antibody tests cannot distinguish between different strains of pathogens, which is crucial to determine the proper treatment plans. For example, some clonal lineages of *Phytophthora infestans*, such as US-8 and US-11, are resistant to mefenoxam, a commonly used fungicide [3]. In addition, *P. infestans* has been known to undergo population shifts, in which an emerging lineage dominates and replaces previously extant lineages [4, 5]. As a result, a rapid field-portable nucleic acid detection platform is immediately needed to maximize the diagnostic capability of growers and field workers to more efficiently protect the crops. A nucleic acid assay usually consists of three steps: (1) nucleic acid extraction, (2) nucleic acid amplification, and (3) amplicon detection. Although portable PCR systems [6, 7], isothermal nucleic acid amplification assays (e.g., LAMP and RPA) [1, 8], and colorimetric [9] lateral flow-based amplicon detection systems [10] have been presented in the literature to perform nucleic acid amplification in the field settings, the nucleic acid extraction step is still posing a major challenge for developing a portable "sample-to-answer" molecular detection platforms for plant pathogens.

Isolation of nucleic acids from infected plant specimens is a complicated multistep process because the plant cells are surrounded by rigid polysaccharide cell walls [11]. Usually, plant sample preparation relies on bulky instruments for mechanical grinding and purification, and skilled personnel to operate them [12]. The representative laboratory plant nucleic acid extraction methods for DNA and RNA are cetyltrimethylammonium bromide (CTAB) extraction method [13] and TRIzol extraction method [14], respectively, which are both time-consuming and require toxic chemicals (e.g., phenol and chloroform).

Alternative to these conventional methods, we have recently developed a polymeric microneedle (MN) patch-based extraction technology to rapidly extract DNA and RNA molecules from plant leaves [11, 15, 16]. We demonstrate that the MN patch can effectively isolate both DNA and RNA molecules from hard-to-lyse plant tissues by a simple compression and retraction process on the infected leaf. During compression, the conical MNs break the plant cells and release DNA, RNA, and other intracellular molecules. As the MN patch is made of polyvinyl alcohol (PVA), a highly water-swellable polymer, the MNs instantly absorb intracellular water molecules and accumulate the released DNA, RNA, and other molecules on the surfaces of needle tips. Compared with conventional laboratory-based sample preparation protocols, the MN patch-based nucleic acid extraction method provides several advantages: (a) it is rapid (extract nucleic acids <1 min without

using laboratory equipment); (b) it is low-cost (approximately 1 cent per MN patch); (c) it extracts both DNA and RNA and can potentially isolate protein biomarkers or small molecules as well; (d) the method is minimally invasive, and the extracted sample can be used for various molecular analysis without further purification; (e) the MN method can be applied to various plant species (i.e., potato, tomato, pepper [11]); (f) MN-extracted DNA and RNA are stable on the patch for minimally 1 week and 3 days, respectively, when stored at room temperature [16]; (g) single microneedle tip extracts sufficient nucleic acid to detect a plant pathogen [16]; (h) the needle array format can be easily reconfigured or patterned for multiplex detection [16]; (i) the surface chemistry of the MN patch can be rationally tuned to enable selective target extraction in future; (j) MN extraction also has the potential to be combined with DNA sequencing analysis in the field. In this chapter, we describe the detailed protocol for the MN patch-based nucleic acid extraction to isolate *P. infestans* DNA and TSWV RNA from infected tomato leaves. Moreover, we discuss the fabrication process of the MN patch, as well as the steps to perform PCR, RT-PCR, LAMP, and RT-LAMP reactions with MN-extracted samples to amplify the ribulose-bisphosphate carboxylase (*rbcL*) gene of host tomato DNA, internal transcribed spacer (ITS) region 2 of *P. infestans DNA*, and nucleoprotein (N) gene of TSWV RNA.

2 Materials

Prepare all solutions using DNase-/RNase-free distilled water; store all reagents and solutions at room temperature (unless indicated otherwise).

2.1 Microneedle Patch Fabrication

1. Polydimethylsiloxane (PDMS) MN mold (*see* **Note 1**).
2. PVA, fully hydrolyzed, molecular weight approximately 31,000–50,000 (*see* **Note 2**).
3. 10% PVA solution: Add 85-g deionized water to a glass beaker. Weigh 15-g PVA. Heat water on a magnetic stirrer hot plate to 80–90 °C. Keep the temperature constant. Transfer PVA to water gradually and stir continuously until PVA is fully dissolved. Cool and store at room temperature for up to 6 months.
4. Kimwipes.
5. Petri dish.
6. Parafilm.

2.2 MN Patch-Based Nucleic Acid Extraction

1. Healthy, *P. infestans*- and TSWV-infected tomato leaves (*see* **Note 3**).
2. MN patch.
3. 1× Tris–EDTA (TE) buffer: 10-mM Tris–HCl, 0.1-mM EDTA, pH 8.
4. Deionized (DI) water.
5. 200-µL micropipette.
6. 200-µL micropipette tips.
7. 1.5-mL microcentrifuge tube.
8. Parafilm.

2.3 Real-Time PCR and RT-PCR Amplification of MN-Extracted Nucleic Acid Samples

1. cDNA synthesis kit: 5× cDNA synthesis buffer, random hexamer, RT enzyme mix, RT enhancer; store at −20 °C.
2. 10× PCR buffer; store at −20 °C.
3. 50-mM magnesium chloride; store at −20 °C.
4. dNTPs (5 mM each); store at −20 °C.
5. 20× EvaGreen dye; store at −20 °C.
6. 50-mg/mL BSA; store at −20 °C.
7. Taq DNA polymerase; store at −20 °C.
8. Primers for RT and PCR reactions [11, 17, 18]:
   - RT reaction

     RNA primer (TSWV-N (cDNA)) [17]:

     5′-ATGTCTAAGGTTAAGCTCACTAAGG-3′.
   - PCR reactions
     (i) *rbcL* gene detection [11] (amplification product = 103 bp):
     Forward primer (*rbcl*_Rf5′- GTAACTCCT CAACCTGGAGTTC-3′).
     Reverse primer (*rbcl*_Rb): 5′- GTAAGTC CATCGGTCCATACAG-3′.
     (ii) *P. infestans* detection [18] (amplification product = 100 bp):
     Forward primer (PINF2): 5′-CTCGCTACAATAG CAGCGTC-3′.
     Reverse primer (HERB2): 5′-CGGACCGACTGC GAGTCC-3′.
     (iii) TSWV detection [17] (amplification product = 140 bp):
     Forward primer (TSWV-N_For): 5′- GCTTCCCACCCTTTGATTC-3′.
     Reverse primer (TSWV-N_Rev): 5′- ATAGCCAA GACAACACTGATC-3′.

9. Deionized (DI) water.
10. PCR tube.
11. Real-time PCR instrument.

2.4 Real-Time LAMP and RT-LAMP Amplification of MN-Extracted Nucleic Acid Samples

1. 10× isothermal amplification buffer; store at −20 °C.
2. 100-mM magnesium sulfate; store at −20 °C.
3. dNTPs (10 mM each); store at −20 °C.
4. 5-M betaine; store at 4 °C.
5. 20× EvaGreen; store at −20 °C.
6. 2.5-mM hydroxynaphthol blue (HNB).
7. Bst 2 WarmStart DNA polymerase; store at −20 °C.
8. WarmStart RTx Reverse Transcriptase; store at −20 °C.
9. LAMP and RT-LAMP primers [8, 16] (*see* **Note 4**).
   - LAMP amplification
     (i) *rbcL* gene detection [16]
     F3: 5′-GAGAGATCGTTTCTTATTTTGTG-3′.
     B3: 5′-CCAAGGTAGTATTTGCGGTAA-3′.
     FIP: 5′-TGCAGTAGCATTCAAGTAATGCCCG AAGCACTTTTT AAAGCACA-3′.
     BIP: 5′-ATGCGAAGAAATGATCAAAAGAG CCCCCCGTTAAG TAGTCATG-3′.
     Loop B: 5′-TGTATTTGCTAGAGAATTGGGCG-3′ (*see* **Note 4**).
     (ii) *P. infestans* detection [8]
     F3: 5′-CTCCAAAAGTGGTGGCATTG-3′.
     B3: 5′-GCAACAGCAAAGCCGATTC-3′.
     FIP: 5′-TCTCCATTAACGCCGCAGCAGTGG ACGCTGCTATTG.
     TAGC-3′.
     BIP: 5′-CGTGGTATGGTTGGCTTCGGCATGG TTCACCAGTCC
     ATCAC-3′.
     Loop F: 5′-ACAAACCGGTCGCCAACTC-3′.
     LoopB: 5′- ATGCGCTTATTGGGTGATTTTCCTG -3′.
   - RT-LAMP amplification [16]
     (i) TSWV detection
     F3: 5′-TCAAGCCTATGGATTACCTCT-3′.
     B3: 5′-TCTCACTGTAATGTTCCATAGC-3′.
     FIP: 5′-GGTCGATCCCGAGATCCTTGTAGCTT CAGTTGATAGCTTTGAG-3′.

BIP: 5′-ACACCAGGGAAGCCTTAGGAACCTT CTTCACCTGATCTT.
CATT-3′.
Loop F: 5′-AGCCAAGACAACACTGATCAT-3′.
Loop B: 5′-AAGTTTGCACTGTGCTGAAA-3′.

10. Deionized (DI) water.
11. PCR tube.
12. Real-time PCR instrument.

3 Methods

Wear gloves during all procedures, use RNase- and DNase-free filter tips and plastics, and carry out all experimental steps at room temperature unless otherwise specified.

3.1 Microneedle Patch Fabrication

1. Sonicate PDMS mold for 5 min to dislodge PVA fragments in needle cavities from previous MN fabrication (*see* **Note 5**).
2. Gently dry PDMS mold with a Kimwipes tissue.
3. Place PDMS mold in the vacuum chamber. Ensure the cavity side of the mold is facing up. This can be checked by gently scraping the surface of the mold with a pipette tip.
4. Pipette 500-μL 10% PVA solution onto the mold (*see* **Note 6**).
5. Seal the vacuum chamber, select −100-kPa vacuum pressure, and turn on the vacuum pump for 10 min for PVA to be drawn into cavities of the MN mold (*see* **Notes 7** and **8**).
6. Open the vacuum chamber and transfer the mold to a chemical hood (*see* **Notes 9** and **10**).
7. Store mold overnight at 25 °C in the hood to dry the PVA solution (*see* **Notes 11** and **12**).
8. Gently peel the MN patch from mold by lifting it at the corner (*see* **Note 13**). Observe the surface integrity of the MNs. A representative image of a MN patch, where all MNs are formed properly, is showed in Fig. 1a. The MN patch has 225 conical MNs, and these MNs are arranged in an array of 15 × 15. The height of each needle is 800 μm, and the diameters at the tip and base are 10 μm and 300 μm, respectively.
9. Store MN patch at room temperature in a Petri dish sealed with Parafilm for future use (*see* **Note 14**).

3.2 MN Patch-Based Nucleic Acid Extraction

1. Obtain a fresh plant leaf and determine a suitable area for nucleic acid extraction. If testing for pathogen DNA/RNA, choose a location of plant leaf that displays a visible sign of infection (e.g., water-soaked area for *P. infestans* infection and yellow spots for TSWV infection).

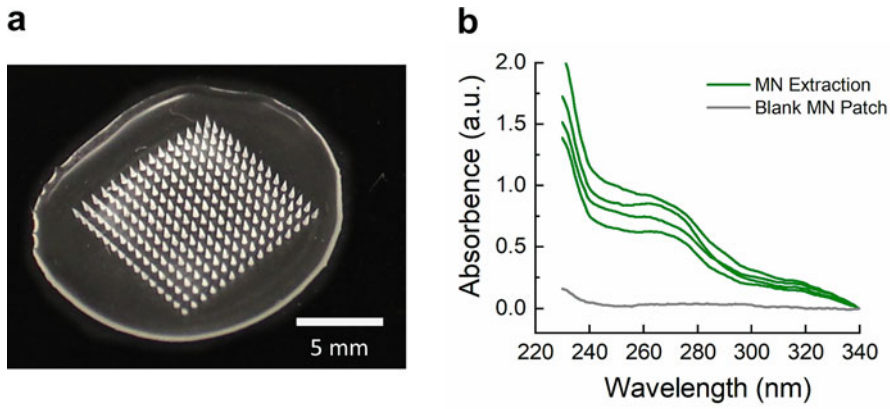

Fig. 1 (**a**) A photograph of a microneedle (MN) patch made of polyvinyl alcohol (PVA). (**b**) The UV–vis absorption spectra of MN-extracted nucleic acid solutions (green curves). The gray line represents the rinsing solution of a blank MN patch without leaf pressing

2. Cut a piece of Parafilm appropriately sized for a work surface to contain the leaf and MN patch. Place the Parafilm on a hard flat surface, such as a table, hardcover book, or the back of a phone.
3. Place the leaf on top of the Parafilm.
4. Place a MN patch on top of the leaf area selected for DNA/RNA extraction. Ensure needle tips are facing downward.
5. Gently press the MN patch into the leaf using pressure from the finger. Hold for approximately 10 s to capture plant materials (e.g., DNA and RNA) on microneedle tips (*see* **Notes 15** and **16**).
6. Gently lift the MN patch from the leaf and place it on Parafilm. Ensure needles are facing upward.
7. Rinse the MN patch eight to ten times using 100-μL TE buffer (for plant and *P. infestans* DNA isolation) or DI water (for TSWV RNA isolation) to obtain the extracted nucleic acid samples in solution. Pipette TE buffer or DI water onto the MN patch surface, and draw back into the pipette repeatedly (*see* **Note 17**).
8. Transfer the rinsing solution to a 1.5-mL microcentrifuge tube.
9. Measure the UV–vis absorbance spectrum of the rinsing solution with a NanoDrop spectrophotometer (*see* **Notes 18** and **19**). Typical absorption spectra for MN-extracted solutions are showed in Fig. 1b. The absorbance at 260-nm wavelength (A260 values) indicates that MN patches extracted significant amounts of nucleic acids from plant leaves (Fig. 1b, green curves). In contrast, no absorption at 260 nm was observed for rinsing solution from a blank MN patch without leaf pressing (Fig. 1b, gray curve).

10. Store the solution at −20 °C, or run molecular assays (e.g., PCR, RT-PCR, LAMP, and RT-LAMP) to detect the pathogen (*see* **Note 20**).

3.3 Real-Time PCR and RT-PCR Amplification of MN-Extracted Nucleic Acid Samples

1. Thaw all reagents on ice.
2. Vortex thawed reagents (*see* **Note 21**).
3. Centrifuge reagents at 100 × g for 5–10 s to collect reagents at tube bottoms.

3.3.1 Reverse Transcription (RT) Reaction (Required Only for RNA Viruses Such as TSWV)

1. In a PCR tube kept on ice, prepare 15 μL of RT master mix. The recipe is given below: 4-μL 5× cDNA synthesis buffer; 2-μL dNTPs (5 mM each); 0.5-μL random hexamer; 0.5-μL 10-μM gene-specific RNA primer; 1-μL RT enhancer; 1-μL RT enzyme mix; 6-μL DI water.
2. Add 5-μL MN-extracted solution.
3. Mix everything by pipetting up and down.
4. Close the cap and place the PCR tube in a thermal cycler set at 42 °C for 30 min for cDNA synthesis.
5. Set the temperature to 95 °C for 2 min to deactivate the RT enzyme.
6. Store cDNA solution on ice.

3.3.2 PCR Amplification

1. In a PCR tube kept on ice, prepare 23 μL of PCR master mix. Add DNA polymerase last. The recipe for PCR master mix is given below: 2.5-μL 10× PCR buffer; 0.5-μL dNTPs (5 mM each); 0.5-μL 10-μM forward primer; 0.5-μL 10-μM reverse primer; 1.25-μL 20× EvaGreen; 1.25-μL 50-mM magnesium chloride; 0.05-μL 50-mg/mL BSA; 16.35-μL deionized water; 0.1-μL 5-U/μL *Taq* DNA polymerase.
2. Add 2-μL MN-extracted solution (for *rbcL* and *P. infestans* detection) or cDNA sample (for TSWV detection).
3. Mix everything by pipetting up and down.
4. Close the cap and place PCR tubes in a thermal cycler to perform real-time PCR amplification. For *rbcL* gene amplification, perform 1 cycle of 94 °C for 2 min (initial denaturation); 35 cycles of 94 °C for 30 s (denaturation), 56 °C for 30 s (annealing), 72 °C for 30 s (extension), and fluorescence detection; and 1 cycle of 72 °C for 5 min (final extension). For *P. infestans* detection, perform 1 cycle of 94 °C for 2 min (initial denaturation); 35 cycles of 94 °C for 15 s (denaturation), 56 °C for 15 s (annealing), 72 °C for 15 s (extension), and fluorescence detection; and 1 cycle of 72 °C for 5 min (final extension). For TSWV detection, perform 1 cycle of 95 °C for 30 s (initial denaturation); 35 cycles of 95 °C for 10 s

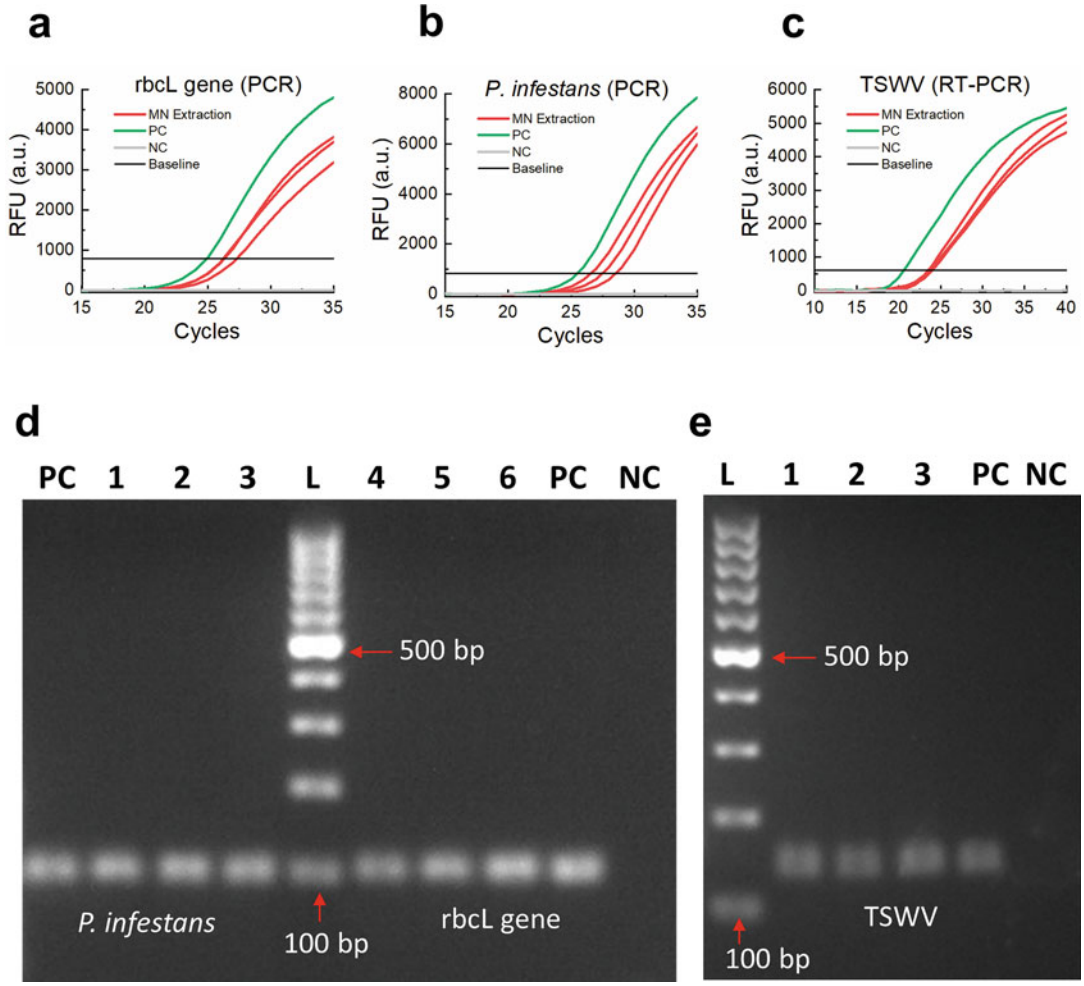

Fig. 2 PCR and RT-PCR amplification of MN-extracted nucleic acid samples. Real-time amplification curves for (**a**) *rbcL* gene, (**b**) *P. infestans*, and (**c**) TSWV detection. Gel electrophoresis showing amplified PCR products for (**d**) *P. infestans*, *rbcL* gene, and (**e**) TSWV detection. The amplicon lengths for the *rbcL* gene, *P. infestans*, and TSWV were 102 bp, 100 bp, and 140 bp, respectively. Lanes 1–3 in (**d**), three different amplified bands of *P. infestans* ITS DNA; lanes 4–6 in (**d**), three different amplified bands of *rbcL* gene; and lanes 1–3 in (**e**), three different amplified bands of TSWV N gene. L, 100-bp ladder; PC, positive control; NC, negative control (no DNA, blank rinsing solutions)

(denaturation), 55 °C for 15 s (combined annealing and extension), and fluorescence detection; and 1 cycle of 72 °C for 5 min (final extension). During PCR, EvaGreen dye binds to the amplified PCR products and emits fluorescence signals, which are recorded by a real-time PCR machine for monitoring the progress of the amplification reaction. Real-time amplification curves for the *rbcL* gene, *P. infestans*, and TSWV are reported in Fig. 2a–c.

5. Confirm the presence of a specific amplicon by gel electrophoresis in 2% agarose gel running at 120 volts for 45 min. The

amplicons' lengths for the *rbcL* gene, *P. infestans*, and TSWV were 103 bp, 100 bp, and 140 bp, respectively (Fig. 2d, e). Alternatively, the melt curve analysis can be performed in a real-time PCR machine to determine the melt temperatures of PCR amplicons (*see* **Note 22**). Single melt temperature indicates the presence of a specific amplicon in the PCR reaction. The amplicons' melt temperatures for the *rbcL* gene, *P. infestans*, and TSWV detection were 84.5 °C, 82.5 °C, and 82 °C, respectively.

3.4 Real-Time LAMP and RT-LAMP Amplification of MN-Extracted Nucleic Acid Samples

1. Thaw all reagents on ice.
2. Vortex thawed reagents (*see* **Note 21**).
3. Centrifuge reagents at 100 × g for 5–10 s to collect reagents at tube bottoms.
4. Store all reagents on ice.
5. In a PCR tube kept on ice, prepare 23 μL of LAMP or RT-LAMP master mix. The recipe for the master mix is given below: 2.5-μL 10× isothermal amplification buffer; 1.5-μL 100-mM magnesium sulfate; 3.5-μL dNTPs (10 mM each); 4-μL 5-M betaine (*see* **Note 23**); 0.50-μL 10-μM F3 primer; 0.50-μL 10-μM B3 primer; 0.8-μL 50-μM FIP primer; 0.8-μL 50-μM BIP primer; 0.2-μL 50-μM loop F primer; 0.2-μL 50-μM loop B primer; 1.25-μL 20× EvaGreen; 1.2-μL 2.5-mM HNB; 1-μL 8-U/μL Bst 2 WarmStart DNA polymerase; 0.5-μL 15-U/μL WarmStart RTx Reverse Transcriptase (only for TSWV detection); 4.55-μL nuclease-free water.
6. Add 2-μL MN rinsing solution.
7. Place the PCR tube in a real-time PCR thermal cycler set at 65 °C for 60 min, and capture fluorescence signals at 1-min intervals. Real-time LAMP amplification curves for detecting the *rbcL* gene, *P. infestans* ITS DNA, and TSWV N gene are showed in Fig. 3a, c.
8. Set the temperature at 80 °C for 5 min to deactivate Bst 2.0 WarmStart DNA Polymerase and WarmStart RTx Reverse Transcriptase.
9. Inspect the color of the reaction tube. For a positive sample, color changes from violet to sky blue due to the incorporation of HNB in the LAMP assay (Fig. 3d).
10. Confirm the presence of a specific target amplicon either by melt curve analysis or gel electrophoresis in 2% agarose gel running at 120 volts for 60 min (*see* **Note 20**). Gel images of LAMP amplicons for detecting the *rbcL* gene, *P. infestans*, and TSWV are reported in Fig. 3e, g. The amplicons' melt temperatures for the *rbcL* gene, *P infestans* ITS DNA, and TSWV N gene were 81.5 °C, 86.5 °C, and 83 °C, respectively.

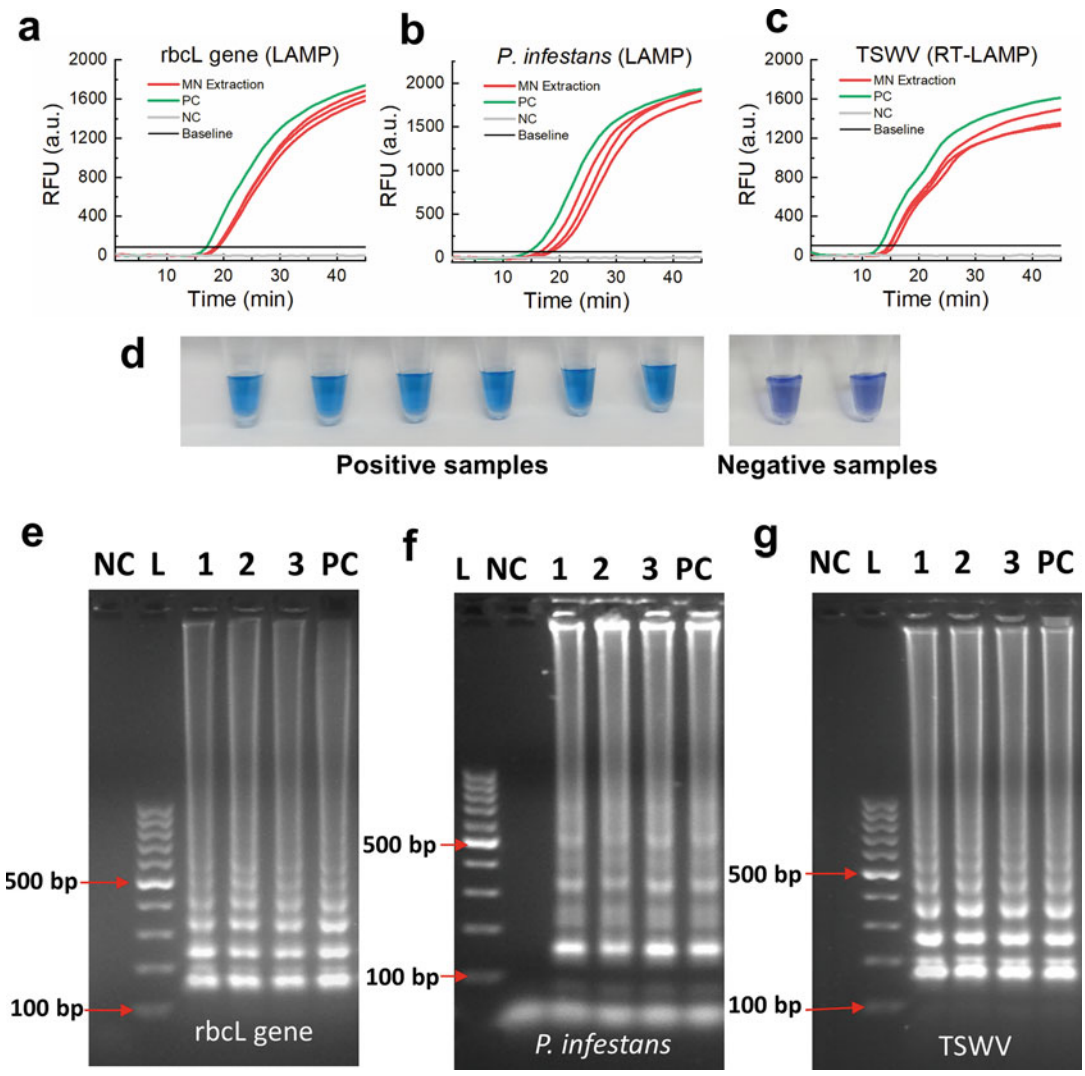

Fig. 3 LAMP and RT-LAMP amplification of MN-extracted nucleic acid samples. Real-time LAMP amplification curves for (**a**) *rbcL* gene, (**b**) *P. infestans* ITS DNA, and (**c**) TSWV N gene detection. (**d**) Visualization of the positive LAMP amplification from the color change of HNB dye. Gel electrophoresis showing LAMP amplicons for (**e**) *rbcL* gene, (**f**) *P. infestans* ITS DNA, (**g**) TSWV N gene detection. Lanes 1–3 in (**e**), LAMP amplicons for three different MN extractions from healthy leaves; lanes 1–3 in (**f**), LAMP amplicons for three different MN extractions from *P. infestans*-infected tomato leaves; and lanes 1–3 in (**g**), LAMP amplicons for three different MN extractions from TSWV-infected tomato leaves. L, 100 bp ladder; PC, positive control; NC, negative control

4 Notes

1. PDMS MN molds are purchased from Blueacre Technology, Ireland, and Micropoint Technologies Pte Ltd, Singapore. In addition, a user can also make their own MN mold with a metal MN patch [19].
2. Please select a fully hydrolyzed PVA to dissolve it entirely in DI water for MN patch fabrication.

3. Procedures for infecting tomato leaves with *P. infestans* and TSWV can be found in our previous publications [8, 15, 16].
4. Loop primers are optional. One or two loop primers are usually used in the LAMP assay to speed up the amplification reaction. For *P. infestans* and TSWV detection, two loop primers (loop F and loop B) are used in LAMP assays. On the other hand, only one loop primer (loop B) is used for *rbcL* gene detection.
5. Fragments from previous MN fabrications can remain on the PDMS mold and block microneedle formation in subsequent fabrication procedures. These fragments must be removed by sonication. If necessary, increase the sonication time to fully clean the MN mold.
6. Ensure PVA solution is pipetted in the center of mold above the MN pattern.
7. The vacuum time needs to be optimized based on vacuum pressure. An insufficient vacuum time may result in an incomplete formation of the MN patch.
8. The vacuum time also depends on the concentration and molecular weight of PVA. Increasing these parameters increases the solution viscosity. High viscosity will prevent PVA from entering mold cavities and result in incomplete MN formation. Adjust vacuum time according to the solution viscosity.
9. When releasing the vacuum, let air enter the chamber very slowly to prevent the disruption of the mold.
10. It may require waiting for another 10–15 min after releasing the vacuum for air bubbles to settle.
11. MN molds can also be kept on a laboratory bench at room temperature for drying.
12. MN molds can also be placed in an oven at 37 °C to speed up the drying process.
13. Microneedles may break when the PVA patch is peeled from the PDMS mold. Peel slowly and gently to prevent breakage. An adhesive tape can be used instead of removing patches by hand. Breakage may also occur due to insufficient drying time.
14. Ensure the storage container for MN patches is sealed from air to prevent loss of mechanical strength. The absorption of moisture can weaken the integrity of the MNs.
15. A MN patch can also be cut into two or multiple smaller pieces for nucleic acid extraction. If a smaller piece of MN patch is used, then adjust the rinsing buffer or DI water volume in the next step, accordingly.
16. The MN patch must be pressed into the plant leaf long enough (e.g., 10 s) to obtain sufficient DNA/RNA yield. If the yield is low, try to hold the pressure for a longer time.

17. The volume of washing buffer or DI water can be reduced to increase the concentration of MN-extracted nucleic acid sample.
18. If the MN patch cannot isolate enough nucleic acids for the amplification reaction, it may be due to broken needle tips or improper storage and age. Use newly fabricated MN patches with intact needles for the best results. If newly fabricated patches are mechanically weak to penetrate the leaf, use more PVA solutions or a higher concentration of PVA when fabricating.
19. Increasing the rinsing times with TE buffer may also improve the extraction yield.
20. Perform the DNA extraction, PCR, and LAMP amplification at different lab benches to prevent cross-contamination and false positives.
21. Do not vortex RT enzyme, *Taq* DNA polymerase, Bst 2.0 WarmStart DNA Polymerase, and WarmStart RTx Reverse Transcriptase, which would cause deactivation.
22. For melt curve analysis, the temperature of a reaction tube is raised in an increment of 0.5 °C from 72 °C to 95 °C. For each increment, the temperature is held constant for 5 s before measuring the fluorescence signal.
23. Betaine is optional for the LAMP assay. It is usually used to reduce false-positive amplification.

Acknowledgments

The authors thank Dr. Jean Ristaino and Dr. Anna Whitfield (Department of Entomology and Plant Pathology, NC State University) for providing *P. infestans*- and TSWV-infected tomato leaves. The authors also gratefully acknowledge the funding support from the NCSU Chancellor's Faculty Excellence Program, the Kenan Institute for Engineering, Technology & Science (KIETS), NCSU Game-Changing Research Incentive Program for the Plant Science Initiative (GRIP4PSI), USDA (No. 2019-67030-29311), USDA APHIS Farm Bill grant (No. 3.0096), and BASF.

References

1. McCoy AG, Miles TD, Bilodeau GJ et al (2020) Validation of a preformulated, field deployable, recombinase polymerase amplification assay for *Phytophthora* species. Plan Theory 9:466
2. Paul R, Ostermann E, Wei Q (2020) Advances in point-of-care nucleic acid extraction technologies for rapid diagnosis of human and plant diseases. Biosens Bioelectron 169: 112592–112592
3. Saville A, Graham K, Grünwald N et al (2015) Fungicide sensitivity of U.S. genotypes of *Phytophthora infestans* to six oomycete-targeted compounds. Plant Dis 99:659–666

4. Saville A, Ristaino JB (2019) Genetic structure and subclonal variation of extant and recent U.S. lineages of *Phytophthora infestans*. Phytopathology 109:1614–1627
5. Fry WE, Birch PRJ, Judelson HS et al (2015) Five reasons to consider *Phytophthora infestans* a reemerging pathogen. Phytopathology 105: 966–981
6. Koo C, Malapi-Wight M, Kim HS et al (2013) Development of a real-time microchip PCR system for portable plant disease diagnosis. PLoS One 8:e82704
7. Julich S, Riedel M, Kielpinski M et al (2011) Development of a lab-on-a-chip device for diagnosis of plant pathogens. Biosens Bioelectron 26:4070–4075
8. Ristaino JB, Saville AC, Paul R et al (2020) Detection of *Phytophthora infestans* by loop-mediated isothermal amplification, real-time LAMP, and droplet digital PCR. Plant Dis 104:708–716
9. Hansen ZR, Knaus BJ, Tabima JF et al (2016) Loop-mediated isothermal amplification for detection of the tomato and potato late blight pathogen, *Phytophthora infestans*. J Appl Microbiol 120:1010–1020
10. Lu X, Zheng Y, Zhang F et al (2020) A rapid, equipment-free method for detecting *Phytophthora infestans* in the field using a lateral flow strip-based recombinase polymerase amplification assay. Plant Dis 104:2774–2778
11. Paul R, Ostermann E, Gu Z et al (2020) DNA extraction from plant leaves using a microneedle patch. Curr Protoc plant Biol 5:e20104
12. Yadav S, Ram C, Singh S, Rana M (2018) Plant genomic DNA isolation-the past and the present, a review. Indian J Plant Genet Resour 31: 315–327
13. Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321–4326
14. Chomczynski P (1993) A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. BioTechniques 15:532–534
15. Paul R, Saville AC, Hansel JC et al (2019) Extraction of plant DNA by microneedle patch for rapid detection of plant diseases. ACS Nano 13:6540–6549
16. Paul R, Ostermann E, Chen Y et al (2021) Integrated microneedle-smartphone nucleic acid amplification platform for in-field diagnosis of plant diseases. Biosens Bioelectron 187: 113312
17. Nachappa P, Margolies DC, Nechols JR et al (2013) Tomato spotted wilt virus benefits a non-vector arthropod, Tetranychus urticae, by modulating different plant responses in tomato. PLoS One 8:1–14
18. Ristaino JB, Hu CH, Fitt BDL (2013) Evidence for presence of the founder Ia mtDNA haplotype of *Phytophthora infestans* in 19th century potato tubers from the Rothamsted archives. Plant Pathol 62:492–500
19. Caffarel-Salvador E, Kim S, Soares V et al (2021) A microneedle platform for buccal macromolecule delivery. Sci Adv 7:1–12