

Development of Recombinase Polymerase Amplification–Lateral Flow Dipstick (RPA–LFD) as a Rapid On-Site Detection Technique for *Fusarium oxysporum*

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Abstract

Fusarium oxysporum can cause many important plant diseases worldwide, such as crown rot, wilt, and root rot. During the development of strawberry crown rot, this pathogenic fungus spreads from the mother plant to the strawberry seedling through the stolon, with obvious characteristics of latent infection. Therefore, the rapid and timely detection of *F. oxysporum* can significantly help achieve effective disease management. Here, we present a protocol for the recombinase polymerase amplification–lateral flow dipstick (RPA–LFD) detection technique for the rapid detection of *F. oxysporum* on strawberry, which only takes half an hour. A significant advantage of our RPA–LFD technique is the elimination of the involvement of professional teams and laboratories, which qualifies it for field detection. We test this protocol directly on plant samples with suspected infection by *F. oxysporum* in the field and greenhouse. It is worth noting that this protocol can quickly, sensitively, and specifically detect *F. oxysporum* in soils and plants including strawberry.

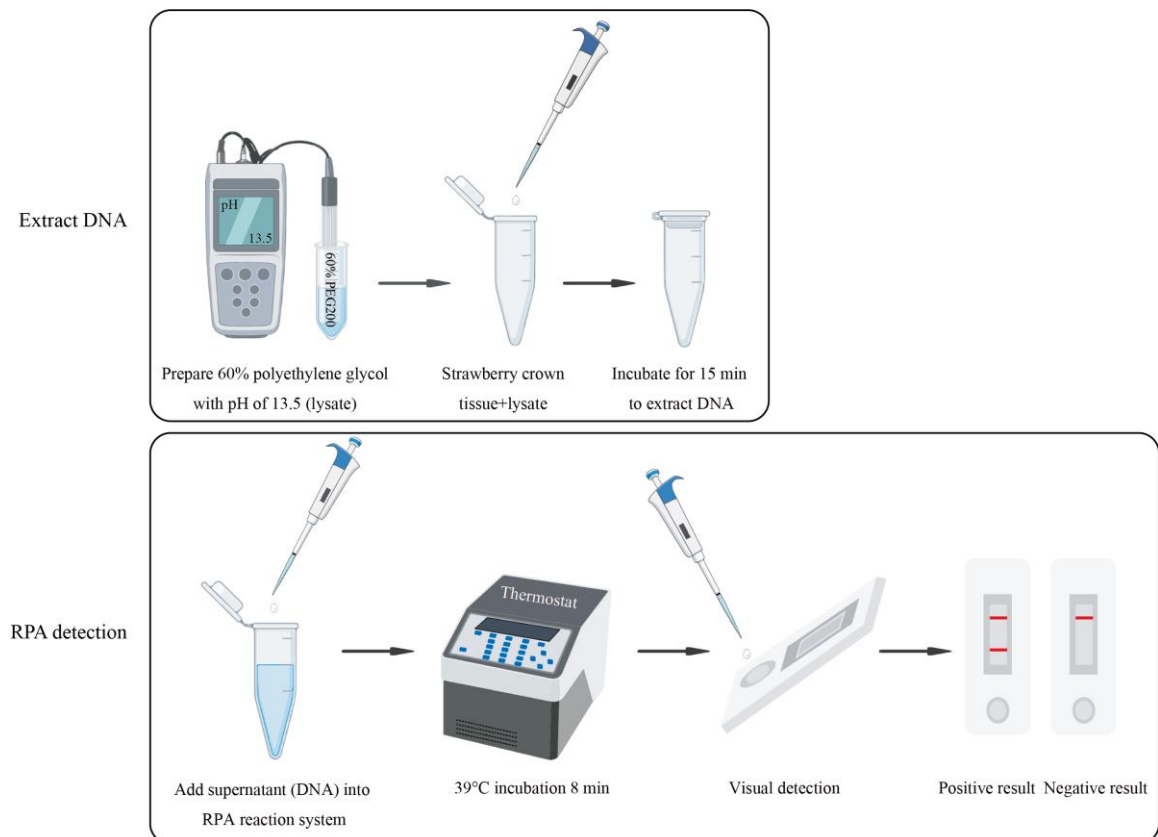
Key features

- This protocol is used to detect whether plants such as strawberry are infected with *F. oxysporum*.
- This protocol has potential for application in portable nucleic acid detection.
- It can complete the detection of samples in the field within 30 min.

Keywords: *Fusarium oxysporum*, Strawberry, Crown rot, RPA-LFD, Rapid detection

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Graphical overview



Background

Strawberry crown rot caused by *Fusarium oxysporum* has seriously affected strawberry yield [1]. The pathogen is transmitted from the mother plant to the strawberry seedling through the stolon [2]. Due to the particularities of pathogen transmission, the control of this disease is difficult. Therefore, rapid and accurate detection of *F. oxysporum* in the early stages is of utmost importance for preventing and controlling crown rot disease.

Koch's postulates are the traditional method for pathogen detection through isolation, culture, and pathogenicity testing, being time-consuming and requiring professional training. Molecular detection and identification of the pathogen are highly accurate and sensitive alternative methods for disease diagnosis. Polymerase chain reaction (PCR), quantitative PCR, and other molecular detection techniques have been invented to detect pathogens [3, 4]. However, these techniques require professional experience in performing the tests and expensive equipment, which may not be possible in on-site detection. Altogether, these create a bottleneck for early-disease diagnosis. Fortunately, other molecular biological methods can overcome these limitations, such as the recombinase polymerase amplification (RPA) detection technique [5]. RPA is performed at constant temperature, and its most significant advantage is that the detection results can be visualized by lateral flow dipstick (LFD) [6]. This method is simple to operate and requires little laboratory equipment. Therefore, it has potential for application in portable nucleic acid detection.

Here, we develop and evaluate the RPA-LFD detection technique that can quickly, sensitively, and specifically detect *F. oxysporum* infection in strawberry [7]. By comparing the genomes of *F. oxysporum* with other strains common in soil and strawberry plants, we found that the sequence of *CYP51C* gene was unique and highly variable in *Fusarium* spp.; therefore, we chose the *CYP51C* gene to design primers and probes for *F. oxysporum* detection. We demonstrate how *F. oxysporum* can be accurately detected on-site. This protocol requires no professional

experience, is easy to operate, and the results can be observed within 8 min at 39 °C. This advantage makes it a valuable diagnostic tool, especially in the absence of experienced microscopists. The sensitivity of this protocol is consistent with PCR, and it is accurate enough to detect *F. oxysporum*. In summary, this protocol is validated on the early field diagnosis of strawberry crown rot. Additionally, it can be applied to detect *F. oxysporum* in soils and other plants that are damaged by this fungus and can be adjusted to detect other pathogens, only by redesigning specific primers and probes according to the genome sequence of the pathogen to be detected.

Materials and reagents

Biological materials

1. DNA of *F. oxysporum*, *F. tricinctum*, *F. proliferatum*, *F. fujikuroi*, *F. graminearum*, *F. solani*, *F. equiseti*, *Colletotrichum siamense*, *C. gloeosporioides*, *C. aenigma*, *C. fruticola*, *Botrytis cinerea*, *Phoma* sp., and *Stagonosporopsis* sp.
2. Strawberry plant
3. Potato

Reagents

1. KOH (SangonBiotech, catalog number: A610441)
2. Polyethylene glycol (PEG200) (SangonBiotech, catalog number: A601780-0500)
3. EDTA disodium salt dihydrate (SangonBiotech, catalog number: A610185-050)
4. CTAB, cetyltrimethylammonium bromide (SangonBiotech, catalog number: A600108-0500)
5. Sodium chloride (NaCl) (SangonBiotech, catalog number: A610476-0001)
6. Tris (SangonBiotech, catalog number: A610195-0500)
7. Ethanol absolute (SangonBiotech, catalog number: A500737-0500)
8. Isopropanol (Shanghai Hushi, catalog number: 180109218)
9. Trichloromethane (Shanghai Hushi, catalog number: 10006818)
10. Dextrose (SangonBiotech, catalog number: A610219-0500)
11. Agar (SangonBiotech, catalog number: A505255-025)
12. Primer and probe:
 - a. F91:5'-TCAACTGGCATCGTCAACATCACCGAAGTAA-3'
 - b. R91:5' Biotin-CCAGGCATGACGAAGTTGATAGGTTGAAAGC-3'
 - c. Probe:5' 6-FAM-AGGCTCCCTCCTCGGTAACGAAGTCCGCTCCA/idSp/GTTTGACAGCACATT-3' C3 Spacer

Solutions

1. CTAB extracting solution (see Recipes)
2. 70% Ethanol absolute (see Recipes)
3. 60% PEG200 with pH 13.2–13.5 (see Recipes)
4. 2 M KOH (see Recipes)
5. Potato dextrose agar (PDA) (see Recipes)

Recipes

1. CTAB extracting solution

*Note: The solute is first mixed and then ddH₂O is added to the constant volume of 1,000 mL.

Reagent	Final concentration	Quantity or Volume
CTAB	2%	20 g

Tris	100 mM	12.114 g
EDTA	20 mM	7.448 mL
NaCl	1.4 M	81.816 g
H ₂ O	n/a	to 1,000 mL
Total	n/a	1,000 mL

2. **70% ethanol absolute**

Reagent	Final concentration	Quantity or Volume
Ethanol absolute	70% (v/v)	70 mL
H ₂ O	n/a	30 mL
Total	n/a	100 mL

3. **2 M KOH**

**Note: Store at 4 °C.*

Reagent	Final concentration	Quantity or Volume
KOH	2 M	1.12 g
H ₂ O	n/a	10 mL
Total	n/a	10 mL

4. **60% PEG200 with pH 13.2–13.5**

**Note: This solution needs to be prepared and used immediately.*

Reagent	Final concentration	Quantity or Volume
KOH (Recipe 3)	n/a	0.33 mL
PEG200	60% (v/v)	8 mL
H ₂ O	n/a	11.67 mL
Total	n/a	20 mL

5. **Potato dextrose agar (PDA)**

**Note: Boil the potato in 1,000 mL of H₂O using an induction cooker and pan and filter. Then, add the dextrose and agar to the filtrate.*

Reagent	Final concentration	Quantity or Volume
Potato	200 g/L	200 g
Dextrose	20%	20 g
Agar	15%	15 g
H ₂ O	n/a	1,000 mL
Total	n/a	1,000 mL

Laboratory supplies

1. 1.5 mL microcentrifuge tubes (Axygen, catalog number: MCT-150-C)
2. HybriD DNA thermostat rapid amplification kit (APWL, catalog number: WLN8203KIT)
3. HybriDetect lateral flow dipstick (APWL, catalog number: WLFS8201)
4. Forceps (Shanghai Leigu, catalog number: W-002903)
5. Sterile blade (VWR International, catalog number: 55411-050)
6. Beakers (Shanghai Leigu, catalog number: B-000103)
7. Stir bars (Shanghai Leigu, catalog number: B-040315)
8. Graduated cylinders (Shanghai Leigu, catalog number: B-031603)
9. Glass bottles (Shanghai Leigu, catalog number: B-W00323)
10. 90 mm Petri dish (Nantong Baiyao, catalog number: YJ-90-12g)
11. Inoculating needle (Shanghai Leigu, catalog number: W-006001)

12. Forceps (Shanghai Leigu, catalog number: W-002806)
13. Quartz sand (SangonBiotech, catalog number: A500823-0001)

Equipment

1. Micropipettes 0.1–2.5 µL, 2–20 µL, 20–200 µL (Eppendorf, model: 3123000217, 3123000233, 3123000250)
2. Incubator, 25 °C (Shanghai Boxun, model: MJX-250B-Z)
3. 4 °C refrigerator (Haier, model: SC-339JN)
4. Grinding machine (Shanghai Jingxin, model: JXFSTPRP-24L)
5. Ultramicro accounting protein analyzer (BioDrop, model: BioDrop µlite+)
6. Centrifuge (ThermoFisher, model: 75002440)
7. Thermostat (Hangzhou Aosheng, model: 028-14484-20110001)
8. Balance (Shanghai Haosheng, model: JA303A)
9. Induction cooker (Midea, model: RT21E0105)
10. Pan (Supor, model: ST22P1)
11. Vortex shaker (SangonBiotech, model: WH-861)

Software and datasets

1. DNAMAN v6.0.3.99
2. Snapgene v.5.2.4

Procedure

A. Design of primers and probes

1. The sequence of the *CYP51C* gene was unique and highly variable in *Fusarium* spp. Therefore, we used DNAMAN v6.0.3.99 to design primers based on the *CYP51C* sequence of the common *Fusarium* species in soil and plants, such as *F. tricinctum*, *F. proliferatum*, *F. fujikuroi*, *F. graminearum*, *F. solani*, and *F. equiseti*. The specific sequence of *F. oxysporum* was found by *CYP51C* gene sequence to design specific primers (Figure 1).
2. Recommended primers length was between 31 and 32 bp. Shorter primers can affect amplification speed and detection sensitivity, while longer primers can form secondary structures that affect amplification. The pairs of primers will be as follows:
F91: 5'-TCAACTGGCATCGTCAACATCACCGAAGTAA-3'
R91: 5'-CCAGGCATGACGAAGTTGATAGGTTGAAAGC-3'
Biotin label was applied to the 5' end of the screened downstream primers R91. Tailed primers will be as follows:
5' Biotin-CCAGGCATGACGAAGTTGATAGGTTGAAAGC-3'.
3. The probe was designed with an antigen marker (6-FAM) modification at the 5' end, a modified group (C3 Spacer) at the 3' end, and a dSpacer (tetrahydrofuran, THF) labeled at the middle of the 5' and 3' ends as the recognition site for NFO. C3 Spacer was blocking groups that prevent the DNA strand from extending, FAM was a fluorophore. Exonuclease NFO (buffer) recognized dSpacer (tetrahydrofuran, THF) and cut it to form a free hydroxyl end extending at the 3' end. Finally, the amplification product of 5' FAM-RPA-3' Biotin was obtained. Gold particle of biotin antibody and FAM antibody fixed on LFD (T line), 5' FAM-RPA-3' biotin will be captured to appear bands.
The labeled probe will be as follows:

Common sequence

▶ F. oxysporum
 ▶ F. oxysporum
 ▶ F. oxysporum
 ▶ F. oxysporum
 ▶ F. oxysporum
 ▶ F. equiseti
 ▶ F. graminearum
 ▶ F. proliferatum
 ▶ F. pseudograminearum
 ▶ F. solani
 ▶ F. tricinatum
 ▶ F. verticillioides

ATGTTTGCACGACATTCGCAACCTCTACCGCCATCTC6ATGGCTTCCAACCATCAACTTCGTCATGCTG6TCTT
 R91

B. DNA extraction

- ### C. Specific detection of primers and probes

1. Set up the reaction system as follows:
Buffer A 29.4 μL
10 μM upstream F91 2 μL
10 μM downstream R91 2 μL
10 μM probe 0.6 μL
ddH₂O 11.5 μL
Template DNA 2 μL
Buffer B 2.5 μL
Buffer B needs to be added last. Buffer A and Buffer B are supplied with the HybriD DNA thermostat rapid amplification kit.

2. Incubate at 38 °C for 10 min.
3. Dilute the reaction product 20 times using ddH₂O and add 50 µL of the diluted reaction product to the sample hole of HybriDetect LFD. Interpretation of test results is available within 5 min. If the test results are not read within 5 min, the test is not successful.
4. If the control line is visible and the detection line is not, this means a negative result. If both the detection and control lines are visible, this means a positive result. If neither the detection and control lines are visible, this means that the experimental operation was performed incorrectly or that the kit was damaged (Figure 2).

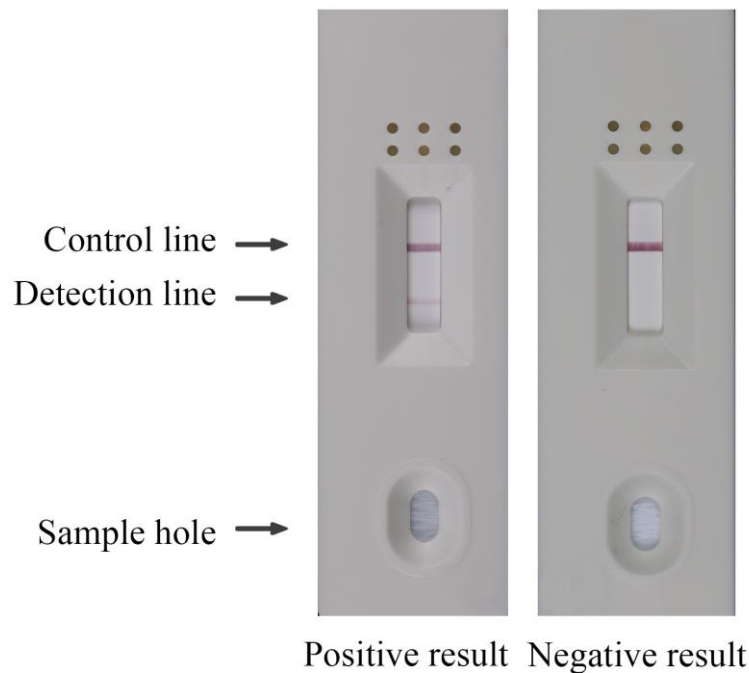


Figure 2. Assessment of specificity of primers pair F91/R91 and probe for recombinase polymerase amplification–lateral flow dipstick (RPA–LFD) detection of *F. oxysporum*

D. RPA–LFD reaction condition optimization

1. According to kit instructions, set the reaction temperature to 33, 36, 39, 42, and 45 °C and the reaction time to 10 min.
2. After determining the optimal reaction temperature, set the reaction time to 4, 6, 8, 10, and 12 min.
3. Visualize the amplification results according to the above method.
4. The best reaction condition was determined according to the color depth of the detection line (Figure 3).

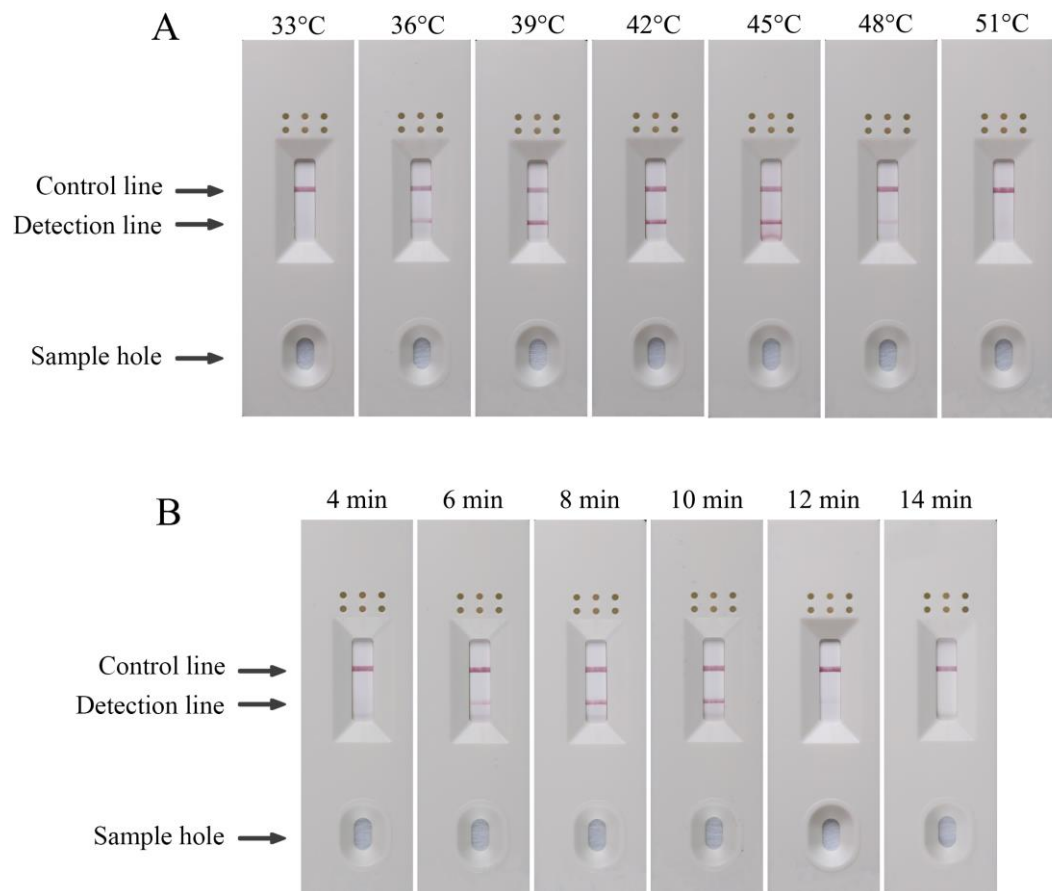


Figure 3. Optimization of recombinase polymerase amplification–lateral flow dipstick (RPA–LFD) detection for *F. oxysporum*. (A) Optimization of the RPA reaction temperature (33, 36, 39, 42, 45, 48, and 51 °C). (B) Optimization of the RPA reaction time (4, 6, 8, 10, 12, and 14 min).

E. Detection system sensitivity verification

1. Set the DNA concentration to 10 ng/μL, 1 ng/mL, 100 pg/μL, 10 pg/μL, 1 pg/μL, and 100 fg/μL. DNA concentration was determined using Ultramicro accounting protein analyzer.
2. Add different concentrations of DNA templates to the reaction system.
3. Incubate at 39 °C for 8 min.
4. Visualize the amplification results according to step C3.
5. The detection limit was determined by the presence or absence of the detection line.

F. Field sample detection

1. Take 100 mg of strawberry crown tissue (approximately 125 mm³ of crown tissue) and place in a 1.5 mL tube.
2. Add 100 μL of 60% PEG200 .
3. Incubate at room temperature for 15 min to lyse plant tissue and release DNA.
4. Add the lysate supernatant as a template into the RPA reaction system. Set up the reaction system as follows:
Buffer A 29.4 μL
Upstream F91 2 μL
Downstream R91 2 μL

Probe 0.6 μ L

ddH₂O 11.5 μ L

Template DNA 2 μ L

Buffer B 2.5 μ L

Buffer B needs to be added last. Buffer A and Buffer B are supplied with the kit.

5. Incubate at 39 °C for 8 min.

Visualize the amplification results according to step C3.

6. If the control line is visible and the detection line is not, this indicates that the strawberry was not infected by *F. oxysporum*. If both the detection and control lines are visible, this indicates that the strawberry was infected by *F. oxysporum* (Figure 4).

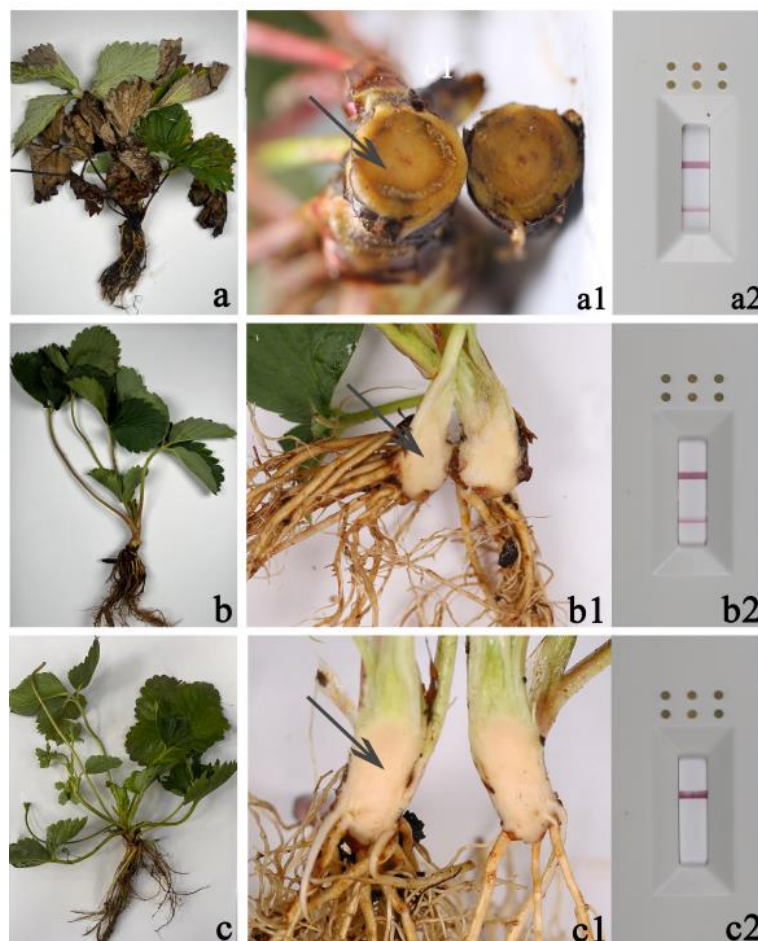


Figure 4. Symptoms of strawberry crown rot and the result of recombinase polymerase amplification–lateral flow dipstick (RPA–LFD) detection. (a–a2) Plant with crown rot symptoms and positive test results. (b–b2) Plant without crown rot symptoms and positive test results. (c–c2) Plant without crown rot symptoms and negative test results. The tissue indicated by the arrow is used for detection.

Data analysis

1. Determination of primers and probes specificity:

Judge the specificity of primers and probes by the presence or absence of detection lines of HybriDetect LFD. The detection line was visible only for *F. oxysporum* and the detected result is positive; the detection lines were not visible for other genomic DNA, and the detected result is negative (Table 1). These results indicate that the primers F91/R91 and probe had good specificity for *F. oxysporum*.

Table 1. Results of other isolates detected by RPA-LFD

Species	Detection result
<i>F. oxysporum</i>	+
<i>F. tricinctum</i>	-
<i>F. proliferatum</i>	-
<i>F. fujikuroi</i>	-
<i>F. graminearum</i>	-
<i>F. solani</i>	-
<i>F. equiseti</i>	-
<i>C. siamense</i>	-
<i>C. gloeosporioides</i>	-
<i>C. aenigma</i>	-
<i>C. fructicola</i>	-
<i>Botrytis cinerea</i>	-
<i>Phoma</i> sp.	-
<i>Stagonosporopsis</i> sp.	-

“+” represents positive result, “-” represents negative result.

2. Determination of optimal reaction conditions:
According to the manufacturer’s instructions, the optimal reaction temperature was determined with 10 min as the reaction time. The result showed that 39 °C is the optimal reaction temperature, and the optimal reaction time was 8 min.
3. Determination of detection limits:
The detection limit was determined by the presence or absence of the detection line. The HybriDetect LFD still had bands in the detection line at a template concentration of 1 pg/μL, and we concluded that the detection limit of the RPA-LFD detection was 1 pg/μL.
4. Field application of RPA-LFD detection:
The appearance of the test line indicated a positive result, suggesting that the plant was infected with *F. oxysporum*.

Validation of protocol

Hu et al. (2023) [7]. Establishment of the recombinase polymerase amplification-lateral flow dipstick (RPA-LFD) detection technique for *Fusarium oxysporum*. Plant Disease. (Figure 4).

General notes and troubleshooting

General notes

1. The length of primers for RPA detection was 31–32 bp; too-short primers will affect the amplification speed and detection sensitivity.
2. Before the positive test, the negative test was performed to rule out the formation of false positives due to the primers probe.
3. Care should be taken to avoid cross contamination during the experimental operation.

4. This operation was effective only when the HybriDetect LFD control line is striped.
5. HybriDetect LFD interpretation results were completed within 5 min.

Troubleshooting

Problem 1: The combination of primers and probe could not specifically detect the target fungus.

Possible cause(s): Poor specificity of primers and probe.

Solution(s): The specific primers need to be screened again, and the probes need to be screened after the specific primers are obtained.

Problem 2: The test line of negative control showed banding.

Possible cause(s): The primers bound to the probe itself.

Solution(s): Re-screen primers and probe.

Problem 3: The color of the detection line was weak.

Possible cause(s): The reaction conditions were not optimal, or the DNA concentration is low.

Solution(s): Optimize reaction conditions and increase DNA concentration.

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The protocols described here are adapted from our previous work [7].

Competing interests

The authors declare no competing interests.

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