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Rapid Serodiagnostics for Plant Disease Identification and Management-Lateral Flow Assay

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Abstract

Detection is the best protection and disease diagnosis plays an important factor in crop protection and management of the plant diseases. Among the different diagnostic tests for plant pathogens, serological based Lateral Flow Assay (LFA) was very widely adopted due to its simple use coupled with high sensitivity. In the recent past, several LFAs were developed for rapid diagnosis of several plant pathogens including, fungi, bacteria and viruses, across the globe.

1. Introduction

Plant diseases account for 14% towards total yield loss all over the world. Therefore, every plant pathologist's concern is to manage the diseases and reduce this yield loss and achieve food security. One of the ways to manage the disease/epidemic is to forecast the disease well in advance, by diagnosing them at the earliest possible. Different diagnostic techniques for plant pathogens have been grouped into (i) biological techniques (ii) serological techniques and (iii) nucleic acid based techniques. Among these, serology is widely used method to diagnose the disease since it is simple and robust. Since the first report of plant virus detection by Enzyme linked immunosorbent assay (ELISA) by Clark and Adams in 1977, the incorporation of serological methods into routine diagnosis for plant pathogens has improved the sensitivity and reliability of disease diagnosis. ELISA is suitable for large scale testing in the field samples. However, there remains the need for the detection of pathogens on-site, in the field condition, using a test that can rapidly and reliably confirm the presence or absence of a particular pathogen in symptomatic tissues. The characteristics required for on-site tests are different from that of tests for laboratory use:

- The end user may have no appropriate expertise
- May be unfamiliar with handling of diagnostic equipment
- No facilities or equipment to handle
- Not prepared to wait a long time for the test result and require a

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clear and unambiguous result

The first reported field tests for the detection of plant viruses were chlorophyll agglutination and latex agglutination tests. Both were not widely accepted because not being robust enough for the routine use in the field and it was difficult to distinguish false positives from true positives. One of the methods to for the on-site detection of pathogens is Lateral Flow Assay (LFA) or Lateral flow device (LFD).

Immuno-chromatographic assays, also called lateral flow tests or simply strip tests, have been around for some time. They are a logical extension of the technology used in latex agglutination tests, the first of which was developed in 1956 by Plotz and Singer. In LFA, enzymes can be replaced by binding antibodies to microscopic labels, such as latex, gold or silica. These labels can be visualised by capturing them in a line or dot. As the amount of captured antigen increases the concentration of accumulated particles also increases. Then the reaction site can be seen by eye once a high enough density of particles is reached. This is the basis of all LFDs, rapid immune filter paper assays or immune-chromatographic assays. The most famous use of this technique is home pregnancy test first used by Unipath in 1988 (<https://en.wikipedia.org/wiki/Unipath>). The one-step LFD technology is already proven and fully commercialised in the pharmaceutical field and human medicine.

2. Application of Lateral Flow Assay

Lateral flow assays can be applied to many fields such as:

- Agriculture
- Aquaculture
- Environment
- Forensic science
- Therapeutic monitoring
- Medical diagnosis
- Food safety
- Military biodefence
- Consumer diagnosis
- Animal health
- Blood banking
- Industry

3. Lateral Flow Assay in Plant Pathology

The adoption of LFAs in the field of plant pathology,

across varied pathogens is well documented and was presented in Table 1.

Table 1: Adoption of lateral flow assay for detection of plant pathogens

Sl. No.	Plant pathogen/s detection	Reference
1	Cucumber mosaic cucumo virus (CMV), and Tobacco mosaic tobamo virus (TMV)	Tsuda et al. (1992)
2	One-step LFD test kit for bacteria: <i>Ralstonia solanacearum</i> , <i>Xanthomonas hortorum pv pelargonii</i> and <i>Clavibacter michiganensis</i> subsp <i>sepedonicus</i> and <i>Erwinia amylovora</i>	El-Badry (2005)
3	Multi-RIPA for the detection of 3 Tobamo viruses; <i>Cucumber green mottle mosaic virus</i> (CGMMV), <i>Kyuri green mottle mosaic virus</i> (KGMMV) and <i>Zucchini green mottle mosaic virus</i> (ZGMMV) in cucurbitaceous crops.	Choi et al. (2001)
4	One-step lateral flow tests for 2 potato viruses PV-Y and PV-X	Danks and Barker (2000)
5	One-step LFD test kit for Fungi: <i>Rhizoctonia solani</i> , <i>Trichoderma hamatum</i> , <i>Phytophthora ramorum</i>	Thornton et al. (2004) Thornton (2008) Lane et al. (2006)
6	One-step LFD test kit for viruses: <i>Potato viruses X, Y, A, S and V</i> , <i>Plum pox virus</i> , <i>Pepino mosaic virus</i> , <i>Tomato spotted wilt virus</i> , <i>Impatiens necrotic spot virus</i>	http://www.pocket-diagnostic.com

4. Principle of LFA

LFA involves two sources of antibody (polyclonal or monoclonal), one of which is immobilised onto a nitrocellulose-based membrane, using a sophisticated reagent dispenser, and the other is sensitised onto blue-dyed latex particles. The sensitised latex is then airbrushed onto a conjugated release pad and sealed together with the membrane and an absorbent pad into a plastic housing (Figure 1)

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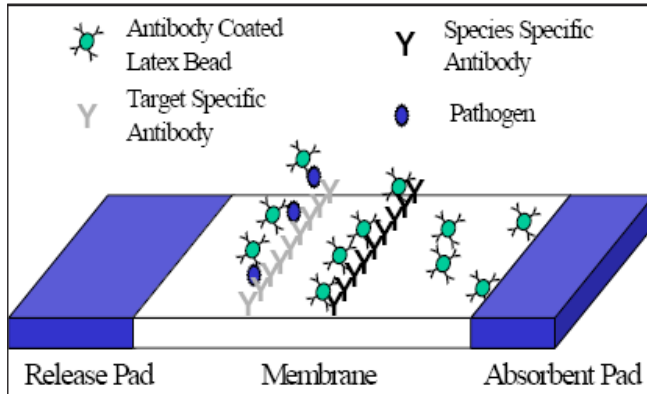


Figure 1: Format of one-step lateral flow assay (Danks and Barker, 2000)

Kits for diagnosis distributed for the final operators includes: Lateral flow device; A plastic bag/bottle for extraction (Figure 2), a plastic pipette; 5 ml extraction buffer consisting of PBS, 0.02% of Tween-20, 2% PVP (MW 24,000) and 0.5% Triton X-100. On addition of a few drops of plant extract to the well, the latex is released and flows along the membrane. If target antigen is present, the latex-antigen complex deposits as a blue line over the immobilised antibody line (T). An anti-species antibody, which is immobilised ahead of the target line, captures excess sensitised latex and produces an internal control line (C). The results are easily interpreted visually within 3 minutes. The development of two lines indicates positive detection of the target pathogen, whilst a valid negative result is indicated by the development of only one line at the control position (Figure 3).

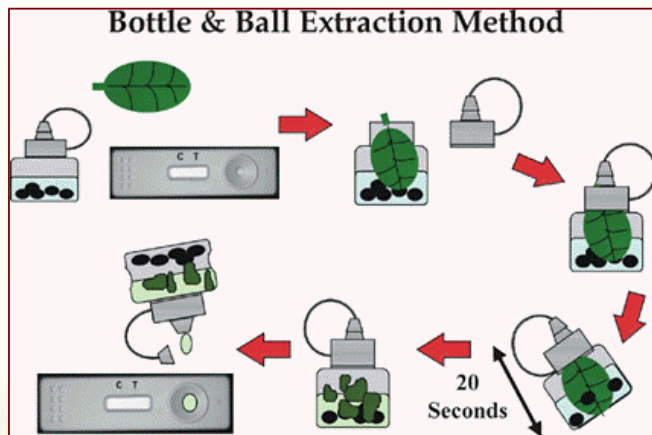


Figure 2: The sample extraction method takes 30 seconds (Danks and Barker, 2000). Whole or parts from plant leaves are placed into the bottle containing extraction buffer. The bottle is shaken for 20 seconds, then a few drops are added to the LFD kit



Figure 3: Development of the LFD test result (Danks and Barker, 2000). An actual positive and negative result using a Pocket Diagnostic device (results obtained after 3 minutes after sample addition) +ve (2 blue lines - control and test (C and T)) indicates a positive result; -ve (1 blue line - control only (C)) indicates a negative result

5. Lateral Flow Assay Components

The key components of a typical LFA are depicted in figure 4.

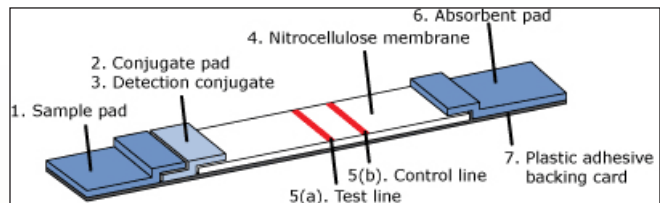


Figure 4: Lateral-flow assay/device components. (Korf and Amerongen, 2009; El-Badry, 2005; <http://www.rapid-diagnostics.org/index.htm>)

5.1. The Sample Pad

The sample pad is made of cellulose, glass fibre or other material where the fluid sample is applied to the lateral flow device and, if necessary modifies it to improve the results of the assay. These allow for a steady flow and prevent non-specific binding of sample components to the pad.

5.2. The Conjugate Pad

The conjugate pad is made of a non-absorbent material such as fibreglass pad, polyester, rayon or a similar material. Pre-treatment of the conjugate pad helps to ensure the conjugate releases at the proper rate and enhances its stability. The pre-treatment is performed in the same way as with the sample pad.

5.3. Detection Conjugate

The signal reagent used in lateral flow tests have become much more varied as the technology advances. Test may use colloidal metals such as gold or silver, carbon, a visible or florescent dye, magnetic particles, enzymes, latex beads impregnated with visual or fluorescent dyes, or a combination of these which are conjugated to either an antibody or antigen to generate signal.

5.4. Nitro-Cellulose Membrane

The nitrocellulose (NC) membrane consists of a very thin Mylar sheet coated with a layer of NC.

5.5. Test and control reagent lines

The complex of gold conjugate and analyte then moves onto the membrane strip and migrates towards the capture binding protein, where it becomes immobilized and produces a distinct signal in the form of a sharp red line. A second line, a control, may also be formed on the membrane by excess gold conjugate, indicating the test is complete. The standard for lateral flow tests is one test line and one control line are placed on the NC membrane.

5.6. Absorbent Pad

The absorbent pad, also called a wick or wicking pad, pulls fluid off of the membrane to allow the capillary flow of the membrane to keep flowing in the proper direction and at the proper rate. These pads can be manufactured in a variety of thicknesses and densities to suit the needs of the assay.

5.7. Plastic-adhesive backing card

Due to the delicate nature of the materials used in an ICS assay as well as the need to maintain a precise, direct contact between components to ensure proper reagent and sample flow a backing card of some sort is always necessary. Usually these are made pre-treated with pressure-sensitive adhesive selected for its stability in the assay and to insure it doesn't leach chemicals that may interfere with results.

5.8. Laminate Cover Tape

The Laminate Cover Tape is an adhesive tape the acts as a protective barrier and prevents evaporation of reagents and helps to limit back-flow of reagents.

5.9. Strip housing/Cassette

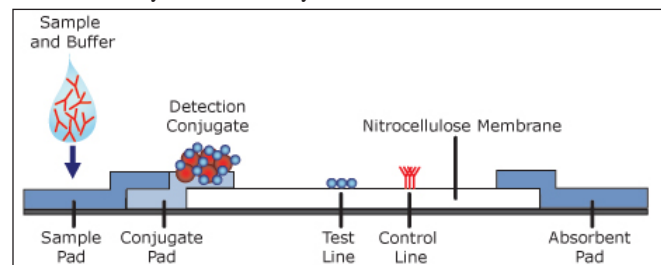
A plastic housing typically made of two pieces that snap together and protect the assembly. The test strip and absorbent pad are contained within this housing that allows the unit to be handheld more easily and protects

the strip from damage and environmental contamination.

6. Lateral-flow: How it Works?

6.1. Step 1

To perform the test, a sample is placed on the sample pad at one end of the strip. The sample may be used alone as is commonly done with urine or serum compatible tests, or it may be mixed with a buffer specific to the test. This buffer may simply be a diluent/running buffer or it may be much more complex and have specific components or properties required to make the strip perform properly, such as a cell lyses buffer. In the following description we are assuming a gold conjugate is being used. While this is one of the most common detection methods; however it is certainly not the only one available.



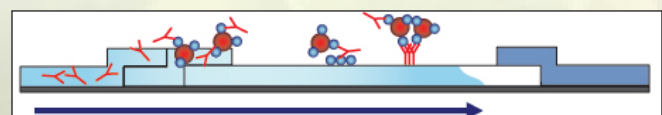
6.2. Step 2

With the addition of the sample, the detector molecules are solubilized. When solubilized the detector molecules mix with and bind to the analyte in the sample (if analyte is present).



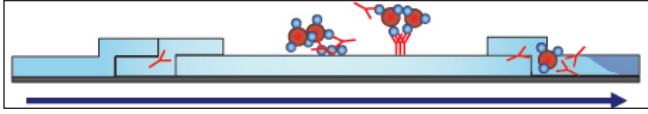
6.3. Step 3

Then capillary action draws the fluid mixture up the sample pad and into the membrane. The sample/detector molecule mix continues to move up the membrane until it reaches the analyte capture molecule. In these lines a second (and third) antibody or antigen, immobilized as a thin stripe in the nitrocellulose will then capture the complex if it is positive for the target analyte. The control line should always show as a visible line, otherwise the test is invalid and must be repeated. If the test is positive, a colored (typically pink or purple) line develops along with the control line.



6.4. Step 4

Excess buffer along with any reagents not captured at the test of control line will then move into the absorbent wicking pad.



7. Application of LFA in Detection of Plant Pathogens - A case Study

7.1. Development and Validation of a Lateral Flow Device (LFD) Field Test Kit for Diagnosis of Potato Ring Rot (El-Badry, 2005)

Monoclonal antibodies were produced from a series of selected cell lines of the ring rot bacterium (*Clavibacter michiganensis* subsp. *sepedonicus*) (*cms*). Of the monoclonal antibodies produced, preliminary evaluation suggested that one (IgG 287/8.F5.C2.D10) showed the highest potential for use in lateral flow format. This antibody was purified using protein G columns “High Trap™ (Gaynor)” (CSL, York, UK) and used in all further LFD validation work conducted within this study. Prototype LFD kits were assembled according to the methods described by Danks and Barker (2000). A latex conjugate was developed using a 0.43- μm blue latex particle, passively coated with anti-*Cms* monoclonal antibody immunoglobulin (IgG 287/8.F5.C2.D10) (at approx. 1 mg per ml). The *Cms* MAb was precision sprayed onto 135M membranes (HF135 Hi-Flow Plus Membranes, Millipore) to form the target line (T). For the control line (C), anti-mouse antibodies which recognise the *Cms* antibody were similarly sprayed 5 mm ahead of the target line using a Biodot dispenser. The membrane was then cut into strips of the required dimensions to fit into the plastic housing. The latex conjugate was applied onto the release region of the device (sample pad) by air jet. The processed strips were assembled onto cards, cut to dipsticks and then sealed in plastic housings. An absorbent pad was also included at the opposite end of the membrane to the release pad to ensure the sample is efficiently drawn along the membrane. A proprietary blocking buffer (Buffer C, CSL Pocket Diagnostics, York, UK) containing phosphate buffered saline, Tween 20 detergent, 0.05% sodium azide as a preservative and polyvinylpyrrolidone (PVP) was also included in the prototype LFD kit. This blocking buffer facilitates 47 sample movements along the membrane and inhibits

non-specific binding to the antibodies. Sensitivity and specificity of detection of *Cms* using the prototype kit was evaluated. For specificity testing, suspensions containing approximately 10^6 CFU per ml in Buffer C were used and 65 μl of suspension was added to the wells of each LFD test. For sensitivity testing, tenfold serial dilutions of *Cms* isolates in buffer C were tested. Finally, to simulate on-site field testing, stem and petiole sections from *Cms*-infected and healthy potato plants and eggplant seedlings (CSL, York, UK) were shaken for 2 minutes in 5 ml buffer C in extraction bottles provided with the LFD kit and three drops of the resulting suspension was added to wells of each LFD test.

Sensitivity of detection of 5 of the 7 *Cms* isolates, in the prototype LFD test, was equivalent to that observed when tested by IFAS, with the limit of reliable detection at 10^4 cells per ml and unreliable detection at 10^3 cells per ml or lower (Figure 6). However, two *Cms* isolates were not detected at any concentration. The optimum concentration for detection was around 10^6 cells per ml. No cross-reactions were observed with any of the closely related bacteria to *Cms* or with the other bacterial pathogens of potato when tested at 10^6 cells per ml.

8. Advantages and Disadvantages

8.1. General advantages of rapid diagnostic tests

- Easy to use, with minimal training required.
- Relatively rapid; same-day results are possible, resulting in fewer patients lost to follow-up and quicker treatment.
- A shelf life as long as 1-2 years at ambient temperatures, with no need for refrigeration.
- Limited or no need for instrumentation, allowing these tests to be performed at the periphery of health systems, often where there is no laboratory or electricity, thus increasing the number of testing sites.
- In some cases, rapid tests are more accurate than existing reference-level laboratory tests.

8.2. General disadvantages of rapid tests

- Cost per test for rapid tests may exceed traditional testing methods such as microscopy.
- Most rapid tests have limited shelf lives that place increased demands on procurement and distribution systems.
- They are mainly qualitative, producing only “yes/no” answers that may yield less information than the existing laboratory-based quantitative tests.

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- They require subjective interpretation, which may result in reader variation in results.
- In many cases, rapid tests are less sensitive or less accurate compared to existing reference-level laboratory tests.
- Are not amenable for high throughput testing.
- Requires extensive and robust quality control and quality assurance mechanisms.

9. Conclusion

- Serological tests are usually used for a presumptive identification in a presumptive diagnosis
- Since there is a risk of errors due to serological cross reactions, further laboratory testing is always required to confirm the results obtained with test kits
- Nevertheless an immediate identification of the presence of a particular target organism in a growing area can assist on-site decision making
- This will hopefully enable a more efficient front-line defence against the entry and spread of key plant pathogens.

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