THE UNIVERSITY OF QUEENSLAND

AUSTRALIA

Development of point-of-care and multiplex diagnostic methods for the detection of plant pathogens

Han Yih Lau

Master of Plant Biotechnology

Bachelor of Molecular Biology (Hons)

A thesis submitted for the degree of Doctor of Philosophy at

The University of Queensland in 2016

School of Agriculture and Food Sciences

Abstract

New diagnostic technologies for the detection of plant pathogens with high multiplexing ability and point-of-care (POC) capability are an essential tool in the fight to reduce the large agricultural production losses caused by plant diseases. The main desirable characteristics for such diagnostic assays are high specificity, sensitivity, reproducibility, quickness, cost efficiency and high-throughput multiplex detection capability. The aim of this project is to develop new methods combining nucleic acid amplification, multiplexing and point-of-care application for the detection of plant pathogens.

Isothermal amplification methods, such as recombinase polymerase amplification (RPA) which operates at low constant temperature are especially suited for POC applications. However, the development of a rapid and simplified detection method for the isothermal amplification products in resource-poor settings is still challenging. A new method to visualize the success of the amplification step, and therefore the presence of pathogen DNA in a sample, was developed based on bridging flocculation. This method requires minimal equipment and can be assessed by the naked eye allowing its use in POC settings. The method was initially applied to the rapid and sensitive detection of several important plant pathogens and subsequently extended to animal and human pathogens to demonstrate the wide applications of the approach. A nanoparticle based electrochemical biosensor was also developed for rapid and sensitive detection of amplified plant pathogen DNA on screen-printed carbon electrodes. Gold nanoparticles were employed as a probe for electrochemical assessment by differential pulse voltammetry (DPV). This method was 10,000 times more sensitive than conventional polymerase chain reaction (PCR)/gel electrophoresis and could readily identify *P. syringae* infected plant samples even before the disease symptoms were visible.

To allow multiplex detection of plant pathogen, a novel method that can screen for thousands of plant pathogens with high specificity and sensitivity using molecular inversion probes (MIPs) was been developed. As proof of concept, a MIP targeting a unique DNA sequence present in the *F.oxysporum* f.sp. *conglutinans* (Foc) genome was designed. The specificity, sensitivity and detection limit of the assay were assessed and used to detect the presence of pathogen in infected *Arabidopsis thaliana* samples. This methodology successfully detected as little as 2.5 ng of pathogen DNA and it was highly specific, being able to accurately differentiate *Fusarium oxysporum* f.sp. *conglutinans* from other fungal pathogens such as *Botrytis cinerea* and even pathogens of the same species such as *Fusarium oxysporum* f.sp. *lycopersici*. Finally, a diagnostic platform for POC plant pathogen detection using a combination of surface-enhanced Raman

scattering (SERS) and RPA was developed for rapid multiplex detection. The RPA-SERS method was faster, more sensitive than polymerase chain reaction and could detect as little as 2 copies of *B. cinerea* DNA. Furthermore, multiplex detection of *Fusarium oxysporum* f.sp. *conglutinans*, f.sp. *lycopersici* and *Botrytis cinerea* was demonstrated *in vivo* in *Arabidopsis thaliana* and tomato. The assay was subsequently simplified into a single tube assay and tested outside of a laboratory setting to detect pathogens in tomato plants.

Declaration by author

This thesis ***is composed of my original work, and contains*** no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted ***to qualify for the award of any*** other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

I acknowledge that an electronic copy of my thesis must be lodged with the University Library and, subject to the policy and procedures of The University of Queensland, the thesis be made available for research and study in accordance with the Copyright Act 1968 unless a period of embargo has been approved by the Dean of the Graduate School.

I acknowledge that copyright of all material contained in my thesis resides with the copyright holder(s) of that material. Where appropriate I have obtained copyright permission from the copyright holder to reproduce material in this thesis.

Publications during candidature

Peer-reviewed papers

Lau, H. Y., Palanisamy, R., Trau, M. & Botella, J. R. Molecular inversion probe: a new tool for highly specific detection of plant pathogens. ***PloS one*** 9, e111182, doi:10.1371/journal.pone.0111182 (2014)

Wee, E. J. H., **Lau, H. Y.**, Botella, J. R. & Trau, M. Re-purposing bridging flocculation for on-site, rapid, qualitative DNA detection in resource-poor settings. ***Chem Commun*** 51, 5828-5831, doi:10.1039/c4cc10068a (2015)
This work contributed to the WO2015095929 A1 Patent

Lau, H. Y., Wang, Y, Wee, E. J. H., Botella, J. R. & Trau, M. Field Demonstration of a Multiplexed Point-of-care Diagnostic Platform for Plant Pathogens. ***Anal Chem*****.** Just accepted manuscript. DOI: 10.1021/acs.analchem.6b01551 (2016)

Poster Presentation

Lau, H. Y., Wee, E. J. H., Botella, J. R. & Trau, M. Re-purposing bridging flocculation for on-site, rapid, qualitative DNA detection in resource-poor settings.18th International Plant Protection Congress (Aug 2015)

Publications included in this thesis

1. Wee, E. J. H., **Lau, H. Y.**, Botella, J. R. & Trau, M. Re-purposing bridging flocculation for on-site, rapid, qualitative DNA detection in resource-poor settings. ***Chem Commun.*** 51, 5828-5831, doi:10.1039/c4cc10068a (2015) – Incorporated in Chapter 3

Contributor	Statement of contribution
Han Yih Lau (Candidate)	Conceptualisation (20%), experimental plan (30%), experimental design (40%), performing experiments (50%), data analysis (40%) and manuscript writing (50%).
Eugene J.H. Wee	Conceptualisation (40%), experimental plan (40%), experimental design (40%), performing experiments (50%), data analysis (60%) and manuscript writing (50%).

José Ramón Botella (Jimmy Botella)	Conceptualisation (20%), experimental plan (15%) and manuscript proofreading (50%).
Matt Trau	Conceptualisation (20%), experimental plan (15%) and manuscript proofreading (50%).

2. **Lau, H. Y.**, Palanisamy, R., Trau, M. & Botella, J. R. Molecular inversion probe: a new tool for highly specific detection of plant pathogens. ***PloS One*** 9, e111182, doi:10.1371/journal.pone.0111182 (2014) – Incorporated in Chapter 4

Contributor	Statement of contribution
Han Yih Lau (Candidate)	Conceptualisation (20%), experimental plan (40%), experimental design (30%), performing experiments (50%), data analysis (40%) and manuscript writing (50%).
Ramkumar Palanisamy	Conceptualisation (20%), experimental plan (30%), experimental design (30%), performing experiments (50%), data analysis (60%) and manuscript writing (50%).
Matt Trau	Conceptualisation (30%), experimental plan (20%) and manuscript proofreading (50%).
José Ramón Botella (Jimmy Botella)	Conceptualisation (30%), experimental plan (20%) and manuscript proofreading (50%).

3. **Lau, H. Y.**, Wang, Y, Wee, E. J. H., Botella, J. R. & Trau, M. Field Demonstration of a Multiplexed Point-of-care Diagnostic Platform for Plant Pathogens. ***Anal Chem*.** Just accepted manuscript. DOI: 10.1021/acs.analchem.6b01551

Contributor	Statement of contribution
Han Yih Lau (Candidate)	Conceptualisation (30%), experimental plan (50%), experimental design (40%), performing experiments (80%), data analysis (40%) and manuscript writing (40%).
Yuling Wang	Conceptualisation (20%), experimental plan (30%), experimental design (40%), performing

	experiments (15%), data analysis (40%) and manuscript writing (40%).
Eugene J.H. Wee	Conceptualisation (15%), experimental plan (10%), experimental design (40%), performing experiments (5%), data analysis (20%) and manuscript writing (20%).
José Ramón Botella (Jimmy Botella)	Conceptualisation (5%), experimental plan (5%) and manuscript proofreading (50%).
Matt Trau	Conceptualisation (10%), experimental plan (5%) and manuscript proofreading (50%).

Contributions by others to the thesis

Samples involved in Chapter 3 project: the HIV samples were provided by David Harrich and Dongsheng Li, the Bovine HPV-1 samples were provided by Timothy Mahony, MTB samples were provided by Nick West, the malaria samples were provided by Christopher Peatey, the F. oxysporum cubense Race 1 infected banana samples were provided by Elizabeth Aitken and Juliane Henderson, and the influenza H1N1 samples were provided by Paul Young and Daniel Watterson.

Statement of parts of the thesis submitted to qualify for the award of another degree

None

Acknowledgements

I would like to show my appreciation to **Malaysian Agriculture Research & Development Institute (MARDI)** for providing me the 3.5 years of PhD scholarship to pursue my study in The University of Queensland.

It is a great privilege to express my gratitude to **Prof. Jimmy Botella** and **Prof. Matt Trau** for giving me a wonderful opportunity and supporting me throughout my PhD study. I immensely revere their leadership, encouragement and enthusiasm that has made my PhD journey a wonderful experience.

I would like to express my profound thanks to **Dr. Eugene Wee**, **Dr. Yuling Wang** and **Dr. Ramkumar Palanisamy** for their constant support, guidance and the generosity with which they share their expertise throughout my research career. I sincerely appreciate their encouragement, patience and concern they have shown during my candidature.

I would like to thank to all the past and present members of the **Botella group** and **Trau group** especially all the postdoctoral research fellows over the years for their constant support and encouragement throughout my PhD.

I will forever be grateful to my family and all my friends who have gave me moral support throughout my candidature. I dedicate this work to my family especially to my husband **Mr. Tze Leong Lee**, my son **Ambrose Lee**, my dad **Mr. Hua Ding Lau** and mom **Lee Ha Siaw** who have sacrificed a lot over the years to put me where I am right now.

Keywords

Plant pathogen, point-of-care testing, multiplex detection, recombinase polymerase amplification, molecular diagnostics, electrochemical sensor, bridging flocculation, molecular inversion probe, surface enhanced Raman scattering, gold nanoparticles

Australian and New Zealand Standard Research Classifications (ANZSRC)

ANZSRC code: 100101, Agricultural Biotechnology Diagnostics (50%)

ANZSRC code: 100703, Nanobiotechnology (30%)

ANZSRC code: 030102, Electroanalytical Chemistry (20%)

Fields of Research (FoR) Classification

FoR code: 1001, Agricultural Biotechnology (70%)

FoR code: 0301, Analytical Chemistry (30%)

Table of contents

List of Figures & Tables

Chapter 1

Literature review

Chapter 2

Specific and Sensitive Recombinase Polymerase Amplification Electrochemical Biosensor for Plant Pathogen DNA Detection with Colloidal Gold Nanoparticles as Probes

Chapter 3

Re-purposing bridging flocculation for on-site, rapid, qualitative DNA detection in resource-poor settings

Chapter 4

Molecular inversion probe: A new tool for highly specific detection of plant pathogens

Chapter 5

Field demonstration of a multiplexed point-of-care diagnostic platform for plant pathogens

List of Abbreviations used in the thesis

A, T, G, C	Adenine, thymine, guanine and cytosine nucleotide bases
AuNPs	Gold nanoparticles
dai	Day after inoculation
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
DPV	Differential pulse voltammetry
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
HDA	Helicase dependent amplification
hr, min, s	Hours, minutes, seconds
HPLC	High performance liquid chromatography
LAMP	Loop-mediated isothermal amplification
LFD	Lateral flow device
MAb	Monoclonal antibody
MBA	4-Mercaptobenzoic acid
MIP	Molecular inversion probe
MMC	2,7-mercapto-4-methylcoumarin
nt	Nucleotide
Pab	Polyclonal antibody
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PLP	Padlock probe
POC	Point-of-care
qPCR	Quantitative polymerase chain reaction
RCA	Rolling circle amplification
RPA	Recombinase polymerase amplification
RNA	Ribonucleic acid
RSD	Relative standard deviation
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SERS	Surface-enhanced Raman scattering
SPCE	Screen printed carbon electrode
SPRI	Solid Phase Reversible Immobilization

SSB	Single stranded binding proteins
TFMBA	2,3,5,6-tetrafluoro-4-mercaptobenzoic acid
TCEP	Tris(2-carboxyethyl)phosphine
V	Volt
µg, ng, pg, fg	Microgram, nanogram, picogram, femtogram
µL	Microliter
µM, nM, pM	Micromolar, nanomolar, picomolar
µTAS	Micro-Total Analysis System

Chapter 1

Literature review

1.1 Plant pathogen detection

Agriculture contributes $1500 billion US dollars (USD) to the world economy annually.[1] However, an estimated $220 billion USD worth of agricultural product is lost every year due to crop disease outbreaks, especially in developing countries[1] making crop health a critically important issue in agricultural based countries. In the absence of resistant varieties, the ideal management strategy is to detect pathogens in an early infection stage in order to prevent the spread of the disease. Hence, the effectiveness of crop disease management is highly dependent on the speed, sensitivity and specificity of the diagnostic methods. It is therefore important to develop more efficient technologies to detect crop diseases and effectively link them to decision bodies in order to efficiently deploy the necessary responses and safeguard agricultural systems.

1.2 Existing plant pathogen detection methods

1.2.1 Traditional detection and identification methods

Many methods have been developed to identify plant pathogens.[2] The earliest conventional methods use symptoms observation, involving field inspections to identify disease symptoms as well as laboratory tests such as pathogen culture on selective media followed by physiological, biochemical and pathogenicity tests.[3] For a long time, Koch's postulates[4] have been used as an effective way to identify unknown plant pathogens in diseased samples.[1] Koch's postulates use four criteria to establish the causal agent of a plant disease: (1) the pathogen must be present in all cases of the disease; (2) the pathogen must be isolated form the sick plant and grown in pure culture; (3) The pathogen from the pure culture must be re-inoculated into a healthy plant causing the same disease symptoms and (4) the pathogen must be re-isolated from the newly inoculated plant and proven to be the same than the original one.[5] Although conventional methods are reliable, they are time consuming and require experienced plant pathologists to identify the pathogen responsible for the disease. These reasons made it desirable to develop detection methods with higher sensitivity, specificity and speed for plant pathogen identification.

1.2.2 Antibody-based detection

Antibody technology has been well established in plant pathogen diagnostic since the 1980s and many reviews on this technology have been published.[6,7] Antibody-based diagnostic methods for plant pathogen detection has become a popular and powerful tool because of their speed, sensitivity and inexpensive nature. However, polyclonal antibodies (PAbs) against plant pathogens produced by animal immunization may show cross-reactivity with unrelated pathogenic species due to the

limited specificity of PAbs.[8] With the development of monoclonal antibodies (MAbs), specificity was improved since they target a single epitope in a pathogen protein.[9] Hence, various antibody-based diagnostic methods such as enzyme-linked immunosorbent assays (ELISAs)[10,11], immunoblot[12], immunofluorescent test[13] and lateral flow devices (LFD)[14] have been developed and widely used to identify plant pathogens. However, MAb are expensive to produce and it has been reported that the closely related species may share common epitopes and cause MAbs to react positively.[15,16]

1.2.3 DNA-based detection

The discovery of the polymerase chain reaction (PCR) in the 1980s enabled scientists to explore and develop DNA-based approaches for plant pathogen detection. As a result many PCR-based diagnostic methods for the identification of plant pathogens have been reported.[17,18] Furthermore, the amplification of pathogen-specific sequences and the coupling of PCR with other techniques have been described in order to improve specificity and sensitivity.[19-21] For example highly specific immunocapture-PCR (IC-PCR) has been used for viral detection which combines the conventional PCR amplification with antibody-captured viral particles. This approach increased sensitivity by 250-fold compared to direct PCR amplification[22] which enabled the successful detection of the bacterial blight disease in anthurium propagation material (*X. axonopodis* pv. *dieffenbachiae*).[23]

A combination of conventional PCR and enzyme-linked immunosorbent assay (ELISA) termed PCR-ELISA was developed in the early 1990s.[24] The assay involves hybridizing the labelled PCR product to an immobilized probe on an ELISA plate followed by the addition of an enzyme conjugate and a substrate to analyze the captured PCR product using a photometric measurement. This assay enabled the detection of a single amplicon population among several others that were generated in a multiplex reaction.[25] This technique has been reported to successfully detect viruses[26], bacteria[27] and fungi[28,29] with higher specificity than conventional PCR. However, despite its high specificity, the assay generated false positive results while detecting *Neisseria meningitidis*[30] and *Mycobacterium tuberculosis*[31,32] because the PCR amplified DNA was found to hybridize with the ELISA probe from other species.

An important improvement in DNA-based diagnostic methods came with the development of real-time quantitative PCR which allowed not only to detect the presence of pathogens but also to quantify pathogen levels in tissue samples thus allowing to determine the severity of the pathogen infection.[33] A drawback of this technology is the requirements of expensive equipment and reagents

which limits its use as a rapid cost-effective diagnostic method. In addition, the high sensitivity of the assay has the inherent risk of contamination resulting in the detection of false positives creating the need for normalization steps or pre-read runs to guarantee accuracy of results.[34,35]

Although PCR based assays have improved the sensitivity and have been used for multiplex pathogen detection,[36-38] the assays are still prone to non-specific DNA amplification resulting in false positive results while performing multiplex detection on unknown pathogens in diseased plants tissues.[39,40]

1.3 Point-of-care testing for plant pathogens

Nucleic acid POC bioassays that can be performed on-site with minimal equipment, rapidly and at low cost are in high demand.[41] PCR based methods have multiple advantages over other technologies but require a thermal cycler to achieve DNA amplification and hence are not suitable for field or on-site applications.[42] Nonetheless, recent developments in isothermal DNA amplification methods may potentially overcome this limitation.[43,44] For instance, isothermal amplification methods combined with lateral flow strips and portable fluorometers have been developed to enable POC detection of pathogenic DNA.[45-51] Such readout methods whilst convenient are still dependent on the use of relatively sophisticated equipment and may still present financial and technical obstacles for worldwide adoption. A field-ready comprehensive assay incorporating the complete process from on-site sample preparation to results display is still elusive. Agriculture is one area that can benefit from the use of low cost on-site assays as crop field locations can be far away from analytical laboratory setups and sample transport can pose logistical problems.

1.4 Point-of-care DNA extraction methods

An effective POC DNA extraction method is essential to develop rapid and user friendly molecular diagnostic assays but *on-field* sampling is rarely discussed when describing DNA-based diagnostic methods. This non-trivial task of consistently generating a fixed DNA input and good quality DNA has repercussions on any assays' performance. When dealing with plant tissues, the DNA extraction method requires the ability to efficiently remove chemicals that can inhibit the DNA amplification reaction.[52]

A LFD DNA extraction method has been reported as rapid and efficient for POC testing and has been successfully used in plant pathogen detection.[53,54] This method involves sample disruption in

extraction buffer using metal ball bearings before transferring the lysate onto the release pad of a LFD nitrocellulose membrane. A small piece of membrane is then excised and added into the DNA amplification reaction such as PCR or other isothermal amplification methods. The isolated DNA is very stable on the membrane at room temperature which allows the extraction to be performed in the field.[53]

Another DNA extraction method with potential POC application use Solid Phase Reversible Immobilization (SPRI).[55] A low cost DNA/RNA purification process using common filtered pipette tips in conjunction with SPRItechnology[56] to consistently extract DNA/RNA to a precise concentration that can be used immediately for downstream isothermal amplification has been reported.[55] Magnetic SPRI bead-based extraction is ideal for POC applications because the only equipment required are a magnet and micropipette. After macerating a single leaf disc with a plastic pestle in lysis buffer, the plant lysate was cleared of cellular debris by passing the lysate through a filtered pipette tip. DNA was then purified from the lysate using SPRI and the extracted DNA was directly used in the amplification reaction. This DNA extraction method has been used on various diagnostic applications including human and plant diseases.[55,57]

1.5 Application of nucleic acid isothermal amplification techniques in plant diseases detection

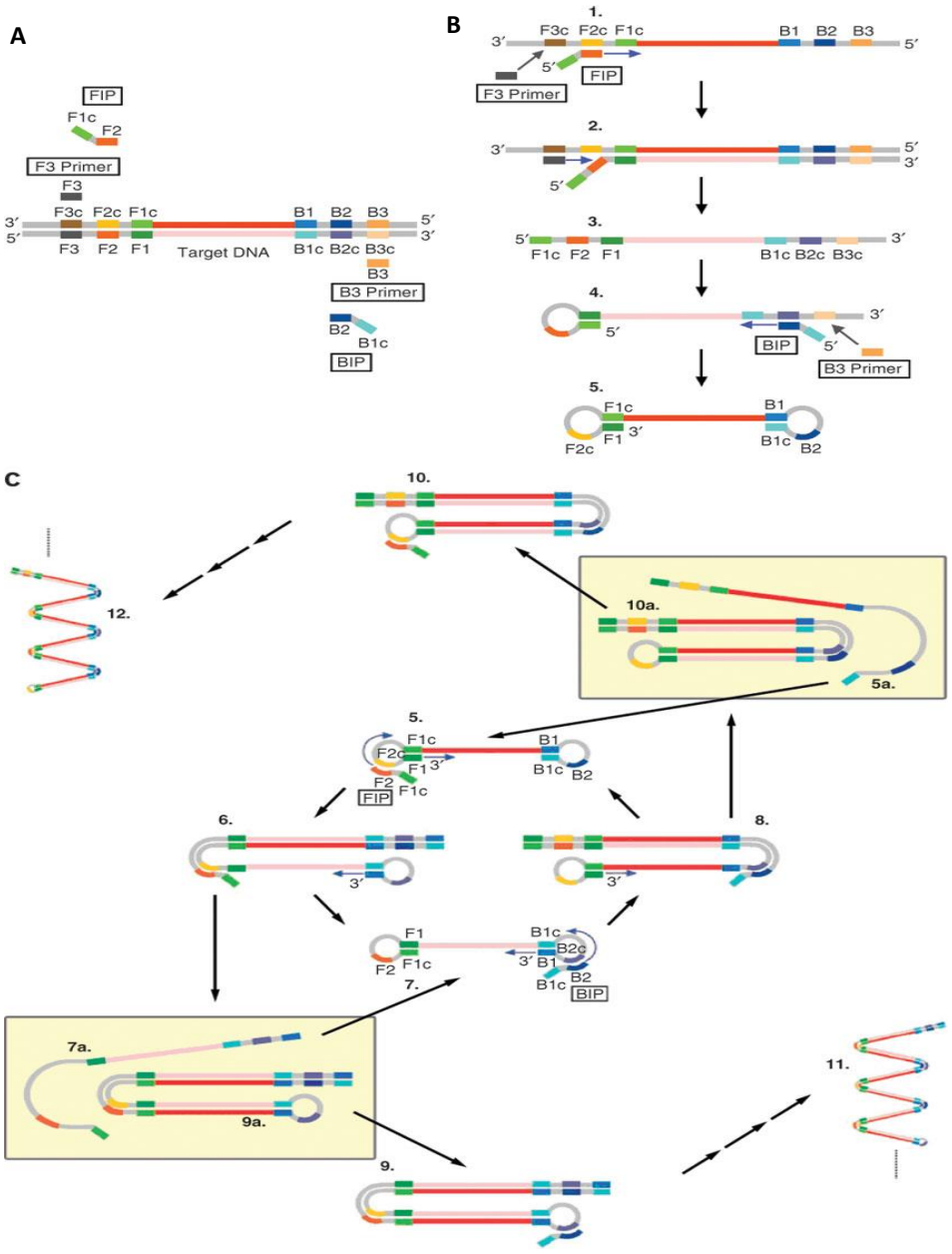
Enzyme mediated *in vitro* amplification of nucleic acid has become an essential tool in molecular biology since 1980's.[58] PCR is one of the most widely used methods for the detection and identification of pathogens by targeting specific sequences in their genomic DNA. Although PCR is highly sensitive and robust, it is constrained by a number of technical limitations. For instance the specificity of the assay is highly dependent on the primers used and its inherent sensitivity makes it prone to false positives due to cross-contamination of samples. Besides, PCR also requires electrically powered equipment to thermally regulate the reaction which limits its use for point-of-care diagnostics especially in resource poor countries. A number of alternative isothermal techniques are now available that can obviate the need for a thermal cycle although each has strengths and weaknesses.

1.5.1 LAMP

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method developed in 2000.[47] It has been widely used due to its high efficiency, specificity, simplicity and quickness. LAMP requires two long outer primers and two short inner primers that recognize six specific sequences of the target DNA. The first inner primer containing sense and antisense sequences of the target DNA hybridizes to the targeted sequence and initiate DNA synthesis (Fig 1.1). Next, the outer primer carries out the strand-displacement DNA synthesis and produces a single stranded DNA which works as a template for the second inner and outer primers producing a DNA molecule with a loop structure. The unremitting cycling reaction accumulates products with repeated sequences of target DNA in different sizes.

LAMP has three major advantages. Firstly, it can be carried out at a constant temperature with a short reaction time. This rapid isothermal process makes it ideal for point-of-care detection of plant pathogens in the field[59] and has been used to detect the plum pox virus in 2.5 hrs.[60] Secondly, it has very high amplification efficiency and sensitivity as it generates large amounts of PCR product with low amounts of input DNA.[61,62] Finally, this method is relatively cost effective as it requires simple equipment (isothermal) to perform the assay. Furthermore, there have been reports stating that LAMP generates PCR amplicons with several inverted repeats which could be potentially used to increase the sensitivity in hybridization assays, such as LAMP-ELISA hybridization[63] and LAMP incorporated with colorimetric gold nanoparticle hybridization probes.[64]

Although LAMP is an ideal isothermal method for on field POC plant pathogen detection, it does possess certain limitations. Firstly, the design of the LAMP primers is complicated and non-intuitive, making it difficult for newcomers. Even though there is available software for LAMP primer design, optimal primers performance is not guaranteed as it is usually the case for PCR. In addition, LAMP PCR amplicons contain a mixture of stem-loop DNA molecules of different sizes which are not suitable for gene cloning purposes or to identify specific targets based on size differences. However the size limitation was overcome by Nagamine et al. who developed a modified LAMP method that generates uniform and single stranded DNA amplicons through *Tsp*RI digestion and then the products were extended using the primer to produce the strand-specific DNA fragments.[65]



Source: Tomita et al. 2008[66]

***Figure 1.1*:** Schematic outline of loop-mediated isothermal amplification (LAMP). A: LAMP involves two sets of primers to target six distinct regions. B: The inner primer containing sense and antisense sequences of the target DNA hybridizes to the targeted sequence and initiates DNA synthesis. The outer primer carries out the strand-displacement DNA synthesis and produces a single stranded DNA which works as a template for second inner and outer primers for DNA synthesis that hybridize to the other end of the target to form a DNA loop structure. C: From the double stem-loop structure, the inner primer binds to the loop and synthesizes a new strand. The extension of the primer opens the loop at the 5' end and again the outer primer strand displaces the newly form longer DNA to produce ssDNA to form a DNA loop. LAMP produces loop structure DNA in various sizes.

Since LAMP has a potential for POC diagnostic purposes, several simple read-out methods have been combined with LAMP to replace the traditional gel electrophoresis analysis in order to detect the presence of amplicons. The simplest and cheapest methods use metal-ion indicators such as hydroxynaphthol blue (HNB) or SYBR green as DNA dyes to visualize the final products.[67,68] By adding color indicators into the LAMP reaction prior to amplification, the products can be detected by the naked eye using a simple colorimetric assay. Using this read-out method, it was possible to detect as low as 10 copies of target DNA.[68] The colorimetric readout has been successfully used to detect potato leafroll virus and *Fusarium oxysporum* with naked-eyes.[69,70]

Alternatively LAMP has also been combined with ELISA by incorporating antigen-labeled nucleotides into the LAMP amplicons during the amplification process. The generated amplicons are then hybridized to specific immobilized oligo nucleotide probes which are later analyzed by immunoassay. The main advantage of LAMP-ELISA is its ability to process hundreds of samples simultaneously within a few hrs.[63,71] The combination of LAMP with ELISA provides very high sensitivity with a single copy of the DNA target being successfully detected.[63] However, this technique requires skilled labour as it involves complicated ELISA steps.

Following the use of optical and colorimetric readout systems, electrochemical sensors that capable of detecting signal changes caused by the electron transfer in double stranded DNA were also used to detect the presence of amplified DNA.[72] The integration of LAMP with the electrochemical sensor offered a robust platform for pathogen detection as it was highly sensitive, detecting as low as 10 copies of pathogen genomic DNA.[73] The applications of LAMP-biosensor technology are increasing in the range from clinical molecular diagnostics to environmental monitoring, but its application is still fairly new in plant pathogen detection. Therefore, LAMP-biosensor technology has a strong potential for on field testing, detection and identification of plant diseases.

1.5.2 HDA

Helicase dependent amplification (HDA) is an alternative isothermal technique developed by New England Biolabs in 2004.[46] This isothermal technique is very similar to the standard PCR but it does not require heat denaturation to separate the double stranded DNA and allow primers to anneal to its complementary target sequences. HDA uses DNA helicase to generate single stranded DNA for primer annealing followed by primer extension at isothermal conditions (Fig 1.2). Single stranded binding protein (SSB) and MutL endonuclease are added to the reaction to prevent the re-hybridization of complementary ssDNA to reform the dsDNA. Detectable amounts of PCR amplicons for downstream analysis are generally generated within 60 min by the HDA method.[46]

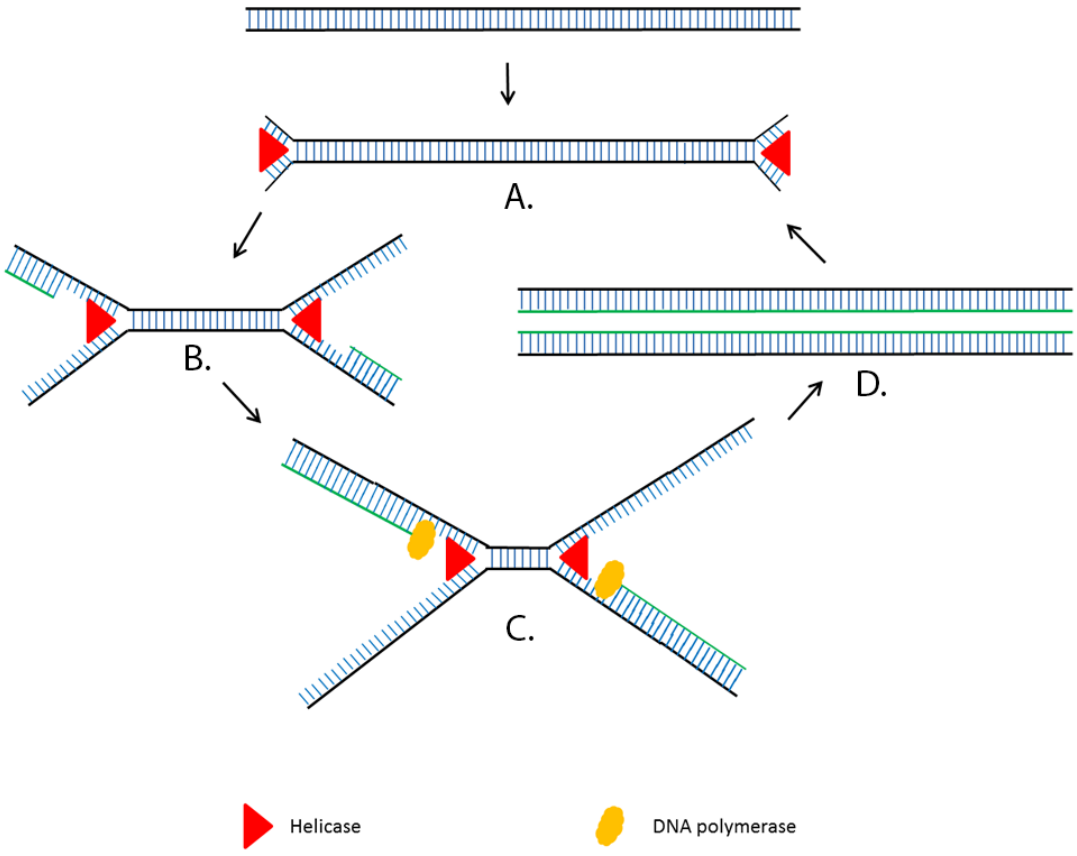


***Figure 1.2*:** Schematic outline of helicase dependent amplification (HDA). A: Helicase opens the dsDNA. B: The primers anneal to the target sequences. C: Primer extension by DNA polymerase. The newly formed dsDNAs are opened by helicase and the process starts again.

HDA has become a popular isothermal technique due to its simple reaction steps. Although it amplifies the target sequences using a pair of primers using the same principle as PCR, the kinetics involved are much simpler as it does not need multiple temperature regulated steps. Although LAMP has the same isothermal feature as HDA, it requires 4 intricately designed long primers which need an initial heat denaturation step before amplification at lower temperature.[65] HDA has been successfully used to detect pathogen genomic DNA present in a crude mixture of human blood sample with high sensitivity.[46] Although the characteristics of the HDA seem perfect for the development of simple point of care diagnostic assays, the main drawback is that it requires complex optimization to ensure a coordinated enzyme activity between the helicase and DNA polymerase.

Additionally, the presence of SSB and MutL, which are essential to prevent ssDNA from re-hybridizing to form dsDNA potentially affect the final results significantly.[46] Furthermore, there are some reports stating that HDA is inefficient when amplifying long targets,[74] probably due to the fact that the UvrD helicase has limited unwinding speed (20bp/s) and process less than 100bp per binding.[75] MutL is able to enhance the UvrD unwinding activity but not increase the processing rate.[76] A significant improvement has been made with the discovery of a thermostable UvrD helicase (Tte-UvrD) suitable for amplification at a higher temperature.[77] The benefit of using Tte-UvrD is to allow the HDA to be performed at a higher temperature, decreasing the re-annealing of single stranded DNA and therefore obviating the need for SSB and MutL.

Currently, HDA is commonly used in human clinical applications such as the diagnosis of *Salmonella paratyphi*[78] and diarrhea-causing pathogens,[79,80] as well as veterinary applications such as detection of *Streptococcus equi* causing strangles in horses.[81] However, its application to plant pathogen detection still limited and has only been used to identify citrus leprosis virus C & tobacco mosaic virus[82]. In order to improve the sensitivity, HDA has been combined with other technologies such as ELISA[83] and gold nanoparticles[84] to detect *Helicobacter pylori*. The results of both HDA-ELISA and HDA-nanoparticles showed a 90% increase in sensitivity and specificity compared to the original HDA assay.

1.5.3 RCA

The principle of isothermal amplification has been also used to amplify circular DNA in a process known as rolling circle amplification (RCA).[85] RCA involves using a DNA polymerase with strand displacement activity (such as ϕ29 DNA polymerase) to extend a single primer annealed to a circular DNA template. The strand displacement activity allows the newly synthesized DNA to displace the previously generated DNA releasing ssDNA.[86] This enzymatic process of primer extension combined with strand displacement generates a long single stranded DNA containing a repeated sequence complementary to the circular template (Fig 1.3).

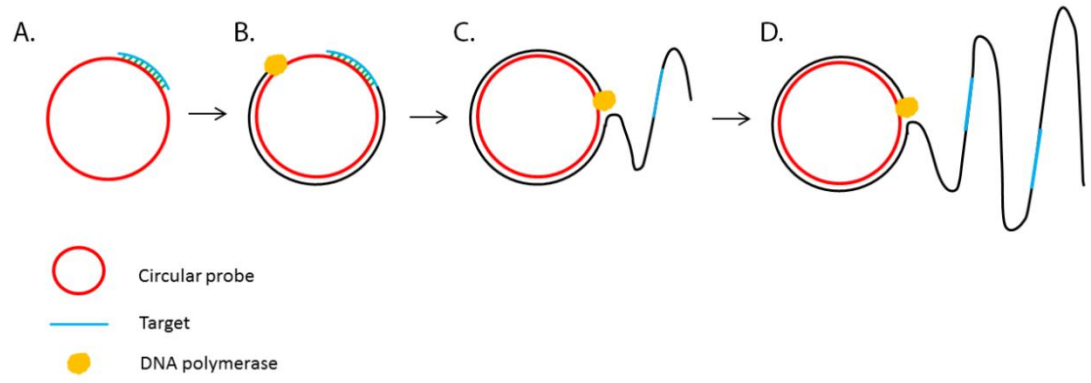


***Figure 1.3*:** Schematic outline of rolling circle amplification (RCA). A: A primer complementary to a region of a circular probe anneals to the circular template. B: DNA polymerase initiates the DNA synthesis. C: Strand displacement allows the continuation of DNA synthesis along the circular template. D: DNA synthesis continues to generate a long ssDNA product.

RCA offers the simplest available isothermal reaction mechanism. With additional manipulation, linear DNA is also suitable as a template for the RCA reaction. A linear ssDNA probe can be designed in such a way that it can be initially hybridized to the target sequence forming a loop and ligated to form a circular probe prior to performing RCA (Fig 1.4). This process termed as the padlock probe assay has been used in the detection of many plant diseases.[87-89] High multiplexity and specificity of padlock probes followed by RCA also increases the popularity of its application in multiplex detection of plant pathogens. In addition, RCA has been reported to have higher specificity and be less prone to nonspecific amplification than PCR. Another advantage of the RCA method is that it allows to amplify the circular probe sequence at up to 0.5 megabases[90] and generates 10^9 or more copies of each circle in 90 min.[91] Generating multiple copies of repetitive sequences offer an advantage in hybridization based readouts where the repetitive sequences can be easily captured to increase the sensitivity.[92-94] Furthermore, RCA is resistant, or at least less prone

to carry-over contamination of the amplification products because there is no new 3´-end ssDNA product generated throughout the RCA process, which could be a potential primer for non-specific amplification.[95]

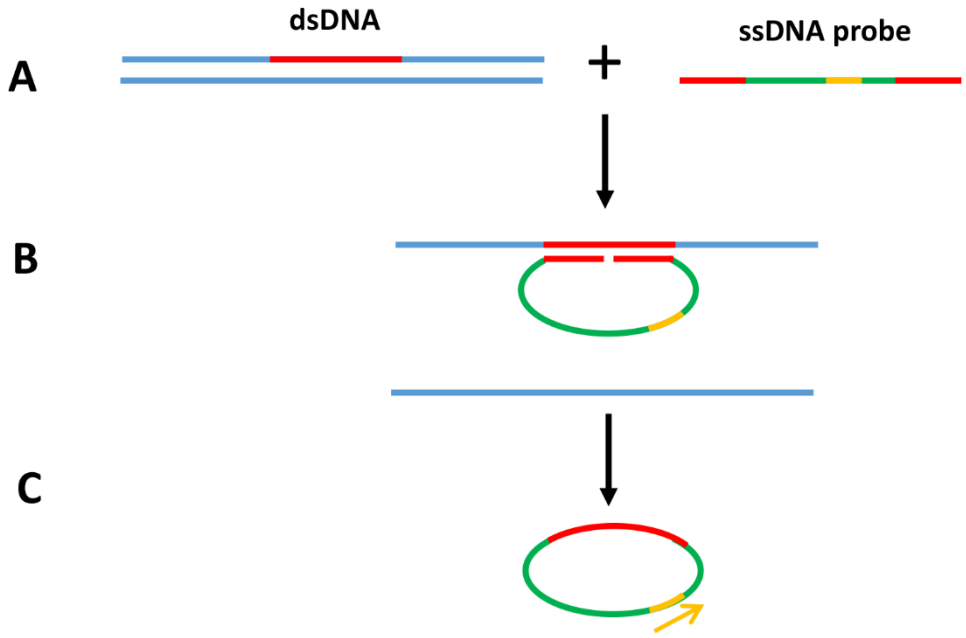


***Figure 1.4*:** Padlock probe assay with RCA. A: Linear ssDNA probes contain two binding sites at both 3´ and 5´ ends to target the specific sequences. B: Denaturation of dsDNA target sequence and hybridization of ssDNA probe towards the target region forming a loop. C: After the ligation to form a circular probe, RCA primer binds to the primer target region and starts the RCA.

RCA has been widely used for plant pathogen detection since early 2000s. Several techniques have been used in combination with RCA such as restriction fragment length polymorphism (RFLP) and direct sequencing to identify and classify plant pathogen efficiently with significantly lower effort and cost than conventional technologies.[96] Visualization of RCA products using naked eye by adding fluorescent dye has been used to detect more than 40 strains of *Fusarium* spp.[97] Ligation of padlock probes followed RCA has also been developed for identification of fungal pathogens.[98] Incorporating RCA with a variety of readout technologies such as microarrays, DNA biosensors and immune assays has significantly improved the sensitivity when compared to the commonly used gel electrophoresis.[92-94,99,100] Although these read out methods seem as an ideal alternative for RCA based assays, but they are expensive and involve complicated steps compared to simple monitoring a color change using naked eye.

1.5.4 RPA

Recombinase polymerase amplification (RPA) is another isothermal technique that, like HDA, does not require an initial heating step to denature the target DNA[45] as it relies on an enzymatic activity to separate the dsDNA in order to assist primer binding to the target sequences. The reaction begins with the integration of a recombinase protein with the primers prior to their annealing to specific sequences in the target. Following the primer annealing, the recombinase dissociates from the primers and leaves their 3' end accessible to the DNA polymerase to initiate the amplification. This creates a d-loop which will be stabilized by a single stranded binding protein (SSB) to keep the DNA open as the DNA polymerase with strand displacement activity continues the amplification (Fig 1.5). Using RPA, billions of DNA copies can be generated efficiently in 60 min with an incubation temperature between 37 °C to 42 °C.[45]

The low incubation temperature and short reaction time (15 – 30 min) make RPA an ideal assay for use in point-of-care diagnostic applications. Furthermore, primer design is simple without consideration of annealing temperature as primers form a complex with the recombinase to target the homologous sequences. RPA is a highly sensitive tool in amplifying target sequences where the detection limit is as low as 6.25 fg of genomic DNA input with specificity >95 %.[101]

However, as with all the other discussed technologies, RPA has some drawbacks and RPA can only amplify small DNA fragments (<500 bp) and is therefore not suitable in cases where amplification of full length genes is required. In addition, the longer primers (30 – 35 nt) required for RPA are prone to generating non-specific amplification at low temperature. Furthermore, the primers used in the RPA reaction frequently generate high background noise on negative and non-template control samples due to the formation of primer dimers thus affecting the sensitivity of the assay.

Many reports describing clinical applications of the RPA have been recently published.[101-103] In plants, a number of RPA-based applications have been described to detect plant pathogens with high sensitivity, specificity and cost effectiveness. A combination of reverse transcriptase RPA (RT-RPA) and lateral flow has successfully detected little cherry virus from crude extracts samples being more cost effective than RT-PCR and more suitable for resource limited settings.[104] RT-RPA has also been used to detect plum pox virus and proven to have a higher sensitivity than conventional ELISA and immunostrip.[105] RPA-ELISA has been developed for the sensitive, specific and cost effective identification of plant pathogens.[106] A naked eye assay which incorporates RPA with bridging flocculation of magnetic beads has been recently developed for

efficient POC detection of plant and human pathogens.[55] Given that RPA is a fairly new isothermal technique, there is still more exploration to be done for point-of-care detection of plant pathogens.

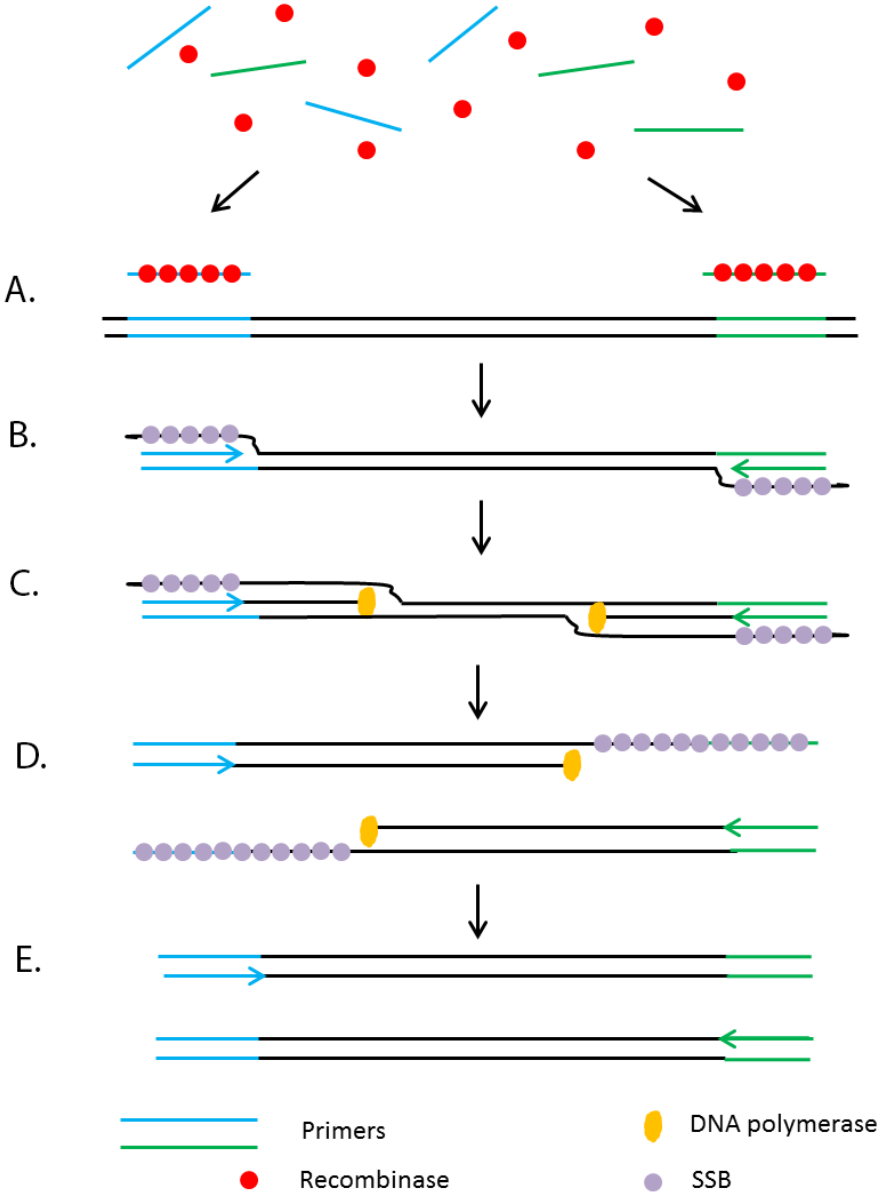


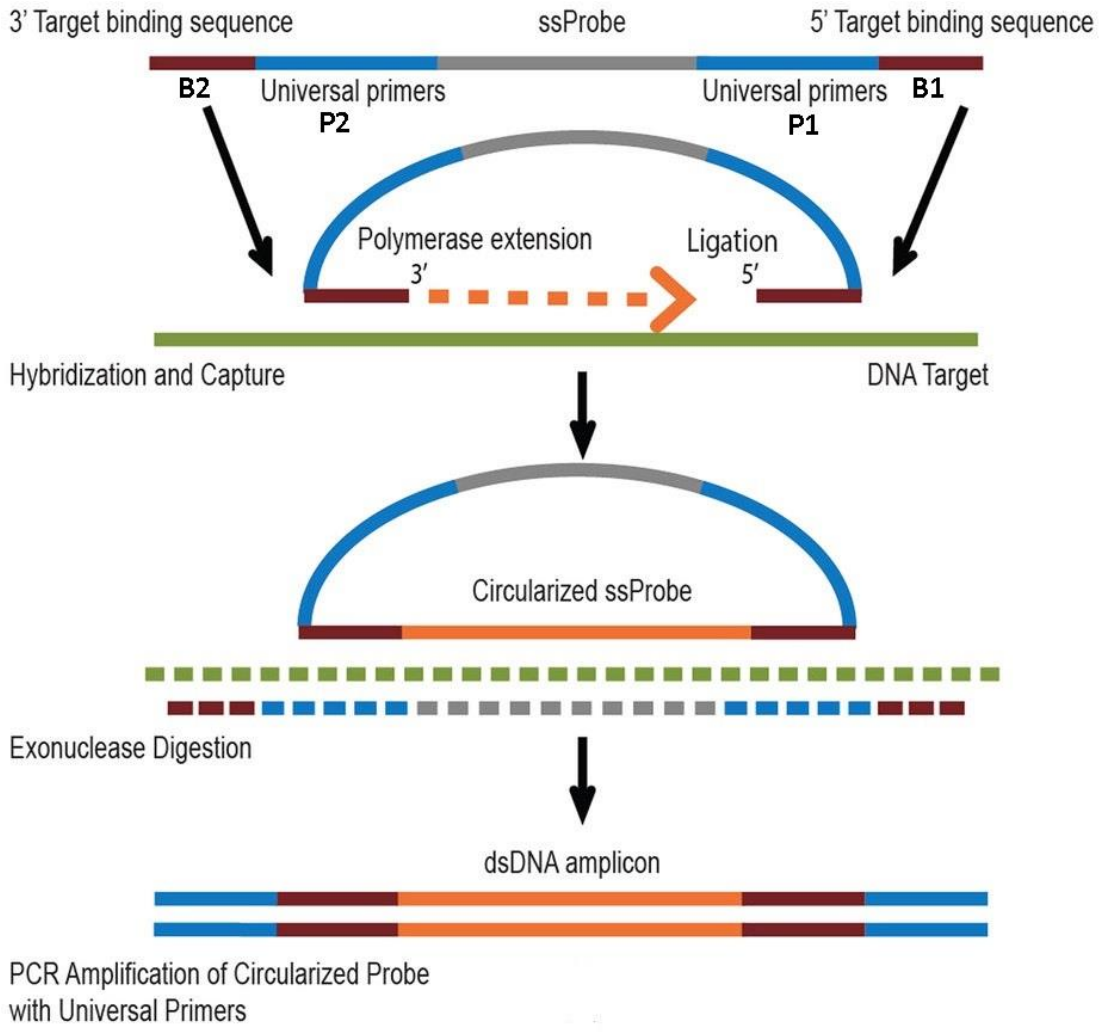
***Figure 1.5*:** Schematic outline of the recombinase polymerase amplification (RPA). A: Recombinase integrates with primers to form recombinase-primer complexes and target specific DNA sequences. B: Strand exchange occurs and single stranded binding proteins (SSB) bind to the DNA to form a D-loop. C: DNA polymerase initiates DNA amplification. D: Displaced D-loop stabilized by SSB as amplification continues. E: Two dsDNA molecules form and the entire cycle starts again.

1.6 Multiplex detection of plant pathogens

Development of technologies with multiplex detection capability is another challenge in plant disease diagnostics as they are more cost effective, reduce assay time and require minimal amount of sample. High throughput multiplex detection has been successfully achieved using real-time PCR on an OpenArray™ platform.[107] microsphere immunoassay technology[108] and microarrays[87]. However, simpler but efficient multiplex detection methods capable to identify multiple pathogens simultaneously are still needed.

Molecular inversion probes (MIPs) enable cost effective multiplex diagnostics and has been often used in clinical applications. A MIP is a long single stranded probe in excess of >100 nt long with two binding domains at the 5' and 3' ends (B1 and B2 in Fig 1.6). The binding domains are designed to be complementary to the target region of interest (Fig 1.6). This enables the MIP to form a circular loop with a single stranded gap in between the two binding regions. After annealing of the MIP, a DNA polymerase without 5'-3' exonuclease and strand displacement activities initiates DNA synthesis from the 3' end of the gap in a gap-fill reaction. DNA ligase is then used to ligate the newly synthesized strand and generate a circular DNA molecule. To ensure the sensitivity of the assay, an exonuclease digestion step is performed to remove the linear probes. The ligated MIPs are then amplified in PCR reaction using a universal primer set targeted P1 and P2.

The highly multiplexable nature of MIP probes, which are able to distinguish thousands of targets in a single reaction is a major advantage of this assay. The binding domains at the 3' and 5' ends are connected by a DNA backbone; this design feature physically restricts the binding domains to a small region of the genome that needs to contain both recognition sequences, dramatically increasing the specificity of the MIP assays. In addition, the noise signal of the assay is decreased by enzymatically degrading the unligated linear MIPs by the exonuclease digestion with up to 99% efficiency. MIPs have been designed to detect up to 330 000 targets in a single reaction.[109] The MIP multiplexing capability has been demonstrated in various clinical studies such as high-throughput analysis of single nucleotide polymorphisms, DNA methylation, detection of genomic copy number changes and other genotyping applications.[109-111] However, applications for plant diagnostics are still limited with the first report describing the successful detection of *Fusarium oxysporum* f.sp. *conglutinans* in infected *Arabidopsis thaliana* with a detection limit of 2.5 ng.[112] In order to improve the efficiency of MIP assays, the MIPgen software has been developed for the design and performance prediction of individual MIPs.[113]



Source: Stefen et al. 2016[114]

***Figure 1.6*:** Schematic outline of MIP assay. MIP consists of two binding sites at 3' and 5' ends (B1 and B2) which are complementary to target sequences, and two universal primer sites (P1 and P2). B1 and B2 hybridize to specific sequences on the target with single stranded gap between two binding regions. B1 and B2 bind to specific sequences on the target DNA creating single stranded gap between the binding domains of the MIP. A DNA polymerase that lacks exonuclease and strand displacement activities synthesizes DNA from 3' end of the MIP to 5' end until the single stranded gap is filled. A DNA ligase ligates the 3' end the 5' end of the MIP creating a circular DNA. Exonucleases I and III digest the linear MIPs and the DNA target in the reaction mixture leaving the circularized MIPs for amplification. A pair of universal primers (P1 and P2) amplifies the circularised MIP using the universal primer binding domains to generate PCR amplicons.

Serious limitations of these assays are the requirement for several temperature settings throughout the assay and a tedious yet complicated experimental process that makes it unsuitable for POC testing. Besides, the assay is time consuming taking in excess of 10 hrs to complete a reaction, making MIP assays only suitable for applications in the laboratory. MIPs with lengths of >100 bp require HPLC purification which increases the cost of oligonucleotide synthesis. Achieving a high level of multiplex detection involving thousands of MIPs in a single reaction requires a large initial investment. In addition, multiplex detection using MIP assays needs a complex read-out platform such as next generation sequencing, microarrays or biosensors,[115-117] further increasing the cost of the assay.

1.7 Conclusions

In this chapter, some of the commonly used nucleic acid-based methods for plant pathogen detection were discussed. Although the vast majority of applications in PCR-based, numerous research reports have established that the existing isothermal techniques have been proven to perform as well as or even better than PCR-based assays. Each of the isothermal and non-isothermal technologies discussed above has inherent advantages and limitations. However, the final decision on the best technology to adopt will depend on the specific problem; i.e. the nature of the pathogen, the crop being monitored and the technological capability of the country. The ability to perform reactions at a constant temperature makes the isothermal techniques a favorable candidate for a point-of-care diagnostic assay suitable for a low resource setting. The main advantage of using POC diagnostic testing for crop diseases is to provide rapid results *in situ* and enable farmers to make immediate management decisions to minimize crop loses. An additional advantage of POC diagnostic testing is the reduction in logistic problem associated with sample transportation to centralized laboratories for disease analysis and the concomitant labor costs. In addition, POC diagnostic kits should be portable and user friendly allowing a single operator with no scientific skills to carry out the test.

Among the available isothermal techniques, LAMP and RCA are the best candidates for hybridization based applications because the repeated sequences present in the amplification products are able to increase the sensitivity of detection.[73,92,94] However, both techniques produce isothermal amplification products in various sizes and are therefore not suitable for applications where a specific DNA fragment size is needed for identification. The HDA and RPA isothermal techniques do not require a heat denaturation step and as a result can be performed directly at a constant temperature which is a big advantage for on field applications. Nevertheless, HDA and

RPA are limited to targets shorter than 100bp and 500bp, respectively. Furthermore, despite the sensitivity and specificity of these isothermal techniques, they are not highly multiplexable limiting their application to the detection of a single, or at best a limited number of pathogens in a single assay.

Micro-Total Analysis System (μTAS) has been developed to simplify POC rapid detection of nucleic acids. Initial efforts had been focused in the use of PCR but the advent of isothermal amplification has changed the focus and simplified the process aiming to miniaturize the amplification reaction and couple it with sample preparation and detection of results in a single miniaturized portable device.[118] These systems require only a small volume of reagents, enzymes and primers making the detection process more cost effective. Furthermore, pre-stored reagents in chips along with fewer manual steps significantly reduce the sources of contamination from human error. However, μTAS devices using isothermal technologies for plant pathogen detection are still in an early developmental stage.

In conclusion, traditional laboratory methods involve time consuming culture steps for pathogen identification by experienced plant pathologists.[3] The advent of antibody-based methods offered multiple advantages over traditional techniques but several studies have reported high error rates due to cross reactivity.[119] The limitations of antibody-based methods are further conpounded by their short shelf life and variable performance between batches.[120] Therefore, DNA-based diagnostic methods, with potentially better specificity, have therefore been proposed to overcome the limitations of antibody-based diagnostics.[17,18,121,122] Polymerase chain reaction (PCR) is the most widely used nucleic acid technique for identifying plant pathogens.[39,40] However, it requires temperature cycling which limits its application in the field. Hence to address the limitations of PCR, isothermal amplification systems have since been developed. In this study, several diagnostic methods with better sensitivity, specificity as well as capability in point-of-care applications were developed by incorporating isothermal amplification systems with other technologies as rapid readout methods to analyse the DNA amplification products.

1.8 Aim and objectives of this study

The aim of this study was to develop new diagnostic methods for multiplex and on-site detection of plant pathogens.

Specific objectives were:

1. To investigate the use of Solid Phase Reversible Immobilization (SPRI) beads in plant pathogen genomic DNA extraction. (Chapter 2 and 3)

2. To establish a RPA assay for on-site detection of plant pathogens. (Chapter 2 and 3)

3. To develop a sensitive and specific POC detection method using electrochemical sensor. (Chapter 2)

4. To develop a novel quantitative read-out method for isothermal products using bridging flocculation of SPRI beads. (Chapter 3)

5. To develop a MIP assay and validate the assay parameters using plant pathogens. (Chapter 4)

6. To develop a multiplexable POC detection method for the detection of plant pathogens using surface enhanced Raman scattering (SERS) technology. (Chapter 5)

Chapter 2

Specific and Sensitive Recombinase Polymerase Amplification Electrochemical Biosensor for Plant Pathogen DNA Detection with Colloidal Gold Nanoparticles as Probes

Full article: Lau, H.Y., Wu, H., Wee, E.J.H., Wang, Y., Botella, J.R. and Trau, M. Specific and Sensitive Recombinase Polymerase Amplification Electrochemical Biosensor for Plant Pathogen DNA Detection with Colloidal Gold Nanoparticles as Probes. *Analytical and Bioanalytical Chemistry*. Submitted (2016)

2.1 Introduction

Plant diseases are one of the major causes of economic losses for the agricultural industry with losses estimated to exceed USD150 billion annually worldwide.[1] Many disease management strategies have been implemented to control crop losses globally and, in the absence of resistant varieties, early disease detection is paramount to avoid losses and reduce the spread of the disease to neighboring farms. Therefore, rapid, sensitive and specific diagnostic methods for plant pathogen detection are crucial in facilitating effective disease management practices.

Traditionally, experienced plant pathologists diagnose diseases by observing typical disease symptoms and identify the pathogen by culturing in specialized media.[3] This method is mostly accurate but it is time consuming and therefore not suitable for rapid disease management practices. Immunoassays have hence been widely used for plant pathogen detection since 1980s to address the limitations of symptomatic diagnosis. However, antibody based methods are prone to have cross reactivity with closely related pathogen species having similar epitopes for antibody recognition.[119] In addition, antibodies tend to have short shelf life and are prone to variations between production batches.[120] DNA-based molecular diagnostic methods have been proposed to increase the reliability, sensitivity and specificity of plant pathogen detection. The polymerase chain reaction (PCR) is currently the most popular and reliable molecular technique used in plant pathogen diagnostic assays.[17,18] As an alternative to PCR, recombinase polymerase amplification (RPA) using DNA repair enzymes to replace high temperature heating in DNA denaturation[45] achieves rapid DNA amplification at low constant temperature (37°C - 42°C) and PCR-like sensitivity, making it uniquely suited for point-of-care (POC) applications. While it is evident that RPA has high potential in POC diagnostics,[57,123-125] there is still a need to develop simpler rapid DNA-based diagnostic assay for plant pathogen detection in the field comprising all steps, from DNA isolation to visualization of the amplification results.

All molecular-based electrochemical sensors are claimed to be highly specific to recognize their target analytes. Electrochemical sensors provide a suitable platform to detect the electronic signal which triggered by the binding event between the probe and target.[126] A molecular recognition layer and a signal transducer that coupled with a readout device are the basic elements of an electrochemical sensor. DNA based electrochemical sensors are commonly used because of the high specificity from the base-pairing interactions between complementary sequences. Single-stranded DNA probe is immobilized on the surface of recognition layer to provide the condition of

base-pairing interactions with the target DNA. These interactions generate an electronic signal and the signal is further amplified to a readable signal.

Thus, electrochemical DNA biosensors have been widely used in disease diagnosis as they potentially offer high sensitivity, rapid analysis and portability at potentially lower cost than traditional technologies.[127] For this reason, the use of screen printed electrodes has gained popularity in POC development platforms. Several DNA biosensors have been reported in previous studies which involve the labeling of PCR products with enzymes,[128] redox active components [129] or nanoparticles[130,131] to enhance the electrochemical signal. One of the nanoparticle-base strategies, gold nanoparticles (AuNPs) are the most widely used and have gained significant attention recently as an electrochemical label. This is because they provide a large surface area for chemical reactions and have outstanding surface immobilization advantages which can enhance the efficiency of the electrochemistry detection assay.[132-134] Therefore, AuNPs-based DNA biosensors can provide a promising platform for the development of rapid, sensitive, specific and portable diagnostic tools for detection of DNA targets.[135-137]

Herein, a rapid and highly sensitive diagnostic method coupling RPA with AuNPs as electrochemical probes to detect the presence of plant pathogen DNA by differential pulse voltammetry (DPV) on disposable screen printed carbon electrodes (SPCE) was described. Simultaneously, the performance of the assay was compared with conventional PCR and agarose gel electrophoresis. *Pseudomonas syringae* was used as a model system in this study because it infects wide variety of crops and is an economically important plant pathogen.[138] This assay is believed to have wide applications to detect other DNA targets in agriculture and human diagnostics.

2.2 Experimental section

2.2.1 Reagents and materials

Plant and pathogen materials

Arabidopsis thaliana ecotype Columbia (Col-0) was obtained from the Arabidopsis Biological Resource Center (ABRC; Ohio State University). *Pseudomonas syringae* DC3000 (Psy) was obtained from the Department of Agriculture, Fisheries and Forestry, Queensland, Australia.

Materials and Chemical reagents

KAPA2G Robust HotStart PCR kit was purchased from KAPA Biosystems (Cat#KK5516). Streptavidin coated magnetic beads were obtained from New England Biolabs (Cat#S1420S). Oligonucleotides were synthesized by Integrated DNA Technologies. TCEP (tris(2-carboxyethyl)phosphine), PBS (Phosphate-buffered saline) and Tween 20 (Polysorbate 20) were from Sigma-Aldrich. Screen-printed carbon electrodes (SPCE, DS 110) were purchased from Dropsens.

Instrumentations

Bio-Rad thermo cycler (MJ Mini Personal Thermal Cycler) and 254 nm transilluminator (Vision-capt version 14.2) were used for PCR amplification and PCR products visualization. DPV responses were measured on a workstation potentiostat (CH Instruments).

2.2.2 Methods

DNA sample preparation

The *Pseudomonas syringae* infected Arabidopsis plants were prepared according to literature.[139,140] *P. syringae* pv *tomato* strain DC3000 was cultured on King's B plate at room temperature for overnight. The culture plate was washed with 10 mM $MgCl_2$ to resuspend the bacteria. Six weeks old *Arabidopsis thaliana* seedlings were sprayed with 2 to 5 X 10^8 cfu/mL of *P. syringae* suspension in 10 mM $MgCl_2$ and 0.03 % Silwet L-77. The infected leaves with symptoms were collected after 7 days inoculation. The infected leaves were rinsed with 80 % ethanol to remove the existing pathogen on the surface before DNA extraction. The total genomic DNA were extracted from infected (D) and healthy (H) leaves using solid-phase reversible immobilization (SPRI) beads as published in Wee et al. 2015[55] and briefly described below.

Plant genomic DNA was extracted from a single Arabidopsis leaf (~300 mg) using an optimized lysis buffer (50 mM Tris-HCl pH 8.0, 1.5 M guanidium-HCl, 2% w/v PVP40 and 1% v/v Triton-X).

RNase A (400 ng) was added to the lysis buffer. Plant tissue was macerated in a 1.5 mL tube with a disposable plastic pestle in the presence of 200 µL of lysis buffer. After 10 min incubation at room temperature, the lysate was cleared using a homemade filtration device made from a common filtered pipette tip. Using this approach further enhanced the potential for low resource applications. Nucleic acids were then purified using a modified SPRI protocol.[56,141] Briefly, a single drop (~10 µL) of the cleared lysate was incubated with 1.8 volumes of 1 micron carboxylic acid coated magnetic beads (Thermo Fisher, Cat# 4515-2105-050250) in a binding buffer (10 mM Tris-HCl pH 8.0, 20% PEG8000, 2.5 M NaCl) for 5 min. DNA bound beads were then separated from the lysate with a magnet and washed twice with 100 % isopropanol, two 80 % ethanol washes and eluted in one drop of water (~10 µL). All chemicals were purchased from Sigma Aldrich unless stated otherwise.

Preparation of AuNPs-DNA probe

AuNPs with the diameter of 16 nm were synthesized by the citrate reduction of $HAuCl_4$ according to the summarized below.[142] A volume of 100 mL 1 mM chloroauric acid ($HAuCl_4$) was heated to boil and then added with 10 mL of 1 % 38.8 mM sodium citrate ($Na_3C_6H_5O_7$). The mixture was heated for another 10 min and stirred for 30 min. DNA probe was designed as the capture oligonucleotides to target the specific sequence as described in table 2.1. The DNA probe was modified with thiol-group (-SH) for the purpose of immobilization on the surface of gold nanoparticles. To prepare the AuNPs-DNA probe, 100 µL of TCEP and DNA probe mixture was prepared by adding 50 µL of 10 mM TCEP into 50 µL of 100 µM DNA probe and then incubated at room temperature for 2 hrs. The mixture was then mixed with 1 mL of freshly prepared AuNPs solution and continuously shaken overnight at room temperature. After that, the mixture was centrifuged at 17500 x g and 4 °C for 25 min, and then the supernatant was removed. The AuNPs were resuspended in 200 µL of 0.1 M PBS.

Polymerase chain reaction (PCR)

Amplification of DNA targets were performed in a 25 µL reaction containing 1ng of genomic DNA, 1 unit of KAPA Hotstart DNA polymerase, 1X KAPA Taq hotstart buffer (contains of the buffer was not disclosed by manufacturer), 25 µM $MgCl_2$, 0.2 mM each dNTP and 0.25 µM for each forward and reverse primers (Table 2.1). The forward primer containing a 10 nt barcode at 5' end which complementary to the DNA probe and the reverse primer was labeled with biotin. PCR reaction was carried out in a Bio-Rad thermo cycler using the following conditions: denaturation at 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s and final elongation at 72 °C for 1 min. The products were validated by agarose gel electrophoresis using 1.5%

agarose gels containing GelRedTM 1X staining solution in sodium borate buffer. Then the gel was visualized under a 254 nm transilluminator.

***Table 2.1*:** Sequences of oligonucleotides involved in this study

Target / GenBank Accession	*Pseudomonas syringae* pv. tomato str. DC3000 AE016853.1
5'-Forward-3'	TACACAGCAC(C3)TTTGTCCGAAACGACGTACAGCCATTTAACCTT
5'-Reverse-3'	Biotin-TTC TAC GTC GGG GTA TTT ACT AGC TGG AAA AG
Capture probe	GTGCTGTGTATTTTT-SH

Recombinase polymerase amplification (RPA)

The TwistAmp Basic RPA Kit (TwistDX) was used as recommended by the manufacturer with some modifications. The RPA reaction was performed in the total volume of 12.5 µL at 37 °C for 20 min using 1 µL of the nucleic acid extracted and 480 nM of primer set (Table 2.1). Finally 2.5 µL of the RPA reaction was verified by agarose gel electrophoresis. 10 µL of RPA product was used in the electrochemistry detection.

Electrochemical bioassay

Electrochemical bioassay for plant pathogens detection involved first incubation of the amplified target, AuNPs-DNA probes and the streptavidin beads. Briefly, 5 µL of AuNPs/DNA probe was mixed with 10 µL of PCR/RPA product and topped up to 20 µL in final volume using distilled water. The mixture was then incubated at 37 °C for 20 min. Next, 5 µL of streptavidin coated magnetic beads were added into the solution and was further incubated at room temperature for 10 min. Beads were then captured by a magnetic plate, and the supernatant was removed. The beads were washed 3x with 50 µl of PBST buffer (1 mM PBS, 0.01% Tween20), and resuspended in 10 µL of 10 mM PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4), followed by 95 °C heating for 10 min. The solution was then transferred to the surface of working electrode on SPCE and air dried for electrochemical read-out.

Electrochemistry detection

The AuNPs dried on the working electrode was electrochemically activated by adding 100 µL of 0.1 M HCl and pre-oxidized at a potential of 1.3 V for 30 s to oxidize Au^0 to Au^{3+}. Then the

reduction of this species was analyzed with differential pulse voltammetry (DPV) response. The electrochemistry response was taken from 0.45 V to ending potential of 0.20 V with the incremental potential of 0.004 V, amplitude potential of 0.05 V, and pulse period of 0.2 sec.

2.3 Results and Discussion

2.3.1 Assay design

A flow diagram showing the main steps in the assay for plant pathogen DNA detection is illustrated in Fig 2.1. Total genomic DNA was extracted from plant samples using the solid phase reversible immobilization (SPRI) method.[143] RPA was then used to amplify the target sequence using a biotin labeled reverse primer and a forward primer containing a 10 nt barcode covalently linked to the 5' end via a carbon spacer. After amplification, the resulting amplicons, containing a 10 nt overhang (barcode sequence) on one end and biotin on the other, were hybridized to AuNPs-DNA tags which carried probes complementary to the barcode sequence. Streptavidin magnetic beads were then used to enrich for AuNPs/DNA/biotin products. In order to prevent iron ion contamination from interfering the DPV signal, the magnetic beads were heat treated at 95 °C to denature the dsDNA amplicons and to release any bound AuNPs into solution where the electrochemical reduction of Au (III) to Au (0) was measured. The amount of released AuNPs is proportional to the amount of amplified target DNA which, in turn, denotes a successful RPA amplification and thus indicating the presence of the pathogen.

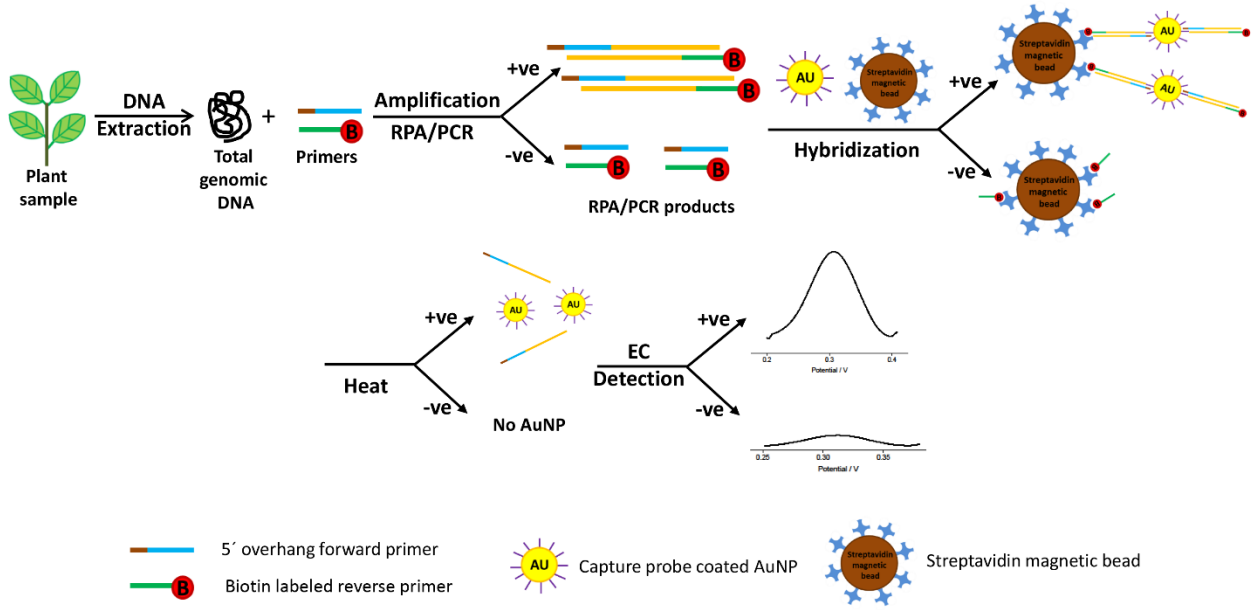


***Figure 2.1*:** Schematic illustration of the electrochemical bioassay for plant pathogen DNA detection.

2.3.2 Specificity study

Specificity of a diagnostic method is essential in identifying a particular pathogen from other species to avoid false positive results. To this end, a *P. syringae*-specific RPA/electrochemistry assay was challenged with *P. syringae* and two unrelated pathogens: *Botrytis cinerea* and *Fusarium oxysporum* f.sp. *conglutinans* (Fig 2.2). As expected, *P. syringae* samples produced a strong DPV signal while no signal was detected from *B. cinerea* and *F. oxysporum*. Electrochemical results were verified using agarose gel electrophoresis where the expected 144 bp band was seen only in the *P. syringae* sample but not the controls. Similarly, the PCR-based assay could also accurately identify the *P. syringae* sample albeit producing a lower DPV signal (Fig 2.3). As only the pathogen-specific DPV peaks were observed, the assay was indeed specific and thus a viable system for plant pathogen detection.

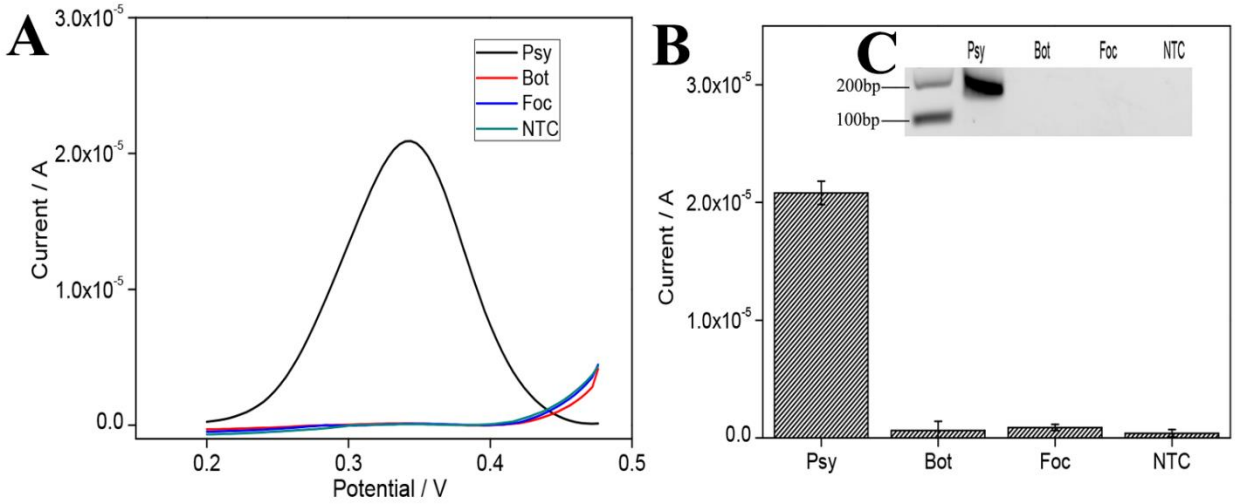


***Figure 2.2*:** Specificity study for plant pathogen DNA detection via electrochemical assay after RPA amplification. (A) DPV curve and (B) Current-response to *P. syringae* (Psy), *B. cinerea* (Bot) and *F. oxysporum* f.sp. *conglutinans* (Foc) as well as a no template control (NTC). Error bars represent ±SD, n = 3. (C) Electrophoresis gel image of RPA products.

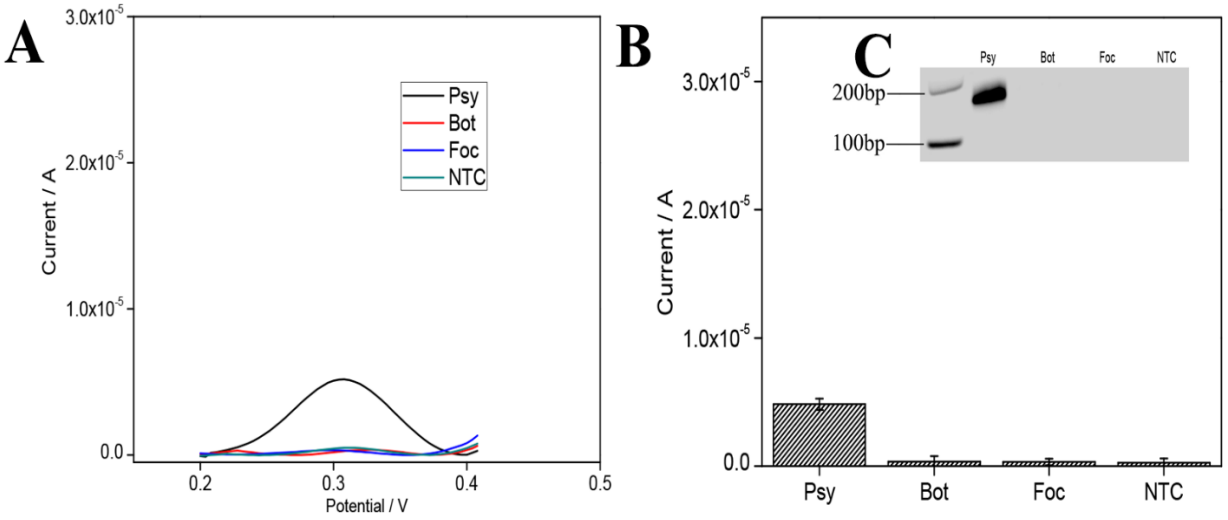


***Figure 2.3*:** Specificity study for plant pathogen DNA detection via electrochemical assay after PCR amplification. (A) DPV curve and (B) Current-response to *P. syringae* (Psy), *B. cinerea* (Bot) and *F. oxysporum* f.sp. *conglutinans* (Foc) as well as a no template control (NTC). Error bars represent ±SD, n = 3. (C) Electrophoresis gel image of PCR products.

2.3.3 Sensitivity studies

To prevent diseases from spreading to neighboring plants, sensitive diagnostic assays are advantageous for early infection detection even before the symptoms are visible. Hence, to identify a suitable amplification method, the sensitivity of RPA was compared with PCR (Fig 2.4) using same amount of *P.syringae* genomic DNA and primers. It was found that RPA (15 copies) was 100 times more sensitive than PCR (1500 copies) based on agarose gel electrophoresis, and thus RPA is a more sensitive yet convenient (by virtue of being an isothermal amplification) alternative compared to PCR.

Next, the detection limit of the electrochemical assay in detection amplified DNA was determined by titrating RPA products and measuring the corresponding DPV signal (Fig 2.5). The data indicated that the electrochemical assay (214 pM) was 100 times more sensitive than agarose gel electrophoresis (21,400 pM). Together, the data thus suggested that the combination of RPA with an electrochemical readout could potentially result in a rapid, sensitive and convenient DNA detection platform. In addition, the relative standard deviation (RSD) of the RPA/electrochemistry assay was 10.7 % which indicated good assay reproducibility.

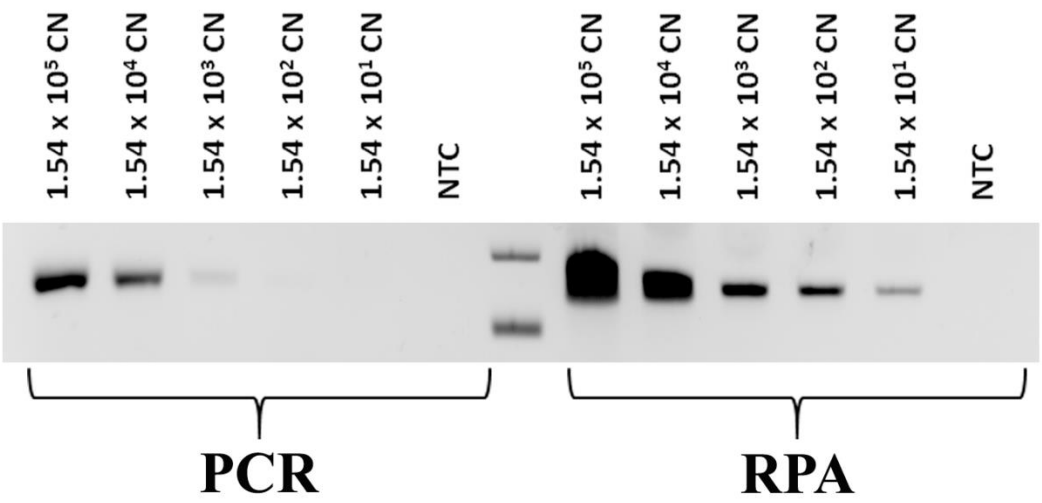


***Figure 2.4*:** Electrophoresis gel image for the sensitivity comparison between RPA and PCR over a range of gDNA inputs. Titration of *P.syringae* gDNA from 1.54 x 10^5 DNA copies number to 1.54 x 10^1 DNA copies number as well as a no template control (NTC).

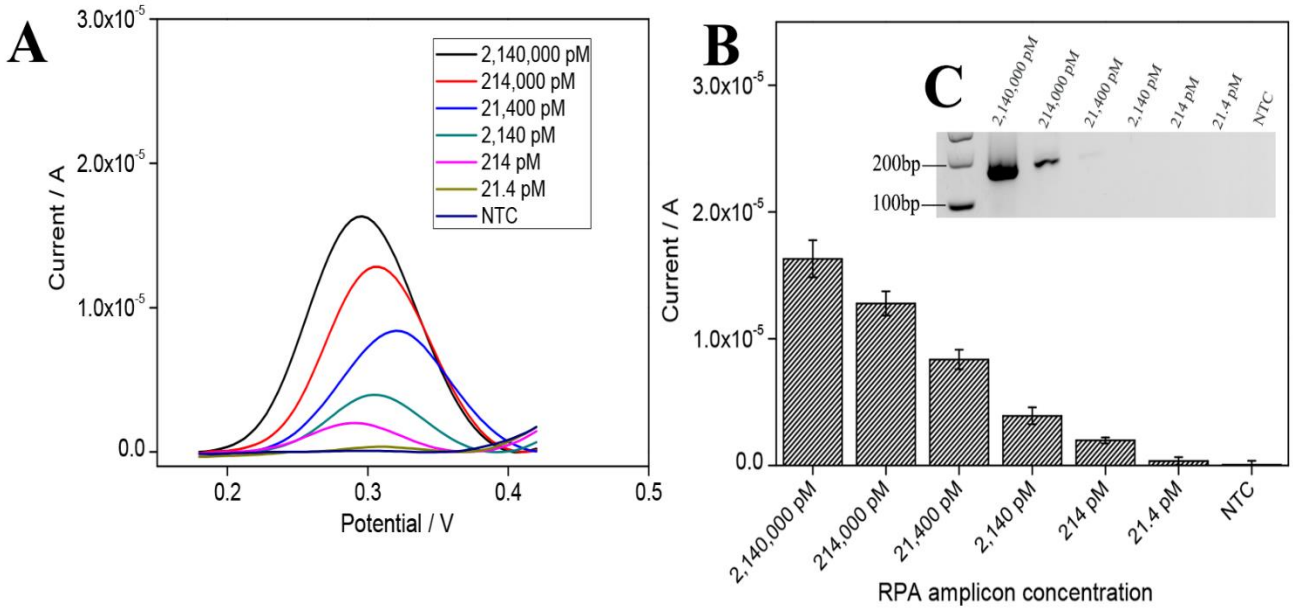


***Figure 2.5*:** Sensitivity study for plant pathogen DNA detection via RPA/electrochemical assay. (A) DPV curve and (B) Current-response to different amounts of amplification products, the error bars represent ±SD, n = 3. (C) Electrophoresis gel image of the RPA products.

2.3.4 Electrochemical detection of infected *A. thaliana*

Although the initial results with AuNPs-based DNA biosensor were very promising, we wondered if the approach could be applied to complex samples such as diseased plants. To this end, total genomic DNA was extracted from the leaves of a healthy plant and those infected with *P. syringae* at two different degrees of infection (Stage 1: before visible symptoms and stage 2: just symptoms become visible) (Fig 2.6). With this approach, *P. syringae* was successfully detected disease from as early as Stage 1; before symptoms were visible. The DPV signal obtained from Stage 2 sample was two times higher than Stage 1 sample and no signal was detected from the healthy sample. This demonstrated the assay had potential for early detection of pathogens in infected plant samples.

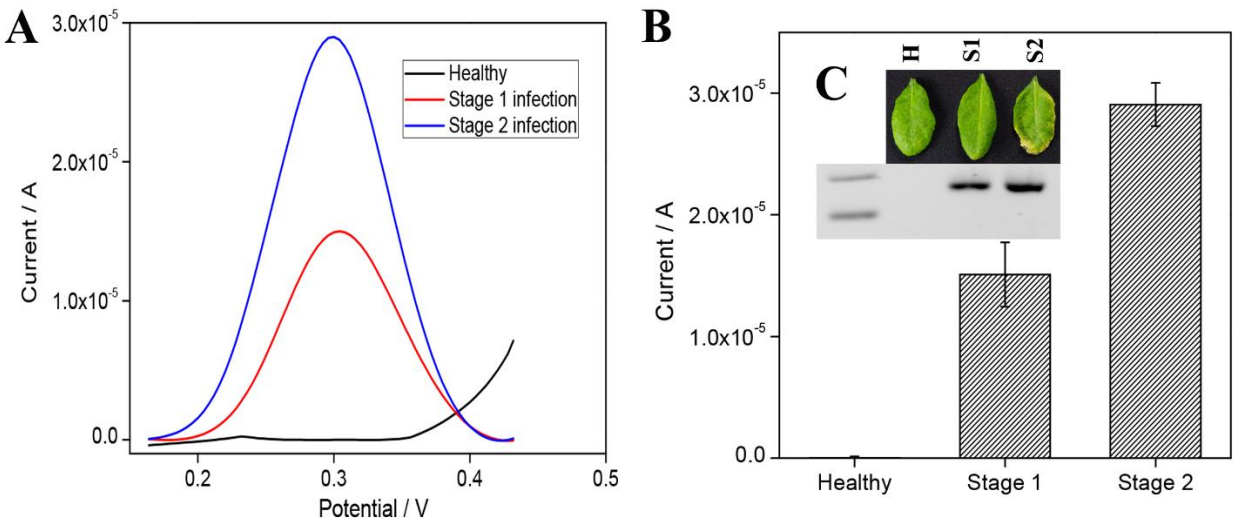


***Figure 2.6*:** RPA/electrochemistry detection on healthy and *P. syringae* infected *A. thaliana* plant tissue samples (Stage 1 and Stage 2). (A) DPV curve and (B) Current-response of healthy and infected samples. Error bars represent ±SD, n = 3. (C) Images of healthy and diseased *A. thaliana* leaves (Stage 1 and Stage 2) with the electrophoresis gel image of RPA products.

2.4 Conclusions

In conclusion, a highly sensitive method for plant pathogen detection by combining RPA with nanoparticle-electrochemistry was successfully developed. The results indicated that the electrochemical assay was suitable to detect pathogens with high efficiency, specificity and sensitivity. The biosensor was trialed on *P. syringae* infected *A. thaliana* successfully and could potentially detected infections even before disease symptoms appear. This method was 10,000 times more sensitive than conventional PCR/gel electrophoresis and could readily identify *P. syringae* infected plant samples even before the disease symptom was visible. We believe that this method of coupling RPA with an electrochemical readout performed on disposable screen printed electrodes could form the basis of a rapid, sensitive and cost effective POC platform for nucleic acid detecting applications.

Chapter 3

Re-purposing bridging flocculation for on-site, rapid, qualitative DNA detection in resource-poor settings

Full article: Wee, E. J. H., Lau, H. Y., Botella, J. R. & Trau, M. Re-purposing bridging flocculation for on-site, rapid, qualitative DNA detection in resource-poor settings. *Chem Commun* 51, 5828-5831, doi:10.1039/c4cc10068a (2015)
http://pubs.rsc.org/en/Content/ArticleLanding/2015/CC/C4CC10068A#!divAbstract
Patent: https://www.google.com/patents/WO2015095929A1?cl=en22

3.1 Introduction

Nucleic acid point-of-care (POC) bioassays that can be performed on-site with minimal equipment, rapidly and cost effective are in high demand.[41] Classical techniques and methods for detecting disease DNA/RNA biomarkers including the polymerase chain reaction (PCR) and ligase chain reaction (LCR).[42] However, these methods require a thermal cycler to achieve rapid DNA detection and hence are not suitable for field or on-site applications. Nonetheless, recent developments in isothermal DNA amplification methods may potentially overcome this limitation.[43,44] For instance, isothermal amplification methods combined with lateral flow strips and portable fluorometers have been developed to enable POC detection of pathogenic DNA.[45-51] Such readout methods whilst convenient are still dependent on the use of relatively sophisticated equipment and may still present financial and technical obstacles for worldwide adoption. A field-ready comprehensive assay incorporating the complete process from on-site sample preparation to results display is still elusive.

Agriculture is one area that can benefit from the use of low cost on-site assays. While being a major contributor the world economy, crop diseases are still a major concern in agriculturally reliant economies.[1] Traditionally, an experienced plant pathologist identifies the disease by a subjective visual examination of disease symptoms.[3] To address this, more analytical diagnostic methods have since been developed.[10,37,144-148] However, these detection methods require expensive and sophisticated equipment and can only be performed in specialized laboratories by well-trained technicians. As early detection is the ideal method for controlling disease outbreaks, the current lack of on-site detection protocols delays deployment of disease control strategies result in huge crop losses. Similarly, early detection of livestock pathogens is essential to avoid the spread of diseases, especially in modern farms using intensive production methods. Therefore the challenge is to purpose-build a cheap, sensitive and reproducible disease diagnostic approach detailing sampling, detection/amplification and evaluation that can be applied in the field without sophisticated laboratory equipment and highly trained personnel especially in resource-poor situations.

As the distinction between infected and healthy samples is binary, a readout method mirroring a digital yes/no result may be useful. Herein a method to economically visualize amplified disease-specific DNA/RNA without equipment by utilizing bridging flocculation was described. In this report, a protocol for pathogen genetic material detection from a single drop of extract (hence the acronym SDG), in under 90 min with minimal equipment by bridging flocculation for diagnostic display was explained. Various plant pathogens were detected accurately in *Arabidopsis thaliana* and commercial crops such as bananas. Next, to demonstrate its efficacy in detecting a wide range

of pathogens in species from several kingdoms such as plant and cattle viruses, *E.coli*-laced water samples, HIV infected cells, malaria infected blood, influenza virus infected cells and *mycobacterium tuberculosis* (MTB) bacteria. This approach has major advantages over any other existing protocols: (1) it can be potentially being performed on-site; (2) requires only minimal equipment; 3) it is rapid (under 90 min); (4) it is cheap (< USD 2); (5) no refrigeration needed (kits can be stored at room temperature); and (6) it does not need highly trained personnel.

3.2 Experimental section

3.2.1 Sampling inoculated plants

Pathogen-inoculated *Arabidopsis thaliana* were sampled at various times after infection and scored (S1 to S5) based on their severity of symptom development.[149] For *Pseudomonas syringae*, S1: 1 day, S2: 2-3 days, S3: 4-5 days, S4: 6-7 days, S5: 8-9 days. For *F. oxysporum* f. sp. *conglutinans*, S1: 3-6 days, S2: 7-10 days, S3: 11-15 days, S4: 16-20 days, S5: 21-25 days. For *Botrytis cinerea*, S1: 2-4 days, S2: 5-7 days, S3: 8-10 days, S4: 11-13 days, S5: 14-16 days.

3.2.2 Nucleic acid extraction

Plant genomic DNA was extracted from a single Arabidopsis leaf (~300 mg) using an optimized lysis buffer (50 mM Tris-HCl pH 8.0, 1.5 M guanidium-HCl, 2 % w/v PVP40 and 1 % v/v Triton-X) . For DNA applications, 400 ng/µL RNase A was added to the lysis buffer but not for RNA applications. Plant tissue was macerated in a 1.5 mL tube with a disposable plastic pestle in the presence of 200 µL of lysis buffer. After 10 min incubation at room temperature, the lysate was cleared using a homemade filtration device made from a common filtered pipette tip. Using this approach further enhanced the potential for low resource applications. For non-plant applications, 4 volumes of lysis buffer without PVP40 were used with every volume of sample. Nucleic acids were then purified using a modified SPRI protocol where the 0.5 M EDTA pH 8.0 washing buffer was replaced with 100 % isopropanol and 80 % ethanol.[56,141] Briefly, a single drop (~10 µL) of the cleared lysate was incubated with 1.8 volumes of 1 micron carboxylic acid coated magnetic beads (Thermo Fisher, Cat# 4515-2105-050250) in a binding buffer (10 mM Tris-HCl pH 8.0, 20% PEG8000, 2.5 M NaCl) for 5 min. DNA bound beads were then separated from the lysate with a magnet and washed twice with 100% isopropanol, two 80 % ethanol washes and eluted in one drop of water (~10 µL). All chemicals were purchased from Sigma Aldrich unless stated otherwise.

3.2.3 Nucleic acid amplification

The TwistAmp Basic RPA Kit (TwistDX, cat# TABAS01kit) was used as recommended by the manufacturer with some modifications. Briefly 12.5 µL reactions were performed at 37 °C for 30 min using 1 µL of the nucleic acid extraction and 480-600 nM of each primer (Table 3.1). For RNA applications, 50 units of MMuLV reverse transcriptase (New England Biolabs, cat# M0253S) were added to the RPA reaction. Following amplification, 5 µL of the RPA reaction was verified by agarose gel electrophoresis.

***Table 3.1*:** List of RPA primers used. GenBank Accession numbers are as given. All primers were purchased from Integrated DNA Technologies (IDT).

Target/ GenBank Accession	5'-Forward-3'	5'-Reverse-3'
F.oxysporum f.sp. *conglutinans* AGNF01000001.1	GCTCTTGATTTAGGTACAACTCTTTCCCTCGTC	ATATATCTGTATAGGAATCCCACTGAATTTTTC
Botrytis cinerea ALOC01000004.1	TTTCCACAGGGTTTGTGTACGAGATTGGTATTC	TTCTCCGGTGTCCGTTCGCACTGTAGACAATCG
Pseudomonas syringae AE016853.1	TTTGTCCGAAACGACGTACAGCCATTTAACCTT	TTCTACGTCGGGGTATTTACTAGCTGGAAAAG
F.oxysporum cubense AMGP01000029.1	ATTGAAGGACTCATACAAGGTTGCATCAAAATA	TTTCCTTTTGCAACTCCTACAGAGTGTCTATAA
Cucumber mosaic virus RNA3 Coat AJ585517.1	AGTTAATCCTTTGCCGAAATTTGATTCTAC	GTGCTCGATGTCAACATGAAGTACTAGCTC
Bovine HPV TK1 NC_001847.1	GGAAGATCTGCTCATGCTCGCGGCCGCCATGCC	GAGCGCGTAAGCATTGCGCACAGCGACCAGAAA
Bovine HPV Glycoprotein B NC_001847.1	AAGTGGCGCGAGGCGGACGAAATGCTGCGAGAC	ACGTGCGTGCCGTTGTAGCGCTCGCGGTAGACG
E.coli uidA gene NC_017635.1	CTGTGACGCACAGTTCATAGAGATC	AAAAGCAGTCTTACTTCCATGATTTCTTTAACT
HIV M19921.2	AAATTAACAATTACACAAGCTTAATACACTCC	TATAGAAAGTACAGCAAAAACTATTCTTAAACC
Plasmodium falsiparum MSP1 XM_001352134.1	TTGAAGGAAGTAAGAAAACAATTGATCAAAATA	CTAAAACGCTTATTAAATTATGTGCTTCTTCTA
Tuberculosis CFP10 CP003248.2	ATTTTGGCGAGGAAGGTAAAGAGAGAAAGTAGT	GAGTTCCTGCTTCTGCTTATTGGCTGCTTCTT
Tuberculosis ESAT-6 CP003248.2	CAATCCAGGGAAATGTCACGTCCATTCATTCC	CCTATGCGAACATCCCAGTGACGTTGCCTTC
Influenza A H1N1 CY058490.1	CCATTAATAAGACATGAGAACAGAATGGTTC	AAATTTTCAAGGAGATCATTTTTCAGACCAGTG

3.2.4 Bridging Flocculation Assay

5 µL of amplified product was used in the flocculation assay by incubating with 1.5 - 1.8 volumes of SPRI bead solution for 5 min. After bead separation with a magnet and an 80 % ethanol wash, 30 µL of flocculation buffer (100 mM sodium acetate, pH 4.4, 1 % v/v Tween20) was added to the beads and gently agitated.

3.3 Results and discussion

On field sampling is rarely, if ever, discussed when describing diagnostic methods. This non-trivial task of consistently generating a fixed input has repercussions on any assays' performance. A low cost DNA purification was developed using common filtered pipette tips in conjunction with SPRI technology[56] to consistently extract DNA to a precise concentration that can be used immediately for downstream RPA amplification (Fig 3.1A). Magnetic SPRI bead-based DNA extraction is ideal for POC applications because only a magnet is required. After manually macerating a single leaf (~300 mg or four 6 mm diameter discs) with a plastic mortar in 200 µL of lysis buffer, the plant lysate was cleared of cellular debris by passing the lysate through a filtered pipette tip. DNA was then purified using SPRI from a single drop (~10 µL) of lysate with consistent typical yields of 3-5 ng/µL of high molecular weight DNA in a 10 µL elution. As DNA concentration was at the detection limit for spectrometric evaluation (Nanodrop), DNA yield and integrity was estimated by ethidium bromide staining (Fig 3.1B) and validated using fluorescence (Qubit® assay). Although DNA evaluation was not possible using spectrometry, near baseline absorbance at 230 nm indicated minimal contaminants in the sample (Fig 3.1C). For RNA extractions, typical yields were 30-40 ng/µL in a 10 µL elution with 260/280 and 260/230 ratios of 1.9-2.2 and 1.8-2.3 respectively (Fig 3.1C), indicating good purity.

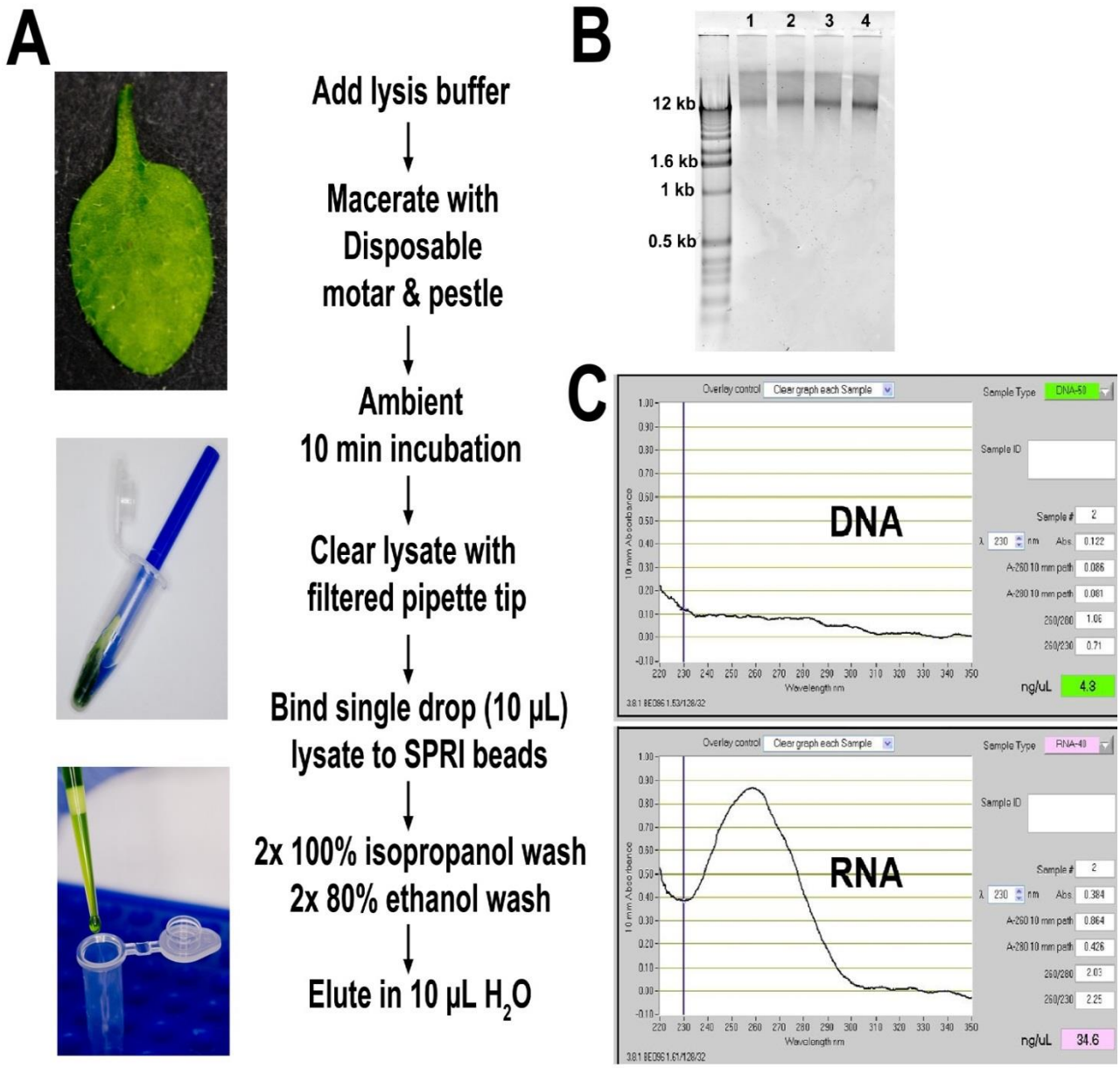


***Figure 3.1*:** Precision nucleic acid extraction protocol. (A) Graphical representation of the extraction protocol. Photographs show the typical leaf used for extraction, manual maceration of the leaf using a disposable mortar and pestle and clearing the lysate of cellular debris using a common pipette tip. (B) Agarose gel electrophoresis of four independently extracted DNA samples, all 10 µL elute was used. The high molecular weight suggests the good integrity of the extracted DNA. (C) Spectrometry analysis of extracted nucleic acids. Top: DNA, Bottom: RNA.

A key characteristic of flocculation is the abrupt transition from solution phase to flocculate which makes this phenomena ideal for binary yes/no applications. To the best of our knowledge, the detection of DNA/RNA has not yet been demonstrated via a DNA-mediated bridging flocculation mechanism which can be readily observed by the naked eye (Fig 3.2A). Indeed, it is the unique feature of the bridging flocculation process to discriminate between long and short DNA polymer segments which lie at the heart of enabling a very attractive, versatile, field-ready system for the detection of any pathogen DNA or RNA sequence.

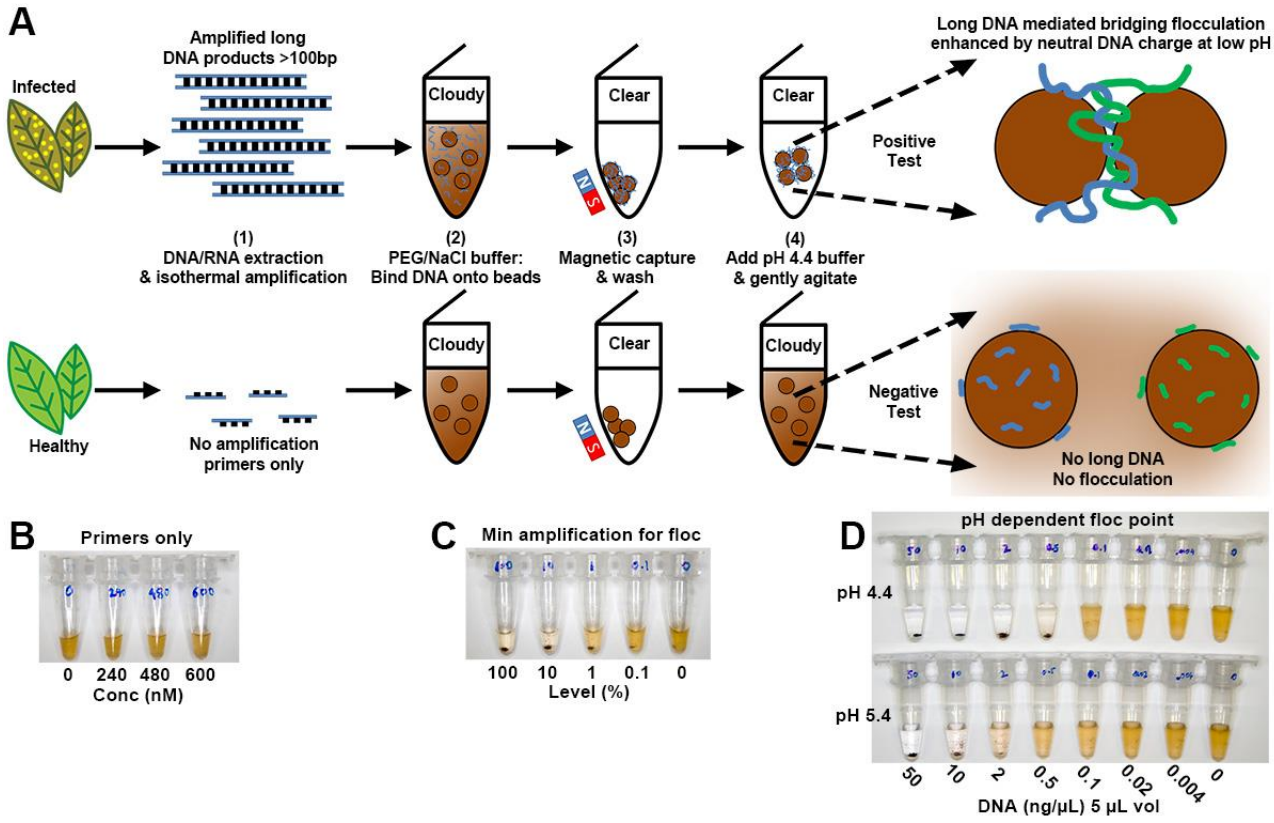


***Figure 3.2*:** The bridging flocculation assay. (A) Conceptual representation of the DNA-mediated bridging flocculation assay which only occurs in the presence of amplified pathogen DNA which is in high molecular weight DNA amplicons/polymers. (B) Excess primers (low molecular DNA polymers which have not been amplified) do not induce flocculation. (C) Only 10% or higher amplification results in flocculation. (D) Cut-off concentration of amplified DNA for DNA-mediated bridging flocculation is pH-dependent. Each figure is a representative of at least 3 experimental replicates.

Bridging flocculation is a well-known phenomenon in colloid chemistry and is used for a wide variety of colloidal separation processes (e.g., to clarify contaminated water). The phenomenon was first described in the 1950's by Ruehrwein, R.A[150] and explained in the 1960's by La Mer and Healy[151-153] to be the result of the surface adsorption of polymers which are long enough to cross-link multiple particles together and thus (reversibly) flocculate out of solution. A key aspect of this phenomenon is that the polymers (in this case DNA amplicons) must be of sufficient length to induce this flocculation. DNA primer pairs, by contrast, are typically too short to enable this type of particle cross-linking, hence flocculation can only occur if the primer pairs are successfully amplified to create long polymer strands, which in turn, represents the presence of a target DNA sequence. Another key aspect of bridging flocculation is that the solution conditions (e.g., salt concentration and pH) must be adjusted so that polymer/surface interactions are stronger than the polymer/solution interaction (as defined by the relevant Flory-Huggins Parameters[154-156]). Under, such conditions longer polymer chains (DNA amplicons) will displace surface adsorbed shorter polymers (primers) to induce a spontaneous flocculation. As a consequence, the bridging flocculation process is also reversible and may have the added versatility to "tune" the assay possibly for quantitative applications.

As a proof-of-concept, the Solid Phase Reversible Immobilization (SPRI)[56] method of DNA purification was used to first select for DNA lengths above 100 bp. Briefly, high molecular weight DNA were first precipitated onto the bead surface in a high polyethylene glycol (PEG)/NaCl buffer. DNA loaded beads were next enriched with a magnet and the PEG/NaCl was removed with an ethanol wash. Then instead of eluting the captured DNA, a low pH acetate buffer was used to trigger DNA loaded magnetic beads to flocculate while long amplicon-free beads are readily dispersed back into solution. To confirm that only amplified amplicons of lengths longer than primers could trigger flocculation, the assay was tested using only primers (Fig 3.2B). As expected, even up to 600 nM of primers were inert to the flocculation assay. Next, to determine how much amplification was required to trigger the flocculation, various ratios of product to primers mixes representing various levels of amplification were evaluated. As little as 10 % amplification efficiency for a 250 bp amplicon was enough to trigger a visually distinct positive response (Fig 3.2C). This was estimated by assuming that the maximum amount of amplified products was equivalent to the initial primer amount (480 nM) in the reaction. Long single stranded DNA such that produced by rolling circle amplification, could also mediate a flocculation response was also observed (Fig 3.3). Therefore, this flocculation assay could potentially be a universal readout of high molecular weight DNA produced by a plethora of amplification systems.

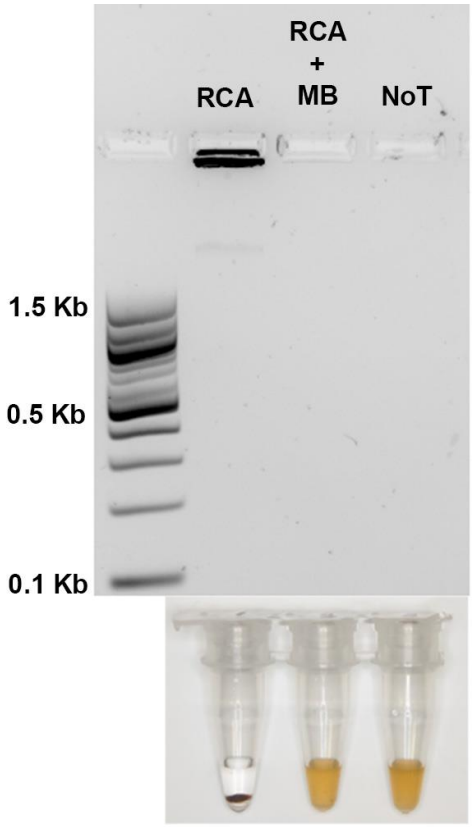


***Figure 3.3*:** Using the flocculation assay to detect long single stranded DNA generated by rolling circle amplification (RCA). Mung bean nuclease (MB), a single stranded DNA exonuclease, was used to demonstrate that RCA generated DNA was indeed single stranded. First lane indicates 100 bp DNA ladder. As expected, flocculation occurred only when long single stranded DNA was present but not in MB digested and no template (NoT) controls. Circle sequence: TGGTCTTAAAAACTCTTTCGTTGTCATTGGGATAGGCGATTCTAAATTTCTCAACGAAA TCTGG was purchased from IDT with a 5'phosphate modification and circularized using the CircLigase II ssDNA Ligase Kit (Epicentre, Cat# CL9021K) following the manufacturer's recommendation**.** The Primer sequence: AGAATCGCCTATCCCAATGACA was used to trigger RCA.

Another interesting feature of the assay was its sensitivity to pH changes (Fig 3.2D). At pH 4.4, 0.5 ng/µL (5 µL volume) of amplified product could be detected. However, at pH 5.4, the cut-off concentration for clear and distinct flocculation increased 20-fold to 10 ng/µL (5 µL volume). In addition, titrating pH with NaOH reversed flocculation (Fig 3.4). Thus, this feature could offer some level of "tuning" and may be beneficial for certain applications. This pH versatility is however, absent in current nanoparticle approaches. Considering these observations: (1) the requirement for long DNA polymers, (2) pH dependence, (3) reversible agglutination and (4) sharp transition between solution phase and flocculate, the mechanism was concluded as a DNA-mediated bridging flocculation of the particles.[157,158] Therefore, it was hypothesized that the accumulation of beads to the magnet facilitates the DNA/bead entanglement by bringing neighboring DNA/beads into close proximity such that DNA strands from one bead may also facilitate interaction with adjacent beads. On introduction of an acidic buffer e.g. acetate buffer pH 4.4, two mechanisms may occur to enhance flocculation: (1) DNA charge is neutralized at pH 4.4, thus making the bead/DNA surface hydrophobic and will spontaneously flocculate in an aqueous environment. (2) Precipitated high molecular weight DNA "intertwine" on the beads surface to form an aggregate or flocculate. However, DNA-free beads which have the COOH groups exposed remain negatively charged thus electrostatically repel each other and readily dispersing into solution.

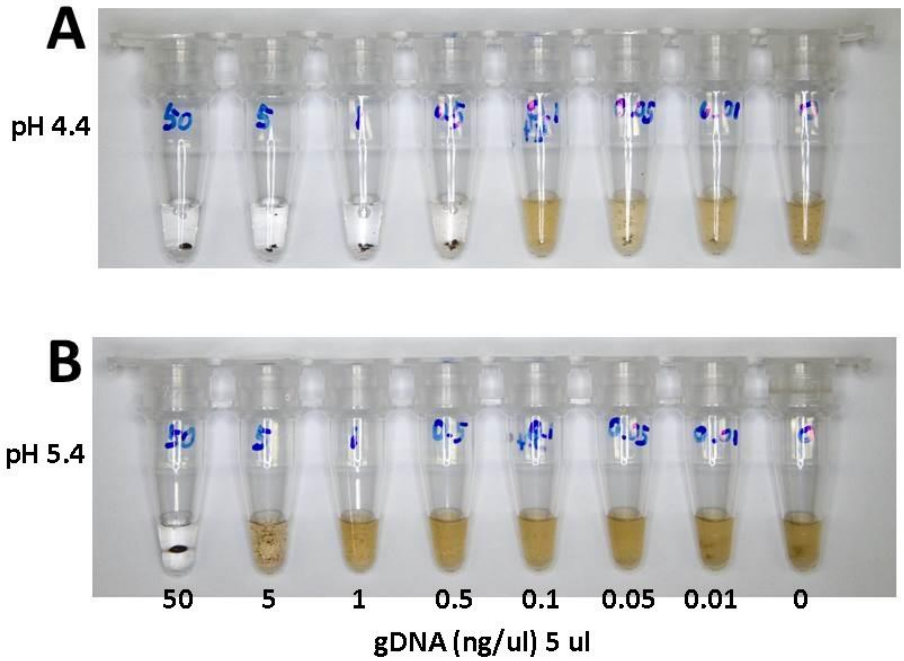


***Figure 3.4*:** Reversible flocculation assay by pH changing with NaOH. (A) Flocculation assay using gDNA at pH4.4. (B) NaOH was added to the same tubes from result A until pH turned to 5.4.

Besides the mechanism of aggregation, bridging flocculation is also distinct in many ways from many recently described aggregation assays for biomolecule detection using gold nanoparticles via various strategies including antibody, DNA probe modified and electrostatic-mediated aggregation.[159-162] For instance, unlike gold nanoparticle methods, the larger size and variety of colloidal particles/material which can be manipulated by a bridging flocculation process (e.g., the 1 μm sized particles used here) allows for better naked eye contrast and therefore does not require the use of spectrometry equipment to verify flocculation hence making the bridging flocculation assay ideal for resource-poor applications.

To enable a meaningful application, the robust isothermal recombinase polymerase amplification (RPA),[45] as a proof of concept, was incorporated with the flocculation assay to detect some examples of agriculturally important pathogens. The RPA was used to facilitate the generation of large amounts of long DNA polymers (amplicons) that could trigger a flocculate only if the pathogen was present. To this end, the model plant *Arabidopsis thaliana* infected with different pathogens at various degrees of infection severity was analyzed. This was achieved by collecting leaf samples at various time points post infection (S1-S5).[149] This approach also served both as a typical traditional visual diagnosis method, and to emulate situations when a farmer would want a diagnosis performed.

To enable a sampling procedure with minimal equipment, a modified SPRI approach was used to extract total DNA from leaf cuttings. This was then followed by the isothermal RPA amplification of pathogen-specific sequences at 37 °C for 15 min. With this approach the bacterial pathogen *Pseudomonas syringae* could be detected at very early in the infection process, even before disease symptoms manifested (Fig 3.5A). To verify that a flocculation was indeed a result of successful RPA amplification, an aliquot of the RPA reactions was also visualized via agarose gel electrophoresis. As expected, flocculation occurred only when there was a successful RPA amplification, therefore confirming that the flocculation assay could be used a viable proxy to evaluate successful amplification which in turn, indicates the presence of the offending pathogen. Compared with current methods in the literature for detecting *P. syringae* in plants, this approach is the fastest with comparable, if not better, sensitivity as other previously described methods (Table 3.2).

In addition, the presence of two other important and devastating pathogenic fungi, *Fusarium oxysporum f. sp. conglutinans* and *Botrytis cinerea* were also detected very early in the infection process when symptoms were just visible to the human eye (Fig 3.5B and C). In contrast, an

additional validation by qPCR could only detect Fusarium infection at stage 5 using the same amount of starting material and primers (Fig 3.6). While detections at the earliest (S1) time point for *F. oxysporum* and *B. cinerea* were not reproducible (Fig 3.5B and C), it was not viewed as a major limitation since plants at these early disease stage were virtually symptomless, hence would have gone unnoticed by the farmer. In actual farming situations, disease diagnostic assays are only performed when potential disease symptoms appear.

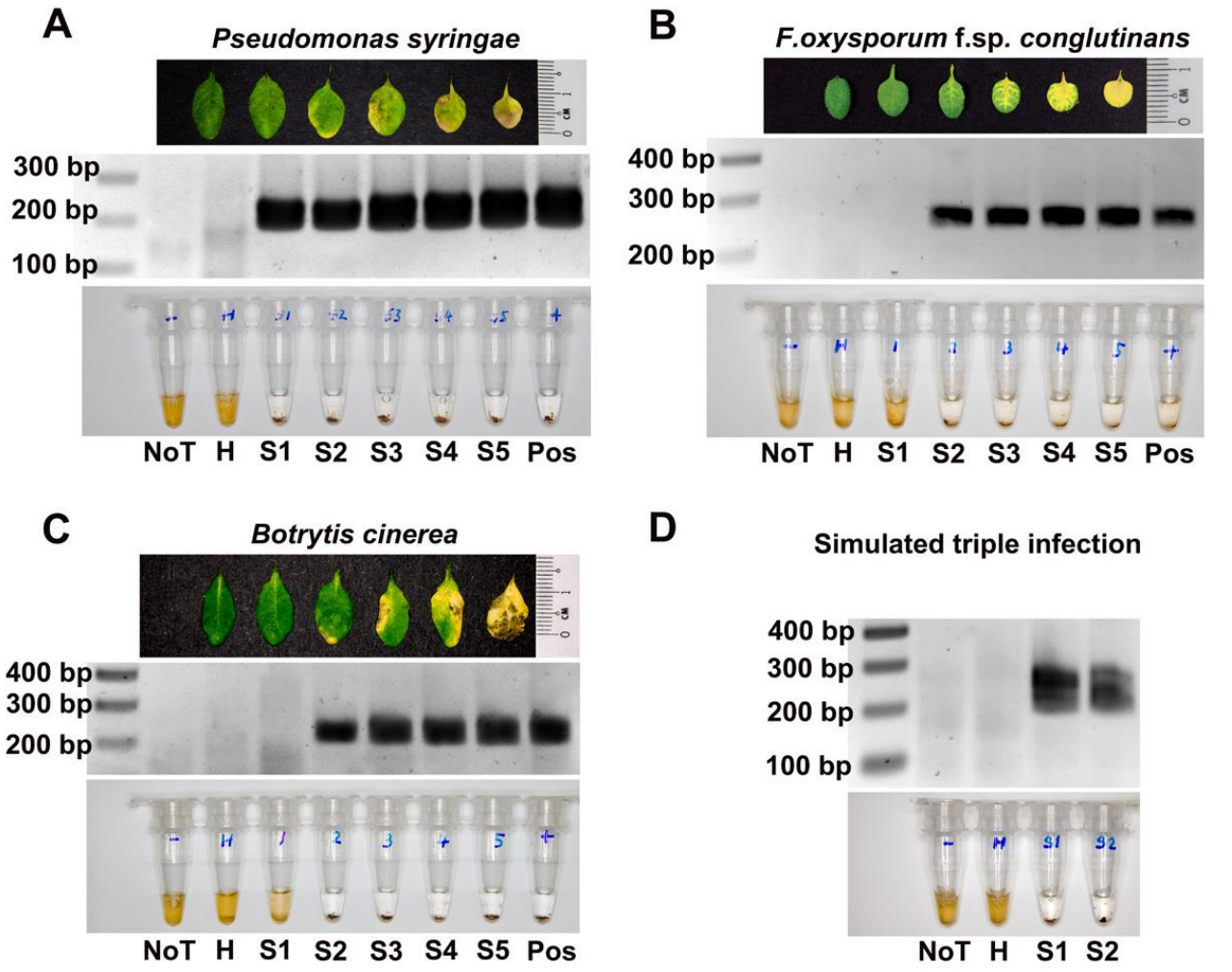


***Figure 3.5*:** Performance at detecting three plant pathogens in *Arabidopsis thaliana*. (A) *Pseudomonas syringae*, (B) *F. oxysporum* f. sp. *conglutinans*, (C) *Botrytis cinerea*, (D) simulated triple infection. Top row: photographs of leaves at various times after infection, S1 to S5. H: healthy sample. Pos: positive control. NoT: no template control. Middle row: agarose gel electrophoresis images of corresponding RPA reactions performed on the same leaf. Bottom row: photographs of the flocculation assay corresponding to the RPA reactions. Each figure is representative of at least 3 experimental replicates.

***Table 3.2*:** A brief comparison of various methods detecting *Pseudomonas syringae* in literature

Methods	Detection limit	Detection time (From sampling to detection)	References
Flocculation Assay	Less than 0.3 pg/µL	90 min	This study
Koch's postulate	-	~20 days	Opgenorth, 1983[163]
ELISA	0.01 µg of antigen	~ 1.5 day	Fogliano, *et. al.* 1999[164]
Immunofluorescence colony staining	70 cfu/L	~3days	Riffaud, and Morris 2002[165]
Dot blot hybridization	1×10^2 cfu	~1.5day	Fanelli, *et. al*.2007[166]
Real time PCR	1pg of gDNA	~ 3 hrs	Green, *et. al.* 2009[167]
PCR (gDNA)	Not stated	~ 6 hrs	Schmidt, *et. al.* 2009[168]
PCR (cell)	1.41×10^3 copies/µL	~ 2 hrs	Choi, *et. al.* 2013[169]
PCR/RFLP	102 cfu/mL	~ 3 days	Biondi, *et. al.* 2013[170]
Gold nanoparticle probe	15 ng/µL	~ 2.5 µ	Vaseghia, *et. al.* 2013[171]
Selective medium (seed)	0.08% contamination	~ 7 days	Suzuki, *et. al.* 2014[172]

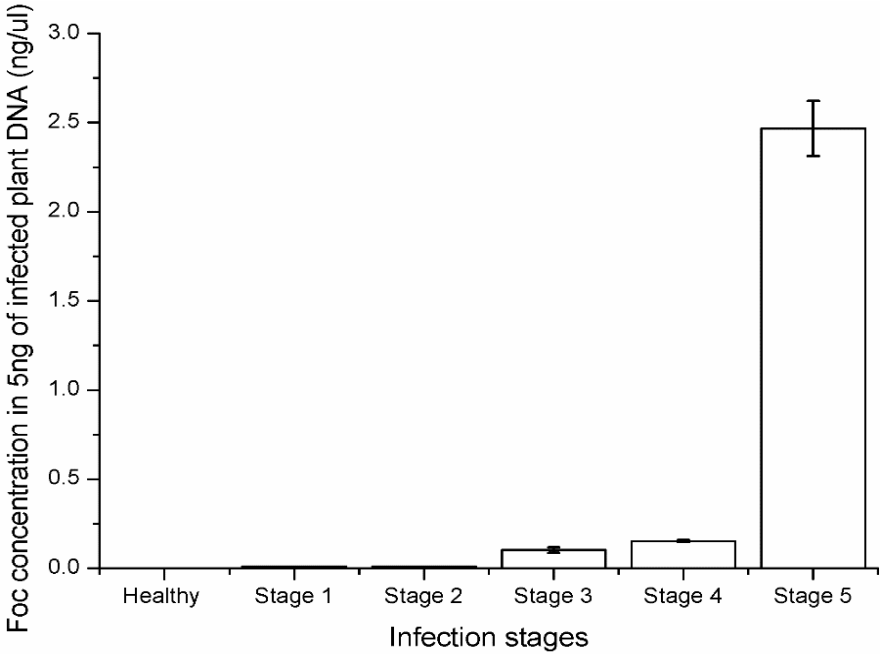


***Figure 3.6*:** qPCR quantification of *F. oxysporum conglutinans* (FOC) in 5 ng of extracted DNA from leaves at various stages of infection. The amount of pathogen DNA was estimated from calibration plot of known target concentrations. Pathogen DNA was detectable from stage 3 infection onwards using the same primers for RPA. Error bars represent the standard deviation of three replicates.

To demonstrate a triplex detection assay, leaves from three plants inoculated with different pathogens were pooled together at their respective times post infection to simulate a triple infection. The presence of pathogens in plants with early signs of infection were reliably detected (Fig 3.5D, Fig 3.6). These results demonstrated both the feasibility and sensitivity of the assay in early detection of plant infections i.e. when phenotypic symptoms were just beginning to manifest. While the assay performed well on the *A. thaliana* plant model system, we wondered if the assay could be applicable to commercial crops and non-leaf tissues with different composition and putative assay inhibitors. To this end, the approach was tested on stem cuttings of banana cv. Lady Finger from diseased field plant samples to detect *F. oxysporum* f. sp. *cubense* Race 1. The healthy sample was able to be distinguished from diseased sample, thus supporting the potential for a viable on-site field test for agricultural applications (Fig 3.7A). Current practices for crop pathogen identification are done via ELISA or PCR-based methods performed off-site at central facilities.[1,10,37,144-148] While useful, the time delay (days) to obtaining results is not ideal as timely interventions are crucial for preventing catastrophic crop losses.[1] In contrast, the approach has the potential for on-site applications because it uses an isothermal amplification method coupled with a simple naked eye evaluation method that can be performed with minimal equipment within 90 min. Recently, RPA has been performed using only body heat.[173] This coupled to a low resource DNA mediated bridging flocculation evaluation assay such as the one described herein could further advance low cost on-site molecular diagnostics.

As a large number of important pathogens use RNA as their genetic material, the attention was focused on RNA-based pathogens. The RT-RPA was performed by adding MMLV reverse transcriptase (RT) to the RPA mix[174,175] on *Nicotiana benthamiana* plants infected with cucumber mosaic virus, a RNA virus that affects a multitude of commercial vegetable crops. As similar to the results from DNA-based pathogens, only samples with RNA viral infection but not healthy plants shown positive (Fig 3.7B). Finally, the application of the approach was extended to the detection of a wider variety of targets. These included bovine herpes virus 1 in bovine cells (Fig 3.7C), *E. coli* in water (Fig 3.7D); and of human diseases such as proviral HIV (Fig 3.7E), malaria (Fig 3.7F), *Mycobacteria tuberculosis* (Fig 3.6G) and influenza virus H1N1 (Fig 3.7H), all of which the approach could successfully discriminate between infected and uninfected samples using the flocculation assay to visualize positive amplifications.

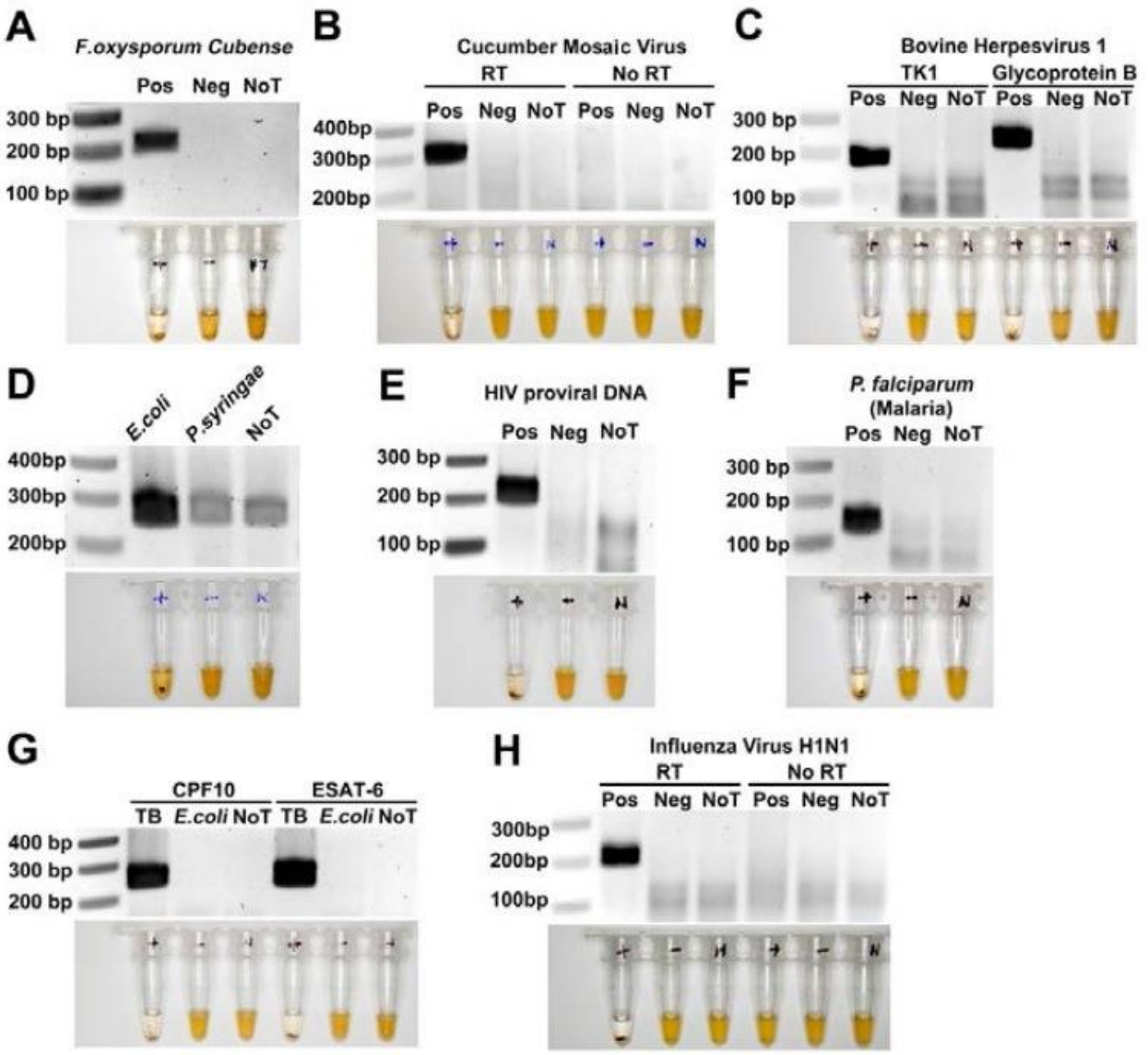


***Figure 3.7*:** Detecting multiple disease pathogens from across various host kingdoms. (A) *F. oxysporum cubense* Race 1 in banana stems. (B) *Cucumber mosaic virus* in *Nicotiana benthamiana* leaves. (C) *Bovine Herpesvirus 1* in bovine cells using two pathogen target genes, tyrosine kinase 1 (*TK1*) and Glycoprotein B. (D) *E. coli* in water using *P. syringae* as an unrelated control. (E) *HIV* proviral DNA in Jurkat cells. (F) *P. falciparum* blood cultures. (G) *Mycobacteria Tuberculosis* in cultures using two pathogen target genes, CFP10 and ESAT-6. *E. coli* was used as an unrelated control. (H) Influenza virus H1N1 in culture media. Top row: agarose gel electrophoresis images of RPA reactions performed. Bottom row: photographs of the flocculation assay corresponding to the RPA reactions. Pos: positive sample. Neg: negative sample. NoT: no template control. RT: reverse transcriptase. Each figure is representative of at least 3 experimental replicates.

3.4 Conclusions

In conclusion, a novel bridging flocculation assay for naked eye qualitative evaluation of amplified DNA was described. The combination of RPA with the flocculation assay then formed the basis of a simple strategy for on-site nucleic acid diagnostics with minimal equipment that may find wide applications. This strategy was first applied successfully to detect economically important plant pathogens and then extended to detect a suite of pathogens in a variety of sources. While promising, a current limitation of the method is the need for multiple wash steps which can be resolved in future improvements to the method. However, considering the wide range of pathogens and samples demonstrated here, the assay is believed to have the potential for on-site, low resource applications.

Chapter 4

Molecular inversion probe:

A new tool for highly specific detection of plant pathogens

Full article: Lau, H. Y., Palanisamy, R., Trau, M. & Botella, J. R. Molecular inversion probe: a new tool for highly specific detection of plant pathogens. *PloS One* 9, e111182, doi:10.1371/journal.pone.0111182 (2014)
http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0111182

4.1 Introduction

Agriculture is a major economic activity with an estimated total annual value of $1500 billion US dollars.[1] However, up to a third of the agricultural production is lost due to three major causes: disease outbreaks, insect attack and weed competition.[1] Among them, losses caused by crop diseases are the most important issue globally, especially in agriculturally reliant countries. In the absence of resistance, the ideal method to control disease outbreaks is by early detection in the field before it spreads to neighboring farms. It is therefore essential to develop new disease diagnostic technologies that are sensitive, reproducible, highly specific and able to detect multiple pathogens in a single assay.

Numerous methods have been evaluated to diagnose plant diseases and detect plant pathogens.[2] The conventional approaches involve identifying the morphological changes in the plant, followed by culturing the pathogens *in vitro* for further characterization.[3] Despite the high accuracy of these approaches, they are time consuming, labor intensive and most importantly, it requires experienced plant pathologists, a luxury in many developing countries, to identify and classify the plant pathogens responsible for the disease. Hence antibody-based rapid diagnostic approaches such as enzyme-linked immunosorbent assays (ELISAs),[10,11] immunoblot[176] and immunofluorescent tests[13] have been developed and widely used to identify a number of plant pathogens. However, these antibody-based methods have been reported to be cross-reactive and sometimes yield false-negative results.[119] To increase sensitivity and specificity, DNA based molecular techniques have recently become the most powerful tool in plant disease diagnostics.[17,18] Amplifying DNA regions unique to a specific pathogen using the polymerase chain reaction (PCR) is one of the most widely used molecular biology techniques and has become a foundation of DNA-based plant pathogen detection. Although PCR based assays demonstrate improved sensitivity and specificity compared to older technologies,[36-38,177] they have limited multiplexing capability when detecting and identifying unknown pathogens in diseased plant samples. Indeed, multiplex PCR assays containing multiple primer sets are more prone to non-specific amplification resulting in false positive results.[39,40]

Furthermore, most of the DNA/PCR-based diagnostic methods have been designed to target the internal transcribed spacer (ITS) regions in the ribosomal RNA genes because of their high copy number and the easy access to large amounts of sequence information in databases.[87,178,179] As a result, the ITS region has been widely used to identify and classify plant pathogens.[180] However, the ITS region is highly conserved in same species and therefore not an ideal target region for intra-species pathogen identification such as differentiating between the *conglutinans* and *lycopersici*

formae speciales from *Fusarium oxysporum*. Therefore, an assay that can readily screen for a particular pathogen or identify the presence of several pathogens in a given sample with high specificity, sensitivity, reproducibility and also amenable to high-throughput multiplex detection is highly desirable.

Currently the most effective way to identify unknown pathogens still involves symptom observation, characterization of the pathogen and proof of Koch's postulates which requires one to few weeks to confirm the identity of the pathogen. Herein, a novel method of plant pathogens detection that can screen for pathogens with high specificity and sensitivity using molecular inversion probes (MIPs) was described. Molecular inversion probes have been previously used for various clinical applications such as high-throughput analysis of single nucleotide polymorphisms, DNA methylation, detecting genomic copy number changes and other genotyping applications.[109-111] MIPs offer a number of advantages over other genome based PCR technologies. Firstly, the MIP backbone physically restricts the two binding domains for localized interaction which increases the specificity of the detection assay. Secondly, the exonuclease digestion step prior to PCR amplification digests all the non-circularized MIPs further increasing the specificity of the assay. Finally, MIPs are highly multiplexable with several thousands of targets can be interrogated in a single multiplex assay.

In this study, the MIP technology was used as a diagnostic tool to screen for plant pathogens in infected tissues. Three economically important plant pathogens, including two from the same species, have been selected for this study (*Fusarium oxysporum* f.sp. *conglutinans*, *Fusarium oxysporum* f.sp. *lycopersici* and *Botrytis cinerea*). As proof of concept, we designed a MIP to target a unique sequence present in the *F. oxysporum* f.sp. *conglutinans* (Foc) genome. The specificity, sensitivity and detection limit of the assay were assessed and used to detect the presence of pathogen in infected *Arabidopsis thaliana* samples.

4.2 Experimental section

4.2.1 Plant and pathogen materials

Arabidopsis thaliana ecotype ***Columbia (Col-0) was obtained from the*** Arabidopsis Biological Resource Center (ABRC; Ohio State University). *Fusarium oxysporum* f.sp. *conglutinans* (Foc), *Fusarium oxysporum* f.sp. *lycopersici* (Fol) and *Botrytis cinerea* (Bc) were obtained from the Department of Agriculture, Fisheries and Forestry, Queensland, Australia.

***Arabidopsis thaliana* growth**

Arabidopsis seeds were sown on soil in a small pot (50 mm diameter) and kept at 4 ^{o}C for three days before transferring to short day growing conditions (photoperiod 8/16 light/dark; 23 ^{o}C) for another 7 days. The seedlings were then carefully removed from the soil by immersing in water and transplanting them into trays. The seedlings were grown in short day conditions for an additional 7 – 14 days until the size of plant reached 25 mm in diameter.

***Fusarium oxysporum* f.sp. *conglutinans* and *Fusarium oxysporum* f.sp. *lycopersici* cultures preparation**

A small agar block containing Foc / Fol hyphae was placed on a plain agar plate and incubated at room temperature for 4 – 6 days until the hyphae were visible. Three agar blocks (5 mm x 5 mm) were cut from the fresh culture plate and transferred into 200 mL of potato dextrose broth (PDB) in a 1 litre bottle. The culture was incubated at 28 °C with shaking at 5 x g for 3 – 4 days. The culture was then filtered through 4 layers of Miracloth to separate the mycelia and the spores. The elute containing the spores was used for inoculation while the mycelia were used for genomic DNA extraction. The concentration of spores in elution was quantified using a hemocytometer and diluted with distilled water to a final concentration of 10^6 spores per mL for inoculation.

***F.oxysporum* f.sp. *conglutinans* inoculation**

A tray containing 20 Arabidopsis seedlings was immersed in water to remove the soil. Then the seedlings were carefully cleaned and dried on a tissue paper before immersing them into inoculation solution for 30 sec. After the inoculation, the seedlings were transplanted onto soil and grown at 28 °C in short day conditions for disease evaluation.

***Botrytis cinerea* culture preparation**

Hyphae of Bc on a small agar block was placed on potato dextrose agar (PDA) and incubated at room temperature for 10 – 14 days until the agar surface was fully covered by mycelium. The layer containing Bc mycelium on PDA surface was taken and used for genomic DNA extraction.

4.2.2 Genomic DNA preparation

Foc, Fol and Bc genomic DNA was extracted using the protocol as previously described.[181] Four-days-old Foc, Fol cultures were prepared as described above and filtered with four layers of Miracloth, the mycelium trapped on the Miracloth surface was transferred into mortar and immediately frozen with liquid nitrogen. The Bc mycelium was taken directly from the 10 – 14 days old culture on PDA plate. Approximately 100 mg of the finely ground mycelium tissue was

transferred to a 1.5 mL microcentrifuge tube. The ground tissue was mixed with 400 µL of extraction buffer (150 mM Tris base, 2% (w/v) SDS, 50 mM EDTA, 1% (v/v) b-mercaptoethanol, adjusted to pH 7.5 with boric acid) and vortexed for 5 min. Then, the mixture was added with 100 µL of absolute ethanol and 44 µL of 5 M potassium acetate before vortexed for 1 min following the addition of each reagent. The solution was added with 500 µL of chilled chloroform : isoamyl alcohol (24:1, v/v) and vortexed for 1 min before centrifugation (maximum speed, 3 min at room temperature). Approximately 500 µL of upper phase was transferred to a new 1.5 mL tube and 500 µL of phenol : chloroform : isoamyl alcohol (25:24:1, v/v) was added. The mixture was again centrifuged at maximum speed for 3 min at room temperature to separate into an upper and a lower phase. The upper phase (400 µL) was transferred to a new tube and mixed well with 1 mL of cold absolute ethanol. The mixture was then incubated at -80 °C for 30 min before centrifuging at 4 °C for 30 min at 15,000 x g to pellet DNA. The supernatant was removed and the pellet was washed with 80% ethanol. The pellet was resuspended in 100 µL H_2O and 5 µL of RNase A (10 mg/mL) was added, followed by 37 °C incubation for 20 min. After the incubation, 10 µL of 3M sodium acetate and 100 µL of isopropanol were added to the mixture. The genomic DNA was allowed for precipitation at -20 °C for 10 min then centrifuged for 2 min at maximum speed. After washing with 70 % ethanol, the dried pellet was resuspended in H_2O. Finally the concentration of extracted genomic DNA was quantified using NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA).

Genomic DNA from Arabidopsis leaves was extracted using a modified Doyle and Doyle CTAB method.[182] Leaf tissue was collected, frozen with liquid nitrogen and ground into fine powder. Approximately 100 mg of ground leaf tissue was then mixed with 500 µL of pre-heated 50 °C extraction buffer (2% w/v CTAB, 1.42 M NaCl, 20 mM EDTA, 100 mM Tris HCl pH 8.0, 1% w/v PVP 40), containing proteinase K (40 µg/mL) and 0.4% β-mercaptoethanol. The mixture was vortexed for 5 min before incubating at 50 °C in a water bath for 30 min, inverting every 5 min. The homogenate was then transferred to a 65 °C water bath and incubated for another 15 min. The solution was then allowed to cool to room temperature and 500 µL of chilled chloroform : isoamyl alcohol was added (24:1, v/v). After a vigorous shake, tubes were gently rocked for 15 min at room temperature, followed by centrifugation at 15,000 x g for 10 min. Approximately 400 µL of supernatant was transferred to a new 1.5 mL tube, 800 µL of cold absolute ethanol added and gently inverted to precipitate the genomic DNA. After incubation for 1 h in -80 °C, genomic DNA was recovered by centrifugation (30 min, 15,000 x g, 4 °C). After washing with 80% ethanol, the precipitate was dried and resuspended in 100 µL of H_2O. The genomic DNA was treated with

RNase A as described above and the concentration was quantified with DNA-50 setting of NanoDrop ND-1000 spectrophotometer.

4.2.3 Molecular inversion probe assay

Design of MIP: The Foc race 2 genome sequence was obtained from GenBank, National Center for Biotechnology Information (NCBI). Bioinformatic analysis was performed and candidate target Foc sequences were identified. Candidate sequences were subjected to BLAST searches to identify homologous DNA fragments in other organisms. Special attention was devoted to find sequences present in the Foc genome but absent from Fol. A fragment showing no homology to any available sequence in the databank was chosen. Based on the unique sequence, a MIP (named MIPFOC01) was designed with 20 nt and 18 nt of binding regions (B1 and B2, respectively) at 3' and 5' end of MIP, respectively. The binding regions sequences were complementary to the DNA target. The Tm values of both binding regions were adjusted to obtain slightly lower Tm at B1 in order to allow B1 hybridized to DNA target before B2. When the MIP hybridized to the DNA target, it generated a single stranded gap (48 nt) between the two binding domains. The binding regions of MIP were connected with a 104 nt linker sequence containing primer sites (P1 and P2) for sequence amplification (Fig 4.1). The MIP had a total size of 143 nt and was analyzed using the OligoAnalyzer 3.0 software (Integrated DNA Technologies) (http://www.idtdna.com/analyzer/Applications /OligoAnalyzer/) to avoid any secondary structures that might interfere with the hybridization process. DNA oligonucleotides listed in Table 4.1 were purchased from Integrated DNA technologies (IDT, Iowa, USA).

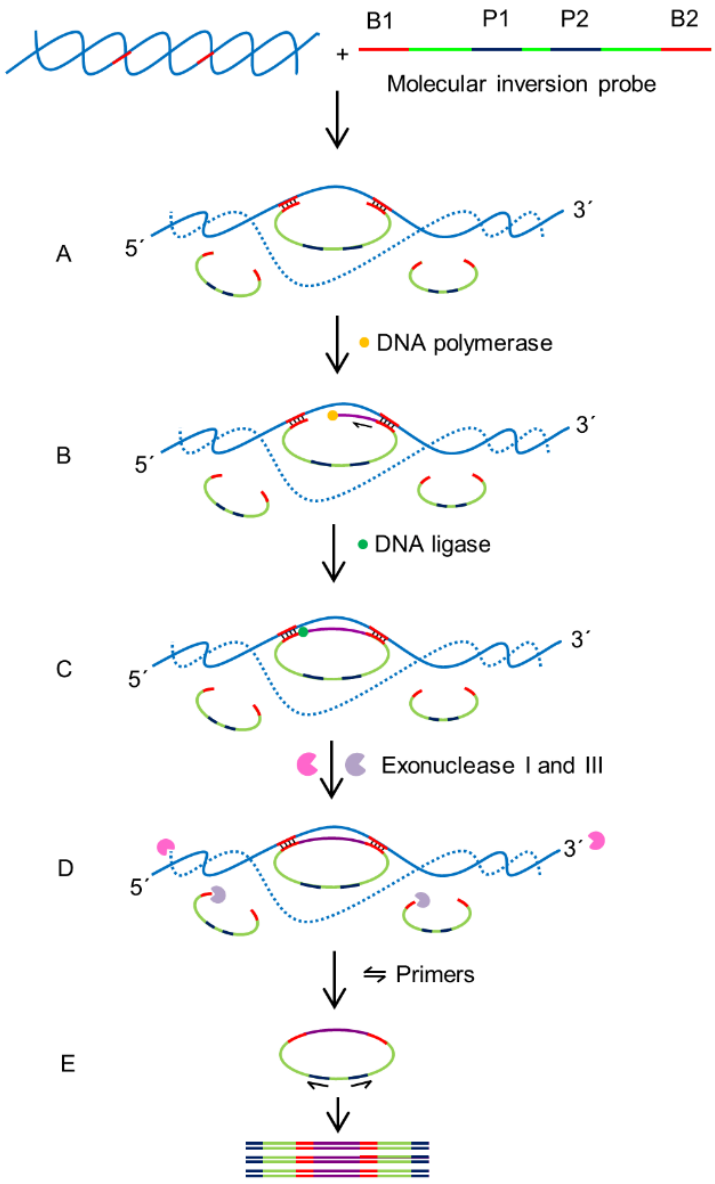


***Figure 4.1*:** Schematic outline of molecular inversion probe (MIP) assay. The MIP consists of two binding domains at its 3' and 5' ends which are designed complementary to target sequences in DNA. The MIPs also contained two universal primer binding domains (P1 and P2) in its DNA backbone. (A) Hybridization: B1 and B2 bind to specific sequences on the target DNA creating a 48 base single stranded gap between the binding domains of the MIP. (B) Gap filling: A DNA polymerase that lacks exonuclease and strand displacement activities synthesizes DNA from 3' end of the MIP to 5' end until the single stranded gap is filled. (C) Ligation: A DNA ligase ligates the 3' end of the newly synthesized DNA strand with the 5' end of the MIP creating a circular DNA. (D) Digestion: exonucleases I and III digest the linear MIPs and the DNA target in the reaction mixture leaving the circularized MIPs for amplification. (E) Polymerase chain reaction (PCR): A pair of universal primers (P1 and P2) amplifies the circularized MIP using the universal primer binding domains to generate PCR amplicons.

***Table 4.1*:** Oligonucleotide sequences

MIP for *F.oxysporum* f.sp. *conglutinans*	MIPFOC01	5´-**AGTAGAATGAAGCCTCCCCC**AGGGTTTGTTGTGGTCAGAATTCT GTCTG**ATGGCTCTTCAGTCCTATAACG**UUU**CCAAATGCTGTGTAG GTCATCT**CACCAATGCATACCAGGCTCACTTTGGGA**GGACGAGGGA AAGAGTTG**-3´
Universal primers for amplifying circular MIPs	MIP forward primer (P1)	5´-CGTTATAGGACTGAAGAGCCAT-3´
	MIP reverse primer (P2)	5´-CCAAATGCTGTGTAGGTCATCT-3´
Real-time PCR primers for Foc quantification	Foc-F1	5´-GGGGGAGGCTTCATTCTACT-3´
	Foc-R1	5´-TGGGACGAGGGAAAGAGTTG-3´
	Foc-F2	5´-CAGACTTTCCACAGCAATGCGT-3´
	Foc-R2	5´-CCATGGGACGAATAGGCACC-3´

Nucleotides highlighted in red and blue indicate the target binding sites and the PCR amplification primer sites, respectively.

Hybridization: 1 pg of MIP was mixed with genomic DNA in 10 µL reactions containing 1X ligase buffer (200 mM Tris-HCl (pH 8.3), 250 mM KCl, 100 mM $MgCl_2$, 5 mM NAD, and 0.1 % Triton X-100). The genomic DNA was initially denatured at 95 °C for 6 min followed by 85 °C for 10 min. The temperature was then gradually decreased from 70 °C to 56 °C at the rate of 1 °C/30 s, followed by 4 h incubation at 56 °C.

Gap filling and ligation: The gap filling reaction was performed by adding 5 µL of the reaction mixture containing 1 unit ampligase enzyme (Ampligase Thermostable DNA Ligase, Epicentre Biotechnologies, Wisconsin, USA), 1 unit Stoffel fragment DNA polymerase (Applied biosystems, USA), 125 nM of each dNTP (Roche diagnostics, Manheim, Germany) in 1X ligase buffer , 250 mM KCl, 100 mM $MgCl_2$, 5 mM NAD, and 0.1% Triton X-100). The reaction mixture was incubated at 56 °C for 2 h followed by cycling the reaction for four cycles using the following conditions, initial denaturation at 95 °C for 6 min followed by 85 °C for 10 min. The temperature was then gradually decreased from 65 °C to 56 °C at the rate of 1 °C/30 s followed by 4 hrs incubation at 56 °C.

Exonuclease digestion: An enzyme mixture containing 10 units of exonuclease I (NEB, Ipswich, USA) and 50 units of exonucelase III (NEB, Ipswich, USA) was added to the gap-fill reaction mixture to digest non ligated MIPs and liner DNA targets. The reaction mixture was initially incubated at 37 °C for 60 min followed by heat killing the enzymes at 80 °C for 20 min.

Polymerase chain reaction (PCR): Amplification of ligated MIPs was performed in a 60 µL reaction containing 1.5 unit Taq DNA polymerase (AmpliTaq DNA Polymerase, Applied Biosystems, Australia), 0.7X PCR buffer (AmpliTaq 10X PCR buffer with 100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM MgCl2, 0.01% (w/v) gelatin), 0.2 mM each dNTP, 0.1% tween 20 and 125 nm of each universal forward and reverse primers (Table 1). PCR reaction was carried out in a Bio-Rad thermo cycler (MJ Mini Personal Thermal Cycler) using the following conditions: denaturation at 94 °C for 10 min followed by 50 cycles of 94 °C for 30 s, 65 °C for 45 s and 72 °C for 30 s. The products were analyzed by agarose gel electrophoresis using 2.5% agarose gels in sodium borate buffer. The agarose gel was stained with ethidium bromide (0.5 µg/mL) then visualized under a 254 nm transilluminator (Vision-capt version 14.2) and the DNA band intensities on agarose gel were quantified using ImageJ software. The PCR amplicon was purified using a QIAquick PCR purification kit (QIAGEN Pty. Ltd., Venlo, Netherlands) and quantified using a ND-1000 Nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA). The purified DNA

was then used in Sanger sequencing and the sequencing result was analyzed using CLUSTALW multiple sequence alignment (http://www.genome.jp/tools/clustalw/).

4.2.4 Real-time PCR

Two sets of primers were designed with each targeting a 100 bp region in the Foc genome (Table 1). Real-time PCR was performed using the Rotor-Gene® Q-Pure Detection System (Software Ver. 2, Qiagen Inc., Valencia, CA, USA). The standard curve was plotted according to the target DNA concentration against the Threshold cycle (Ct) value observed in the real-time PCR assay. The total reaction volume of 20 µL contained either pure Foc genomic DNA (0.1 - 20 ng) or 100 ng of genomic DNA from infected plant samples, 250 nM of each primer, 1 unit Taq DNA polymerase (AmpliTaq DNA Polymerase, Applied Biosystems, Australia), 1X PCR buffer (AmpliTaq 10X PCR buffer), 0.2 mM each dNTP, 2 mM magnesium chloride and 5.0 µM SYTO-9 (*Invitrogen*, USA). Real-time PCR reactions were carried out at the following conditions: 95 °C for 5 min (denaturation and hot start activation), 35 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s and a final elongation step at 72 °C for 5 min. After the real-time PCR, the temperature increased from 72 °C to 95 °C to analyze the melting curves of the PCR products.

4.3 Results

4.3.1 Assay design

A MIP assay was designed to screen and specifically identify Foc infecting *A. thaliana* plants (Fig 4.1). The MIP was hybridized to Foc genomic DNA forming a circular loop with a single stranded gap between the two binding regions. A *Taq* DNA polymerase, lacking 5'-3' exonuclease and strand displacement activities, was then used to initiate DNA synthesis from the 3' end of the MIP in a gap-fill reaction.[110] Following strand synthesis, a DNA ligase was used to ligate the newly synthesized strand with the 5' end of the binding domain generating a circular DNA molecule. To ensure increased sensitivity, the unligated linear probes were eliminated by digestion with exonuclease. The circularized MIP, which is immune to exonuclease digestion, was amplified in a PCR reaction to detectable levels using universal primers designed for two domains in the MIP backbone. In the absence of the target pathogen DNA, the linear MIP was digested in the exonuclease step and no DNA amplicon was produced.

4.3.2 Design of the Molecular Inversion Probe

The MIP (MIPFOC01) used in this study was designed to recognize a fragment of Foc genomic DNA between genes encoding for hypothetical proteins EGU89127.1 and EGU89126.1 (GenBank

locus AGNF01000001). MIPFOC01 is 143 nucleotides (nt) long oligonucleotide with two binding domains, 20 nt and 18 nt each at the 5' and 3' end, respectively (Table 4.1). The binding domains were designed to be complementary to the target region of interest (Fig 4.1). The melting temperature of the 5' binding domain was designed to be few degree centigrade higher than the melting temperature of the 3' binding domain to enable the MIP to hybridize to the target DNA and form a loop with a 48 nt single stranded gap in between the two binding regions (Fig 4.1A). The backbone of MIPFOC01 was designed manually containing two 'universal primer' binding domains that were designed to be present in the backbone of other MIP probes. In this way a single set of primers can be used for amplifying several MIPs, independently of the probe and the pathogen being analyzed.

4.3.3 Detection limit

During disease evolution, pathogens gradually multiply inside their hosts. On many occasions, by the time the disease symptoms are visible, the pathogen may have already spread to neighboring plants making it crucial to determine the lower detection limit of any diagnostic assay that can reliably generate a detectable signal.

Genomic DNA was extracted from *in vitro* grown Foc pure cultures and replicate assays containing different amounts of purified Foc genomic DNA ranging from 160 ng to 1.25 ng were performed using MIPFOC01. Following hybridization and gap-fill reaction, the circularized MIPs were amplified by PCR. The PCR amplicons were analyzed by agarose gel electrophoresis and the relative intensities of the DNA bands were measured. As expected, the intensity of the DNA band increased with increasing amounts of genomic DNA in the reaction but signal saturation was not reached even at relatively high concentrations of pathogen genomic DNA (Fig 4.2). No bands were observed when the amount of input genomic DNA was decreased below 2.5 ng which was same as the control reaction containing no genomic DNA.

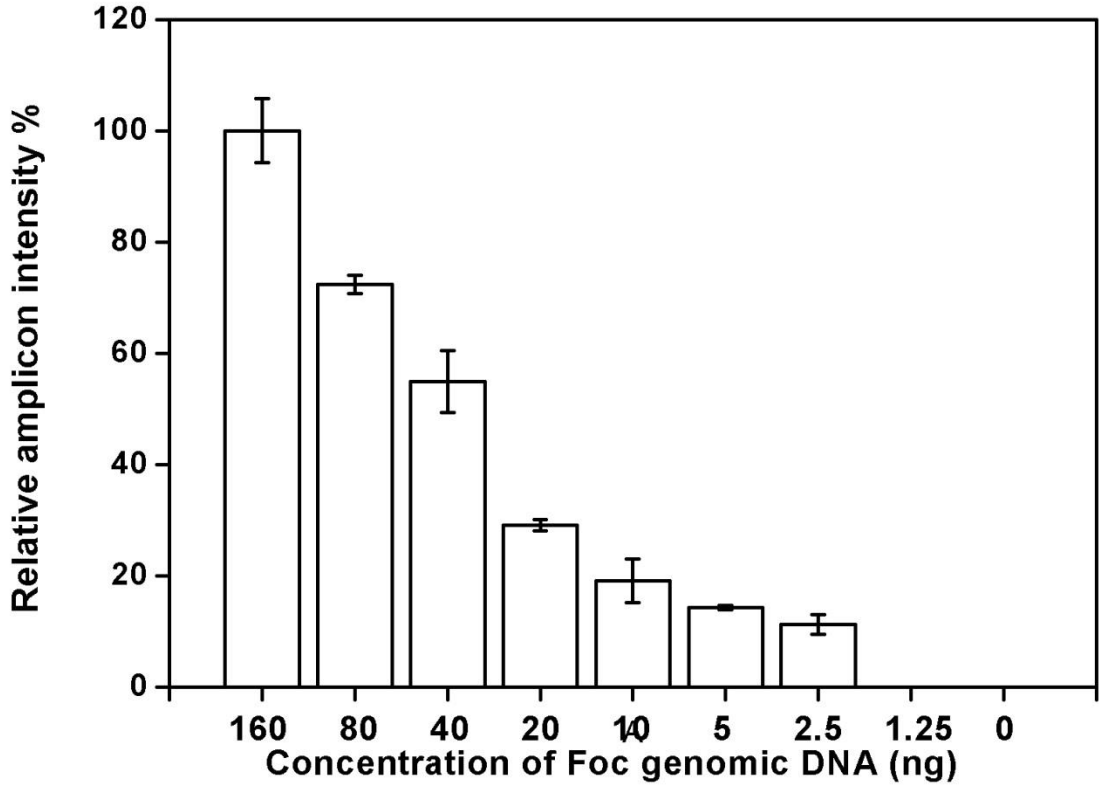


***Figure 4.2*:** Detection limit of the MIP assay. The MIP (MIPFOC01) was mixed with different amounts of Foc genomic DNA (160 ng, 80 ng, 40 ng, 20 ng, 10 ng, 5 ng, 2.5 ng, 1.25 ng and 0 ng) in triplicates and analyzed in a MIP assay. The PCR amplicon was electrophoresed through an agarose gel and the intensity of the DNA bands were measured. Error bars represent the standard deviation of three replicates.

4.3.4 Sensitivity analysis

Even though detection limits for diagnostic methods are usually determined using pure pathogen DNA,[183,184] in a real world situation DNA extracted from an infected plant contains a complex mixture of genomic DNA from the plant as well as the pathogen. The ratio of plant DNA to pathogen DNA in a given sample is variable as it depends on the pathogen and disease progression, but even in advanced stages of infection, plant DNA will always be present in vast excess over pathogen DNA. Therefore, detecting low number of pathogens present in plant tissues during early disease stages has always been a challenge in disease management.

To investigate the sensitivity of the MIP assay, increasing amounts of *A. thaliana* genomic DNA were added to triplicate reactions containing MIPFOC01 and 10 ng of Foc genomic DNA. The PCR

amplicons were analyzed using agarose gel electrophoresis revealing that band intensity decreased significantly ($p < 0.05$) as the amount of *A. thaliana* genomic DNA in the reaction increased (Fig 4.3). The p value was calculated using online p value calculator (http://www.socscistatistics.com) and the statistical analysis was shown in Fig 4.4. As expected, the control reactions containing Arabidopsis genomic DNA but no Foc genomic DNA failed to generate any PCR product. The results indicate that the MIP assay using MIPFOC01 is able to detect Foc DNA even in the presence of 200 fold excess of plant DNA.

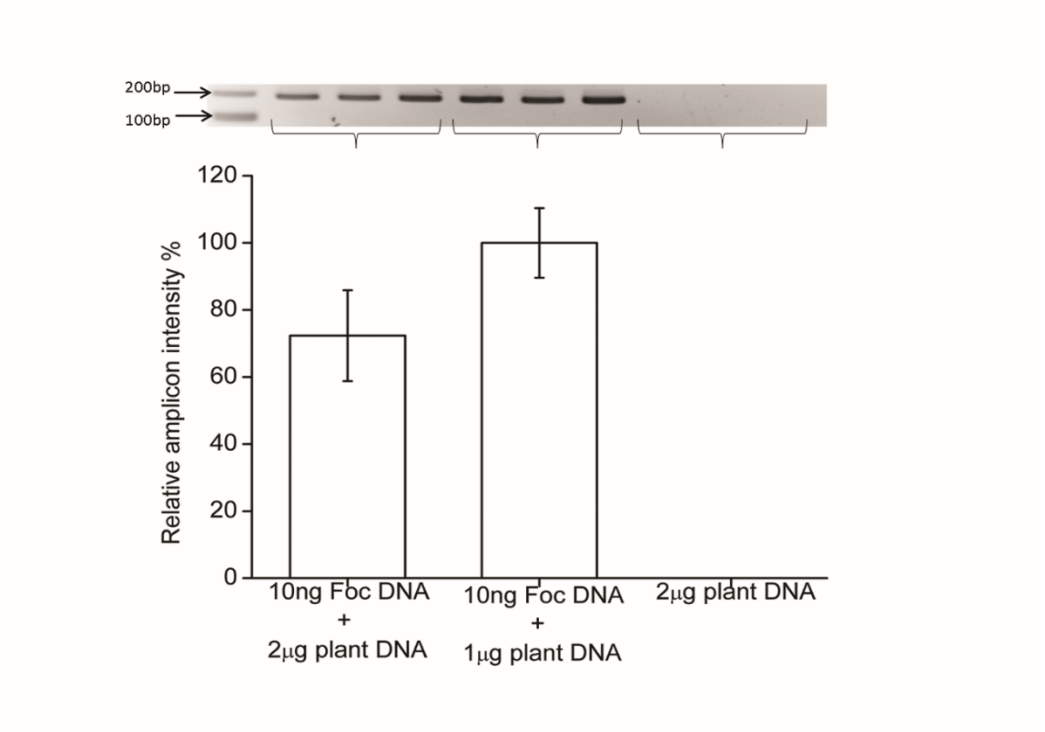


***Figure 4.3*:** The sensitivity of the MIP assay containing of host DNA. Reactions containing 10 ng of Foc genomic DNA were mixed with either 1 µg or 2 µg of genomic DNA from *A. thaliana* in triplicates and analyzed in a MIP assay. A sample containing 2 µg of *A. thaliana* genomic DNA was used as negative control. a) The PCR products were analyzed using agarose gel electrophoresis. b) The intensities of the DNA bands on agarose gel were measured. Error bars represent the standard deviation of three replicates.

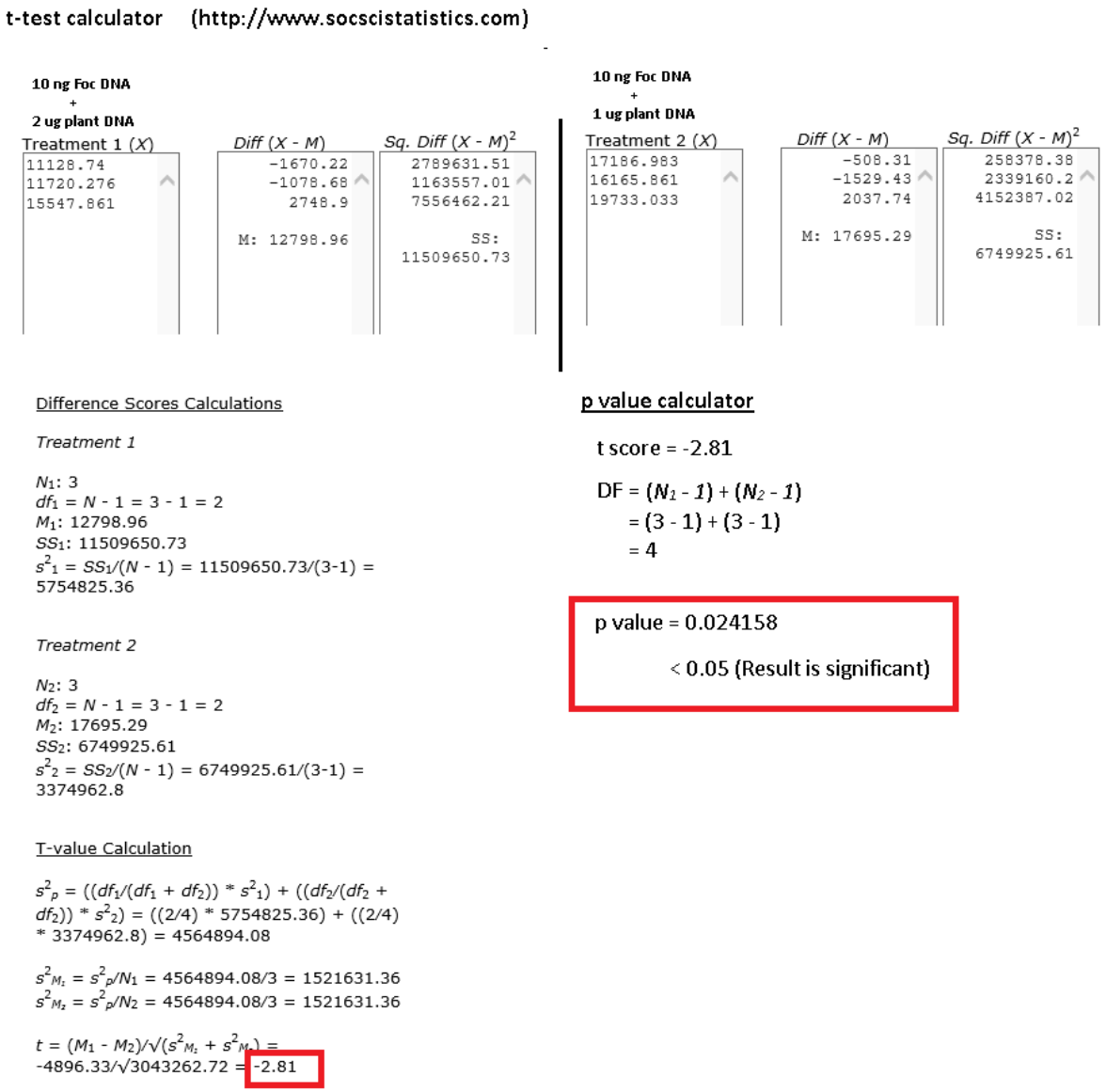


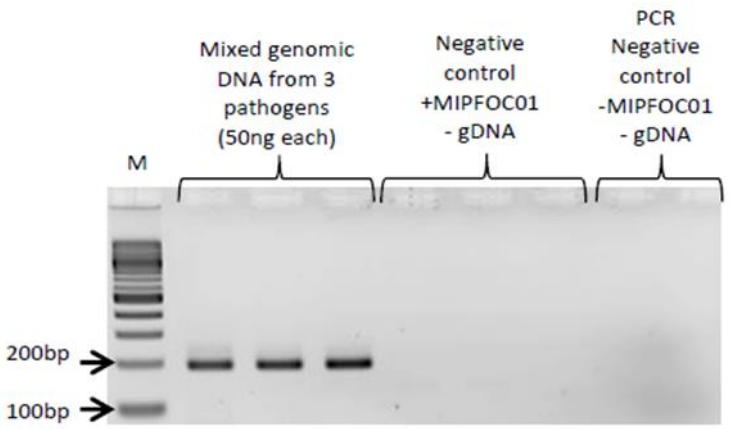
***Figure 4.4*:** Statistical analysis of t-test and p value for the sensitivity test of MIP assay. The result is significant with the p value of 0.024158 which is < 0.05.

4.3.5 Specificity analysis

Specificity, being able to reliably identify a particular pathogen from other species, closely related species or even different races in the same species, is essential for a diagnostic assay. Furthermore, in many instances crops can be infected by multiple pathogens and it is important to avoid false positive results caused by close relatives.[185]

The specificity of the MIP assay and the MIPFOC01 probe was assessed by detecting the presence of Foc DNA in a pool containing DNA from two other pathogens; a close relative (*Fusarium oxysporum* f.sp. *lycopersici*) and a non-related necrotrophic fungal pathogen (*Botrytis cinerea*). The MIP assay was performed in triplicates using the DNA mix extracted from the three pathogens as sample and the reaction products analyzed using agarose gel electrophoresis. A single DNA fragment was observed of the expected size (~200 bp) (Fig 4.5a). The DNA amplicon was purified and sequenced revealing an exact match with the targeted Foc genomic sequence over the entire length of the amplicon (191 bp) (Fig 4.5b). No products were observed when the MIP assay was performed using MIPFOC01 in the absence of the pathogen DNA mix or when both MIPFOC01 and the pathogen DNA mix were omitted. Additionally, the MIP assay failed to amplify any products when *Fusarium oxysporum* f.sp. *lycopersici* or *Botrytis cinerea* were used as source of DNA (Fig 4.6). This result indicates that MIPFOC01 is highly specific for Foc even in the presence of other pathogens from the same species.

a)



b)

```
MIP Product    : GGGGGAGGCTTCATTCTACTAAGCTCTTGT 30
                 ||||||||||||||||||||||||||||||
AGNF01000001.1: GGGGGAGGCTTCATTCTACTAAGCTCTTGT 7892

MIP Product    : CGTTTTGTCCGGCTTGGAATACGCTCTTGA 60
                 ||||||||||||||||||||||||||||||
AGNF01000001.1: CGTTTTGTCCGGCTTGGAATACGCTCTTGA 7922

MIP Product    : TTTAGGTACAACTCTTTCCCTCGTCC 86
                 |||||||||||||||||||||||||
AGNF01000001.1: TTTAGGTACAACTCTTTCCCTCGTCC 7948
```

***Figure 4.5*:** Specificity test of the MIP assay. a) 50 ng of genomic DNA from each of the three infectious plant pathogens (Foc, Fol and Bc) were mixed and analyzed in a MIP assay using MIPFOC01. The PCR products were analyzed using agarose gel electrophoresis. b) The DNA amplicon was purified and sequenced using Sanger sequencing method. The data obtained indicated a perfect match with AGNF01000001.1 (*Fusarium oxysporum* f. sp. *conglutinans* race 2 54008 cont1.1).

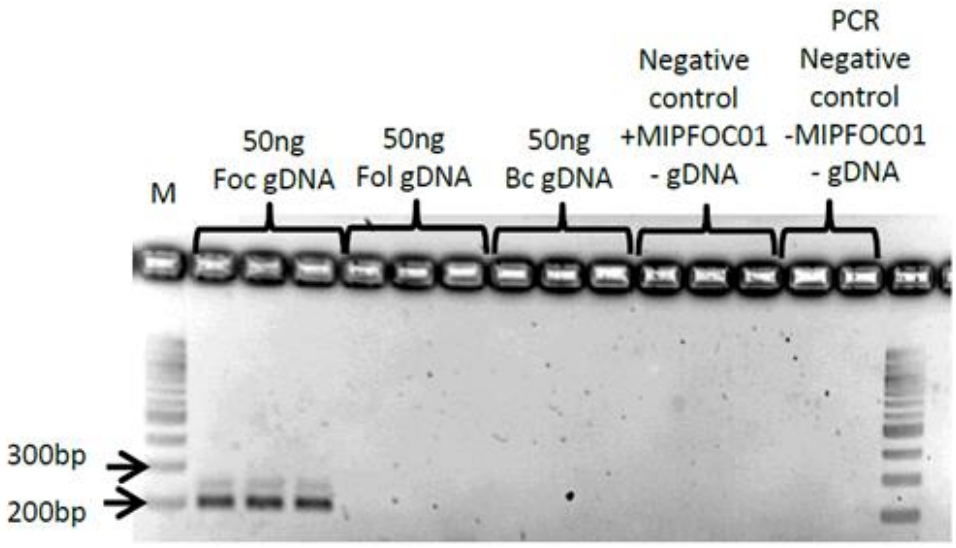


***Figure 4.6*:** Specificity test of the MIP assay. MIP assay was performed on 50 ng of genomic DNA from three infectious plant pathogens (Foc, Fol and Bc) using the MIPFOC01 probe. The MIP products were analyzed using agarose gel electrophoresis.

4.3.6 Disease development and sampling

Arabidopsis thaliana seedlings were manually inoculated with Foc as described by Trusov *et al.*.[186] Inoculated plants started to show chlorosis in leaves and wilt symptoms 7 days after inoculation (dai), and the disease progressed until the fungus invaded all tissues killing the plant 21 - 25 dai. Non-inoculated control plants stayed healthy and no abnormal symptoms were observed over the experimental period.

The infected leaves were harvested based on the sampling method described by Miedaner *et al.* which classified the infection stages by observing and quantifying morphological changes on the leaves.[149] For these experiments, infected leaves were divided into five stages displaying symptoms in 0%, ~25%, ~50%, ~75% and 100% of the leaf surface (Fig 4.7). These stages were observed in plants 3 – 6 dai, 7 – 10 dai, 11 – 15 dai, 16 – 20 dai and 21 – 25 dai, respectively (Fig 4.7). DNA was subsequently extracted from the leaves belonging to each of the 5 different stages and subjected to different analyses.

Infection stage	S1	S2	S3	S4	S5
% symptoms	0	~25%	~50%	~75%	100
dai	3-6	7-10	11-15	16-20	21-25

***Figure 4.7*:** Disease symptom development and classification. The *A. thaliana* leaves were checked for visible symptoms from 1 day after inoculation (dai) until 25 dai and classified into 5 infection stages according to their severity. Stage 1: 0% symptom severity (3 – 6 dai); Stage 2: ~25% symptom severity (7 – 10 dai); Stage 3: ~50% symptom severity (11 – 15 dai); Stage 4: ~75% symptom severity (16 – 20 dai); Stage 5: 100% symptom severity (21 – 25 dai).

4.3.7 Quantification of pathogen DNA in infected plants

Real-time PCR is the most widely used method to quantify the amount of pathogen DNA in a given DNA sample.[33,177,187,188] For the purpose of these experiments, the accuracy of Foc quantification using real-time PCR was validated in several ways. Firstly, quantification was performed using two sets of primers, each set targeting different regions of the Foc genome. Secondly, two standard curves from each of the 2 primer sets were generated by adding known amounts of Foc genomic DNA (20 ng, 10 ng, 5 ng, 1 ng, 0.5 ng and 0.1 ng), while DNA from healthy plants served as negative control. Finally, three replicates of all infected plant samples were analyzed for each specified amount of DNA. The average amount of pathogen DNA was quantified from each primer set and standard deviations were calculated.

The standard curves of the two primer sets (Foc-F1/Foc-R1 and Foc-F2/Foc-R2) resulting from linear regression are shown in Fig 4.8a and 4.8b, respectively. Both standard curves indicated a good correlation between the amount of DNA template and the Ct values (R^2: 0.98 and 0.99, respectively). Melting curve analysis of the PCR amplicons was also performed to confirm successful amplification indicating a maximum Tm of 82.5 °C and 85 °C, respectively (Fig 4.8c and 4.8d). The amount of Foc genomic DNA in all stages of infection was then determined based on the Ct values and averages of the quantified concentrations from the two primer sets (Fig 4.9). Infection in stages 1 and 2 showed very low amounts of Foc genomic DNA while a very significant increase was observed in stage 3 that kept steadily growing until stage 5 (Fig 4.9).

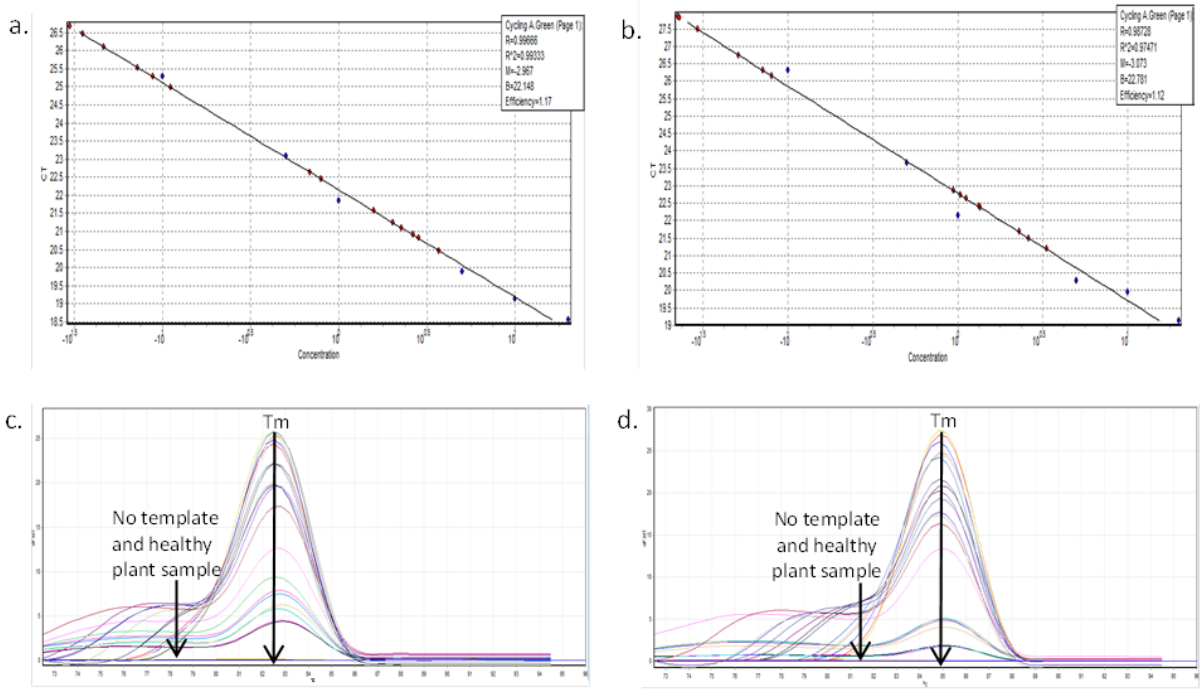


***Figure 4.8*:** Quantification of Foc genomic DNA in infected *A. thaliana* using real-time PCR. a,b) Standard curves using 2 primer sets Foc-F1 / Foc-R1 (2a) and Foc-F2 / Foc-R2 (2b) and known concentrations of purified Foc genomic DNA (20 ng – 100 pg) (blue dots). The concentration of Foc genomic DNA in 5 infection stages as well as in non-inoculated plants was calculated by interpolating on the standard curves (red dots). c,d) Melting curve profiles of the PCR amplicons generated by the 2 primer sets in a real-time PCR.

Infection stages	Average amount of Foc genomic DNA in 1 µg of infected *A.thaliana* genomic DNA (ng)	DNA copy number
Healthy	0	0
Stage 1	0.3 ± 0.12	$5.0 \pm 2.00 \times 10^3$
Stage 2	0.8 ± 0.20	$1.3 \pm 0.333 \times 10^4$
Stage 3	9.2 ± 1.76	$1.5 \pm 0.293 \times 10^5$
Stage 4	16.0 ± 4.64	$2.7 \pm 0.773 \times 10^5$
Stage 5	29.0 ± 5.18	$4.8 \pm 0.863 \times 10^5$

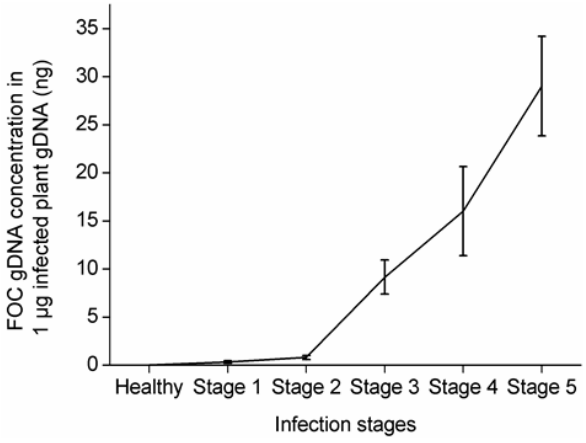


***Figure 4.9*:** Quantification of Foc genomic DNA in infected *A. thaliana* using real-time PCR. The average amount of Foc genomic DNA present in the infected plant samples were quantified using the 2 primer sets (Foc-F1 / Foc-R1 and Foc-F2 / Foc-R2) in the real-time PCR.

4.3.8 MIP assays on infected plants

In order to determine if the MIP assay can reproducibly detect the presence of Foc genomic DNA in infected plant tissues, we analyzed DNA from plant samples at different stages of infection as well as non-infected plants. MIPFOC01 was mixed with 2 µg of DNA extracted from plant tissue at each of the five infection stages described earlier (Fig 4.7). Following hybridization and gap-fill reaction, the circularized MIPs were amplified by PCR. The PCR amplicons were analyzed using agarose gel electrophoresis and followed by quantifying the intensities of the DNA bands (Fig 4.10). No amplification was detected in stages 1 and 2, which according to the RT-PCR quantification (Fig 4.9) contained a concentration of pathogen DNA below the limit of detection shown in Fig 4.2. However, amplification was observed in stages 3, 4 and 5. The amount of amplified DNA increased gradually from stage 3 to stage 5, although in stage 5 the signal seemed to be close to reaching saturation.

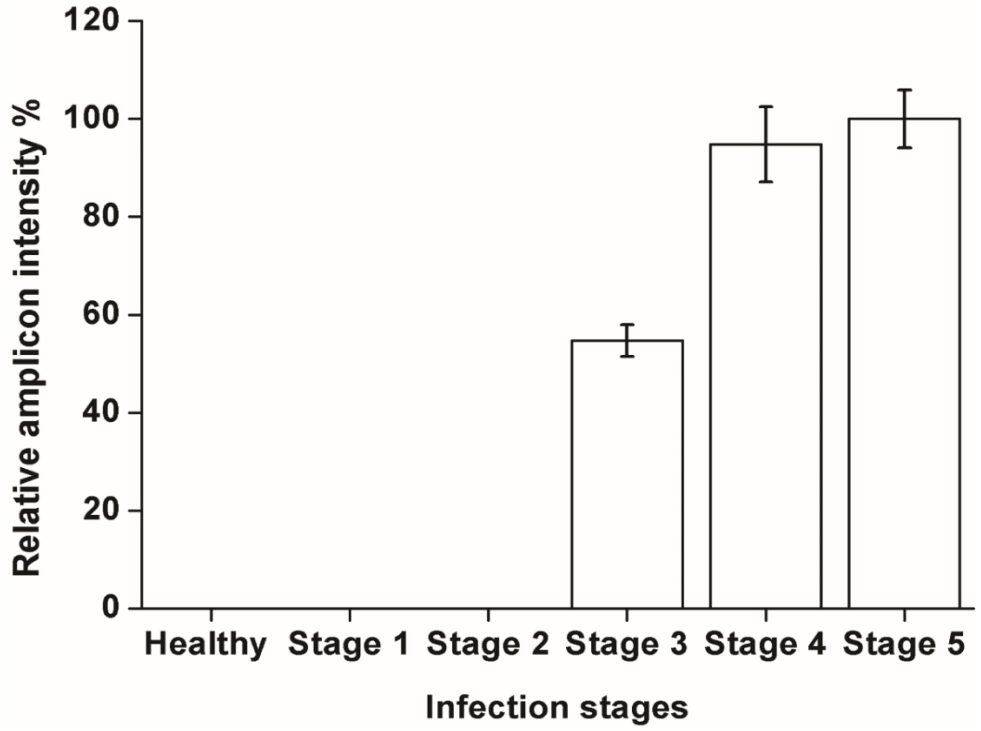


***Figure 4.10*:** MIP assay on infected *A. thaliana* plant tissue samples. Replicate MIP assays were performed on 2 µg of DNA extracted from the *A. thaliana* leaves belonging to each of the five infection stages as well as non-infected (healthy) leaves. Each reaction contained 2 µg of genomic DNA extracted from infected *A. thaliana* leaves. Non-infected *A. thaliana* was used as negative control. MIP assays were performed in triplicates and amplicon abundance was measured by quantifying the intensity of the DNA bands. Error bars represent standard deviation of the three replicates.

4.4 Discussion

As proof of concept, in this work we optimized the molecular inversion probe assay to detect the plant pathogen Foc in pure cultures and infected *A. thaliana* tissues. The sensitivity, specificity and the dynamic range of the assay has been demonstrated in a singleplex MIP assay. MIPs are highly versatile and can be easily tailored to detect virtually any pathogen in a DNA mixture by carefully designing the MIPs binding domains. After hybridizing to the target DNA, the MIP creates a single stranded gap between the binding domains. This looped physical conformation differentiates it from a variant of the MIP technology which does not generate a single stranded gap between the binding domains known as padlock probes (PLP).[189-191] Padlock probes (PLP) have been developed for several applications including plant pathogen identification.[87,107,192,193] However, PLPs exhibited limited accuracy when detecting different pathogens from closely related species[193], mostly due to the fact that the specificity of the PLP assay depends on the fidelity of the ligase which has been known to be promiscuous.[194-196] Therefore, it is likely that the accuracy of the MIP assay which is defined by a polymerization and a ligation component potentially increased the specificity of pathogen detection.

The binding domains at the 3' and 5' end of the MIP are linked by a DNA backbone. This design feature physically restricts the physical distance that can exist in the target genomic DNA for the hybridization of both binding domains, thereby increasing the specificity of the MIPs. In addition, in this approach the signal to noise ratio of the assay was increased by enzymatically digesting the unligated linear MIPs using exonuclease.[197] PCR amplification of the circularized MIPs enabled the MIP assay to detect as little as 2.5 ng of purified Foc genomic DNA. This low detection limit makes the MIP assay an ideal method for detecting pathogens in plants during early stages of infection.

Increasing the relative amount of purified plant DNA to Foc DNA resulted in a decrease in the overall yield of the assay (Fig 4.3) suggesting that the MIP hybridized to the target pathogen DNA more efficiently in the presence of lower amounts of plant DNA. The MIP assay was also able to discriminate among three different pathogens, one of which, *Fusarium oxysporum* f.sp. *Lycopersici,* belonging to the same species of Foc (Fig 4.5). Based on the sequencing data (Fig 4.5b), it can be definitively stated that the MIPF0C01 was highly specific in interrogating the target DNA (Foc gDNA). This is further confirmed by Fig 4.6, where in the absence of the Foc gDNA, the MIP failed to generate PCR amplicons in the presence of Fol gDNA and Bc gDNA. Such level of the inherent specificity of MIPs can be attributed to the use of relatively long sequences for target

recognition (38 bp) and 48 bp of gap fill-in, but even more important, careful design of the MIP can discriminate between almost identical genomes. In this case *in silico* analysis of the *conglutinans* and *lycopersici* genomes was performed and MIPFOC01 was designed to target a region present in Foc but not in Fol. As long as there is enough sequence information available, the same strategy can be used to discriminate between closely related pathogens.

The MIP assay was able to detect the presence of Foc DNA in infected plant tissues from infection stage 3 and forward, while stages 1 and 2 remained undetected. Even though this level of sensitivity is apparently suboptimal, it is mostly due to a very specific characteristic of the pathogen used in this study. In the case of Foc infection of Arabidopsis plants, the appearance of the initial chlorosis symptoms in leaves used for the quantification of the disease development in this work is due to the obstruction of the vascular system in the lower parts of the stem and actually precedes the invasion of the leaves by the fungus.[1,198] This is a relatively unique characteristic of the infection process used by Foc and does not usually happen in diseases caused by other pathogens, where symptom development is strongly linked to pathogen abundance in the infected tissue. Pathogen quantification by RT-PCR confirmed that the leaves belonging to the infection stages 1 and 2 contained extremely low amounts of Foc that challenged even the detection limit of the RT-PCR (Fig 4.9). Roots are nevertheless heavily infected during stages 1 and 2 infection and an obvious question arises as for why do not choose roots as the starting material for the assay. The use of roots in this assay was avoided to preclude the possibility of interference from Foc present in the soil rather than the plant tissues. Due to the nature of the inoculation method, a large amount of Foc is present in the soil and even after extensive washing, contaminating traces of Foc in the root samples could lead to false positive results. As mentioned above, in most crop diseases the development of symptoms is strongly correlated with the presence of the pathogen and it is therefore expected that the MIP assay method will be able to detect the pathogen and the initial stages of disease development allowing for an early diagnosis.

4.5 Conclusions

In conclusion, the MIP technology was presented to detect plant pathogens in infected plants. This method can be a reliable alternative to the existing pathogen detection and identification methods such as morphological identifications which involves traditional *in vitro* culturing and isolation methods. The important advantage of the MIP technology over other available methods is its innate capability for multiplexing using molecular inversion probes as have been previously demonstrated in various clinical studies.[109,199-202] The tailor made backbone of the MIPs provide the opportunity

to incorporate barcode sequences unique for each probe that can be coupled to other technologies to differentiate thousands of amplifications products in a single step.[203-205] Therefore this assay has the potential to be developed into a comprehensive detection system of thousands of pathogens in a single assay, although due to its technical complexity it is unlikely that it will be deployed into a portable device.

Chapter 5
Field demonstration of a multiplexed point-of-care diagnostic platform for plant pathogens

Full article: Lau, H. Y., Wang, Y, Wee, E. J. H., Botella, J. R. & Trau, M. Field Demonstration of a Multiplexed Point-of-care Diagnostic Platform for Plant Pathogens. *Anal Chem*. Just accepted manuscript. DOI: 10.1021/acs.analchem.6b01551 (2016)
http://pubs.acs.org/doi/abs/10.1021/acs.analchem.6b01551

5.1 Introduction

Agriculture contributes $1500 billion US dollars (USD) to the world economy annually. However, an estimated $220 billion USD worth of agricultural products are lost every year due to disease outbreaks, especially in developing countries, thus making crop health a critically important issue in agricultural based countries.[1] In the absence of resistant varieties, the ideal management strategy is to detect pathogens early to prevent the spread of the disease. Hence, the effectiveness of crop disease management is highly dependent on the speed, sensitivity and specificity of the diagnostic methods. Although rapid multiplex diagnostic methods are in high demand for agricultural applications, multiplex detection is currently challenging due to limitations in assay sensitivity and specificity. While numerous diagnostic methods have been evaluated for individual plant disease identification,[2] a rapid and highly specific multiplex detection method has yet to be described. To address this, a rapid multiplex point-of-care (POC) diagnostic method for economically important pathogens in agriculture was demonstrated for the first time.

Traditional laboratory methods involve time consuming culture steps for pathogen identification by experienced plant pathologists.[3] The advent of antibody-based methods offered multiple advantages over traditional techniques but several studies have reported high error rates due to cross reactivity.[119] The limitations of antibody-based methods are further compounded by their short shelf life and variable performance between batches.[120] As such, DNA-based diagnostic methods, with potentially better specificity, have therefore been proposed to overcome the limitations of antibody-based diagnostics.[17,18,121,122] Polymerase chain reaction (PCR) is the most widely used nucleic acid technique for identifying plant pathogens.[39,40] However, it requires temperature cycling which limits its application in the field. Hence to address the limitations of PCR, isothermal amplification systems have since been developed.

Recombinase polymerase amplification (RPA)[45] is an example of an isothermal technique that has seen several novel diagnostic applications in recent years.[55,57,102,123,206-208] RPA, unlike PCR, relies on enzymes, at a single low temperature, to separate dsDNA, assist in primer/target recognition and primer extension.[45] The advantages of RPA include highly efficient amplification and low constant operating temperature,[45] thus making it a candidate for POC applications. Furthermore, RPA is a highly sensitive with a detection limit as low as 6.25 fg of genomic DNA input with specificity >95%.[101] In addition, POC-compatible readouts such as portable fluorometers and equipment-free naked eye strategies[55,123] have also been used with RPA to enable field applications. However, these approaches, while useful, may not be suitable for multiplex RPA assays. To date,

multiplex RPA strategies are rarely described and typically require complicated chip-based assays.[49,209] Thus, development of novel convenient single-tube multiplex RPA strategies could be beneficial to the diagnostic field in general.

Surface-enhanced Raman scattering (SERS) is a technique that can be applied to metal nanoparticles surfaces resulting in enhanced Raman scattering patterns characteristic of the adsorbed molecules upon a single laser excitation.[210] When there is an interaction between an electromagnetic wave and a metal surface, it generates fields from the surface. The wave may excite localized surface plasmons on the surface if the surface is rough and amplify the electromagnetic fields near the surface. Therefore, the enhancement of the electromagnetic field intensity may give a large enhancement of Raman scattering intensity arises.[211] SERS is a potentially powerful molecular spectroscopy detection tool,[212] and has been proposed as a highly promising readout technology for rapid diagnostic assays.[213-215] With its narrow and distinct spectral peaks, SERS could potentially be better suited than standard fluorescent-based methods for highly multiplexed applications.[216-218] Indeed, SERS particles labelled with various Raman reporters have enabled various multiplex detection applications[219] especially in clinical applications.[220,221] However, to the best of our knowledge, SERS has not been applied to any multiplex RPA applications in agriculture.

Herein, a method which entails a simple sampling protocol, followed by a novel single tube multiplexed RPA amplification and SERS detection strategy was demonstrated. The method was first tested on model plant systems and finally demonstrated on commercial tomato samples outside of a laboratory setting.

5.2 Experimental section

5.2.1 Pathogen infection

Arabidopsis thaliana and tomato (Money maker) plants were infected with *F. oxysporum* f.sp. *conglutinans,*[222] *F. oxysporum* f.sp. *lycopersici,*[222] *P. syringae*[139,140] and *B. cinerea*[223] using previously published protocols. *F. oxysporum* was cultured in 200 mL of potato dextrose broth (PDB) for 3-4 days at 28 °C and the culture was filtered using 4 layers of Miracloth to separate the mycelia and the spores. The elution containing 10^6 spores per mL was used for inoculation. *A. thaliana* (14 days old) and tomato (6 weeks old) were immersed in water to remove the soil and dried them on a tissue paper before immersing them into inoculation solution for 30 s. The seedlings were transferred onto soil after the inoculation and grown at 28 °C in short day condition.

Pseudomonas syringae pv *tomato* strain DC3000 was cultured on King's B plate at room temperature for overnight. The culture plate was washed with 10mM $MgCl_2$ to resuspend the bacteria and the bacterial suspension was further diluted to 2 to 5 x 10^8 cfu/mL in 10 mM $MgCl_2$ with 0.03 % Silwet L-77. The bacterial suspension was then sprayed onto seedling leaves. The plants were covered with plastic to maintain high humidity and transferred to 24 °C growth room.

Botrytis cinerea spores suspension was inoculated on PDA plate and cultured for 2 weeks at 22 °C. The spores were harvested by washing the PDA plate surface with 0.01 M KH_2PO_4 (pH 5) to obtain 5 x 10^4 spores/mL suspension. The seedlings were sprayed with the suspension and placed in plastic trays with high humidity at 22 °C in dark conditions.

5.2.2 Preparation of plant samples

Pathogen infected *Arabidopsis thaliana* leaves were sampled at various degrees of symptom severity and scored from S1 to S5.[149,224] Symptom severity was determined according to the percentage of observed symptom on infected leaves which were classified as 0 % (S1), 25 % (S2), 50 % (S3), 75 % (S4) and 100 % (S5). For *P. syringae*, leaves were collected after 7 days infection. For *F. oxysporum* f. sp. *conglutinans* and *B. cinerea*, leaves were collected after 10 days inoculation. Tomato leaves were collected after 7 days infection (*P. syringae*) and 10 days after inoculation (*B. cinerea* and *F. oxysporum* f.sp. *lycopersici*).

5.2.3 Nucleic acid extraction

Total genomic DNA was extracted from *A. thaliana* and tomato leaves using a DNA extraction method described in previous study[55] using a modified SPRI protocol.[56] Briefly, single leaf samples (~300 mg) was homogenized in 200 mL of lysis buffer (50 mM Tris-HCl pH 8.0, 1.5 M guanidium-HCl, 2 % w/v PVP40, 1 % v/v Triton-X and 400 ng RNase) followed by 5 min incubation at 60-65 °C to allow for lysis and for debris to settle to the bottom of the tube. 10 µL of cleared lysate was then incubated with 1.8 volumes of 1µm carboxylic acid coated magnetic beads (Thermo Fisher) in binding buffer (10 mM Tris-HCl pH 8.0, 20 % PEG8000, 2.5 M NaCl) for 5 min at room temperature. The DNA bound beads were then separated using magnetic stand and washed twice with 100 % isopropanol, twice with 80% ethanol washes and eluted in 10 µL of water.

5.2.4 Nucleic acid amplification

The TwistAmp Basic RPA Kit (TwistDX) was used as recommended by the manufacturer with some modifications. The RPA reaction was performed in the total volume of 12.5 µL at 37 °C for

20 min using 1 µL of extracted nucleic acid and 480 nM of each primer set (Table 5.1). Finally, 2.5 µl of the RPA reaction was verified by agarose gel electrophoresis.

***Table 5.1*:** Oligonucleotides sequences involved in this study.

Target / GenBank accession No.	Oligonucleotides	Sequences	RPA products size
B.cinerea AAID02000014.1	Forward primer	ACGATGCATG(C3)TTTCCACAGGGTTTGTGTACGAGATTGGTATTC	203 bp
	Reverse primer	Biotin-TTCTCCGGTGTCCGTTCGCACTGTAGACAATC	
	Capture probe	GCATGCATCGTTTTTT-SH	
P.syringae AE016853.1	Forward primer	TACACAGCAC(C3)TTTGTCCGAAACGACGTACAGCCATTTAACCTT	144 bp
	Reverse primer	Biotin-TTCTACGTCGGGGTATTTACTAGCTGGAAAAG	
	Capture probe	GTGCTGTGTATTTTT-SH	
F.oxysporum f.sp. *conglutinans* AGNF01000001.1	Forward primer	GCTACACGAT(C3)GCTCTTGATTTAGGTACAACTCTTTCCCTCGTC	259 bp
	Reverse primer	Biotin-ATATATCTGTATAGGAATCCCACTGAATTTTTC	
	Capture probe	ATCGTGTAGCTTTTT-SH	
F.oxysporum f.sp. *lycopersici* AAXH01000654.1	Forward primer	GCTACACGAT(C3)ACTCTACTCCAGAGTCTTGTTGATAGTAGC	127 bp
	Reverse primer	Biotin-CCTCATGGGCTGTATACATTTCCCTCAGGACAG	
	Capture probe	ATCGTGTAGCTTTTT-SH	

5.2.5 Preparation of SERS nanotags

SERS nanotags were prepared according to the previous report.[220] Gold nanoparticles (AuNPs) were synthesized by citrate reduction of $HAuCl_4$.[142] SERS nanotags were synthesized by the coating of Raman reporters and DNA probe on the AuNPs surface. Surface coverage of DNA on AuNPs is crucial for the stability of the nanoparticles, which was estimated using a previously described method.[225,226] On average, there were approximately 2235 oligonucleotides strands on each AuNP. Briefly, 1 mL AuNPs were mixed with 10 μL of 50 μM TCEP treated thiolated DNA oligonucleotides (IDT) at RT for 12 hrs. Then, 100 μL of 1 mM Raman reporters (MBA, MMC and TFMBA) were added to the AuNPs and incubated at RT for overnight. Then 0.6 M NaCl in 1 mM PBS was used to age the SERS nanotags at RT for 12 hrs before centrifuged and resuspended into 10 mM PBS solution prior to use on the SERS detection assay.

5.2.6 Surface enhanced Raman scattering (SERS) detection

A small volume of RPA product (10 μL) was used in the SERS detection by incubating with 5 μL of SERS AuNPs for 20 min at 37 °C. The streptavidin magnetic bead (5 μL) was added into the mixture and further incubation at room temperature for 10 min. For the single tube approach, 2 μL of biotin primer/streptavidin magnetic beads and 0.2 μL of SERS nanotags mixture were included in the RPA reaction. After the bead separation with a magnetic stand and 3 washes with 100 μL of 0.1X PBS/0.01 % Tween20 buffer, 60 μL of 1X PBS was added to the beads and the entire bead solution was used for SERS detection on a IM-52 portable Raman microscope (Snowy Range Instruments). The average SERS spectra was obtained from ten of 2-second acquisitions using a 785 nm excitation laser at 70 mW.

5.3 Results and discussion

5.3.1 The multiplex RPA/SERS assay

The principle of the RPA/SERS assay is illustrated in Fig 5.1. Briefly, total genomic DNA was first extracted from plant tissue using a modified Solid Phase Reversible Immobilization (SPRI) method[55], followed by RPA to amplify unique genomic regions of each pathogen using specific primer sets. The primers were designed such that RPA products would contain a biotin handle on one end and a 5' overhang sequence of 10 nt on the opposite end, which functions as a barcode for hybridizing to SERS nanotags. Each SERS nanotag consisted of Raman reporter molecules, a gold nanoparticle core (AuNPs) and DNA capture probes complementary to the barcode sequences of the RPA amplicons. After amplification, biotin/RPA/SERS products were captured by streptavidin magnetic beads.[227] Upon laser excitation, specific Raman signals corresponding to the specific plant pathogens would be generated. The amplification and hybridization of SERS nanotags were eventually optimized to occur simultaneously in a single-tube to enable a faster, simpler assay. In this study, three Raman reporters were used in detecting three economically important plant pathogens, *F. oxysporum*, *B. cinerea* and *P. syringae*. The molecular structures and SERS spectra of the Raman reporters including 4-Mercaptobenzoic acid (MBA), 2,7-mercapto-4-methylcoumarin (MMC) and 2,3,5,6-tetrafluoro-4-mercaptobenzoic acid (TFMBA), are clearly shown in Fig 5.2. Distinct peaks at 1076 cm^{-1}, 1174 cm^{-1} and 1375 cm^{-1} were observed as the characteristic of SERS signatures for detecting *B. cinerea and P. syringae* and *F. oxysporum* f.sp. *conglutinans*, respectively. DNA oligonucleotide sequences used in this study are provided in Table 5.1. The primers were designed based on the unique regions of each pathogen referring to the genome sequences from NCBI database (http://www.ncbi.nlm.nih.gov/).

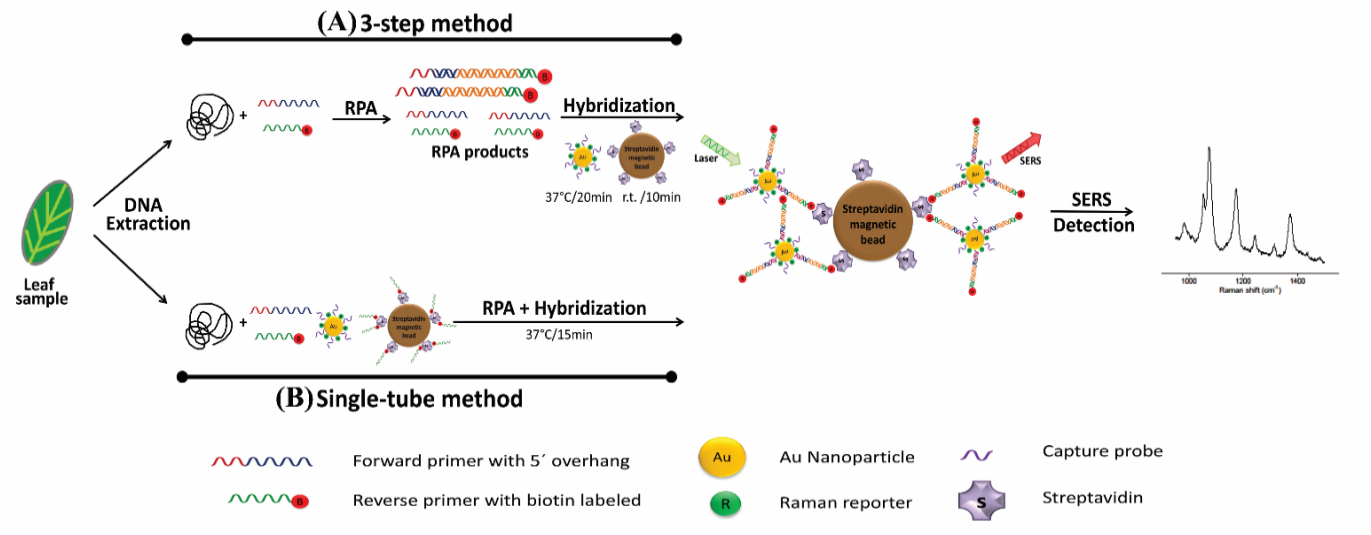


***Figure 5.1*:** Schematic illustration of RPA/SERS multiplex assay. (A) Three steps method of RPA/SERS which involved RPA and 2 times hybridization steps. (B) Single step method of RPA/SERS which performed RPA and hybridization in a single tube.

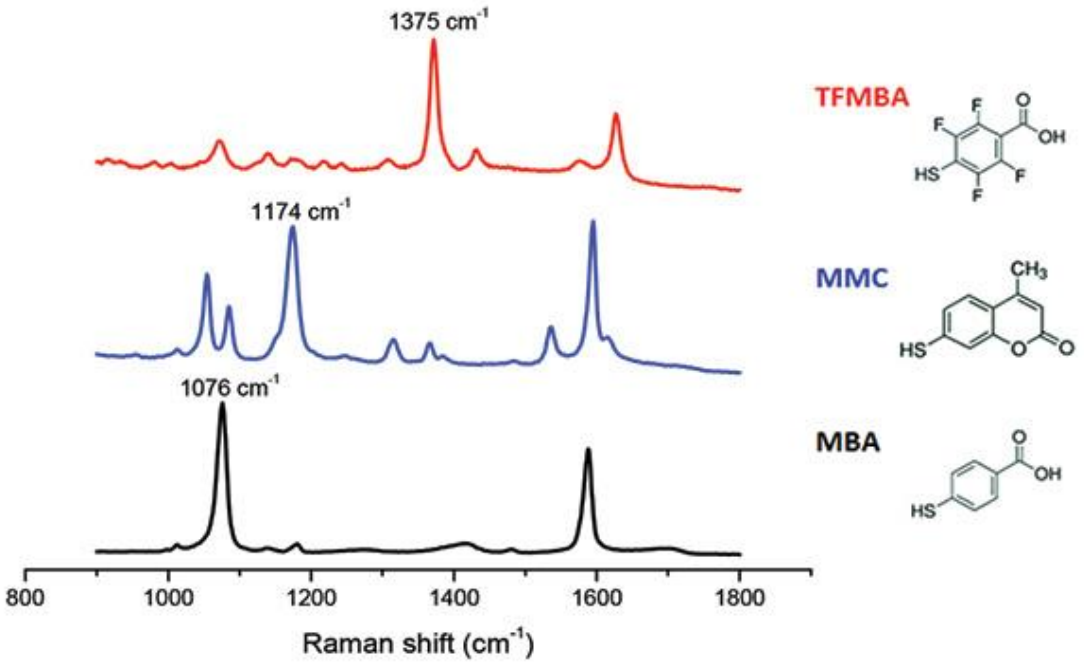


***Figure 5.2*:** SERS signatures of 2,3,5,6-tetrafluoro-4-mercaptobenzoic acid (TFMBA), 2,7-mercapto-4-methylcoumarin (MMC), 4-Mercaptobenzoic acid (MBA), and their corresponding molecular structures.

5.3.2 Specific detection of plant pathogens

Specific multiplex detection of plant pathogens is essential for identifying plant diseases and for prescribing suitable management strategies. Amplification specificity was first tested and verified using agarose gel electrophoresis (Fig 5.3) to demonstrate the specificity of RPA to the specific target. As indicated, the designed primer can specifically amplify the target DNA with negligible background. Next, to evaluate the specificity of the three SERS nanotags, individual pathogen-specific SERS nanotags were challenged with either the cognate or non-cognate RPA products (Fig 5.4). As expected, each assay was highly specific for their respective individual pathogens (Fig 5.4 A-F). Typically, SERS spectra in Fig 5.4 A-B showed that the *B. cinerea*-specific SERS nanotags were able to detect RPA products from *B. cinerea* samples but not *P. syringae* or *F. oxysporum*. Likewise, similar trends were seen in the *P. syringae*-specific (Fig 5.4 C-D) and *F. oxysporum*-specific assays (Fig 5.4 E-F). Furthermore, to demonstrate the multiplexing potential of this method, the assay was applied to all possible pathogen combinations (Fig 5.4 G-H). As only the pathogen-specific SERS peaks were observed, we concluded that the multiplex RPA/SERS assay was indeed specific and thus a viable system for multiplex plant pathogen detection.

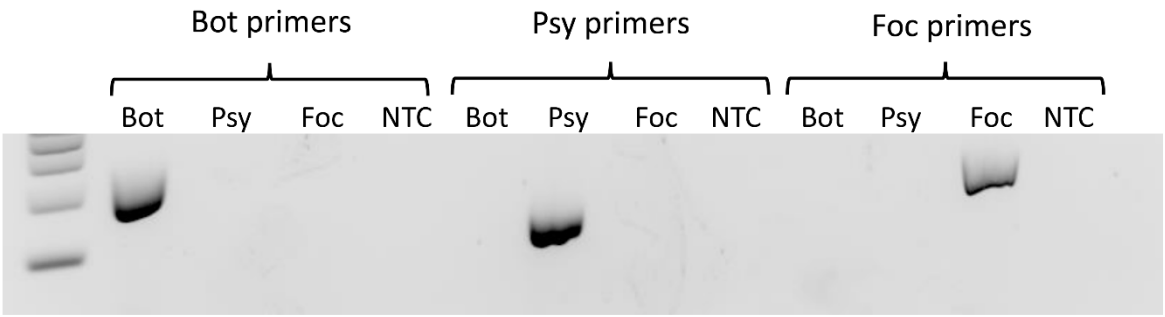


***Figure 5.3*:** Specificity of the primers in RPA amplification. Bot: *B.cinerea*; Psy: *P.syringae*; Foc: *F.oxysporum* f.sp. *conglutinans*; NTC: No template control.

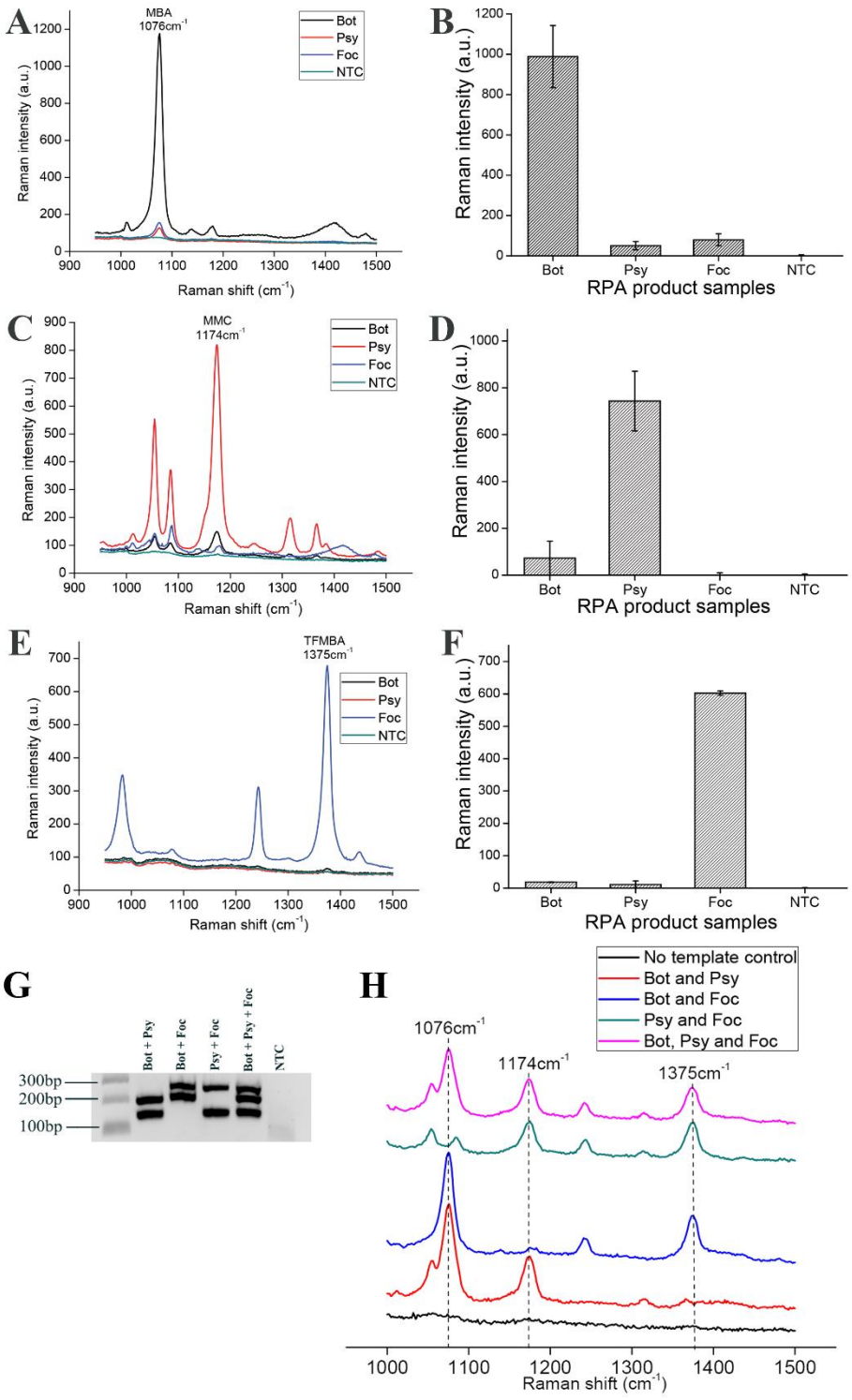


***Figure 5.4*:** Specificity test of the SERS nanotags on three pathogens. (A), (C) and (E): SERS spectra from RPA products of pathogen individually using SERS nanotags labelled by MBA, MMC and TFMBA. (B), (D) and (F): Corresponding target response derived from SERS peaks in (A), (C) and (E). Error bars represent ±SD, n = 3. (G): Electrophoresis agarose gel image of multiplex RPA by combining 3 sets of primers. (H): Multiplex SERS detection with the combination of three SERS nanotags.

5.3.3 Sensitivity of RPA/SERS assay

Highly sensitive assays are essential for early disease detection. To evaluate the performance of this proposed assay, the sensitivity of RPA with PCR (Fig 5.5) was compared using identical *B. cinerea* genomic DNA and primers amounts. The DNA copy number was estimated based on relationship between DNA mass and the genome size of *B. cinerea* (~39Mb).[228] It was found that RPA (2.32 x 10^2 copies) was 100 times more sensitive than PCR (2.32 x 10^4 copies) based on agarose gel electrophoresis. The sensitivity was further enhanced down to 2.32 copies when the RPA amplification was coupled to SERS nanotags (Fig 5.5B and C). It demonstrated the RPA is an alternative amplification system to PCR due to its better suitability for on-site applications and potentially improved analytical performance. Additionally, the sensitivity of the SERS readout was also tested and found that as little as 1.9 fmol of RPA products could be detected over the no input control (t-test $p < 0.05$, Fig 5.6). Therefore, considering the data, an RPA/SERS approach is 10^4 times more sensitive than conventional PCR/gel electrophoresis as a DNA detection platform, making it better suited for POC diagnostics.

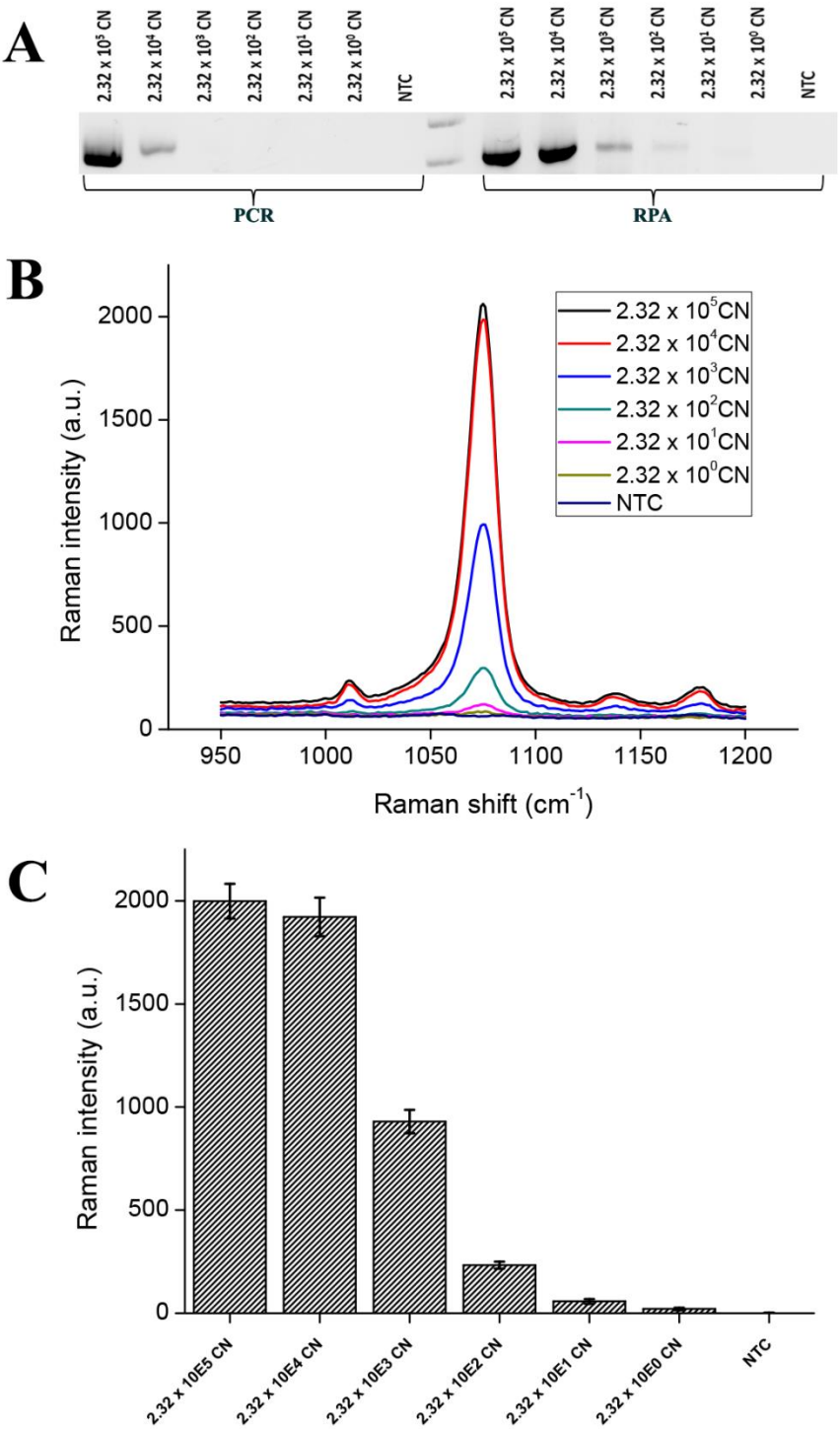


***Figure 5.5*:** Sensitivity study of the assay. (A) Electrophoresis agarose gel image for the sensitivity comparison between RPA and PCR over a range of gDNA inputs. (B) SERS spectral signals of RPA amplicion over a range of gDNA inputs. (C) Corresponding concentration-dependent response derived from SERS peaks in (b). Error bars represent ±SD, n=3 with RSD of 5.25%, n=7.

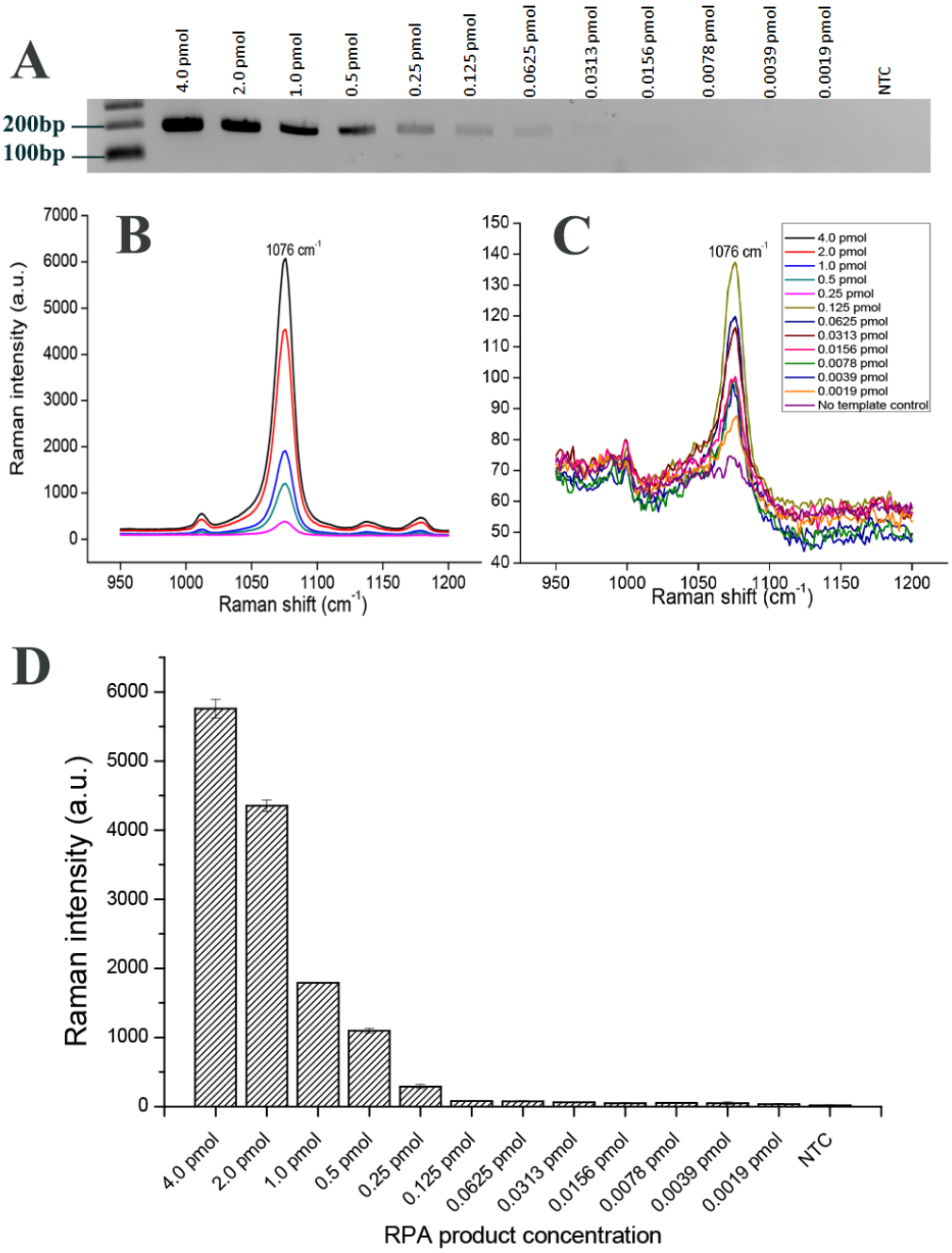


***Figure 5.6*:** Sensitivity of SERS nanotags in detection of RPA amplicons. RPA amplicons were titrated over a range of concentration (A) and SERS signals were measured for each concentration (B-D). As little as 1.9 fmol of RPA amplicons could be detection with SERS.

5.3.4 Early detection of pathogen infections in plants

To demonstrate the suitability of the method for the detection of plant pathogens in infected plant samples, *A. thaliana* plants were inoculated with the three chosen pathogens and the infected leaves were collected at different degrees of infection (S1 – S5) based on symptom development.[112,149] As indicated in Fig 5.7, with this approach, *P. syringae* was successfully detected in infected *A. thaliana* plants at very early infection stage (S1) before any noticeable symptoms were apparent (Fig 5.7B). In *A. thaliana* infected with *B. cinerea* (Fig 5.7A) and *F. oxysporum* f.sp. *conglutinans* (Fig 5.7C), the assay was able to diagnose diseased plants from Stage 2 infection just as symptoms became noticeable although the sensitivity studies showed that the assay was able to detect as low as 2 genomic copies of pure pathogen DNA (Fig 5.6). This might be due to the extracted DNA containing the mixture of plant DNA and pathogen DNA which could slightly interfere with the dynamic range of the assay.

To demonstrate the multiplex capability of this RPA/SERS assay, three S3 infected leaves from individually inoculated plants were pooled together and tested. As expected, three signature SERS peaks representing the three pathogens were observed and validated by the agarose gel electrophoresis (Fig 5.7D). In contrast, no SERS signal was observed in healthy samples and no template controls, further demonstrating the high specificity of the developed assay for multiple plant pathogens detection.

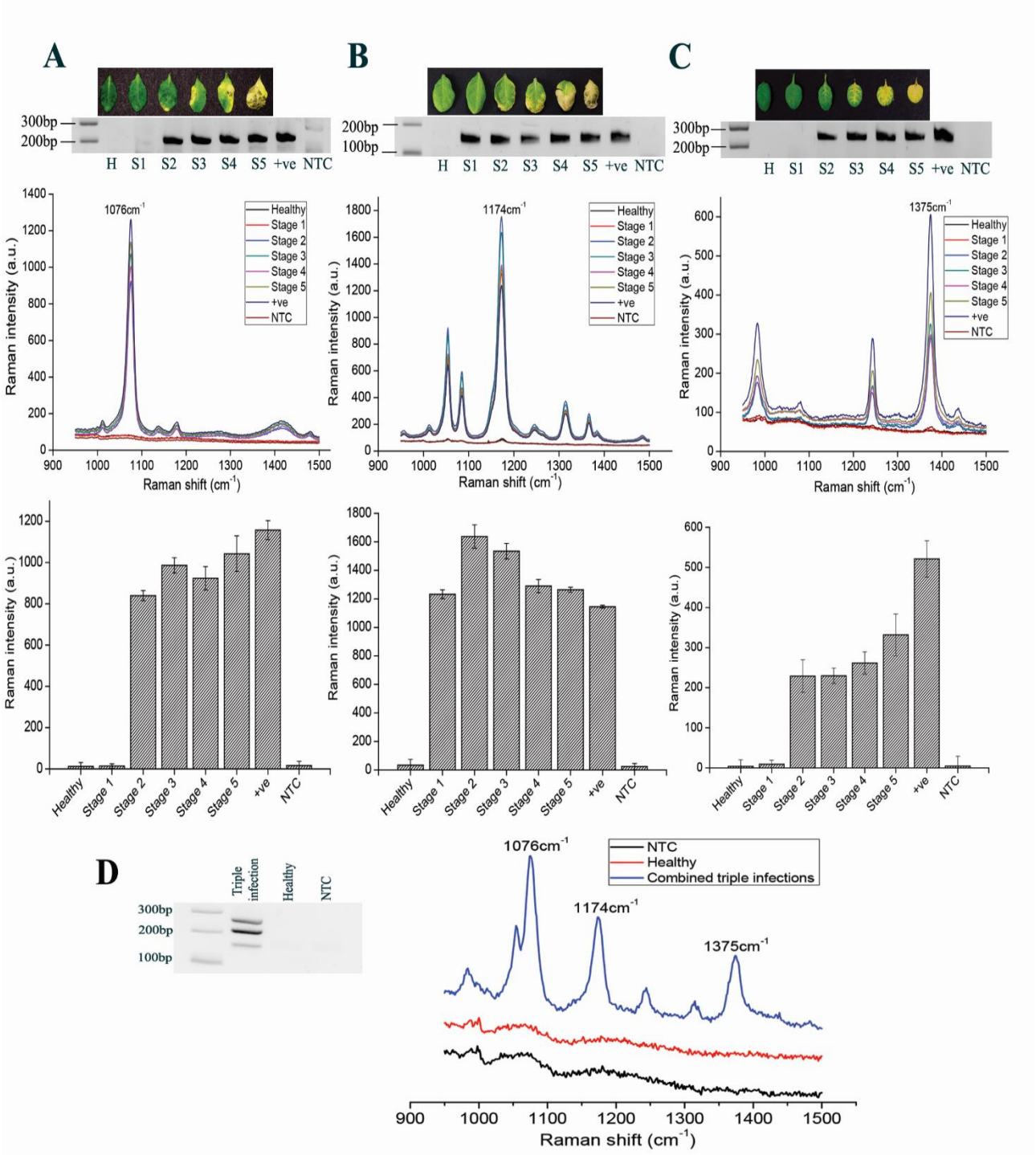


Figure 5.7*:** Performance of SERS detection at three plant pathogens in *Arabidopsis thaliana.** (A) *Botrytis cinerea*, (B) *Pseudomonas syringae*, (C) *F.oxysporum* f.sp. *conglutinans.* Top row: photographs of leaves at various symptoms after infection from S1 to S5. H: healthy sample. +ve: positive control. NTC: no template control. Second row: agarose gel electrophoresis images of corresponding RPA reactions performed on the same leaf. Third row: SERS detection assay corresponding to the RPA reactions. Forth row: Target response derived from signature SERS peaks. Error bar represent ±SD, n=3. (D) Combined triple infection. Electrophoresis gel image of multiplex RPA reactions and SERS spectral corresponding to the RPA reactions.

5.3.5 Detecting disease in commercial crops

The model plant *A. thaliana* has a number of advantages that makes it ideally suited for research and development of new technologies such as the one described in this work. Nevertheless, it is essential to demonstrate the robustness and efficiency of the method on important commercial crops such as tomato. Consequently, the assays were performed on tomato plants infected with *B. cinerea*, *P. syringae* and *F. oxysporum* f.sp. *lycopersici* (Fig 5.8). Tomato plants were inoculated with each of the pathogens and leaves with disease symptoms were harvested (Fig 5.8A). Both SERS and agarose gel electrophoresis results indicated the specificity of the assay to detect the respective pathogens with SERS peaks at 1076cm^{-1}, 1174cm^{-1} and 1375cm^{-1} obtained from tomato samples infected with *B. cinerea*, *P. syringae* and *F. oxysporum*, respectively (Fig 5.8B-C). Furthermore, the capability of multiplexing was demonstrated once again by combining three infected tomato leaves (Fig 5.8B-C). Taken together, the data supports the applicability of this RPA/SERS strategy for multiplexed pathogen detection in commercial crops like tomato.

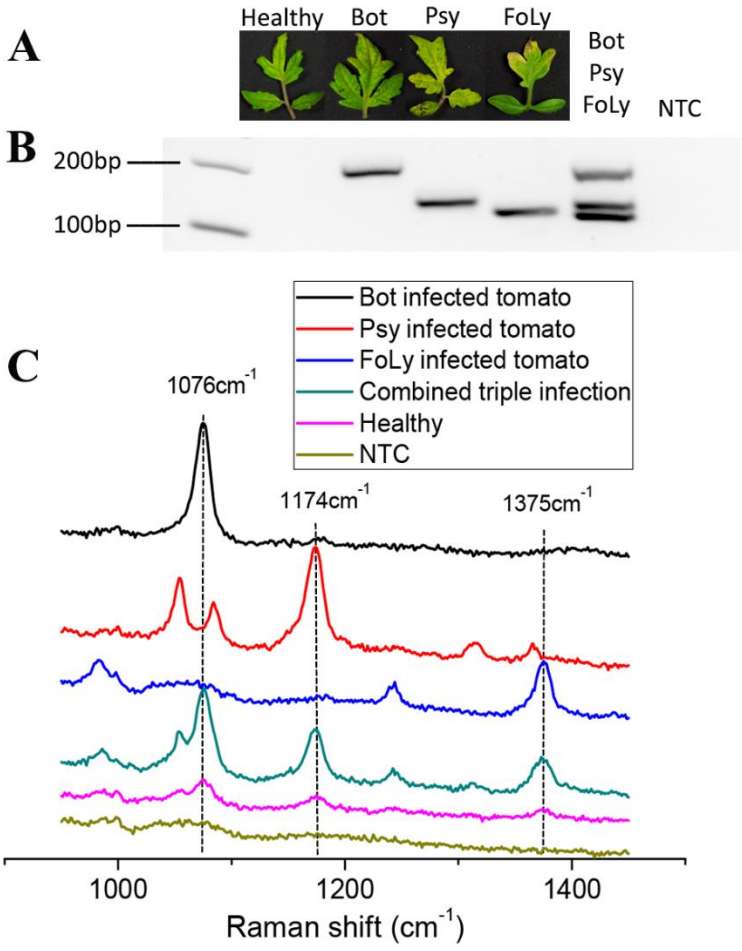


***Figure 5.8*:** Multiplex SERS detection on three pathogens on infected tomato leaves. (A) Photographs of healthy and infected tomato leaves with disease symptoms. (B) Electrophoresis gel image of RPA products. (C) SERS detection on individual infected tomato leaves and combined three infected leaves for multiplex detection.

5.3.6 Field application and development of a single tube RPA/SERS assay

Ideally, diagnostic methods should be able to be performed in the field without the need for a laboratory environment. To demonstrate the POC capability of this method, the assays were performed outside the laboratory and further simplified this diagnostic assay to allow for a more practical field-ready application. To this end, the amplification, hybridization of SERS nanotags and amplicon capture into a single tube reaction were condensed into a single tube reaction (Fig 5.1B). This improvement also reduced assay time by half while maintaining assay performance (Fig 5.9). As RPA uses recombinase enzymes to assist primers/target recognition,[229,230] the same mechanism was believed likely promoted the specific hybridization of the SERS nanotags to the barcode sequences of amplicons. With the help of a portable Raman spectrometer, the improved assay was successfully performed in a garden adjacent to the laboratory to detect single diseased sample (*B. cinerea* infected tomato plants, Fig 5.10) to demonstrate a potential field application. Prior to RPA, the reaction tube initially contained all three SERS nanotags designed to detect each of the three pathogens (indicated by the 3 signature spectral peaks at 1076 cm^{-1}, 1174 cm^{-1} and 1375 cm^{-1}, Fig 5.11A). As expected, after RPA and a quick wash to remove unreacted nanotags, only one SERS peak at 1076 cm^{-1} was observed indicating the accurate on-site diagnosis of the *B. cinerea* infected tomato leaf within 40 min. In comparison, only a very low background signal was generated from a healthy sample (Fig 5.11B), which further underscored the high specificity of the single tube assay.

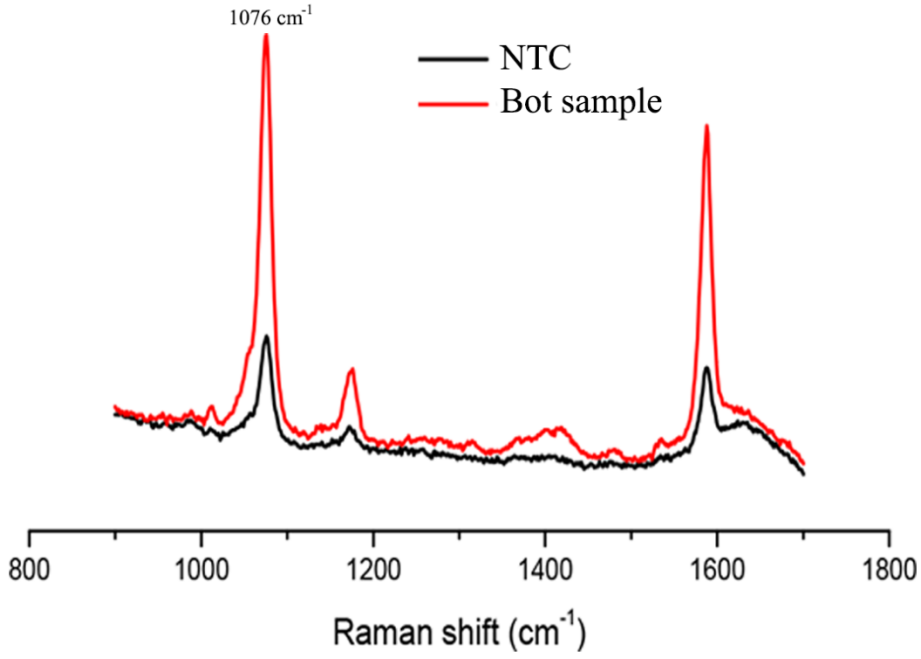


***Figure 5.9*:** Performance of SERS detection on *B.cinerea* gDNA sample using the single-tube method.

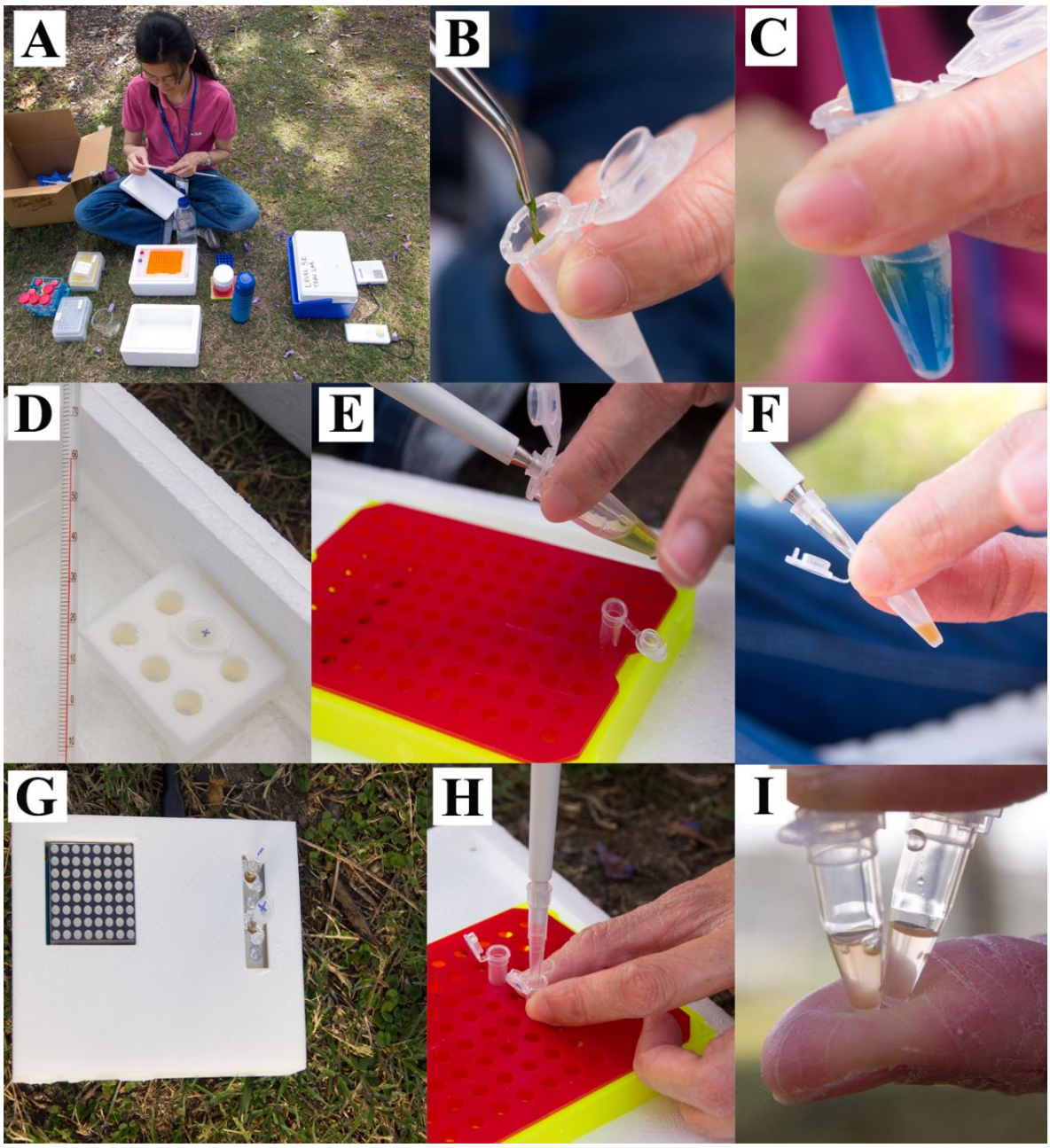


***Figure 5.10*:** Procedures of plant pathogen detection in the garden from DNA extraction to detection. (A): Setting up the experimental platform. (B): Placed a small tomato leaf (5mm x 5mm) into the DNA extraction buffer. (C): The leaf was homogenized in the buffer using disposable pestle. (D): The mixture was incubated at 60-65°C for 5min. (E): 10 µL of the lysate was added into the RPA reaction. (F): Streptavidin magnetic beads and three SERS nanotags were added into the RPA reaction. (G): The RPA mixtures were incubated at 37°C for 15min using a homemade portable isothermal device. (H): The RPA reaction was washed 3x with 1mM PBS/0.01% Tween20 buffer. (I): The streptavidin magnetic beads were resuspended in 10mM PBS buffer and ready for SERS analysis.

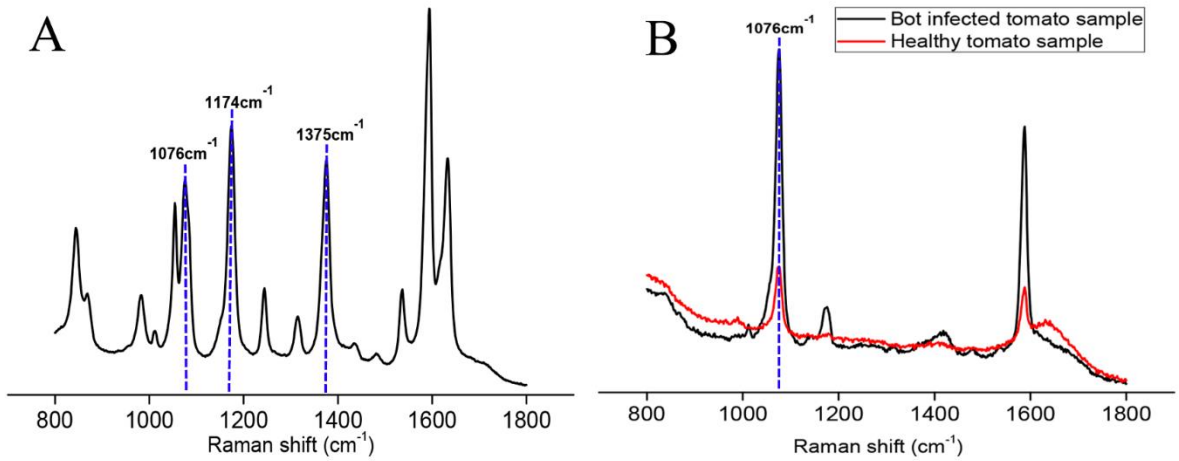


***Figure 5.11*:** SERS spectral signal from the SERS detection of *B. cinerea* in tomato plant in the garden. (A): SERS spectra of the mixture of three SERS nanotags used in the garden experiment. (B): SERS spectra obtained from *B. cinerea* infected tomato sample and healthy sample after the RPA reaction in the garden.

Although several multiplex detection methods have been developed for plant pathogen identification (Table 5.2), in comparison with the published methods,[38,87,107,108,112,193] this approach is the fastest, yet has minimal cross reactivity and has comparable sensitivity to multiplex real-time PCR on the OpenArray™ platform. In addition, this approach is, to the best of our knowledge, the first multiplex detection method using RPA and SERS that has been successfully demonstrated outside of a laboratory setting for plant pathogens detection, underscoring its potential field application. Whilst the assay was simplified into a single tube reaction, a limitation of this method in its current form is the need for some simple sample manipulation. Nonetheless, with advances in engineering, we foresee the development of an integrated protocol which further minimizes pipetting steps and thus enable a simpler user friendly method.

***Table 5.2*:** Comparison of RPA/SERS technology with other multiplex detection methods

Technology	**Total assay time**	**Sensitivity**	**Cross reactivity**	**Tested outside of laboratory**
RPA/SERS	40 min	2.32×10^3 CN	X	√
Multiplex real-time PCR[38]	80 min	$10^{-5} – 10^{-4}$ ng/µL	X	X
Real-time PCR on OpenArrays[107]	75 min	$10^3 – 10^4$ CN	X	X
Microsphere immunoassay technology[108]	120 min	3×10^5 cfu/mL	√	X
Padlock probe[193]	>5 hrs	0.01 pg	√	X
PLP/DNA microarrays[87]	>15 hrs	5 pg	X	X
Molecular inversion probe[112]	>20 hrs	2.5 ng	X	X

5.4 Conclusions

In summary, a multiplex diagnostic platform for POC plant pathogen detection was developed using a synergistic combination of SERS and RPA. This method was applied to the detection of three economically important plant pathogens in *A. thaliana* and tomato. The assay was subsequently simplified into a single tube assay and tested outside of a laboratory setting to identify a specific pathogen in tomato plants. From sampling to results, the assay required only 40 min. In the near future, this SERS/RPA assay could have wide applications as a platform for POC multiplex nucleic acid diagnostics in both agriculture and medical applications.

Chapter 6
General conclusions and future work

6.1 General conclusion

One of the major goals of this thesis was to design robust diagnostic assays for POC testing that could be employed in the field to identify crop diseases. Farmers in resource-poor countries when confronted with a disease in their fields, lack technical knowledge and have virtually no access to government support in terms of technical expertise to deploy disease management strategies. The first and critical step towards devising any management strategy is to identify the disease as fast as possible, ideally on-field, without transporting samples to specialized laboratories and thus avoiding delayed interventions. In real field situations, the harsh environmental conditions (e.g. extreme temperatures, humidity, rain, wind, dust) are not ideal for any laboratory equipment; not to mention the lack of power supply.

To address the problem, this project has successfully developed diagnostics methods for POC testing and multiplex plant pathogen detection. The technologies developed include 1) An optimized DNA extraction method for POC purposes; 2) isothermal nucleic acid amplification for on-site application; 3) DNA biosensor for rapid plant pathogen detection; 4) novel naked-eye assay for DNA detection; 5) high capability multiplex detection method; and 6) field ready multiplex detection method. Summary of the four diagnostic methods was shown in Table 6.1.

One of the major contributions of this research towards POC diagnostic assay development for plant pathogens detection has been the establishment of a DNA/RNA extraction method which requires minimum equipment and is simple enough to be performed outside of a laboratory setting. In this study, a low cost DNA/RNA purification process was developed using common filtered pipette tips in conjunction with Solid Phase Reversible Immobilization (SPRI) technology to simultaneously extract and purify DNA/RNA to a precise concentration which can be directly used for isothermal amplification (Chapter 3). Magnetic SPRI bead-based extraction is ideal for POC applications because only a magnet is required and the maximum amount of purified nucleic acids is determined by the amount of SPRI beads used. This rapid and simple DNA/RNA extraction method was believed to significantly increase the application of nucleic acid based diagnostic methods in the field.

***Table 6.1*:** Comparison of four developed diagnostic methods.

Name of diagnostic method	**Time of assay**	**Sensitivity**	**Advantages**	**Disadvantages**
RPA-EC (Chapter 2)	~ 180 min	15 DNA copy number	- High specificity - Low cost - Portable - Quantitative assay	- Singleplex assay - Require skilled labor - Variation between different batches of electrodes -
Bridging flocculation assay (Chapter 3)	~ 90 min	NIL	- Low cost - Operate at low constant temperature - Minimal equipment - Simple steps - Rapid - Point-of-care detection	- Qualitative assay - Singleplex assay
MIP (Chapter 4)	~ 20 hrs	2.5 ng (3.86×10^4 DNA copy number)	- Highly multiplexable - High specificity	- Time consuming - Laboratory based assay
RPA-SERS (Chapter 5)	~ 40 min	2.32 DNA copy number	- High sensitivity - Multiplex assay - Rapid - Portable - Point-of-care detection - Quantitative assay	- Expensive SERS reader

In Chapters 2 and 3 the potential of RPA for sensitive and rapid isothermal nucleic acid amplification in plant pathogens detection was explored. RPA has many advantages which make it suitable for POC applications including low incubation temperature (37 °C – 42 °C), rapid amplification, PCR-like sensitivity and the freeze-dried pellet format which may be useful for applications in geographically remote areas as it does not need refrigeration. To this end, the suitability of RPA assays for rapid (20 min) and sensitive detection of plant pathogen DNA at the early infection stage was demonstrated successfully.

The combination of the rapid DNA extraction method and RPA to amplify the target sequences provides a good starting platform for POC assays but readout methods for the detection of the amplified DNA usually require relatively complex equipment. To address this point, an electrochemical DNA biosensor for plant pathogen detection was developed in this study (Chapter 2). Electrochemical DNA biosensors offer high sensitivity, rapid analysis and portability at potentially lower cost than traditional technologies. This method successfully detected *Pseudomonas syringae* genomic DNA with high sensitivity and reproducibility in infected plant samples, highlighting the potential of the assays for point of care applications.

To further simplify the readout method, a novel method was developed to quickly and inexpensively visualize amplified disease-specific DNA/RNA with minimal equipment via bridging flocculation using SPRI beads (Chapter 3). This bridging flocculation assay is a naked eye qualitative method for the detection of long DNA molecules and therefore does not pose interference from the primers used in the amplification reaction. As the distinction between diseased and healthy samples is binary, a readout method mirroring a digital yes/no result may be useful. This assay was first applied successfully to the detection of economically important plant pathogens and then extended to detect a suite of pathogens in a variety of sources. Considering the wide range of pathogens and samples demonstrated in this study, the assay was believed to have the potential for on-site, low resource applications.

Multiplex detection of plant pathogens was another focus for this study, although its use in POC applications is still out of our technological reach. A novel molecular inversion probe (MIP) assay was successfully developed that can be potentially developed into a robust multiplex platform to detect and identify plant pathogens simultaneously (Chapter 4). As proof of concept, a MIP was designed for *F. oxysporum* f.sp. *conglutinans* and it successfully detected as low as 2.5 ng of pathogen DNA with high specificity. This method can be a reliable alternative to the existing

pathogen detection and identification methods and the most important advantage of the MIP technology over other available methods is its innate capability for multiplexing using molecular inversion probes.

There are several limitations of MIP assays such as the requirement for several temperature settings throughout the assay and laborious process that makes them unsuitable for POC testing. Besides, the assays are time consuming taking more than 10 hrs to complete a reaction, making them only suitable for applications in the laboratory. Therefore, another multiplex detection method which has more potential for POC testing was developed. The newly developed technology with the combination of RPA and SERS has met the requirements of POC testing (Chapter 5). It is a rapid, highly specific and sensitive POC method for multiplex detection of plant pathogens. This technology was successfully tested in the multiplex detection of *B. cinerea*, *P. syringae* and *F. oxysporum* in *A. thaliana* and tomato. The original method was further modified into a rapid single-tube RPA/SERS assay and successfully performed assays outside of a laboratory setting to prove the potential for on-site field applications.

In summary, this thesis developed two diagnostic methods for highly sensitive and specific singleplex detections (RPA/electrochemistry) as well as naked eye assay (bridging flocculation assay) for resource poor settings. In order to achieve the multiplex detections strategy, two simultaneous multiplex detection technologies have been developed for high multiplex capability application (MIP assay) and rapid, specific assay for POC testing (RPA/SERS). The newly developed technologies were believed to bring significant improvements to plant disease management in the future.

6.2 Future work

There are still limitations of the methods in this study for POC application since they still encompass some sample manipulation, especially the pipetting steps that need to be performed using small volumes (1 – 30 µL). Further improvement of the technologies in the future should include the design of self-contained single use devices that would not require any pipetting and reduce the risk of sample contamination and therefore false positives.

Although the technologies have been used to detect pathogens in plant samples (*A. thaliana* and tomato), they have not yet been tested in real field samples to identify crop diseases. Bringing the developed technologies to commercial use will need collaboration between scientists,

manufacturers, government and end users. A good starting point to implement the technologies into a real scenario could be their use by enforcement bodies such as quarantine departments to monitor import/export operations or in border crossing. An effective disease management strategy is to firstly identify the disease and the potential impact of the disease before considering any suitable treatment to prevent the spreading of the disease and to safe the neighboring farms. Therefore, a specific and sensitive diagnostic method is essential in identifying the plant pathogens. However, the implementation of the new diagnostic methods is closely related to the national, state, local, industry and enterprise policies. Promotion of the new diagnostic methods can be organized concurrently with plant disease conferences or seminars to introduce the new methods to the public. Skill training should be given to the interested end users and the enforcement bodies are ready to help the farmers in troubleshooting when there are technically related problems during the pathogen identification process. Farmers are also well educated by the enforcement bodies to understand and apply the suitable disease treatments to control the disease outbreak. To the purpose of improving the diagnostic methods, it will also be crucial to obtain feedback from the end users for further improvement.

Development of miniature portable devices (MPDs) allowing to perform the entire process, from sampling to detection is another avenue that needs attention. MPDs can significantly reduce the contamination rate caused by human error and it is cost effective since they reduce the volumes of reagents and enzymes used during the DNA amplification and amplicons analysis processes. In addition, pre-stored reagents in the device may reduce the pipetting steps during the sample preparation which is an important issue for a POC diagnostic method. By combining the miniature portable device with electronic components might further improve the technology to achieve fully automatic electronic device and further simply the detection process.

References

1 Agrios, G. N. *Plant pathology*. 5th edn, (Elsevier Academic Press, 2005).

2 Punja, Z. K., De Boer, S. & Sanfaçon, H. l. n. *Biotechnology and plant disease management*. (Cabi Pub., 2007).

3 Horsfall, J. G. & Cowling, E. B. *Plant disease : an advanced treatise*. (Academic Press, 1977).

4 Koch, R. Untersuchungen über Bakterien: V. Die Ätiologie der Milzbrand-Krankheit, begründet auf die Entwicklungsgeschichte des Bacillus anthracis. *Cohns Beitrage zur Biologie der Pflanzen* **2**, 277-310 (1876).

5 Koch, R. *Die Ätiologie der Milzbrand-Krankheit*. (Zentralantiquariat der Deutschen Demokratischen Republik, 1968).

6 Alvarez, A. M. Integrated approaches for detection of plant pathogenic bacteria and diagnosis of bacterial diseases. *Annu Rev Phytopathol* **42**, 339-366, doi:10.1146/annurev.phyto.42.040803.140329 (2004).

7 Narayanasamy, P. *Microbial plant pathogens-detection and disease diagnosis*. (Springer, 2011).

8 Macario, A. J. L. & Conway de Macario, E. *Monoclonal antibodies against bacteria*. (Academic Press, 1985).

9 Thornton, C. R. Production of monoclonal antibodies to plant pathogens. *Methods in molecular biology* **508**, 63-74, doi:10.1007/978-1-59745-062-1_6 (2009).

10 Kokoskova, B. & Janse, J. D. Enzyme-linked immunosorbent assay for the detection and identification of plant pathogenic bacteria (in particular for Erwinia amylovora and Clavibacter michiganensis subsp. sepedonicus). *Methods in molecular biology* **508**, 75-87, doi:10.1007/978-1-59745-062-1_7 (2009).

11 Comstock, J. C. Detection of the Sugarcane Leaf Scald Pathogen, Xanthomonas-Albilineans, Using Tissue Blot Immunoassay, Elisa, and Isolation Techniques. *Plant Dis* **76**, 1033-1035 (1992).

12 Novakova, S., Klaudiny, J., Kollerova, E. & Subr, Z. W. Expression of a part of the Potato virus A non-structural protein P3 in Escherichia coli for the purpose of antibody preparation and P3 immunodetection in plant material. *Journal of virological methods* **137**, 229-235, doi:10.1016/j.jviromet.2006.06.020 (2006).

13 Malin, E. M., Roth, D. A. & Belden, E. L. Indirect Immunofluorescent Staining for Detection and Identification of Xanthomonas-Campestris Pv Phaseoli in Naturally Infected Bean Seed. *Plant Dis* **67**, 645-647, doi:Doi 10.1094/Pd-67-645 (1983).

14 Tomlinson, J. A., Dickinson, M. J. & Boonham, N. Rapid detection of Phytophthora ramorum and P. kernoviae by two-minute DNA extraction followed by isothermal amplification and amplicon detection by generic lateral flow device. *Phytopathology* **100**, 143-149, doi:10.1094/PHYTO-100-2-0143 (2010).

15 Teitelbaum, D., Aharoni, R., Sela, M. & Arnon, R. Cross-reactions and specificities of monoclonal antibodies against myelin basic protein and against the synthetic copolymer 1. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 9528-9532 (1991).

16 Gorris, M. T., Alarcon, B., Lopez, M. M. & Cambra, M. Characterization of Monoclonal Antibodies Specific for Erwinia carotovora subsp. atroseptica and Comparison of Serological Methods for Its Sensitive Detection on Potato Tubers. *Appl Environ Microbiol* **60**, 2076-2085 (1994).

17 Vincelli, P. & Tisserat, N. Nucleic acid-based pathogen detection in applied plant pathology. *Plant Dis* **92**, 660-669, doi:Doi 10.1094/Pdis-92-5-0660 (2008).

18 Ward, E., Foster, S. J., Fraaije, B. A. & McCartney, H. A. Plant pathogen diagnostics: immunological and nucleic acid-based approaches. *Ann Appl Biol* **145**, 1-16, doi:DOI 10.1111/j.1744-7348.2004.tb00354.x (2004).

19 Brasileiro, B. T. R. V., Coimbra, M. R. M., de Morais, M. A. & de Oliveira, N. T. Genetic variability within Fusarium solani specie as revealed by PCR-fingerprinting based on PCR markers. *Braz J Microbiol* **35**, 205-210, doi:Doi 10.1590/S1517-83822004000200006 (2004).

20 Carrasco-Ballesteros, S., Castillo, P., Adams, B. J. & Perez-Artes, E. Identification of Pratylenchus thornei, the cereal and legume root-lesion nematode, based on SCAR-PCR and satellite DNA. *Eur J Plant Pathol* **118**, 115-125, doi:DOI 10.1007/s10658-007-9110-3 (2007).

21 Merighi, M. *et al.* Chemiluminescent and colorimetric detection of Erwinia amylovora by immunoenzymatic determination of PCR amplicons from plasmid pEA29. *Plant Dis* **84**, 49-54, doi:Doi 10.1094/Pdis.2000.84.1.49 (2000).

22 Wetzel, T., Candresse, T., Macquaire, G., Ravelonandro, M. & Dunez, J. A Highly Sensitive Immunocapture Polymerase Chain-Reaction Method for Plum Pox Potyvirus Detection. *J Virol Methods* **39**, 27-37, doi:Doi 10.1016/0166-0934(92)90122-T (1992).

23 Khoodoo, M. H. R., Sahin, F. & Jaufeerally-Fakim, Y. Sensitive detection of Xanthomonas axonopodis pv. dieffenbachiae on Anthurium andreanum by immunocapture-PCR (IC-PCR) using primers designed from sequence characterized amplified regions (SCAR) of the blight pathogen. *Eur J Plant Pathol* **112**, 379-390, doi:DOI 10.1007/s10658-005-7062-z (2005).

24 Nutman, T. B., Zimmerman, P. A., Kubofcik, J. & Kostyu, D. D. A Universally Applicable Diagnostic-Approach to Filarial and Other Infections. *Parasitol Today* **10**, 239-243, doi:Doi 10.1016/0169-4758(94)90127-9 (1994).

25 Laitinen, R., Malinen, E. & Palva, A. PCR-ELISA I: Application to simultaneous analysis of mixed bacterial samples composed of intestinal species. *Systematic and applied microbiology* **25**, 241-248, doi:10.1078/0723-2020-00118 (2002).

26 Shindo, M. *et al.* Detection and Quantitation of Hepatitis-C Virus-Rna in Serum Using the Polymerase Chain-Reaction and a Colorimetric Enzymatic Detection System. *J Virol Methods* **48**, 65-72, doi:Doi 10.1016/0166-0934(94)90089-2 (1994).

27 Daly, P., Collier, T. & Doyle, S. PCR-ELISA detection of Escherichia coli in milk. *Lett Appl Microbiol* **34**, 222-226, doi:DOI 10.1046/j.1472-765x.2002.01074.x (2002).

28 Bailey, A. M., Mitchell, D. J., Manjunath, K. L., Nolasco, G. & Niblett, C. L. Identification to the species level of the plant pathogens Phytophthora and Pythium by using unique sequences of the ITS1 region of ribosomal DNA as capture probes for PCR ELISA. *Fems Microbiol Lett* **207**, 153-158, doi:Pii S0378-1097(01)00470-0, Doi 10.1111/J.1574-6968.2002.Tb11044.X (2002).

29 Somai, B. M., Keinath, A. P. & Dean, R. A. Development of PCR-ELISA for detection and differentiation of Didymella bryoniae from related Phoma species. *Plant Dis* **86**, 710-716, doi:Unsp D-2002-0521-01r, Doi 10.1094/Pdis.2002.86.7.710 (2002).

30 Borrow, R., Guiver, M., Sadler, F., Kaczmarski, E. B. & Fox, A. J. False positive diagnosis of meningococcal infection by the IS1106 PCR ELISA. *Fems Microbiol Lett* **162**, 215-218, doi:DOI 10.1111/j.1574-6968.1998.tb13001.x (1998).

31 Gillespie, S. H., McHugh, T. D. & Newport, L. E. Specificity of IS6110-based amplification assays for Mycobacterium tuberculosis complex. *J Clin Microbiol* **35**, 799-800 (1997).

32 Kent, L., Mchugh, T. D., Billington, O., Dale, J. W. & Gillespie, S. H. Demonstration of Homology between Is6110 of Mycobacterium-Tuberculosis and Dnas of Other Mycobacterium Spp (Vol 33, Pg 2290, 1995). *J Clin Microbiol* **33**, 3082-3082 (1995).

33 Heid, C. A., Stevens, J., Livak, K. J. & Williams, P. M. Real time quantitative PCR. *Genome research* **6**, 986-994, doi:Doi 10.1101/Gr.6.10.986 (1996).

34 Nowrouzian, F. L., Adlerberth, I. & Wold, A. E. High frequency of false-positive signals in a real-time PCR-based "Plus/Minus" assay. *Apmis* **117**, 68-72, doi:DOI 10.1111/j.1600-0463.2008.00010.x (2009).

35 Wong, M. L. & Medrano, J. F. Real-time PCR for mRNA quantitation. *Biotechniques* **39**, 75-85, doi:Doi 10.2144/05391rv01 (2005).

36 Araujo, R., Amorim, A. & Gusmao, L. Diversity and specificity of microsatellites within Aspergillus section Fumigati. *Bmc Microbiol* **12**, doi:Artn 154, Doi 10.1186/1471-2180-12-154 (2012).

37 Dai, J., Peng, H., Chen, W., Cheng, J. & Wu, Y. Development of multiplex real-time PCR for simultaneous detection of three Potyviruses in tobacco plants. *J Appl Microbiol* **114**, 502-508, doi:10.1111/jam.12071 (2013).

38 Price, J. A., Smith, J., Simmons, A., Fellers, J. & Rush, C. M. Multiplex real-time RT-PCR for detection of Wheat streak mosaic virus and Tritcum mosaic virus. *J Virol Methods* **165**, 198-201, doi:10.1016/j.jviromet.2010.01.019 (2010).

39 Markoulatos, P., Siafakas, N. & Moncany, M. Multiplex polymerase chain reaction: a practical approach. *Journal of clinical laboratory analysis* **16**, 47-51 (2002).

40 Sint, D., Raso, L. & Traugott, M. Advances in multiplex PCR: balancing primer efficiencies and improving detection success. *Methods in ecology and evolution / British Ecological Society* **3**, 898-905, doi:10.1111/j.2041-210X.2012.00215.x (2012).

41 Yager, P. *et al.* Microfluidic diagnostic technologies for global public health. *Nature* **442**, 412-418, doi:10.1038/nature05064 (2006).

42 Barany, F. The ligase chain reaction in a PCR world. *PCR Methods Appl* **1**, 5-16 (1991).

43 Craw, P. & Balachandran, W. Isothermal nucleic acid amplification technologies for point-of-care diagnostics: a critical review. *Lab Chip* **12**, 2469-2486 (2012).

44 Gill, P. & Ghaemi, A. Nucleic acid isothermal amplification technologies - A review. *Nucleos Nucleot Nucl* **27**, 224-243 (2008).

45 Piepenburg, O., Williams, C. H., Stemple, D. L. & Armes, N. A. DNA detection using recombination proteins. *Plos Biol* **4**, 1115-1121, doi:ARTN e204, DOI 10.1371/journal.pbio.0040204 (2006).

46 Vincent, M., Xu, Y. & Kong, H. M. Helicase-dependent isothermal DNA amplification. *Embo Rep* **5**, 795-800, doi:DOI 10.1038/sj.embor.7400200 (2004).

47 Notomi, T. *et al.* Loop-mediated isothermal amplification of DNA. *Nucleic acids research* **28**, doi:ARTN e63, DOI 10.1093/nar/28.12.e63 (2000).

48 Rohrman, B. A. & Richards-Kortum, R. R. A paper and plastic device for performing recombinase polymerase amplification of HIV DNA. *Lab Chip* **12**, 3082-3088, doi:Doi 10.1039/C2lc40423k (2012).

49 Lutz, S. *et al.* Microfluidic lab-on-a-foil for nucleic acid analysis based on isothermal recombinase polymerase amplification (RPA). *Lab on a chip* **10**, 887-893, doi:10.1039/b921140c (2010).

50 Mahalanabis, M., Do, J., ALMuayad, H., Zhang, J. Y. & Klapperich, C. M. An integrated disposable device for DNA extraction and helicase dependent amplification. *Biomed Microdevices* **12**, 353-359 (2010).

51 Chow, W. H. A. *et al.* Application of isothermal helicase-dependent amplification with a disposable detection device in a simple sensitive stool test for toxigenic Clostridium difficile. *J Mol Diagn* **10**, 452-458 (2008).

52 Ikeda, S. *et al.* Evaluation of the effects of different additives in improving the DNA extraction yield and quality from Andosol. *Microbes Environ* **23**, 159-166, doi:10.1264/jsme2.23.159 (2008).

53 Tomlinson, J. A., Boonham, N. & Dickinson, M. Development and evaluation of a one-hour DNA extraction and loop-mediated isothermal amplification assay for rapid detection of phytoplasmas. *Plant Pathol* **59**, 465-471, doi:10.1111/j.1365-3059.2009.02233.x (2010).

54 Tomlinson, J. A. *et al.* A five-minute DNA extraction method for expedited detection of Phytophthora ramorum following prescreening using Phytophthora spp. lateral flow devices. *J Microbiol Meth* **81**, 116-120, doi:10.1016/j.mimet.2010.02.006 (2010).

55 Wee, E. J. H., Lau, H. Y., Botella, J. R. & Trau, M. Re-purposing bridging flocculation for on-site, rapid, qualitative DNA detection in resource-poor settings. *Chem Commun* **51**, 5828-5831, doi:10.1039/c4cc10068a (2015).

56 Deangelis, M. M., Wang, D. G. & Hawkins, T. L. Solid-Phase Reversible Immobilization for the Isolation of Pcr Products. *Nucleic acids research* **23**, 4742-4743, doi:DOI 10.1093/nar/23.22.4742 (1995).

57 Ng, B. Y. C., Wee, E. J. H., West, N. P. & Trau, M. Rapid DNA detection of Mycobacterium tuberculosis-towards single cell sensitivity in point-of-care diagnosis. *Sci Rep-Uk* **5**, doi:Artn 15027, 10.1038/Srep15027 (2015).

58 White, T. J., Arnheim, N. & Erlich, H. A. The Polymerase Chain-Reaction. *Trends Genet* **5**, 185-189, doi:Doi 10.1016/0168-9525(89)90073-5 (1989).

59 Fukuta, S. *et al.* Development of loop-mediated isothermal amplification assay for the detection of Pythium myriotylum. *Lett Appl Microbiol* **59**, 49-57, doi:Doi 10.1111/Lam.12244 (2014).

60 Hadersdorfer, J., Neumuller, M., Treutter, D. & Fischer, T. C. Fast and reliable detection of Plum pox virus in woody host plants using the Blue LAMP protocol. *Ann Appl Biol* **159**, 456-466, doi:DOI 10.1111/j.1744-7348.2011.00510.x (2011).

61 Tomlinson, J. A., Dickinson, M. J. & Boonham, N. Detection of Botrytis cinerea by loop-mediated isothermal amplification. *Lett Appl Microbiol* **51**, 650-657, doi:DOI 10.1111/j.1472-765X.2010.02949.x (2010).

62 Bhat, A. I., Siljo, A. & Deeshma, K. P. Rapid detection of Piper yellow mottle virus and Cucumber mosaic virus infecting black pepper (Piper nigrum) by loop-mediated isothermal amplification (LAMP). *J Virol Methods* **193**, 190-196, doi:DOI 10.1016/j.jviromet.2013.06.012 (2013).

63 Lee, M.-F., Chen, J.-Y. & Peng, C.-F. Study on the LAMP-PCR-hybridization-thermal melt-ELISA method for molecular detection of multidrug resistance in Mycobacterium tuberculosis isolates. *Biomarkers and Genomic Medicine* **6**, 126-132 (2014).

64 Watthanapanpituck, K., Kiatpathomchai, W., Chu, E. & Panvisavas, N. Identification of human DNA in forensic evidence by loop-mediated isothermal amplification combined with a colorimetric gold nanoparticle hybridization probe. *International journal of legal medicine* **128**, 923-931, doi:10.1007/s00414-014-1018-9 (2014).

65 Nagamine, K., Kuzuhara, Y. & Notomi, T. Isolation of single-stranded DNA from loop-mediated isothermal amplification products. *Biochem Biophys Res Commun* **290**, 1195-1198, doi:10.1006/bbrc.2001.6334 (2002).

66 Tomita, N., Mori, Y., Kanda, H. & Notomi, T. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nat Protoc* **3**, 877-882, doi:10.1038/nprot.2008.57 (2008).

67 Duan, Y. B., Ge, C. Y., Zhang, X. K., Wang, J. X. & Zhou, M. G. A rapid detection method for the plant pathogen Sclerotinia sclerotiorum based on loop-mediated isothermal amplification (LAMP). *Australas Plant Path* **43**, 61-66, doi:DOI 10.1007/s13313-013-0239-6 (2014).

68 Chen, X. Y. *et al.* Endpoint Visual Detection of Three Genetically Modified Rice Events by Loop-Mediated Isothermal Amplification. *Int J Mol Sci* **13**, 14421-14433, doi:DOI 10.3390/ijms131114421 (2012).

69 Ahmadi, S., Almasi, M. A., Fatehi, F., Struik, P. C. & Moradi, A. Visual Detection of Potato leafroll virus by One-step Reverse Transcription Loop-Mediated Isothermal Amplification of DNA with Hydroxynaphthol Blue Dye. *J Phytopathol* **161**, 120-124, doi:Doi 10.1111/Jph.12037 (2013).

70 Zhang, X. *et al.* Development of a Real-Time Fluorescence Loop-Mediated Isothermal Amplification Assay for Rapid and Quantitative Detection of Fusarium oxysporum f. sp cubense Tropical Race 4 In Soil. *PloS one* **8**, doi:ARTN e82841, DOI 10.1371/journal.pone.0082841 (2013).

71 Ravan, H. & Yazdanparast, R. Development and evaluation of a loop-mediated isothermal amplification method in conjunction with an enzyme-linked immunosorbent assay for

specific detection of Salmonella serogroup D. *Anal Chim Acta* **733**, 64-70, doi:DOI 10.1016/j.aca.2012.04.034 (2012).

72 Ferguson, B. S. *et al.* Integrated Microfluidic Electrochemical DNA Sensor. *Anal Chem* **81**, 6503-6508, doi:Doi 10.1021/Ac900923e (2009).

73 Hsieh, K. W., Patterson, A. S., Ferguson, B. S., Plaxco, K. W. & Soh, H. T. Rapid, Sensitive, and Quantitative Detection of Pathogenic DNA at the Point of Care through Microfluidic Electrochemical Quantitative Loop-Mediated Isothermal Amplification. *Angew Chem Int Edit* **51**, 4896-4900, doi:DOI 10.1002/anie.201109115 (2012).

74 Guatelli, J. C. *et al.* Isothermal, Invitro Amplification of Nucleic-Acids by a Multienzyme Reaction Modeled after Retroviral Replication. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 1874-1878, doi:DOI 10.1073/pnas.87.5.1874 (1990).

75 Ail, J. A., Maluf, N. K. & Lohman, T. M. An oligomeric form of E-coli UvrD is required for optimal helicase activity. *J Mol Biol* **293**, 815-834 (1999).

76 Mechanic, L. E., Frankel, B. A. & Matson, S. W. Escherichia coli MutL loads DNA helicase II onto DNA. *J Biol Chem* **275**, 38337-38346, doi:DOI 10.1074/jbc.M006268200 (2000).

77 An, L. X. *et al.* Characterization of a thermostable UvrD helicase and its participation in helicase-dependent amplification. *J Biol Chem* **280**, 28952-28958, doi:DOI 10.1074/jbc.M503096200 (2005).

78 Faizul Rahman, S., Nor Amalina, Z., Asma, I. & Aziah, I. Asymmetric helicase-dependent amplification combined with lateral flow assay (HDA-LFA) for the detection of S. Paratyphi A. *Asian Pac J Trop Disease* **4**, 243, doi:10.1016/S2222-1808(14)60551-X (2014).

79 Huang, S. C. *et al.* Low Cost Extraction and Isothermal Amplification of DNA for Infectious Diarrhea Diagnosis. *PloS one* **8**, doi:ARTN e60059, DOI 10.1371/journal.pone.0060059 (2013).

80 Eckert, C., Holscher, E., Petit, A., Lalande, V. & Barbut, F. Molecular Test Based on Isothermal Helicase-Dependent Amplification for Detection of the Clostridium difficile Toxin A Gene. *J Clin Microbiol* **52**, 2386-2389, doi:Doi 10.1128/Jcm.00353-14 (2014).

81 Artiushin, S. *et al.* Thermophilic helicase-dependent DNA amplification using the IsoAmp (TM) SE experimental kit for rapid detection of Streptococcus equi subspecies equi in clinical samples. *J Vet Diagn Invest* **23**, 909-914, doi:Doi 10.1177/1040638711416968 (2011).

82 Corona, F. M. O., Arif, M., Caasi, D. & Daniels, J. Application of helicase dependent amplification, 5' non-complementary a/t rich sequences and optimal thermodynamics during

primer design for increased isothermal pcr detection. *Conference of International Organization of Citrus Virulogists* **18**, 70 (2010).

83 Gill, P. *et al.* Detection of Helicobacter pylori by enzyme-linked immunosorbent assay of thermophilic helicase-dependent isothermal DNA amplification. *Diagn Micr Infec Dis* **59**, 243-249, doi:DOI 10.1016/j.diagmicrobio.2007.05.005 (2007).

84 Gill, P., Alvandi, A. H., Abdul-Tehrani, H. & Sadeghizadeh, M. Colorimetric detection of Helicobacter pylori DNA using isothermal helicase-dependent amplification and gold nanoparticle probes. *Diagn Micr Infec Dis* **62**, 119-124, doi:DOI 10.1016/j.diagmicrobio.2008.05.003 (2008).

85 Fire, A. & Xu, S. Q. Rolling Replication of Short DNA Circles. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 4641-4645, doi:DOI 10.1073/pnas.92.10.4641 (1995).

86 Blanco, L. *et al.* Highly Efficient DNA-Synthesis by the Phage Phi-29 DNA-Polymerase - Symmetrical Mode of DNA-Replication. *J Biol Chem* **264**, 8935-8940 (1989).

87 Szemes, M. *et al.* Diagnostic application of padlock probes-multiplex detection of plant pathogens using universal microarrays. *Nucleic acids research* **33**, doi:ARTN e70, DOI 10.1093/nar/gni069 (2005).

88 Tian, Y. L. *et al.* Simultaneous Detection of Xanthomonas oryzae pv. oryzae and X. oryzae pv. oryzicola in Rice Seed Using a Padlock Probe-Based Assay. *Phytopathology* **104**, 1130-1137, doi:Doi 10.1094/Phyto-10-13-0274-R (2014).

89 Slawiak, M. *et al.* Multiplex detection and identification of bacterial pathogens causing potato blackleg and soft rot in Europe, using padlock probes. *Ann Appl Biol* **163**, 378-393, doi:Doi 10.1111/Aab.12075 (2013).

90 Baner, J., Nilsson, M., Mendel-Hartvig, M. & Landegren, U. Signal amplification of padlock probes by rolling circle replication. *Nucleic acids research* **26**, 5073-5078, doi:DOI 10.1093/nar/26.22.5073 (1998).

91 Lizardi, P. M. *et al.* Mutation detection and single-molecule counting using isothermal rolling-circle amplification. *Nat Genet* **19**, 225-232, doi:Doi 10.1038/898 (1998).

92 Nallur, G. *et al.* Signal amplification by rolling circle amplification on DNA microarrays. *Nucleic acids research* **29**, art. no.-e118, doi:DOI 10.1093/nar/29.23.e118 (2001).

93 Russell, C. *et al.* Gold Nanowire Based Electrical DNA Detection Using Rolling Circle Amplification. *Acs Nano* **8**, 1147-1153, doi:Doi 10.1021/Nn4058825 (2014).

94 Gusev, Y. *et al.* Rolling circle amplification - A new approach to increase sensitivity for immunohistochemistry and flow cytometry. *Am J Pathol* **159**, 63-69, doi:Doi 10.1016/S0002-9440(10)61674-4 (2001).

95 Kobori, T. & Takahashi, H. Expanding Possibilities of Rolling Circle Amplification as a Biosensing Platform. *Anal Sci* **30**, 59-64 (2014).

96 Schubert, J., Habekuss, A., Kazmaier, K. & Jeske, H. Surveying cereal-infecting geminiviruses in Germany - Diagnostics and direct sequencing using rolling circle amplification. *Virus Res* **127**, 61-70, doi:DOI 10.1016/j.virusres.2007.03.018 (2007).

97 Davari, M. *et al.* Rapid identification of Fusarium graminearum species complex using Rolling Circle Amplification (RCA). *J Microbiol Meth* **89**, 63-70, doi:DOI 10.1016/j.mimet.2012.01.017 (2012).

98 Najafzadeh, M. J., Sun, J., Vicente, V. A. & de Hoog, G. S. Rapid identification of fungal pathogens by rolling circle amplification using Fonsecaea as a model. *Mycoses* **54**, E577-E582, doi:DOI 10.1111/j.1439-0507.2010.01995.x (2011).

99 Li, X. J. *et al.* Genotyping of multiple single nucleotide polymorphisms with hyperbranched rolling circle amplification and microarray. *Clin Chim Acta* **399**, 40-44, doi:DOI 10.1016/j.cca.2008.08.012 (2009).

100 Ji, H. X., Yan, F., Lei, J. P. & Ju, H. X. Ultrasensitive Electrochemical Detection of Nucleic Acids by Template Enhanced Hybridization Followed with Rolling Circle Amplification. *Anal Chem* **84**, 7166-7171, doi:Doi 10.1021/Ac3015356 (2012).

101 Boyle, D. S. *et al.* Rapid Detection of Mycobacterium tuberculosis by Recombinase Polymerase Amplification. *PloS one* **9**, doi:ARTN e103091, DOI 10.1371/journal.pone.0103091 (2014).

102 Crannell, Z. A., Rohrman, B. & Richards-Kortum, R. Quantification of HIV-1 DNA Using Real-Time Recombinase Polymerase Amplification. *Anal Chem* **86**, 5615-5619, doi:Doi 10.1021/Ac5011298 (2014).

103 Kersting, S., Rausch, V., Bier, F. F. & von Nickisch-Rosenegk, M. Rapid detection of Plasmodium falciparum with isothermal recombinase polymerase amplification and lateral flow analysis. *Malaria J* **13**, doi:Artn 99, Doi 10.1186/1475-2875-13-99 (2014).

104 Mekuria, T. A., Zhang, S. L. & Eastwell, K. C. Rapid and sensitive detection of Little cherry virus 2 using isothermal reverse transcription-recombinase polymerase amplification. *J Virol Methods* **205**, 24-30, doi:DOI 10.1016/j.jviromet.2014.04.015 (2014).

105 Zhang, S. L. *et al.* Rapid diagnostic detection of plum pox virus in Prunus plants by isothermal AmplifyRP (R) using reverse transcription-recombinase polymerase amplification. *J Virol Methods* **207**, 114-120, doi:DOI 10.1016/j.jviromet.2014.06.026 (2014).

106 Santiago-Felipe, S., Tortajada-Genaro, L. A., Puchades, R. & Maquieira, A. Recombinase polymerase and enzyme-linked immunosorbent assay as a DNA amplification-detection

strategy for food analysis. *Anal Chim Acta* **811**, 81-87, doi:DOI 10.1016/j.aca.2013.12.017 (2014).

107 van Doorn, R. *et al.* Quantitative multiplex detection of plant pathogens using a novel ligation probe-based system coupled with universal, high-throughput real-time PCR on OpenArrays (TM). *BMC genomics* **8**, doi:Artn 276, 10.1186/1471-2164-8-276 (2007).

108 Charlermroj, R. *et al.* Multiplex Detection of Plant Pathogens Using a Microsphere Immunoassay Technology. *PloS one* **8**, doi:ARTN e62344, 10.1371/journal.pone.0062344 (2013).

109 Diep, D. *et al.* Library-free methylation sequencing with bisulfite padlock probes. *Nature methods* **9**, 270-272, doi:10.1038/nmeth.1871 (2012).

110 Hardenbol, P. *et al.* Multiplexed genotyping with sequence-tagged molecular inversion probes. *Nature biotechnology* **21**, 673-678, doi:Doi 10.1038/Nbt821 (2003).

111 Wang, Y. K. *et al.* Allele quantification using molecular inversion probes (MIP). *Nucleic acids research* **33**, doi:ARTN e183, DOI 10.1093/nar/gni177 (2005).

112 Lau, H. Y., Palanisamy, R., Trau, M. & Botella, J. R. Molecular inversion probe: a new tool for highly specific detection of plant pathogens. *PloS one* **9**, e111182, doi:10.1371/journal.pone.0111182 (2014).

113 Boyle, E. A., O'Roak, B. J., Martin, B. K., Kumar, A. & Shendure, J. MIPgen: optimized modeling and design of molecular inversion probes for targeted resequencing. *Bioinformatics* **30**, 2670-2672, doi:10.1093/bioinformatics/btu353 (2014).

114 Stefan, C. P., Koehler, J. W. & Minogue, T. D. Targeted next-generation sequencing for the detection of ciprofloxacin resistance markers using molecular inversion probes. *Sci Rep-Uk* **6**, 25904, doi:10.1038/srep25904 http://www.nature.com/articles/srep25904#supplementary-information (2016).

115 Carrascosa, L. G. *et al.* Molecular inversion probe-based SPR biosensing for specific, label-free and real-time detection of regional DNA methylation. *Chem Commun* **50**, 3585-3588, doi:Doi 10.1039/C3cc49607d (2014).

116 Lin, S. R., Wang, W. Y., Palm, C., Davis, R. W. & Juneau, K. A molecular inversion probe assay for detecting alternative splicing. *BMC genomics* **11**, doi:Artn 712, Doi 10.1186/1471-2164-11-712 (2010).

117 Wang, Y., Cottman, M. & Schiffman, J. D. Molecular inversion probes: a novel microarray technology and its application in cancer research. *Cancer Genet-Ny* **205**, 341-355, doi:DOI 10.1016/j.cancergen.2012.06.005 (2012).

118 Asiello, P. J. & Baeumner, A. J. Miniaturized isothermal nucleic acid amplification, a review. *Lab on a chip* **11**, 1420-1430, doi:10.1039/c0lc00666a (2011).

119 Franken, A. A. J. M., Zilverentant, J. F., Boonekamp, P. M. & Schots, A. Specificity of Polyclonal and Monoclonal-Antibodies for the Identification of Xanthomonas-Campestris Pv Campestris. *Netherlands Journal of Plant Pathology* **98**, 81-94, doi:Doi 10.1007/Bf01996321 (1992).

120 Murphy, K., Travers, P., Walport, M. & Janeway, C. *Janeway's immunobiology*. 8th edn, (Garland Science, 2012).

121 Miotke, L., Lau, B. T., Rumma, R. T. & Ji, H. P. High sensitivity detection and quantitation of DNA copy number and single nucleotide variants with single color droplet digital PCR. *Anal Chem* **86**, 2618-2624, doi:10.1021/ac403843j (2014).

122 Hindson, B. J. *et al.* High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Anal Chem* **83**, 8604-8610, doi:10.1021/ac202028g (2011).

123 Wee, E. J. H., Ngo, T. H. & Trau, M. Colorimetric detection of both total genomic and loci-specific DNA methylation from limited DNA inputs. *Clin Epigenetics* **7**, doi:ARTN 65, 10.1186/s13148-015-0100-6 (2015).

124 Liljander, A. *et al.* Field-Applicable Recombinase Polymerase Amplification Assay for Rapid Detection of Mycoplasma capricolum subsp capripneumoniae. *J Clin Microbiol* **53**, 2810-2815, doi:10.1128/Jcm.00623-15 (2015).

125 Abd El Wahed, A. *et al.* Recombinase Polymerase Amplification Assay for Rapid Diagnostics of Dengue Infection. *PloS one* **10**, doi:ARTN e0129682, 10.1371/journal.pone.0129682 (2015).

126 Turner, A. P. Tech.Sight. Biochemistry. Biosensors--sense and sensitivity. *Science* **290**, 1315-1317 (2000).

127 Drummond, T. G., Hill, M. G. & Barton, J. K. Electrochemical DNA sensors. *Nature biotechnology* **21**, 1192-1199, doi:10.1038/nbt873 (2003).

128 Lin, C. S. *et al.* An electrochemical biosensor for the sensitive detection of specific DNA based on a dual-enzyme assisted amplification. *Electrochim Acta* **147**, 785-790, doi:10.1016/j.electacta.2014.10.092 (2014).

129 Yola, M. L., Eren, T. & Atar, N. A novel and sensitive electrochemical DNA biosensor based on Fe@Au nanoparticles decorated graphene oxide. *Electrochim Acta* **125**, 38-47, doi:10.1016/j.electacta.2014.01.074 (2014).

130 Wang, Z. *et al.* Electrocatalytic oxidation of phytohormone salicylic acid at copper nanoparticles-modified gold electrode and its detection in oilseed rape infected with fungal pathogen Sclerotinia sclerotiorum. *Talanta* **80**, 1277-1281, doi:10.1016/j.talanta.2009.09.023 (2010).

131 Vetrone, S. A., Huarng, M. C. & Alocilja, E. C. Detection of Non-PCR Amplified S. enteritidis Genomic DNA from Food Matrices Using a Gold-Nanoparticle DNA Biosensor: A Proof-of-Concept Study. *Sensors-Basel* **12**, 10487-10499, doi:10.3390/s120810487 (2012).

132 Kleijn, S. E. F., Lai, S. C. S., Koper, M. T. M. & Unwin, P. R. Electrochemistry of Nanoparticles. *Angew Chem Int Edit* **53**, 3558-3586, doi:10.1002/anie.201306828 (2014).

133 Riley, D. J. Electrochemistry in nanoparticle science. *Curr Opin Colloid In* **7**, 186-192, doi:Pii S1359-0294(02)00047-X, Doi 10.1016/S1359-0294(02)00047-X (2002).

134 Luo, X. L., Morrin, A., Killard, A. J. & Smyth, M. R. Application of nanoparticles in electrochemical sensors and biosensors. *Electroanal* **18**, 319-326, doi:10.1002/elan.200503415 (2006).

135 Lin, L., Liu, Y., Tang, L. H. & Li, J. H. Electrochemical DNA sensor by the assembly of graphene and DNA-conjugated gold nanoparticles with silver enhancement strategy. *Analyst* **136**, 4732-4737, doi:10.1039/c1an15610a (2011).

136 Liu, G. D. *et al.* Aptamer-Nanoparticle Strip Biosensor for Sensitive Detection of Cancer Cells. *Anal Chem* **81**, 10013-10018, doi:10.1021/ac901889s (2009).

137 Wang, W. T., Fan, X. J., Xu, S. H., Davis, J. J. & Luo, X. L. Low fouling label-free DNA sensor based on polyethylene glycols decorated with gold nanoparticles for the detection of breast cancer biomarkers. *Biosens Bioelectron* **71**, 51-56, doi:10.1016/j.bios.2015.04.018 (2015).

138 Iacobellis, N. S. *Pseudomonas syringae and related pathogens : biology and genetic*. (Kluwer Academic Publishers, 2003).

139 Katagiri, F., Thilmony, R. & He, S. Y. The Arabidopsis thaliana-pseudomonas syringae interaction. *The Arabidopsis book / American Society of Plant Biologists* **1**, e0039, doi:10.1199/tab.0039 (2002).

140 Trusov, Y. *et al.* Heterotrimeric G proteins facilitate Arabidopsis resistance to necrotrophic pathogens and are involved in jasmonate signaling. *Plant Physiol* **140**, 210-220 (2006).

141 Rohland, N. & Reich, D. Cost-effective, high-throughput DNA sequencing libraries for multiplexed target capture. *Genome research* **22**, 939-946, doi:10.1101/gr.128124.111 (2012).

142 Frens, G. Controlled Nucleation for Regulation of Particle-Size in Monodisperse Gold Suspensions. *Nature-Phys Sci* **241**, 20-22 (1973).

143 Hawkins, T. L., Oconnormorin, T., Roy, A. & Santillan, C. DNA Purification and Isolation Using a Solid-Phase. *Nucleic acids research* **22**, 4543-4544, doi:DOI 10.1093/nar/22.21.4543 (1994).

144 Janse, J. D. & Kokoskova, B. Indirect immunofluorescence microscopy for the detection and identification of plant pathogenic bacteria (in particular for Ralstonla solanacearum). *Methods in molecular biology* **508**, 89-99 (2009).

145 Price, J. A., Smith, J., Simmons, A., Fellers, J. & Rush, C. M. Multiplex real-time RT-PCR for detection of Wheat streak mosaic virus and Triticum mosaic virus. *Journal of virological methods* **165**, 198-201, doi:10.1016/j.jviromet.2010.01.019 (2010).

146 Wang, Z. H. *et al.* Development of an ID-ELISA for the detection of Rice black-streaked dwarf virus in plants. *Journal of virological methods* **134**, 61-65, doi:10.1016/j.jviromet.2005.11.019 (2006).

147 Heiny, D. K. G., D.G. Enzyme-linked immunosorbent assay, immunoblot detection, and antibody neutralization of Stemphylium botryosum phytotoxin. *Physiological and Molecular Plant Pathology* **35**, 439 - 451, doi:10.1016/0885-5765(89)90063-5 (1989).

148 Wright, S. F. & Morton, J. B. Detection of vesicular-arbuscular mycorrhizal fungus colonization of roots by using a dot-immunoblot assay. *Applied and environmental microbiology* **55**, 761-763 (1989).

149 Miedaner, T., Gang, G. R. & Geiger, H. H. Quantitative-genetic basis of aggressiveness of 42 isolates of Fusarium culmorum for winter rye head blight. *Plant Dis* **80**, 500-504 (1996).

150 Ruehrwein, R. A. & Ward, D. W. Mechanism of Clay Aggregation By Polyelectrolytes. *Soil Science* **73**, 485-492 (1952).

151 La Mer, V. K. Filtration of colloidal dispersions flocculated by anionic and cationic polyelectrolytes. *Discussions of the Faraday Society* **42**, 248-254, doi:10.1039/DF9664200248 (1966).

152 Healy, T. W. & La Mer, V. K. The energetics of flocculation and redispersion by polymers. *Journal of Colloid Science* **19**, 323-332, doi:http://dx.doi.org/10.1016/0095-8522(64)90034-0 (1964).

153 Smellie Jr, R. H. & La Mer, V. K. Flocculation, subsidence and filtration of phosphate slimes: VI. A quantitative theory of filtration of flocculated suspensions. *Journal of Colloid Science* **13**, 589-599, doi:http://dx.doi.org/10.1016/0095-8522(58)90071-0 (1958).

154 Flory, P. J. Thermodynamics of high polymer solutions. *J Chem Phys* **9**, 660-661, doi:Doi 10.1063/1.1750971 (1941).

155 Flory, P. I. Thermodynamics of high polymer solutions. *J Chem Phys* **10**, 51-61, doi:Doi 10.1063/1.1723621 (1942).

156 Huggins, M. L. Solutions of long chain compounds. *J Chem Phys* **9**, 440-440, doi:Doi 10.1063/1.1750930 (1941).

157 Hunter, R. J. *Foundations of colloid science*. 2nd edn, (Oxford University Press, 2001).

158 Napper, D. Polymeric stabilization of colloidal dispersions, 1983. *Academic, New York*.

159 Xia, F. *et al.* Colorimetric detection of DNA, small molecules, proteins, and ions using unmodified gold nanoparticles and conjugated polyelectrolytes. *P Natl Acad Sci USA* **107**, 10837-10841 (2010).

160 Xu, X. W., Wang, J., Yang, F., Jiao, K. & Yang, X. R. Label-Free Colorimetric Detection of Small Molecules Utilizing DNA Oligonucleotides and Silver Nanoparticles. *Small* **5**, 2669-2672 (2009).

161 Li, H. X. & Rothberg, L. Colorimetric detection of DNA sequences based on electrostatic interactions with unmodified gold nanoparticles. *P Natl Acad Sci USA* **101**, 14036-14039 (2004).

162 Mirkin, C. A., Letsinger, R. L., Mucic, R. C. & Storhoff, J. J. A DNA-based method for rationally assembling nanoparticles into macroscopic materials. *Nature* **382**, 607-609, doi:10.1038/382607a0 (1996).

163 Opgenorth, D. C. Pseudomonas Canker of Kiwifruit. *Plant Dis* **67**, doi:10.1094/PD-67-1283 (1983).

164 Fogliano, V. *et al.* Immunological detection of syringopeptins produced by Pseudomonas syringae pv. lachrymans. *Physiological and Molecular Plant Pathology* **55**, 255-261, doi:http://dx.doi.org/10.1006/pmpp.1999.0227 (1999).

165 Riffaud, C. M. H. & Morris, C. E. Detection of Pseudomonas syringae pv. aptata in Irrigation Water Retention Basins by Immunofluorescence Colony-staining. *European Journal of Plant Pathology* **108**, 539-545, doi:10.1023/A:1019919627886 (2002).

166 Fanelli, V., Cariddi, C. & Finetti-Sialer, M. Selective detection of Pseudomonas syringae pv. tomato using dot blot hybridization and real-time PCR. *Plant Pathology* **56**, 683-691, doi:10.1111/j.1365-3059.2007.01612.x (2007).

167 Green, S., Laue, B., Fossdal, C. G., A'Hara, S. W. & Cottrell, J. E. Infection of horse chestnut (Aesculus hippocastanum) by Pseudomonas syringae pv. aesculi and its detection by quantitative real-time PCR. *Plant Pathology* **58**, 731-744, doi:10.1111/j.1365-3059.2009.02065.x (2009).

168 Schmidt, O., Moreth, U., Dujesiefken, D., Stobbe, H. & Gaiser, O. Fast molecular detection of Pseudomonas syringae pv. aesculi in diseased horse chestnut trees. *Forest Pathology* **39**, 343-348, doi:10.1111/j.1439-0329.2009.00595.x (2009).

169 Choi, H. *et al.* Improved PCR for identification of Pseudomonas aeruginosa. *Appl Microbiol Biotechnol* **97**, 3643-3651, doi:10.1007/s00253-013-4709-0 (2013).

170 Biondi, E. *et al.* Pseudomonas syringae pv. actinidiae detection in kiwifruit plant tissue and bleeding sap. *Annals of Applied Biology* **162**, 60-70, doi:10.1111/aab.12001 (2013).

171 Vaseghi, A., Safaie, N., Bakhshinejad, B., Mohsenifar, A. & Sadeghizadeh, M. Detection of Pseudomonas syringae pathovars by thiol-linked DNA–Gold nanoparticle probes. *Sensors and Actuators B: Chemical* **181**, 644-651, doi:http://dx.doi.org/10.1016/j.snb.2013.02.018 (2013).

172 Suzuki, H. *et al.* Highly sensitive detection of Pseudomonas syringae pv. japonica from wheat seeds by selective medium and membrane filter method. *Annual Report of The Kansai Plant Protection Society* **56**, 43-47, doi:10.4165/kapps.56.43 (2014).

173 Crannell, Z. A., Rohrman, B. & Richards-Kortum, R. Equipment-free incubation of recombinase polymerase amplification reactions using body heat. *PloS one* **9**, e112146, doi:10.1371/journal.pone.0112146 (2014).

174 Euler, M. *et al.* Recombinase polymerase amplification assay for rapid detection of Rift Valley fever virus. *J Clin Virol* **54**, 308-312, doi:10.1016/j.jcv.2012.05.006 (2012).

175 Euler, M. *et al.* Development of a panel of recombinase polymerase amplification assays for detection of biothreat agents. *J Clin Microbiol* **51**, 1110-1117, doi:10.1128/JCM.02704-12 (2013).

176 Novakova, S., Klaudiny, J., Kollerova, E. & Subr, Z. W. Expression of a part of the Potato virus A non-structural protein P3 in Escherichia coli for the purpose of antibody preparation and P3 immunodetection in plant material. *J Virol Methods* **137**, 229-235, doi:DOI 10.1016/j.jviromet.2006.06.020 (2006).

177 Lievens, B., Brouwer, M., Vanachter, A. C. R. C., Cammue, B. P. A. & Thomma, B. P. H. J. Real-time PCR for detection and quantification of fungal and oomycete tomato pathogens in plant and soil samples. *Plant Science* **171**, 155-165, doi:DOI 10.1016/j.plantsci.2006.03.009 (2006).

178 Tooley, P. W., Bunyard, B. A., Carras, M. M. & Hatziloukas, E. Development of PCR primers from internal transcribed spacer region 2 for detection of Phytophthora species infecting potatoes. *Appl Environ Microbiol* **63**, 1467-1475 (1997).

179 Lee, Young-Mi, Choi, Y. K. & Min, B. R. PCR-RFLP and sequence analysis of the rDNA ITS region in the Fusarium spp. *Journal of Microbiology* **38**, 66-73 (2000).

180 Chillali, M. *et al.* Variation in the ITS and IGS regions of ribosomal DNA among the biological species of European Armillaria. *Mycological Research* **102**, 533-540, doi:Doi 10.1017/S0953756297005315 (1998).

181 Cazzonelli, C. I., Cavallaro, A. S. & Botella, J. R. Cloning and characterisation of ripening-induced ethylene biosynthetic genes from non-climacteric pineapple (Ananas comosus) fruits. *Australian Journal of Plant Physiology* **25**, 513-518 (1998).

182 Doyle, J. J. & Doyle, J. L. Isolation of plant DNA from fresh tissue. *Focus* **12**, 13 - 15 (1990).

183 Yamazaki, W., Seto, K., Taguchi, M., Ishibashi, M. & Inoue, K. Sensitive and rapid detection of cholera toxin-producing Vibrio cholerae using a loop-mediated isothermal amplification. *Bmc Microbiol* **8**, doi:Artn 94, 10.1186/1471-2180-8-94 (2008).

184 Ge, Z., Qing, Y., Zicheng, S. & Shiying, S. Rapid and sensitive diagnosis of Acanthamoeba keratitis by loop-mediated isothermal amplification. *Clin Microbiol Infec* **19**, 1042-1048, doi:10.1111/1469-0691.12149 (2013).

185 Clark, J. S. & Hersh, M. H. Inference in incidence, infection, and impact: Co-infection of multiple hosts by multiple pathogens. *Bayesian Anal* **4**, 337-365, doi:Doi 10.1214/09-Ba413 (2009).

186 Trusov, Y., Chakravorty, D. & Botella, J. R. Fusarium oxysporum infection assays in Arabidopsis. *Methods in molecular biology* **1043**, 67-72, doi:10.1007/978-1-62703-532-3_7 (2013).

187 Klosterman, S. J. Real-Time PCR for the Quantification of Fungi In Planta. *Plant Fungal Pathogens: Methods and Protocols* **835**, 121-132, doi:Doi 10.1007/978-1-61779-501-5_8 (2012).

188 Lin, Y. H. *et al.* A molecular diagnosis method using real-time PCR for quantification and detection of Fusarium oxysporum f. sp cubense race 4. *Eur J Plant Pathol* **135**, 395-405, doi:DOI 10.1007/s10658-012-0096-0 (2013).

189 Nilsson, M. *et al.* Padlock probes: circularizing oligonucleotides for localized DNA detection. *Science* **265**, 2085-2088 (1994).

190 Hirani, R., Connolly, A. R., Putral, L., Dobrovic, A. & Trau, M. Sensitive Quantification of Somatic Mutations Using Molecular Inversion Probes. *Anal Chem* **83**, 8215-8221, doi:Doi 10.1021/Ac2019409 (2011).

191 Palanisamy, R., Connolly, A. R. & Trau, M. Epiallele quantification using molecular inversion probes. *Anal Chem* **83**, 2631-2637, doi:10.1021/ac103016n (2011).

192 van Doorn, R. *et al.* Robust Detection and Identification of Multiple Oomycetes and Fungi in Environmental Samples by Using a Novel Cleavable Padlock Probe-Based Ligation Detection Assay. *Applied and Environmental Microbiology* **75**, 4185-4193, doi:Doi 10.1128/Aem.00071-09 (2009).

193 Sikora, K. *et al.* A Universal Microarray Detection Method for Identification of Multiple Phytophthora spp. Using Padlock Probes. *Phytopathology* **102**, 635-645, doi:Doi 10.1094/Phyto-11-11-0309 (2012).

194 Wu, D. Y. & Wallace, R. B. Specificity of the nick-closing activity of bacteriophage T4 DNA ligase. *Gene* **76**, 245-254 (1989).

195 Xu, Y. & Kool, E. T. High sequence fidelity in a non-enzymatic DNA autoligation reaction. *Nucleic acids research* **27**, 875-881 (1999).

196 Cherepanov, A., Yildirim, E. & de Vries, S. Joining of short DNA oligonucleotides with base pair mismatches by T4 DNA ligase. *Journal of biochemistry* **129**, 61-68 (2001).

197 Palanisamy, R. *Towards comprehensive DNA methylation analysis using molecular inversion probes* PhD thesis, The University of Queensland, (2014).

198 Mauchmani, B. & Slusarenko, A. J. Systemic Acquired-Resistance in Arabidopsis-Thaliana Induced by a Predisposing Infection with a Pathogenic Isolate of Fusarium-Oxysporum. *Mol Plant Microbe In* **7**, 378-383 (1994).

199 Li, J. B. *et al.* Multiplex padlock targeted sequencing reveals human hypermutable CpG variations. *Genome research* **19**, 1606-1615, doi:10.1101/gr.092213.109 (2009).

200 Turner, E. H., Ng, S. B., Nickerson, D. A. & Shendure, J. Methods for genomic partitioning. *Annual review of genomics and human genetics* **10**, 263-284, doi:10.1146/annurev-genom-082908-150112 (2009).

201 Lin, S., Wang, W., Palm, C., Davis, R. W. & Juneau, K. A molecular inversion probe assay for detecting alternative splicing. *BMC genomics* **11**, 712, doi:10.1186/1471-2164-11-712 (2010).

202 Deng, J. *et al.* Targeted bisulfite sequencing reveals changes in DNA methylation associated with nuclear reprogramming. *Nature biotechnology* **27**, 353-360, doi:10.1038/nbt.1530 (2009).

203 Akhras, M. S. *et al.* PathogenMip assay: a multiplex pathogen detection assay. *PloS one* **2**, e223, doi:10.1371/journal.pone.0000223 (2007).

204 Hyman, R. W. *et al.* Molecular probe technology detects bacteria without culture. *Bmc Microbiol* **12**, doi:Artn 29, Doi 10.1186/1471-2180-12-29 (2012).

205 Novais, R. C., Borsuk, S., Dellagostin, O. A. & Thorstenson, Y. R. Molecular inversion probes for sensitive detection of Mycobacterium tuberculosis. *J Microbiol Methods* **72**, 60-66, doi:10.1016/j.mimet.2007.10.009 (2008).

206 Ng, B. Y. C. *et al.* Rapid, Single-Cell Electrochemical Detection of Mycobacterium tuberculosis Using Colloidal Gold Nanoparticles. *Anal Chem* **87**, 10613-10618, doi:10.1021/acs.analchem.5b03121 (2015).

207 del Rio, J. S. *et al.* Real-time and label-free ring-resonator monitoring of solid-phase recombinase polymerase amplification. *Biosens Bioelectron* **73**, 130-137, doi:10.1016/j.bios.2015.05.063 (2015).

208 Wee, E. J. H. & Trau, M. Simple isothermal strategy for multiplexed, rapid, sensitive and accurate miRNA detection. *ACS Sensors*, doi:10.1021/acssensors.6b00105 (2016).

209 Kersting, S., Rausch, V., Bier, F. F. & von Nickisch-Rosenegk, M. Multiplex isothermal solid-phase recombinase polymerase amplification for the specific and fast DNA-based detection of three bacterial pathogens. *Microchim Acta* **181**, 1715-1723, doi:10.1007/s00604-014-1198-5 (2014).

210 Schlucker, S. Surface-Enhanced Raman Spectroscopy: Concepts and Chemical Applications. *Angew Chem Int Edit* **53**, 4756-4795, doi:10.1002/anie.201205748 (2014).

211 Stiles, P. L., Dieringer, J. A., Shah, N. C. & Van Duyne, R. P. Surface-enhanced Raman spectroscopy. *Annual review of analytical chemistry* **1**, 601-626, doi:10.1146/annurev.anchem.1.031207.112814 (2008).

212 Moskovits, M. SPECTROSCOPY Expanding versatility. *Nature* **464**, 357-357, doi:10.1038/464357a (2010).

213 Anker, J. N. *et al.* Biosensing with plasmonic nanosensors. *Nat Mater* **7**, 442-453, doi:10.1038/nmat2162 (2008).

214 Bantz, K. C. *et al.* Recent progress in SERS biosensing. *Phys Chem Chem Phys* **13**, 11551-11567, doi:10.1039/c0cp01841d (2011).

215 Sharma, B., Frontiera, R. R., Henry, A. I., Ringe, E. & Van Duyne, R. P. SERS: Materials, applications, and the future. *Mater Today* **15**, 16-25 (2012).

216 Laing, S., Gracie, K. & Faulds, K. Multiplex in vitro detection using SERS. *Chemical Society Reviews* **45**, 1901-1918, doi:10.1039/C5CS00644A (2016).

217 Schlucker, S. SERS Microscopy: Nanoparticle Probes and Biomedical Applications. *Chemphyschem* **10**, 1344-1354, doi:10.1002/cphc.200900119 (2009).

218 Wang, Y. L. & Schlucker, S. Rational design and synthesis of SERS labels. *Analyst* **138**, 2224-2238, doi:10.1039/c3an36866a (2013).

219 Lai, Y. M., Sun, S. Q., He, T., Schlucker, S. & Wang, Y. L. Raman-encoded microbeads for spectral multiplexing with SERS detection. *Rsc Adv* **5**, 13762-13767, doi:10.1039/c4ra16163g (2015).

220 Wang, Y. L., Wee, E. J. H. & Trau, M. Highly sensitive DNA methylation analysis at CpG resolution by surface-enhanced Raman scattering via ligase chain reaction. *Chem Commun* **51**, 10953-10956, doi:10.1039/c5cc03921e (2015).

221 Maiti, K. K. *et al.* Multiplex cancer cell detection by SERS nanotags with cyanine and triphenylmethine Raman reporters. *Chem Commun* **47**, 3514-3516, doi:10.1039/c0cc05265e (2011).

222 Trusov, Y., Chakravorty, D. & Botella, J. R. Fusarium oxysporum Infection Assays in Arabidopsis. *G Protein-Coupled Receptor Signaling in Plants: Methods and Protocols* **1043**, 67-72, doi:10.1007/978-1-62703-532-3_7 (2013).

223 Birkenbihl, R. P., Diezel, C. & Somssich, I. E. Arabidopsis WRKY33 Is a Key Transcriptional Regulator of Hormonal and Metabolic Responses toward Botrytis cinerea Infection. *Plant Physiol* **159**, 266-285, doi:10.1104/pp.111.192641 (2012).

224 Braun-Kiewnick, A., Altenbach, D., Oberhansli, T., Bitterlin, W. & Duffy, B. A rapid lateral-flow immunoassay for phytosanitary detection of Erwinia amylovora and on-site fire blight diagnosis. *J Microbiol Meth* **87**, 1-9, doi:10.1016/j.mimet.2011.06.015 (2011).

225 Demers, L. M. *et al.* A fluorescence-based method for determining the surface coverage and hybridization efficiency of thiol-capped oligonucleotides bound to gold thin films and nanoparticles. *Anal Chem* **72**, 5535-5541 (2000).

226 Cao, Y. C., Jin, R. & Mirkin, C. A. Nanoparticles with Raman spectroscopic fingerprints for DNA and RNA detection. *Science* **297**, 1536-1540, doi:10.1126/science.297.5586.1536 (2002).

227 Wilchek, M., Bayer, E. A. & Livnah, O. Essentials of biorecognition: The (strept)avidin-biotin system as a model for protein-protein and protein-ligand interaction. *Immunol Lett* **103**, 27-32, doi:10.1016/j.imlet.2005.10.022 (2006).

228 Amselem, J. *et al.* Genomic analysis of the necrotrophic fungal pathogens Sclerotinia sclerotiorum and Botrytis cinerea. *PLoS genetics* **7**, e1002230, doi:10.1371/journal.pgen.1002230 (2011).

229 Sena, E. P. & Zarling, D. A. Targeting in Linear DNA Duplexes with 2 Complementary Probe Strands for Hybrid Stability. *Nat Genet* **3**, 365-372, doi:Doi 10.1038/Ng0493-365 (1993).

230 Kaboev, O. *et al.* Improved RecA-assisted fluorescence assay for DNA strand exchange reaction. *Biotechniques* **40**, 736-+, doi:10.2144/000112195 (2006).

Appendix

Cover page

Volume 51 | Number 27 | 7 April 2015 | Pages 5781–6024

ChemComm

Chemical Communications

www.rsc.org/chemcomm

ISSN 1359-7345

ROYAL SOCIETY OF CHEMISTRY

COMMUNICATION

J. R. Botella, M. Trau *et al.*

Re-purposing bridging flocculation for on-site, rapid, qualitative DNA detection in resource-poor settings