Eindrapport Final report

"On-site plantpathogen detection and barcode sequencing for improving plant health and phytosanitary control."

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T&U Nummer: TU-18148

Looptijd: 1 jan 2019 – 31 dec 2022

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The NVWA was no partner in this project, but was present at meetings in lieu of LNV

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1 Samenvatting NL

1.1 Inleiding

Het toenemende internationale transport van plantaardige producten en klimaatverandering zorgen voor problemen voor de Nederlandse tuinbouwsector door de verspreiding van ziekteverwekkers. De ontwikkeling van snelle, nauwkeurige en gevoelige methoden voor identificatie en detectie van (plant)pathogenen die direct in de productieketen on-site kunnen worden toegepast, is essentieel. Vroege detectie maakt tijdige actie mogelijk om verspreiding van de ziekte of vertraging en beperkingen in de export van plantgoed en zaden te voorkomen. Voor een groot aantal plantpathogenen zijn in het verleden veel detectiemethoden ontwikkeld. De uitdaging in dit voorstel was niet alleen het testen op locatie (on-site) voor deze organismen, maar ook het testen op meerdere organismen in één reactie. Ook is on-site sequencing van diagnostische monsters niet eerder getest in de tuinbouw, maar veelbelovend. In dit PPS project is hier aan gewerkt.

1.2 Resultaten

In dit project zijn de diverse activiteiten uitgevoerd in drie werkpakketten (WP1, WP2 en WP3).

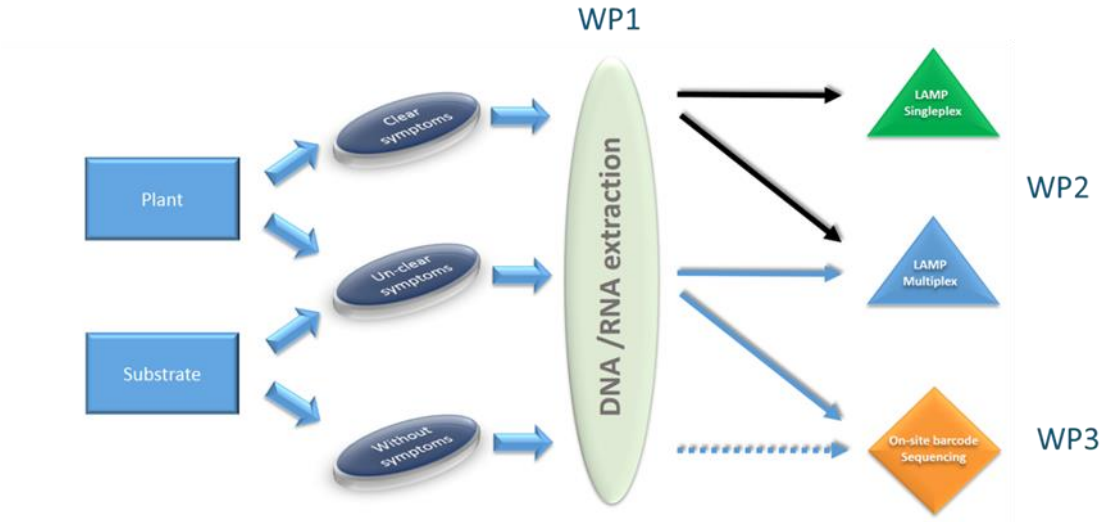


Figure 1-1: Overzicht van het project PPS On-site en barcoding

Bij moleculaire detectie in de productieketen spelen een aantal verschillende aspecten een belangrijke rol. Dit zijn: extractie van DNA en/of RNA, detectiemethode, analyse en gegevensinterpretatie. Voor een goede detectie moet met al deze aspecten rekening worden gehouden.

WP1: DNA/RNA-extractie

Uit verschillende geselecteerde substraten (zoals blad, stengel, water, lucht etc.) wordt op eenvoudige wijze DNA en RNA geëxtraheerd.

WP2: Ontwikkeling van een on-site multiplexmethode met behulp van eerder ontwikkelde LAMP-testen of die beschreven zijn in de literatuur. Deze zullen worden aangevuld met nieuw ontwikkelde testen.

WP3: On-site en barcodesequencing met behulp van de MinION (ONT). Amplicons worden gegenereerd uit geselecteerde barcode gebieden d.m.v. PCR amplificatie en vervolgens voorbereid voor sequencing in een MinION-systeem. Data-analyse laat dan zien welke pathogenen in het monster aanwezig zijn.

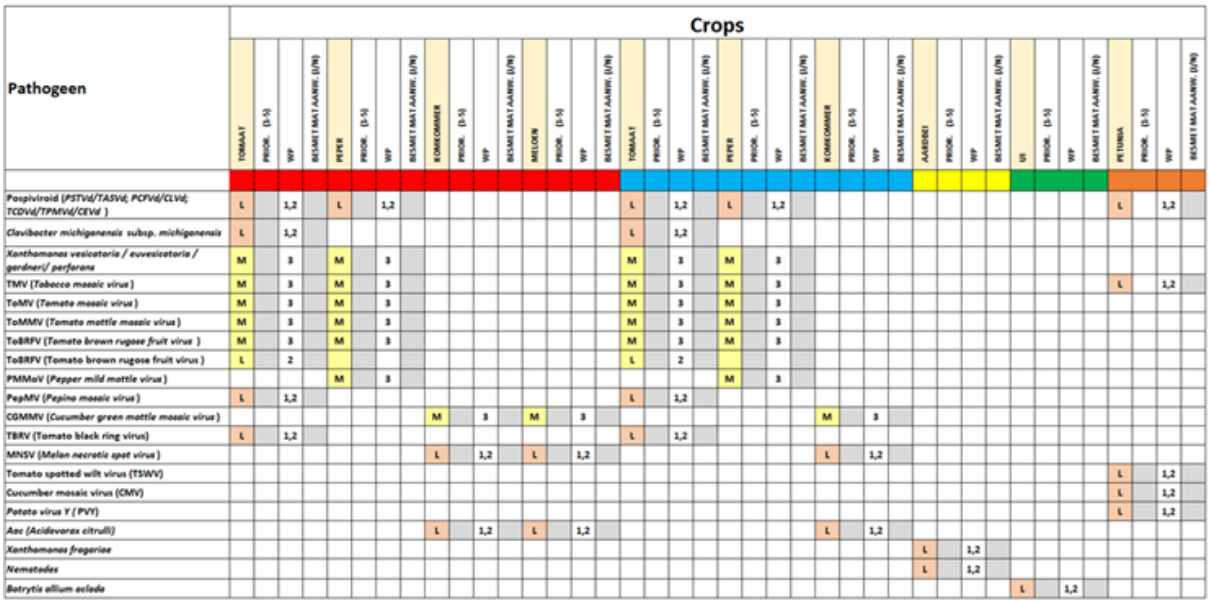


Figure 1-2: Verschillende plantenpathogenen en gewassen en hun detectie methoden.

In Figure 1-2 zijn voor diverse plantenpathogenen en gewassen aangegeven in welk werkpakket (WP) met welke methode is gewerkt. L: LAMP en M: MinION sequencing.

1.2.1 WP1: DNA/RNA extractie

1.2.1.1 Inleiding

Voor een eenvoudige on-site extractie van pathogenen is het belangrijk dat DNA of RNA snel kan worden geëxtraheerd zonder complexe laboratoriumapparatuur. Bovendien moet de extractiemethode geschikt zijn voor een breed scala aan gewassen. En daarnaast mogen de ingrediënten die voor de extractie worden gebruikt de detectie niet remmen en tegelijkertijd moeten remmende plantenstoffen zoveel mogelijk worden verminderd.

1.2.1.2 Resultaten

Vergelijking van extractiebuffers

Verschillende extractiebuffers zijn uitgetest op tomatenblad: OptiGene lysis buffer (OptiGene), Quickextract buffer (Epicenter), USEB buffer en een zelfsamengestelde polyethylene glycol (PEG) buffer. De extractie efficientie was getest in tomaat m.b.v. de ToBRFV-LAMP. Er waren slechts kleine verschillen tussen de verschillende buffers. De beste resultaten werden verkregen met de OptiGene lysisbuffer en de zelfgemaakte PEG-buffer, die de vroegste amplificatie (Tpos: Time of positivity) vertoonden van monsters met slechts 10% geïnfecteerd materiaal.

Extractie op groot aantal gewassen

De zelfgemaakte PEG-lysisbuffer werd vergeleken met de PEG-lysisbuffer van OptiGene. Er zijn verschillende gewassen (rode biet, broccoli, wortel, dahlia, sperziebonen, boerenkool, prei, ossenhart, savooiekool, spinazie, aardbei en duizendschoon) getest die beschikbaar waren op het moment van testen. Analyse werd uitgevoerd op basis van de Cox interne controle. Voor alle gewassen werd aangetoond dat de extractie goed werkt. Beide extractiebuffers bleken even goed te werken en daarom wordt vanwege de lagere kosten de zelfgemaakte PEG-buffer aanbevolen.

FTA cards

FTA cards zijn kaartjes waaop bladmateriaal kan worden gedrukt. Drie verschillende Whatman FTA-kaarten (Figure 1-3,Tabel 1-1), de Classic Card, de Plant Saver Card en de Elute Micro Card zijn getest in combinatie met twee verschillende extractieprotocollen. Deze werden getest op meloenblad geïnfecteerd met *A. citrulli* (DNA) en tomatenblad geïnfecteerd met ToBRFV (RNA). Na extractie werden de ontwikkelde LAMP assays uitgevoerd (WP2).

Figure 1-3: Voorbeeld van een FTA-kaart.

Tabel 1-1: Monsters voor de extractie van FTA-cards met ToBRFV. Alle samples zijn in een 50x verdunning aangebracht op de FTA-card

Monster	**Beschrijving**	**Target**
L1 Rugose	Classic card, LAMP extractie	ToBRFV
L2 Rugose	Plant saver card, LAMP extractie	ToBRFV
L3 Rugose	Elute micro card, LAMP extractie	ToBRFV
W1 Rugose	Classic card, Whatman methode	ToBRFV
W2 Rugose	Plant saver card, Whatman methode	ToBRFV
W3 Rugose	Elute micro card, Whatman methode	ToBRFV
PC	Positieve controle	ToBRFV
NC	Negatieve controle	ToBRFV

Resultaten (Figure 1-4) laten zien dat van alle FTA cards ToBRFV goed kan worden aangetoond.

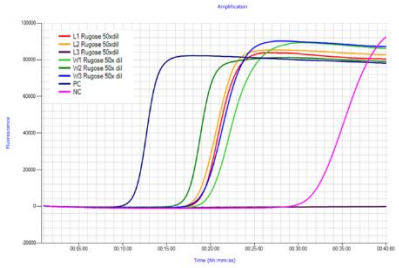


Figure 1-4: LAMP detectie van ToBRFV na extractie van FTA-card.

1.2.1.3 Conclusies

- Er zijn verschillende extractiebuffers uitgetest om zo snel en goedkoop mogelijk goed DNA of RNA uit het plantmateriaal te verkrijgen. Resultaten laten zien dat de gebruikte buffers goed in staat zijn om DNA/RNA snel en efficiënt te extraheren.
- Een tweetal extractiebuffers zijn uitgetest op een groot aantal verschilende gewassen. Uit alle gewassen kon goed DNA worden geëxtraheerd en waren de LAMP resultaten goed.
- Het gebruik van FTA cards is een goede manier om (geïnfecteerd) plantmateriaal te verzamelen en te versturen. Resultaten laten zien dat ToBRFV goed aan te tonen is op bladmateriaal wat gespot is op FTA cards.

1.2.2 WP2: LAMP

1.2.2.1 Inleiding

Voor on-site detectie van ziekteverwekkers in de kas of in het veld is een methode nodig die geen dure laboratoriumapparatuur en zo min mogelijk voorbereiding vereist. Vaak worden PCR of qPCR gebruikt voor detectie. Deze methoden zijn echter gevoelig voor remming door van planten afgeleide verbindingen en vereisen een uitgebreid DNA-extractieprotocol en een thermocycler. Isotherme methoden, zoals LAMP (**l**oop-mediated **amp**lification) daarentegen, zijn minder gevoelig voor remmers en kunnen bij één temperatuur worden uitgevoerd (isotherm). Deze eigenschappen maken de methode geschikt voor toepassing buiten laboratoria. Bovendien kunnen LAMP-assays zeer specifiek zijn voor het doel-DNA of -RNA, aangezien de LAMP-assay uit 4 tot 6 verschillende primers bestaan. Aangezien een LAMP-run een grote hoeveelheid amplicon produceert, is de methode ook nogal gevoelig. Toch verschillen specificiteit en gevoeligheid tussen LAMP-assays en zijn ze afhankelijk van bijvoorbeeld de mate van overeenkomst tussen doelwit en niet-doelwit, de variabiliteit binnen een doelgroep (bijvoorbeeld een soort) en het gebied van het genoom waarvoor de test is ontworpen. Daarom is uitgebreide validatie van nieuw ontwikkelde LAMP-assays nodig.

In een LAMP methode wordt DNA of RNA van de ziekteverwekker (pathogeen) vermenigvuldigd op één temperatuur (65°C). Wij voeren deze reactie uit in een apparaat Genie II of Genie III die de vermenigvuldiging real-time kan volgen m.b.v. fluorescentie.

1.2.2.2 Resultaten

In Tabel 1-2 is aangegeven voor welke pathogenen we LAMP methodes hebben ontwikkeld.

Tabel 1-2: Overzicht van de ontwikkelde LAMP targets

Target	**Waardplant**
Pospiviroiden	Tomaat, Peper, Petunia
Clavibacter michiganensis pv. *michiganensis*	Tomaat
TMV	Tomaat
PepMV	Tomaat
ToBRFV	Tomaat
TBRV	Tomaat
MNSV	Komkommer, Meloen
TSWV	Petunia
CMV	Petunia
PVY	Petunia
Acidovorax citrulli	Komkommer, Meloen
Xanthomonas fragariae	Aardbei
Fusarium, Verticillium	Tomaat, Peper, Ui

Een voorbeeld van een LAMP reactie voor ToBRFV is weergegeven in Figure 1-5.

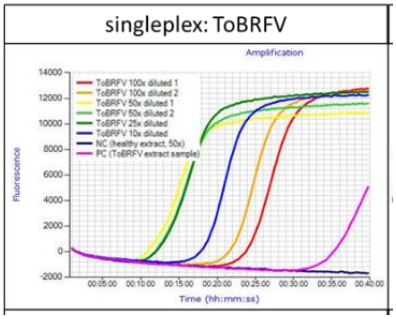


Figure 1-5: Voorbeeld van LAMP methode toegepast op een verdunningsreeks van een ToBRFV geinfecteerd tomatenblad.

De toename van de fluorescentie is te zien in Figure 1-5. Na 11 minuten is er al een positief signaal. Het is ook mogelijk deze vermenigvuldiging colorometrisch te zien. Echter hierbij zijn we alleen het resultaat aan het eind van de vermenigvuldiging en niet real-time (Figure 1-6).

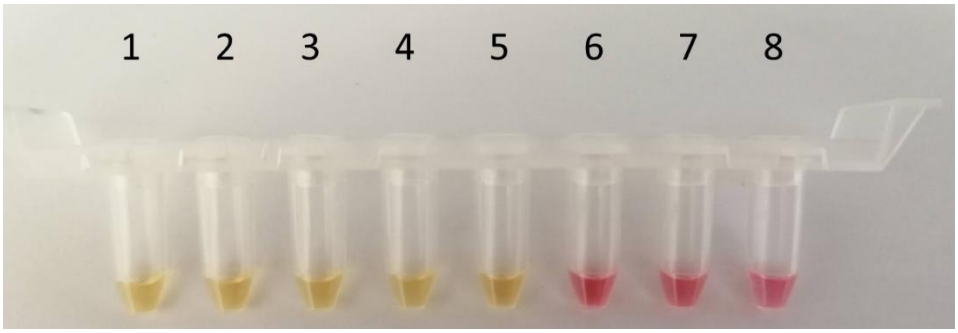


Figure 1-6: Colorimetrische LAMP assay van een 10x verdunningsreeks van ToBRFV geinfecteerd materiaal. Oranje/geel gekleurde oplossing betekent dat de assay positief en er dus ToBRFV in het monster aanwezig is.

Goed te zien is dat de monsters 1, 2, 3, 4 en 5 positief zijn. Dit betekent dat een ToBRFV geinfecteerd blad extract 10,000x verdund kan worden.

De vermenigvuldiging in de LAMP reactie kan ook in een waterbad worden uitgevoerd in een zgn. Tcup (Figure 1-7). Dit is een soort Nespresso cup waarin de temperatuur goed op 65°C kan worden gehouden.

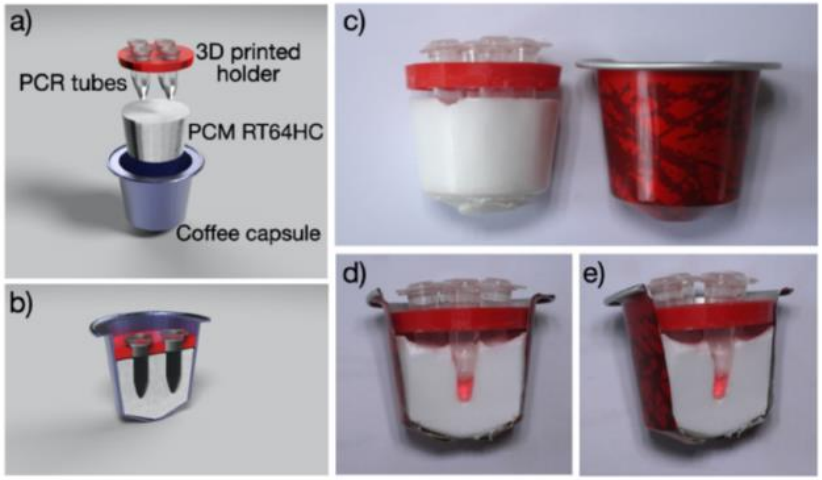


Figure 1-7: Colorimetrische LAMP assay in een Tcup.

1.2.2.3 Multiplex

Multiplex betekent dat meerdere targets tegelijkertijd kunnen worden aangetoond. Hiervoor hebben we experimenten uitgevoerd met een zgn. microfluidic chip. Hierin wordt het monster in 4 aparte reactiekamers geanalyseerd.

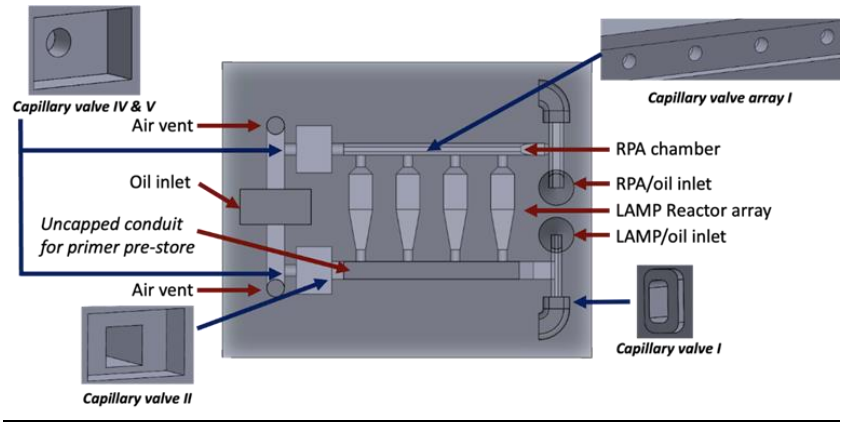


Figure 1-8: Design van de microfluidic chip.

In samenwerking met Pensylvania State University (USA) zijn experimenten met de microfluidic chip uitgevoerd. Er is gewerkt met een drietal LAMP asays (TMV, ToBRFV en Cox). Daar er niet gewerkt kon worden met virussen, zijn de experimenten uitgevoerd met synthetisch DNA (gBlock) met de sequentie van de betrokken virussen (TMV en ToBRFV). Cox wordt gebruikt als controle voor de plant en het 4^{e} reactievaatje gebruiken we als negatieve controle. In Figure 1-9 is te zien dat een mengsel van de twee plantenvirussen en de plant, de reacties in bijbehorende reactievaatje na de LAMP reactier positief kleuren. De negatieve controle blijft negatief.

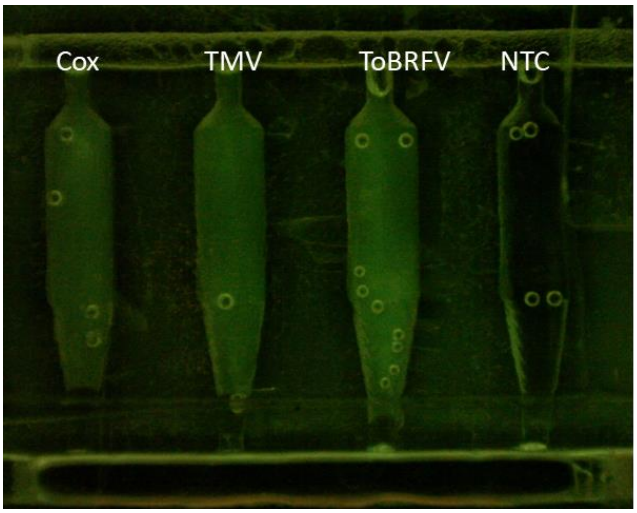


Figure 1-9: Multiplex detectie van TMV en ToBRFV gBlocks in tomatenextract; plaatje van de eindpunt meting van de micro-fluidic chip met 4 reactie kamers.

1.2.2.4 Luchtbemonstering

De beoogde luchtbemonstering en diagnostiek van luchtmonsters moest worden stopgezet vanwege het niet beschikbaar zijn van het prototype van de apparatuur gedurende de looptijd van het project.

1.2.2.5 Conclusies

1. The **ToBRFV** LAMP assay is erg gevoelig en kan ToBRFV infectie detecteren in symptomatisch plantmateriaal, zelfs na grote verdunning (detectie limiet is gemeten in een 1:1000000 verdunning van RNA geëxtraheerd uit plant materiaal). De assay is ook specifiek en geeft geen vals positieven met nauwverwante soorten. Tevens kan de ToBRFV LAMP gebruikt worden in een multiplex assay met de *Cox* assay als een amplificatie controle.
   Een colorimetrische assay kan gebruikt worden in plaats van de normale fluoroscentie meting, maar deze bepaling is minder gevoelig, is tijdrovender en soms afhankelijk van interpretatie door uitvoerder. Colorimetrische detectie vereist echter minder apparatuur en is daarom meer geschikt voor detectie op locatie. Verdere evaluatie van colorimetrische detectie is nodig om de betrouwbaarheid en gevoeligheid van de methode te vergroten. Ook of andere eenvoudige uitleesmethoden zoals laterale-flow-apparaten en fluorescentiemeting met een mobiele telefoon moeten worden onderzocht.
2. De **TMV** LAMP-assay is zowel gevoelig (detectielimiet: 10^3 kopieën van gBlocks / µl) als specifiek en geschikt voor de detectie van het virus in zowel tomaten- als petunia-plantmateriaal. Materiaal van andere plantensoorten is niet getest, maar het is aannemelijk dat detectie ook werkt. Ook kan de TMV LAMP worden gebruikt in een multiplex met het Cox-gen.
3. De **MNSV** LAMP-assay voor MNSV-stammen in komkommer is zeer gevoelig met een detectielimiet van 10 kopieën gBlocks/µl. Het is ook specifiek omdat er geen amplificatie plaatsvond met RNA van verwante virussen die aanwezig zijn in komkommer. Het is geschikt voor detectie in geïnfecteerd en symptomatisch komkommerbladmateriaal. De test kan ook worden gecombineerd met de Cox-test in een multiplex. Deze test is specifiek ontwikkeld voor een MNSV isolaat in komkommer waarvan de genomische sequentie beschikbaar was op het moment van het ontwerp van de primer. Het is niet getest op MNSV-isolaten van meloen.
4. De **TSWV** LAMP-assay is voldoende gevoelig om RNA te detecteren dat is geïsoleerd uit geïnfecteerde planten bij een verdunning van 1:50 en verwante virussen werden niet geamplificeerd. De test presteerde ook goed op geïnfecteerd bladmateriaal.
5. De LAMP-assay voor **PVY** in Petunia is zeer gevoelig met een detectielimiet van een 1:10000 verdunning van RNA geïsoleerd uit geïnfecteerd plantenmateriaal. De assays laten ook een zwakke detectie van SucMoV zien, maar dit virus is niet aanwezig in petunia en zou daarom niet moeten interfereren met de specificiteit van de assay. De test bleek in staat om PVY te detecteren in geïnfecteerd petunabladmateriaal.
6. De LAMP-assay voor ***X. fragariae*** in aardbei is gevoelig (detectielimiet: 10^3 kopieën van gBlocks/µl) en specifiek voor deze ene soort. De test kan infectie detecteren in symptomatisch bladmateriaal en kan worden gecombineerd met de Cox-test in een multiplex.
7. Voor elk van de 4 Fusarium- en Verticillium-targets werden LAMP-assays uit de literatuur geïdentificeerd. Voor ***F. solani, F. oxysporum*** en ***V. dahliae*** waren de testen specifiek voor de doelsoort. Alleen de ***F. proliferatum***-assay vertoont een kruisreactie met *F. oxysporum*. Desalniettemin bleek het de beste test te zijn die momenteel uit de literatuur beschikbaar is. Het testen van de testen op geïnfecteerd plantmateriaal laat zien dat de LAMP-test iets minder gevoelig is dan de TaqMan-test. Voor de meeste monsters was de TaqMan-detectie net boven de detectielimiet (meestal een CT van 40). In de meeste van deze monsters kon *V. dahliae* niet worden gedetecteerd met LAMP-assays. Bij hogere concentraties lukte het echter wel.
8. De multiplex microchip voor de gelijktijdige detectie van drie pathogenen in één monster bleek de gelijktijdige detectie van **ToBRFV** en **TMV** mogelijk te maken. Het vereist alleen eenvoudige apparatuur zoals een zelfgebouwde broedmachine en een USB-camera. Correct gebruik van de chip is echter complex en vereist ervaring, wat suggereert dat de chip verder moet worden ontwikkeld voor meer gebruiksvriendelijkheid.
9. De T65 cup-assay is een eenvoudige methode om een LAMP-assay uit te voeren met alleen kokend water en kan daarom in bijna alle omgevingen worden gebruikt. Een eenvoudige meting van een fluorescentiesignaal met bijvoorbeeld een smartphonecamera is echter nog niet mogelijk vanwege de lage gevoeligheid van deze camera's. Colorimetrische evaluatie is mogelijk, maar de kleurverandering is vaak niet eenduidig. Eenvoudige meettechnieken moeten verder worden ontwikkeld om deze methode gemakkelijk en onbeperkt te kunnen gebruiken.

1.2.3 WP3: MinION seq

1.2.3.1 Inleiding

Oxford Nanopore Technologies (ONT) heeft sinds 2005 de zogenaamde Nanopore sequencing methode (MinION) ontwikkeld. Deze techniek maakt gebruik van stroomcellen die een grote hoeveelheid zeer kleine poriën in een elektroresistent membraan bevatten. Wanneer een DNA- of RNA-molecuul door een porie gaat, veroorzaakt elke passerende base een verstoring in het elektrische signaal in de nanoporie (ruwe gegevens, Figure 1-10). Dit signaal kan worden gedecodeerd in de oorspronkelijke volgorde A.C.T of G). Om een DNA- of RNA-molecuul aan een porie te laten hechten, moeten tijdens de monstervoorbereiding adapters aan de moleculen worden geligeerd.

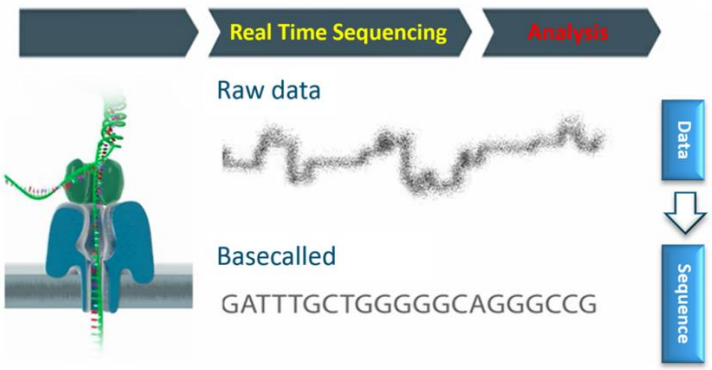


Figure 1-10: Principe van sequentie bepaling met nanoporiën.

Tobamovirussen

Het genoom van een virus van het genus Tobamovirus bestaat uit een enkelstrengs RNA-molecuul met een lengte van ca. 6400 nt met 4 bekende open leesramen (ORF's): 2 die coderen voor het RNA-polymerase, één die codeert voor een bewegingseiwit en één voor het manteleiwit (Pagan et al. 2010). Het geslacht bevat 22 soorten, die kunnen worden onderverdeeld in 3 subgroepen. Subgroep I infecteert voornamelijk solanaceous gastheren, subgroep II infecteert komkommerachtigen en peulvruchten en van subgroep III is bekend dat twee brassica's en asteroïden infecteren. In kassen is subgroep I een serieus probleem bij gewassen als tomaat en paprika. Hoewel detectietesten beschikbaar zijn voor verschillende afzonderlijke soorten, is het vaak niet bekend welke soort de waargenomen symptomen veroorzaakt. In dit geval is het voordelig om op verschillende soorten te kunnen testen. Juiste identificatie kan van belang zijn voor het opsporen van ziekteverwekkers en verspreiding en het toepassen van juiste bestrijdingsmaatregelen. Ook is het mogelijk dat gewassen tegelijkertijd met meerdere ziekteverwekkers besmet zijn.

Om een aantal verschillende tobamovirussen te kunnen detecteren, d.w.z. cucumber green mottle mosaic virus (CGMMV), pepper mild mottle virus (PMMoV), tobacco mosaic virus (TMV), tomato brown rugose fruit virus (ToBRFV), tomato mosaic virus (ToMV) and tomato mottle mosaic virus (ToMMV),wilden we een detectiemethode ontwikkelen op basis van ONT-amplicon-sequencing. Bij deze methode worden sequenties van de zes soorten specifiek geamplificeerd in een multiplex PCR-assay. Deze amplicons worden vervolgens gesequencet door ONT-sequencing. Hierdoor kan de soort of combinatie van soorten die symptomen veroorzaken binnen een relatief kort tijdsbestek worden geïdentificeerd.

FTA card samples

FTA-Card monsters met de virussen CGMMV, PMMoV, TMV en ToMV zijn ontvangen van East West Seed en geanalyseerd met het ontwikkelde extractie protocol en de multiplex PCR gevolgd door de MinION sequencing. De totale procedure is weergegeven in Figure 1-11.

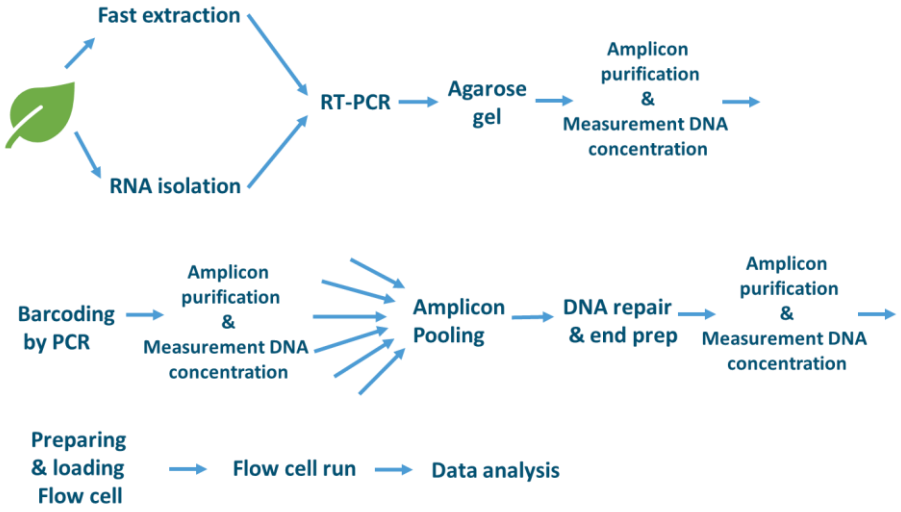


Figure 1-11: Schematische weergave van de gehele procedure MinION sequencing vanaf RNA extractie tot data analyse.

Resultaten van de analyse van de 3 EWS monsters zijn weergegeven in Tabel 1-3. Te zien is dat de 4 monsters tobamovirussen bevatten. Ook werden in twee monsters (EWS-1 en EWS-4) twee virussen aangetroffen.

Tabel 1-3: Resultaten van de analyse mbv MinION sequencing op 4 monsters van East West Seed. Het aantal sequentie reads dat past op de referentie sequentie is weergegeven.

		Reference						Mapping result	
Barcode	Sample	CGMMV	PPMoV	TMV	ToBRFV	ToMMV	ToMV	found	expected
BC01	EWS-1	17,810		63				CGMMV + TMV	CGMMV
BC02	EWS-2		44,160					PPMoV	PPMoV
BC03	EWS-3			16,661				TMV	TMV
BC04	EWS-4			57,528			1,051	TMV + ToMV	ToMV

Nematoden

Een pilotexperiment is uitgevoerd om te kijken of we ook nematodenmengsels kunnen analyseren met ampliconsequencing met het MinION platform. Daartoe werd DNA van *Pratylenchus penetrans* en *Globodera pallida* in verschillende verhoudingen gemengd. De 6 mengsels werden met generieke 18S primers voor nematoden (WU-Nematologie) geamplificeerd en de amplicons werden op het MinION platform gesequenced en geanalyseerd mbv de NCBI database. Resultaten (Tabel 1-4) laten zien dat een 1% aanwezigheid van Globodera zichtbaar is in een achtergrond van 99% Pratylenchus en andersom. Ook zien we dat er andere nematodensoorten aanwezig zijn in het Pratylenchus monster en dat er schimmel (genus Plectosphaerella) aanwezig is in het Globodera monster.

Tabel 1-4: Resultaten van de analyse mbv MinION sequencing op 6 mengsels van DNA van 2 nematodensoorten.

Barcode	Expected species	*Pratylenchus*	*Globodera*	*Chiloplacus*	*Acrobeloides*	*Pseudoacrobeles*	*Geocenamus*	*Rotylenchus*	*Merlinius*	Nematoda	*Plectosphaerella*
07	100% *Pratylenchus*	3375	0	6451	1205	236	96	0	0	620	0
	0% *Globodera*										
08	99% *Pratylenchus*	2263	259	4661	1215	0	170	0	0	332	0

	1% *Globodera*										
09	90% *Praty-lenchus*	2562	1211	4534	1231	0	0	83	89	274	0
	10% *Globodera*										
10	10% *Praty-lenchus*	336	2184	476	0	0	0	0	0	52	180
	90% *Globodera*										
11	1% *Praty-lenchus*	150	25984	161	0	0	0	0	0	0	1362
	99% *Globodera*										
12	0% *Praty-lenchus*	0	18275	0	0	0	0	0	0	0	771
	100% *Globodera*										

1.2.3.2 Conclusies

In deze studie hebben we een set van specifieke PCR primer pairen ontwikkeld voor de simulate detectie van 6 tobamo virussen.

In de 4 monsters van EWS konden de tobamovirussen goed worden aangetoond. Een tweetal monsters bevatten meerdere tobamovirussen.

Voordeel van de MinION sequencing t.o.v. andere sequencing technieken is de korte tijd tussen bemonstering en analyse, omdat het sequencen in huis kon worden uitgevoerd.

We concluderen dat ONT-sequencing voor plantpathogenen nog niet volledig geschikt is voor on-site toepassingen: monstervoorbereiding is arbeidsintensief en vereiste zowel opgeleid personeel als gespecialiseerde apparatuur. PCR stap en voorbereiden bibliotheek is tijdrovend.

Direct sequencen van een monster kan ook geschieden. Zeker als de concentratie van het virus hoog is. Om ook lage concentraties van het virus aan te tonen is derhalve in dit project gekeken naar amplicon sequencing. Echter er moeten dan generieke primers beschikbaar zijn of een mengsel van PCR primers worden samengesteld die in multiplex PCR goed werken. In dit project is multiplex PCR amplicon sequencing van een 6-tal tobamovirussen onderzocht. Goede resultaten zijn behaald met een aantal monsters afkomstig van East West Seed.

Het pilotexperiment met nematoden laat zien dat er potentie zit om ook nematodensuspensies m.b.v. ampliconsequentie analyse met het MinION platform te analyseren.

We concluderen dat Nanopore-sequencing een groot potentieel heeft om ziekteverwekkers en plagen in complexe gemeenschappen op te sporen. De aanpak van het gebruik van zeer specifieke primers voor amplicon-sequencing in een multiplex-benadering is in de praktijk echter niet geschikt voor detectie on-site.

2 Report EN

2.1 Introduction

The increasing international transport of plant products and climate change create problems for the Dutch horticultural sector through the spread of pathogens. The development of rapid, accurate and sensitive methods for identification and detection of (plant) pathogens that can be applied directly in the production chain is essential. Early detection enables timely action to prevent spread of the disease or delay and restrictions in the export of planting material and seeds. In the past, many detection methods have been developed for a large number of plant pathogens. The challenge in this proposal was not only on-site testing for these organisms, but also testing for several organisms in one reaction. Also, on-site sequencing of diagnostic samples has not previously been tested in horticulture but shows great promise.

Wageningen Plant Research, together with partners from private companies, worked on developing rapid on-site detection methods. Following a simple preparation, these methods enable detecting the presence of a pathogen at the point-of-care by a grower, advisor, or inspector within one hour. Good results have been obtained with these kinds of tests on symptomatic material, which enable identification of a wide range of pathogens (viruses, fungi, bacteria, phytoplasmas, viroids and nematodes) and infestations (thrips, white fly, moths etc.). If the symptoms caused by a pathogen are known, the most rapid and simple diagnostic method is visual evaluation. However, symptoms are not always unambiguous or can vary with external factors. Correct and fast diagnosis of suspicious ("symptomatic") plant material is necessary to confirm the health status of the material. Besides testing of actual plants, also planting substrate or irrigation water are a potential source of invisible pathogens.

The on-site tests developed so far are for single use i.e., they are suitable for the detection of only one pathogen. However, there is a need for a rapid and reliable DNA/RNA test for the identification of several pathogens in one test. By using this kind of multipletest (multiplex) the end-user does not have to select a specific test but can use a whole test panel. Thus, the applicability of the on-site test for different material (air, water, seeds, plant material, flowers, vegetables, and fruit) is strongly increased and there is a limited need for expertise in the use of such a test. Therefore, for a number of crops, an on-site general system will be developed for several pathogens simultaneously, using knowledge from earlier projects. First trials with this innovative approach show promise.

At the same time, an innovative on-site detection system will be developed based on determining the DNA sequence of unique regions in the genome of organisms. Detection by sequencing is the most comprehensive technique to identify all (plant) pathogens, as precise sequences can reveal more information about the genetic composition of the pathogen. In spite of this potential advantage, sequencing analysis is seldom used as a first diagnostic test in the case of unknown symptoms because of technical and financial restrictions. A recent development that could lead to an improved analysis is the use of the Oxford Nanopore Technology (ONT), which provides a portable sequencing machine that can be used in a simple laboratory setting. Still, sample preparation by experienced lab personnel is required. However, the intention is that future development will lead to easier, more automated sample preparation that will enable sequencing on-site.

The development and application of these technologies for disease diagnostics in different substrates can be a break-through in this field. These technologies are generic and can also be applied in other agricultural and horticultural sectors.

2.2 Aim

One aim is to simplify the existing method for DNA/RNA extraction in order to be able to use it on-site. Secondly, we aim to develop a range of diagnostic test that can be used on-site for different viral, bacterial, and fungal pathogens in symptomatic infected leaf material. Since symptoms are not always easy to identify, the possibility of using multiplex methods will be tested. Thirdly, we will investigate the

use of ONT sequencing for specific diagnostic testing for a panel of different pathogens that can also be used as a multiplex approach.

2.3 Workplan

In this project the different activities are performed in three workpackages (Figure 2-1).

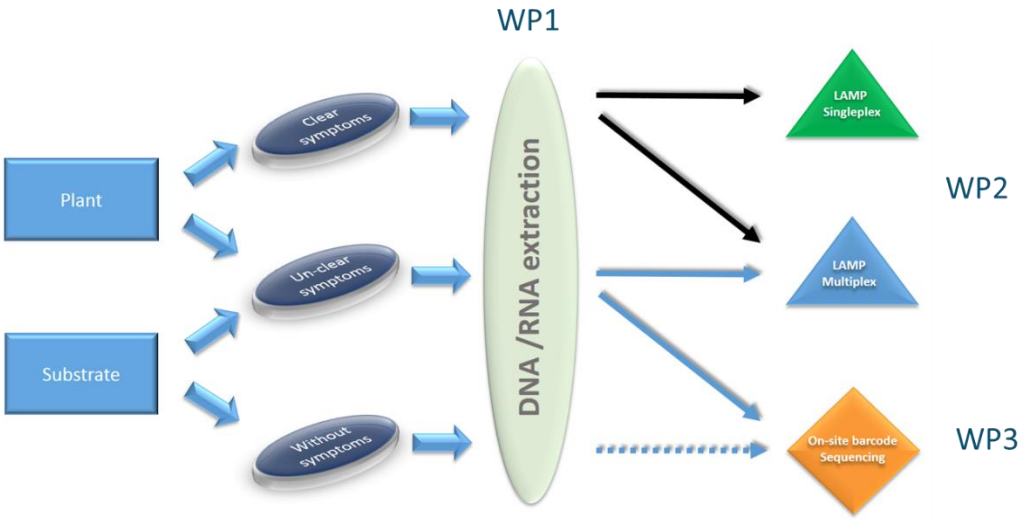


Figure 2-1: Outline Project PPS On-site and barcoding.

During detection in the production chain a number of different aspects play an important role. These are: extraction, detection method, analysis, and data interpretation. For a good detection, all these aspects have to be considered.

WP1: DNA/RNA extraction: From different selected substrates (such as leaf, stem, water, air etc.) DNA and RNA is extracted in a simple way.

WP2: LAMP: Development of an on-site multiplex method using previously developed LAMP tests or those described in literature. These will be complemented by new developed tests.

WP3: On-site and barcode sequencing using the MinION (ONT): PCR amplicons will be generated from selected barcode regions and then prepared for sequencing in a MinION system.

2.4 Targets and Timeline of the project

Targets for the whole project were selected by the partners. Detection assays were developed for either LAMP (L) or the MinION (M) platform (Figure 2-2). For the original timeline see Figure 2-3.

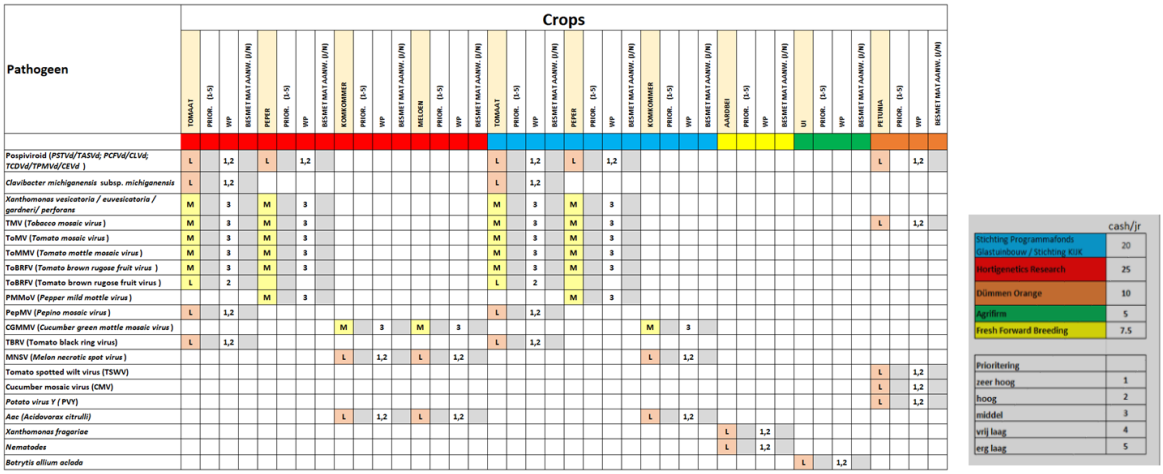


Figure 2-2: Targets and detection methods used in the project

Projectduur	Jaar 2019		Jaar 2020		Jaar 2021		Jaar 2022	
Projectfases en milestones	Mnd 1-6	Mnd 7-12	Mnd 13-18	Mnd 19-24	Mnd 25-30	Mnd 31-36	Mnd 37-42	Mnd 43-48
M 1.1 ontwikkeling NA methode								
M 1.2 vergelijking NA methode								
M 2.1 pathogene en substraat keuze								
M 2.2 ontwikkeling multiplex								
M 2.3 validatie van het multiplex systeem								
M 2.4 detectie pathogenen in de lucht								
M 2.5 implementatie multiplex								
M 3.1 inventarisatie barcode merkers								
M 3.2 amplificatie barcodes								
M 3.3 ontwikkeling en validatie MinIon								
M 3.4 testen van praktijk cases								
M 3.5 implementatie en praktijkdemonstratie								
M 3.6 toepassen software real-time identificatie								
M 4 eindrapportage								

Figure 2-3: Original timeline of the project.

2.5 WP1: DNA/RNA extraction

2.5.1 Introduction

For a simple on-site extraction of pathogens, it is important that DNA or RNA can be rapildy extracted without complex laboratory equipment. Furthermore, the extraction method should be suitable for a wide range of crops. In addition, the ingredients used for extraction should not inhibit the detection and at the same time plant inhibit plant compounds should be reduced as much as possible.

Various buffers are available for extraction and the reagent Chelex-100 was found to bind cell-components and protect DNA. However, no comparison was yet made between the different extraction methods and their effect on detection. The aim of WP1 was to identify the most suitable extraction protocol for on-site LAMP detection.

2.5.2 Methods

Comparison of extraction buffers

For the protocol and samples see Appendix WP.1.

The extraction buffers tested were OptiGene lysis buffer (OptiGene), Quickextract buffer (Epicenter), USEB buffer, and self-made polyethylene glycol (PEG) buffer.

Extraction efficiency was tested in tomato with the ToBRFV-LAMP assay (see WP2) and in melon with the *A. citrulli* LAMP assay (see WP2). Extraction from the remaining plants was tested with a LAMP for the Cox gene. Three samples were created for each pathogen by using either 100% infected material, 10% infected material+90% healthy material or 0% infected material+ 100% healthy material.

Comparison of PEG buffers

For the protocol and sample see Appendix WP.1.

PEG lysis buffer from OptiGene as well as self-made PEG buffer (see Appendix) were tested for LAMP in leaf/fruit material of several crops: beetroot, broccoli, carrot, dahlia, green beans, kale, leek, oxheart cabbage, savoy cabbage, spinach, strawberry en sweet william.

FTA cards

For the protocol see Appendix WP.1.

Three different Whatman FTA-cards (Figure 2-4), the Classic Card, the Plant Saver Card, and the Elute Micro Card were tested n combination with two different extraction protocols (see appendix). These were tested on melon leaf infected with *A. citrulli* (DNA) (Table 2.5-1) and tomato leaf infected with ToBRFV (RNA) (Table 2.5-2). After extraction the developed LAMP assays were performed (WP2).

Figure 2-4: Example of an FTA-card.

Table 2.5-1: Samples for extraction from FTA-cards with A. citrulli.

Sample	Description	Target
L1	Classic card, LAMP extraction	*A. citrulli*
L2	Plant saver card, LAMP extraction	*A. citrulli*
L3	Elute micro card, LAMP extraction	*A. citrulli*
W1	Classic card, Whatman method	*A. citrulli*
W2	Plant saver card, Whatman method	*A. citrulli*
W3	Elute micro card, Whatman method	*A. citrulli*
PC	Positive contro	*A. citrulli*
NTC	Negative controle	*A. citrulli*
L1 Cox	Classic card, LAMP extraction	Cox
L2 Cox	Plant saver card, LAMP extraction	Cox
L3 Cox	Elute micro card, LAMP extraction	Cox
W1 Cox	Classic card, Whatman method	Cox
W2 Cox	Plant saver card, Whatman method	Cox
W3 Cox	Elute micro card, Whatman method	Cox
PC	Positive control	Cox
NTC	Negative control	Cox

Table 2.5-2: Samples for extraction from FTA-cards with ToBRFV.

Sample	Description	Target
L1 Rugose	Classic card, LAMP extraction	ToBRFV
L2 Rugose	Plant saver card, LAMP extraction	ToBRFV
L3 Rugose	Elute micro card, LAMP extraction	ToBRFV
W1 Rugose	Classic card, Whatman method	ToBRFV
W2 Rugose	Plant saver card, Whatman method	ToBRFV
W3 Rugose	Elute micro card, Whatman method	ToBRFV
PC	Positive control	ToBRFV
NC	Negative control	ToBRFV
L1 Cox	Classic card, LAMP extraction	Cox
L2 Cox	Plant saver card, LAMP extraction	Cox
L3 Cox	Elute micro card, LAMP extraction	Cox
W1 Cox	Classic card, Whatman method	Cox
W2 Cox	Plant saver card, Whatman method	Cox
W3 Cox	Elute micro card, Whatman method	Cox
PC	Positive control	Cox
NC	Negative control	Cox

2.5.3 Results

Comparison of extraction buffers

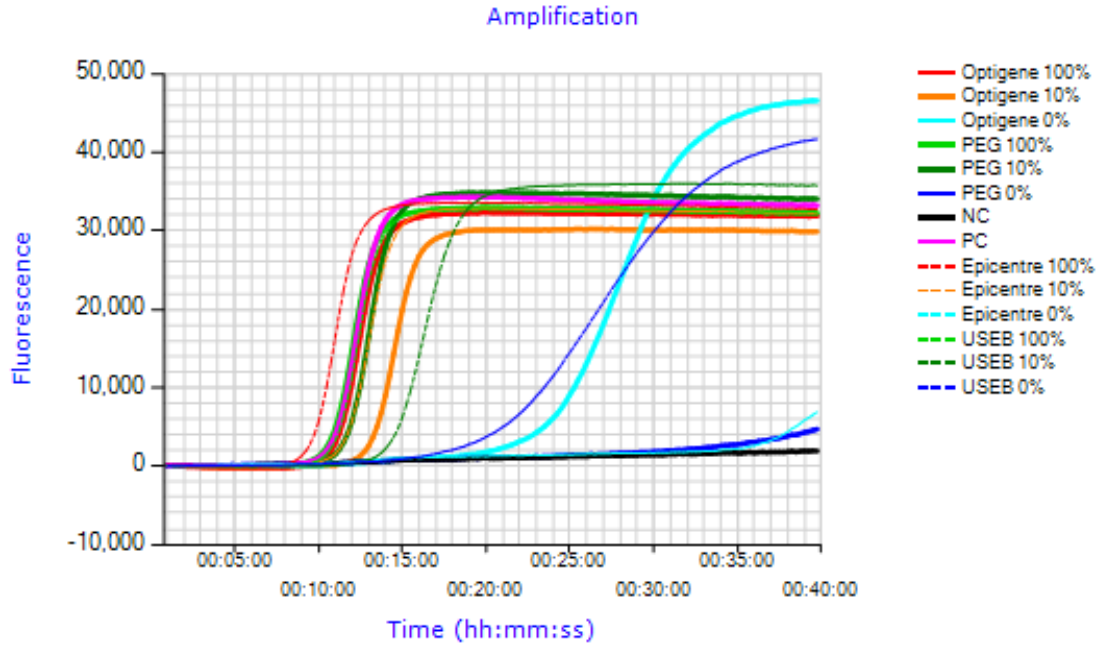
Different buffers for a simple extraction were tested on tomato and melon leaf. There were only small differences between the different buffers (Table 2.5-3,

Table *2.5-4*, 7, Figure 2-5a, Figure 2-7a). The best results were obtained with the OptiGene lysis buffer and the self-made PEG buffer, which showed the earliest amplification (Tpos: Time of positivity) of samples with as low as 10% infected material. Healthy material did not give any amplification with the PEG buffer extraction and the signal with the OptiGene buffer for ToBRFV was aspecific as shown by the difference in melting temperature (Figure 2-5a, Figure 2-7b). Amplification of the Cox gene was similar with all buffers (Figure 2-6, Figure 2-8).

Table 2.5-3: ToBRFV LAMP results after extraction with the different buffers.

Buffers	**ToBRFV**		**Cox**	
	Tpos (min)	**Tm (°C)**	**Tpos (min)**	**Tm (°C)**
OptiGene 100%	12:00	86.9	12:15	84.9
OptiGene 10%	14:15	86.9	12:15	84.8
OptiGene 0%	25:45	67.6	12:45	84.8
PEG 100%	11:45	86.9	13:15	84.8
PEG 10%	12:45	86.8	12:15	84.6
PEG 0%			11:45	84.6
Epicentre 100%	10:45	86.9	12:15	84.9
Epicentre 10%	12:45	87.0	11:30	84.9
Epicentre 0%	38:00		12:00	84.7
USEB 100%	12:00	87.0	12:00	84.8
USEB 10%	15:45	87.0	14:15	84.8
USEB 0%	24:30	67.7	13:00	84.8
NC: neg control	-	-	-	-
PC: pos control	12:00	86.2	10:30	84.3

a



b

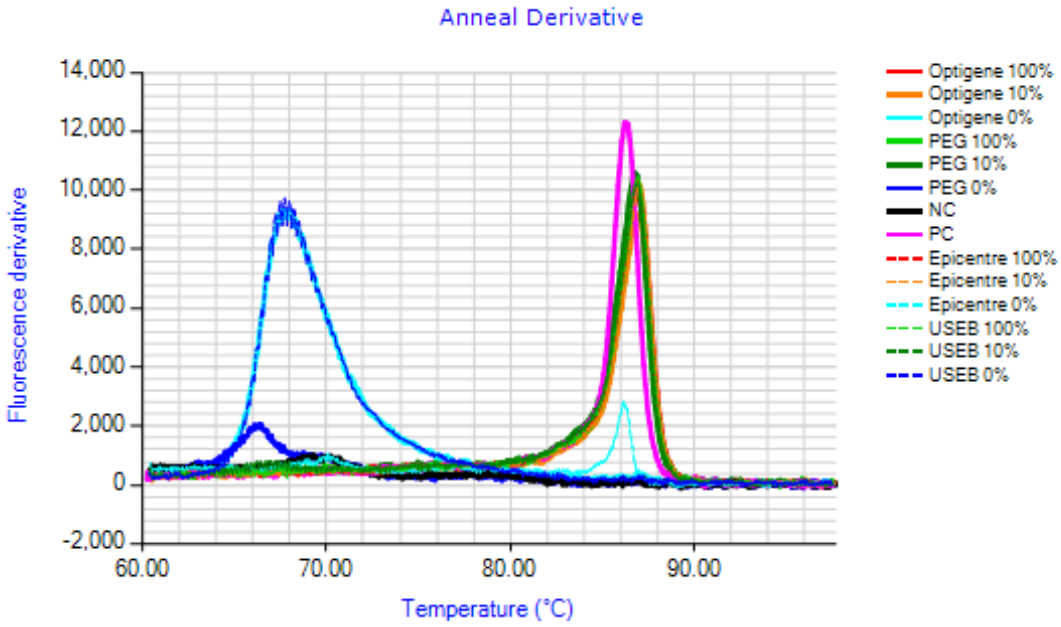
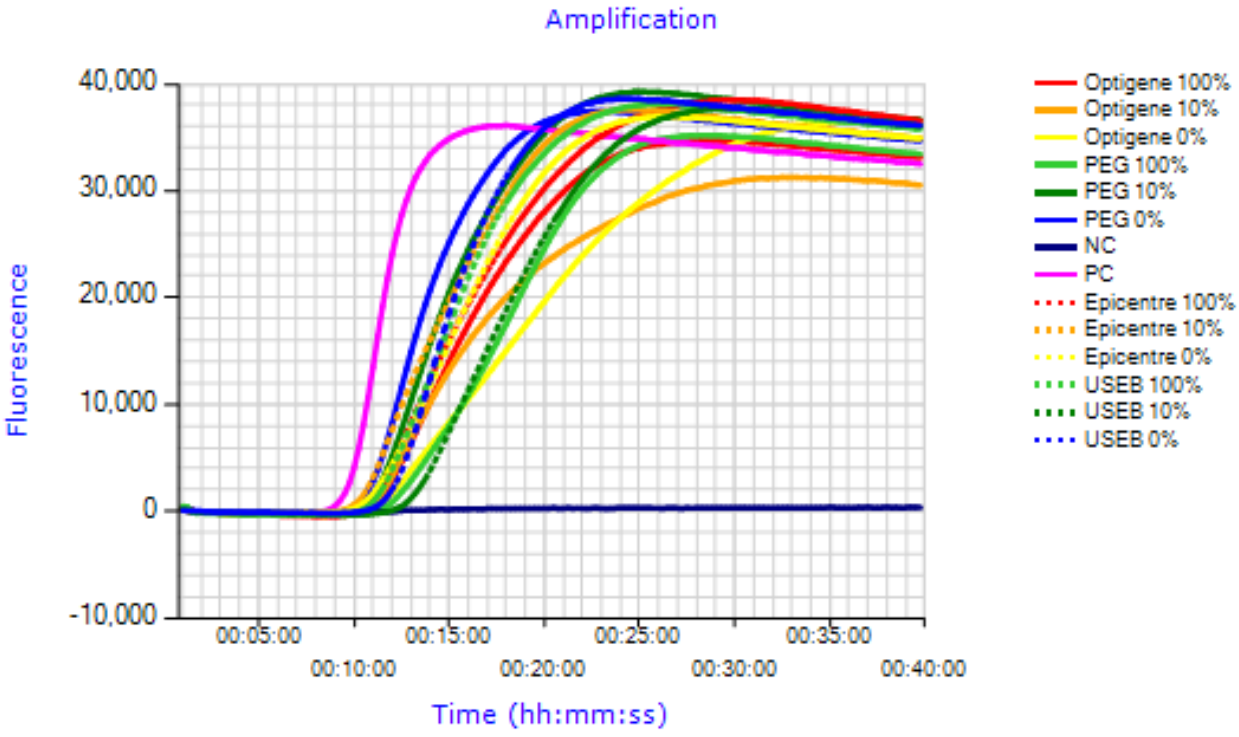


Figure 2-5: ToBRFV LAMP amplification: a) amplification curves, b) melting curves.

a



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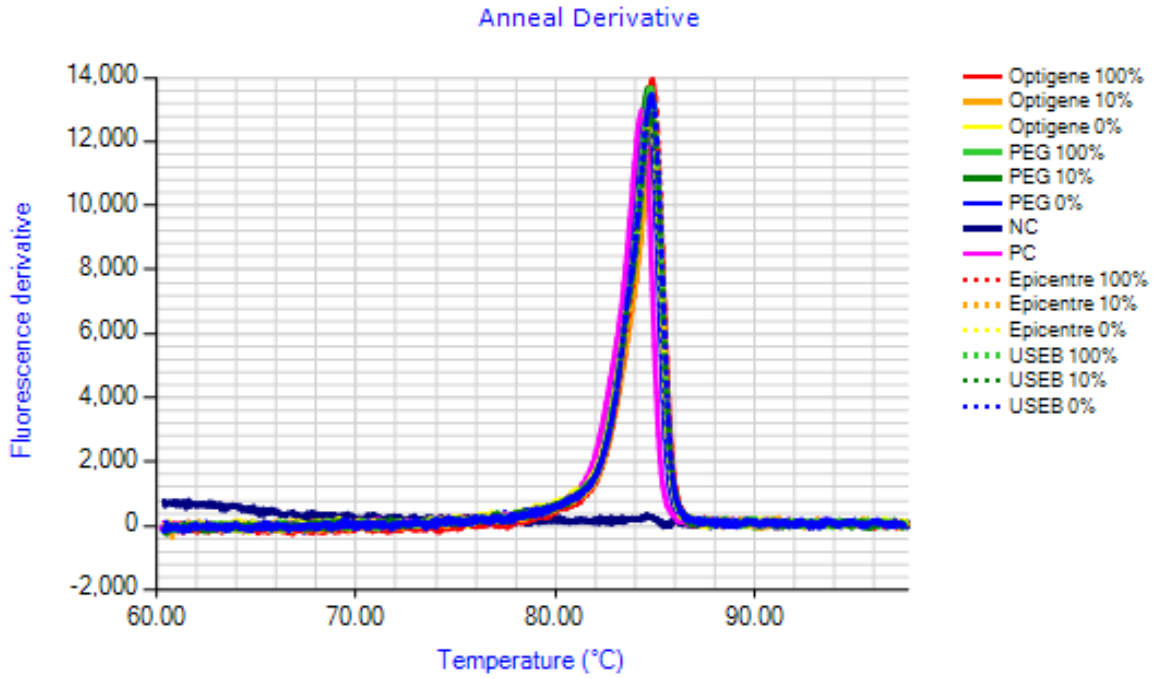
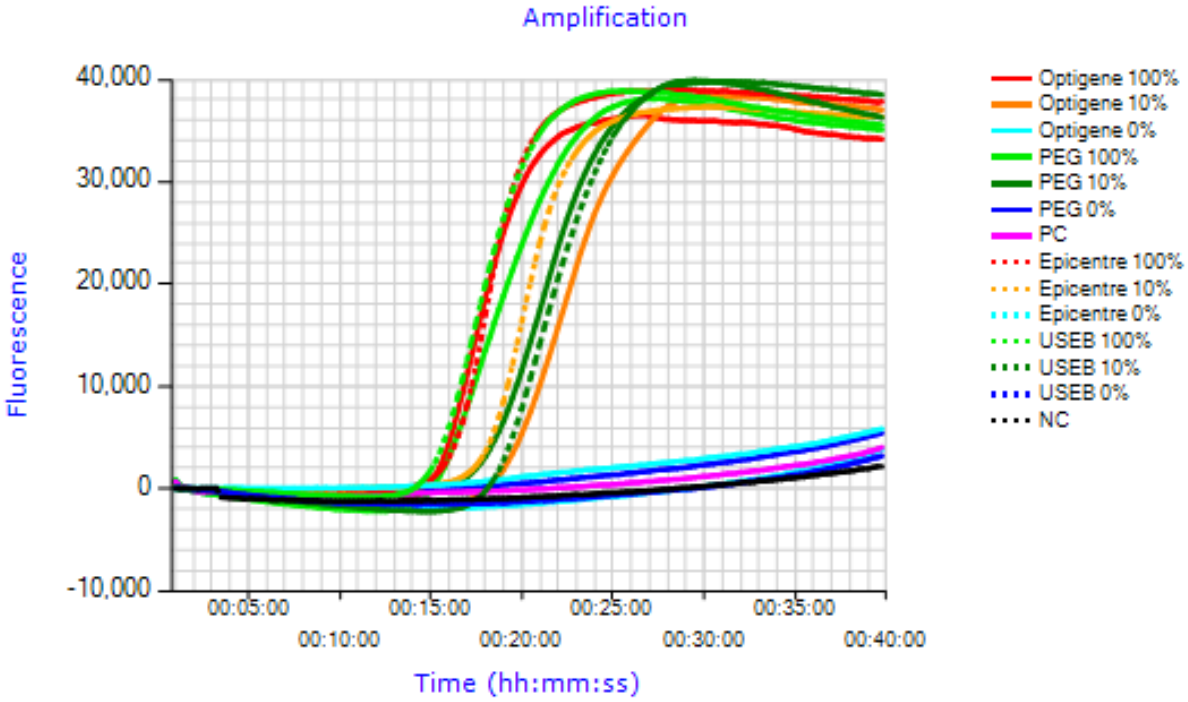


Figure 2-6: Cox amplification: a) amplification curves, b) melting curves.

Table 2.5-4: A. citrulli LAMP results after extraction with the different buffers.

Buffers	***A. citrulli***		**Cox**	
	Tpos (min)	**Tm (°C)**	**Tpos (min)**	**Tm (°C)**
OptiGene 100%	16:45	87.0	16:00	85.7
OptiGene 10%	21:30	87.0	20:15	85.9
OptiGene 0%	-	-	18:30	85.7
PEG 100%	17:00	86.7	19:45	85.6
PEG 10%	20:15	86.8	17:30	85.6
PEG 0%	-	-	19:00	85.6
Epicentre 100%	17:45	87.0	16:15	85.5
Epicentre 10%	19:45	87.0	16:45	85.4
Epicentre 0%			18:15	85.6
USEB 100%	17:15	87.0	16:45	85.4
USEB 10%	20:30	87.0	19:30	85.7
USEB 0%	-	-	21:45	85.6
NC: neg control	-	-	-	-
PC: pos control	-	-	14:45	84.4

a



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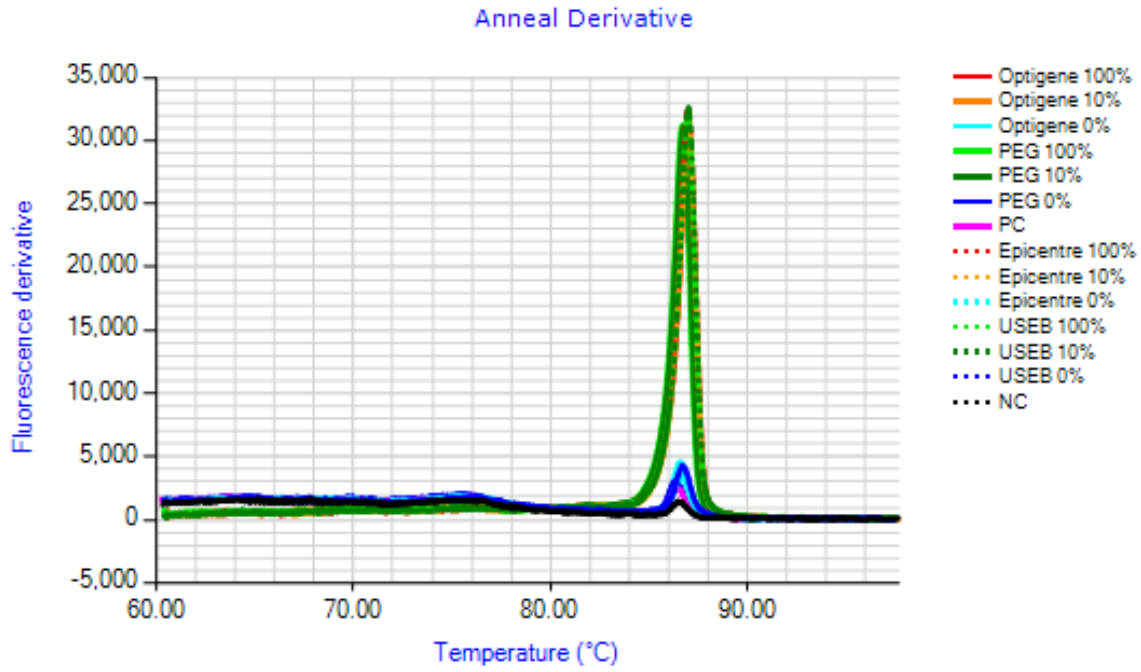
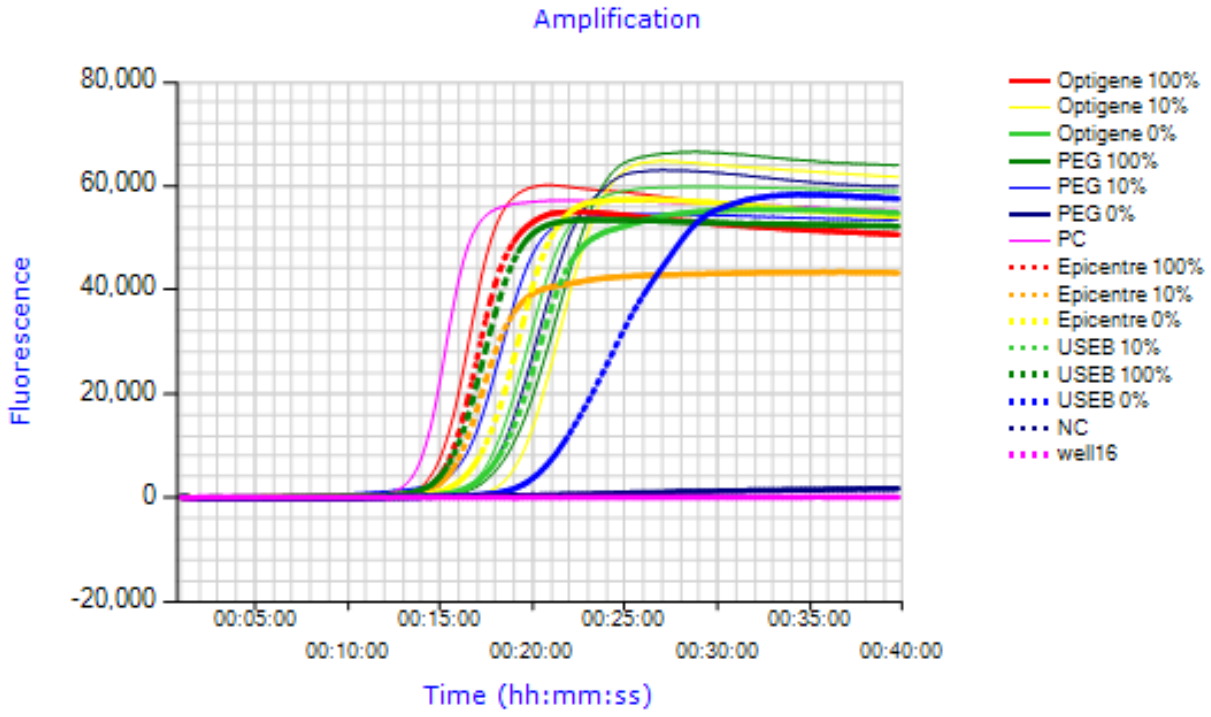


Figure 2-7: A. citrulli LAMP amplification: a) amplification curves, b) melting curves.

a



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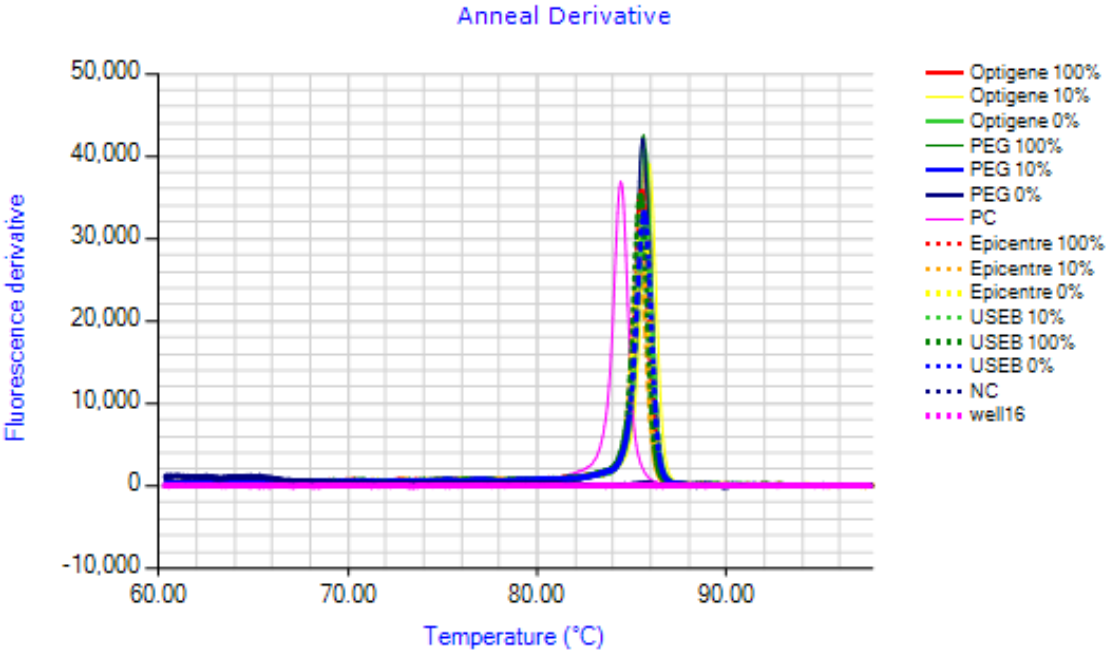


Figure 2-8: Cox LAMP amplification: a) amplification curves, b) melting curves.

Comparison of PEG buffers

Homemade PEG lysis buffer was compared to the PEG lysis buffer from OptiGene. Different crops, available at the time of testing, were tested. Analysis was done based on the *Cox* internal control assay.

Both extraction buffers appeared to work equally well and therefore the self-made PEG buffer is recommended because of the lower costs (Table 2.5-5).

Table 2.5-5: Cox LAMP on leaves from several crops extracted with two different extraction buffers.

Crop	**Extraction buffer**	***COX (Run 1)***		***COX (Run 2)***	
		Tpos (min)	**Tm (°C)**	**Tpos (min)**	**Tm (°C)**
Beetroot	Optigene	26:30	85.3	30:00	84.9
Beetroot	PEG	26:00	85.2	24:00	84.9
Broccoli	Optigene	18:15	84.5	17:45	84.1
Broccoli	PEG	39:00	83.9	27:15	84.1
Carrot	Optigene	15:45	85.4	15:45	85.6
Carrot	PEG	15:30	85.5	16:15	85.5
Dahlia	Optigene	18:45	85.2	20:15	85.1
Dahlia	PEG	19:15	85.2	20:15	85.1
Green Beans	Optigene	16:30	85.2	17:00	85.1
Green Beans	PEG	16:00	85.2	16:45	85.1
Kale	Optigene	28:45	84.2	22:45	84.3
Kale	PEG	25:45	84.1	22:00	84.4
Leek	Optigene	18:00	85.1	21:00	85.2
Leek	PEG	20:45	85.2	23:15	85.2
Oxheart	Optigene	15:45	84.5	20:30	84.3
Oxheart	PEG	16:45	84.5	19:15	84.2
Savoy cabbage	Optigene	26:15	83.5	18:45	84.2
Savoy cabbage	PEG	20:00	84.3	20:15	84.2
Spinach	Optigene	28:45	85.0	29:15	85.4
Spinach	PEG	33:00	84.1	31:00	85.4
Strawberry	Optigene	21:15	85.0	21:15	85.1
Strawberry	PEG	29:30	84.6	-	-
Sweet william	Optigene	-		-	-
Sweet william	PEG	38:45	84.7	-	-

FTA cards

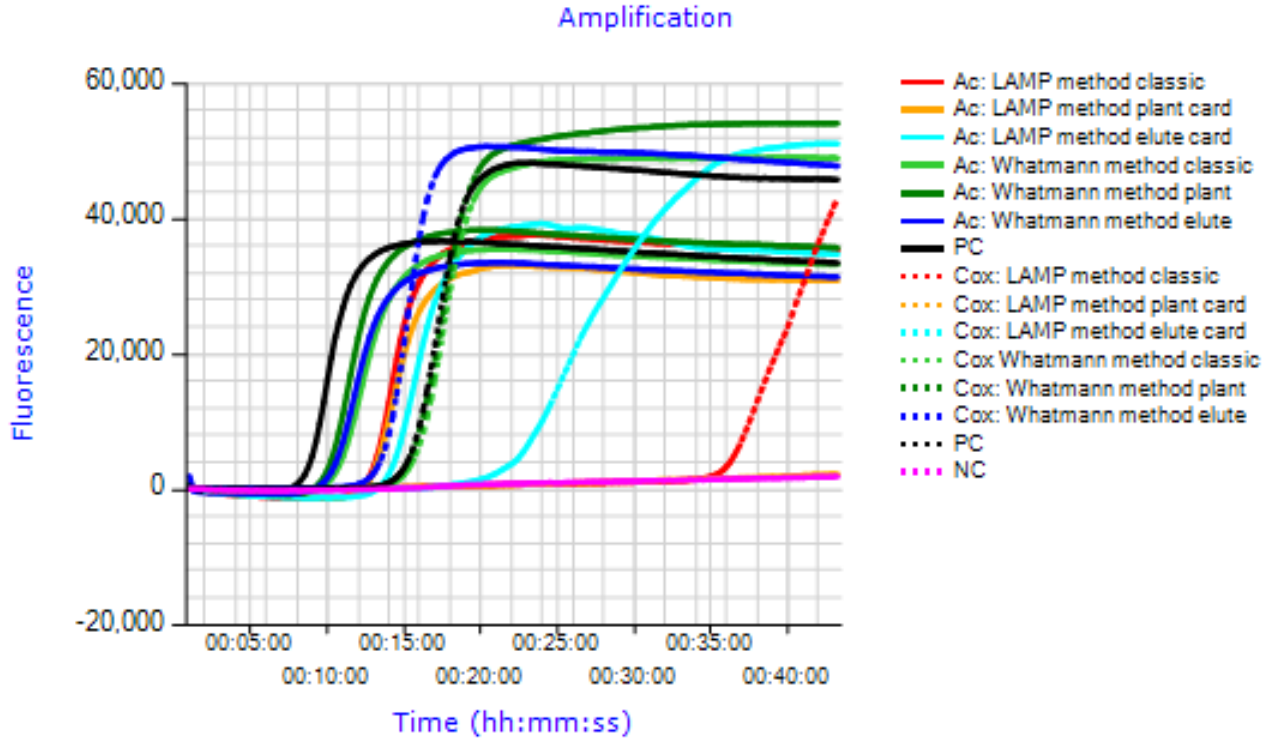
A. citrulli in melon was detected in all samples on all three types of cards and with both extraction methods (Table 2.5-6, Figure 2-9). The *Cox* ampification control was present, too, in most samples. Also, ToBRFV was detected in all samples, save one (Table 2.5-7, Figure 2-10). It could be seen that the Whatman extraction method gave more consistent results. In addition, the *Cox* LAMP only gave positive results with the Whatman protocol (Figure 2-11).

Overall, there was not much difference between the different cards. However, the Plant Saver Card is the most user friendly, especially for wet samples.

Table 2.5-6: Results from a A. citrulli LAMP after extraction from FTA-cards.

Sample	Aac		COX	
	Tpos (min)	**Tm (°C)**	**Tpos (min)**	**Tm (°C)**
L1	14:00	86,3	37:15	84,5
L2	14:00	86,3	-	-
L3	15:15	86,5	23:15	84,9
W1	12:00	86.9	17:00	85.4
W2	11:15	86.7	16:45	85.5
W3	11:30	87	14:45	85.7
PC	09:45	87	16:30	84.4
NTC	-	-	-	-

a



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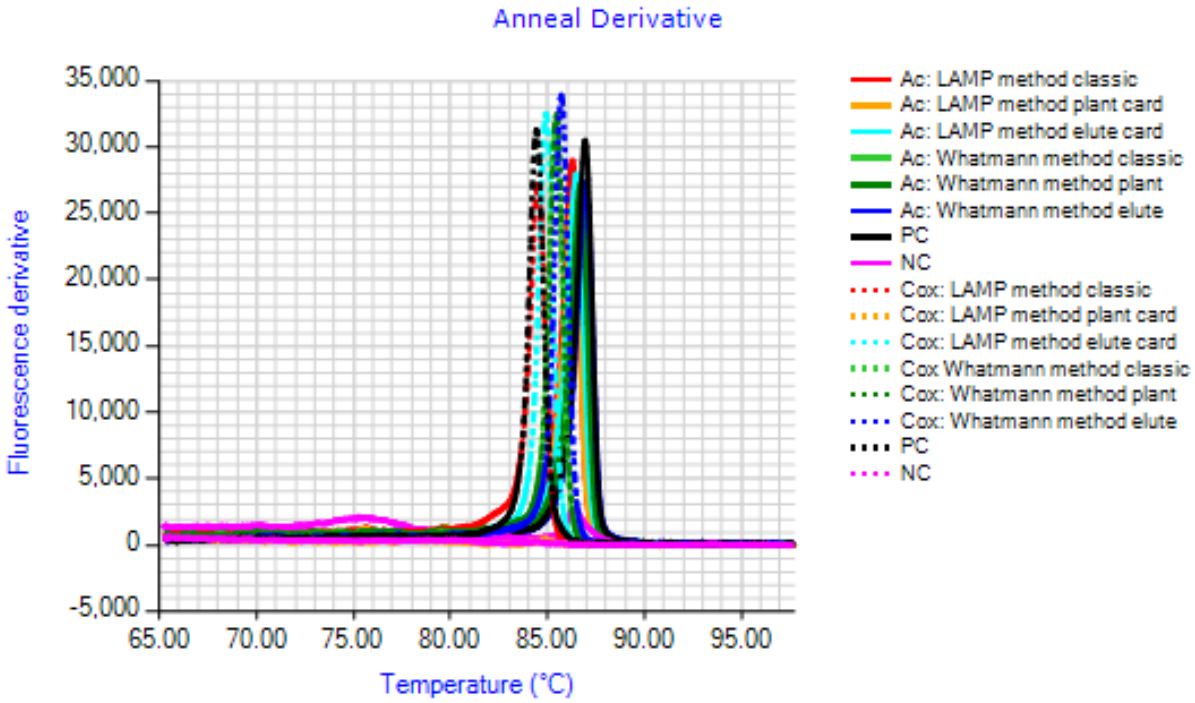
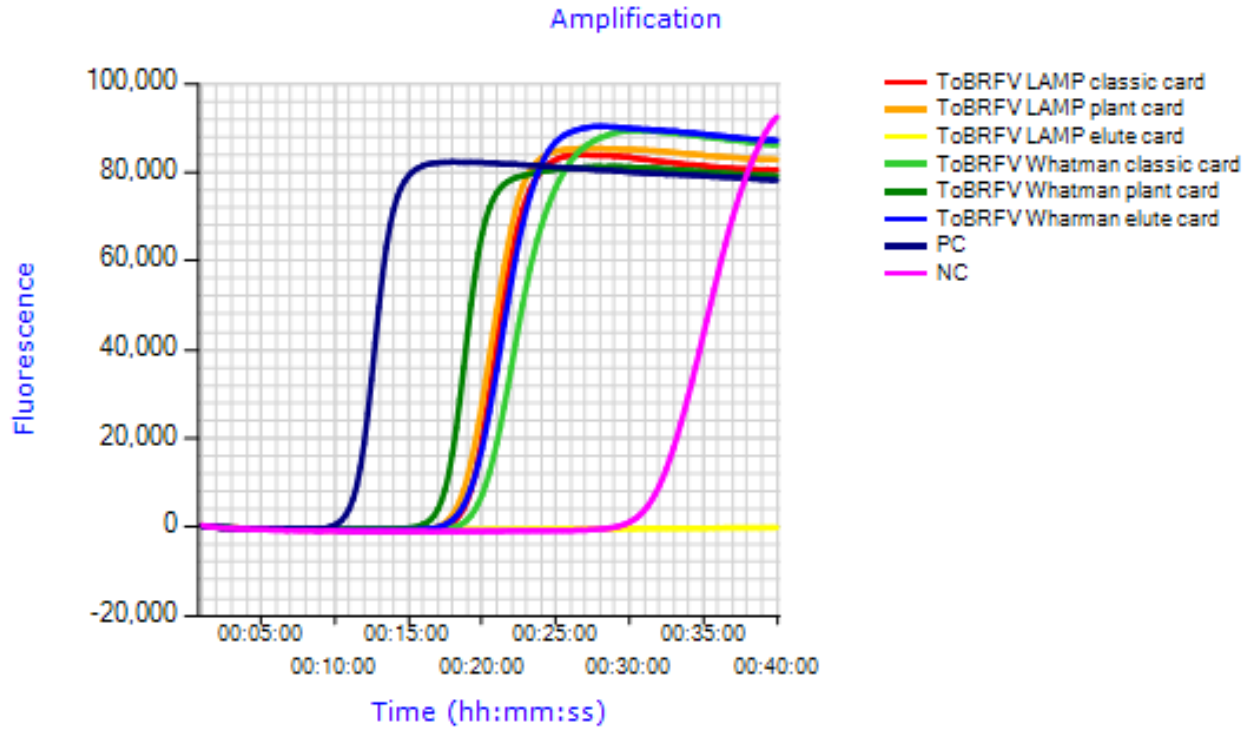


Figure 2-9: Results from a A. citrulli LAMP after extraction from FTA-cards: a) amplification curves, b) melting curves.

Table 2.5-7: Results of a ToBRFV- and Cox LAMP after extraction from FTA-cards.

Sample	**ToBRFV**		**Cox**	
	Tpos (min)	**Tm (°C)**	**Tpos (min)**	**Tm (°C)**
L1	20:15	85.4	-	-
L2	19:45	85.2	-	-
L3	-	-	-	-
W1	21:15	85.4	18:15	84.4
W2	18:30	85.5	-	-
W3	20:30	85.6	13:15	85.1
PC	12:30	85.5	15:30	84.4
NC	33:30	84.8	-	-

a



b

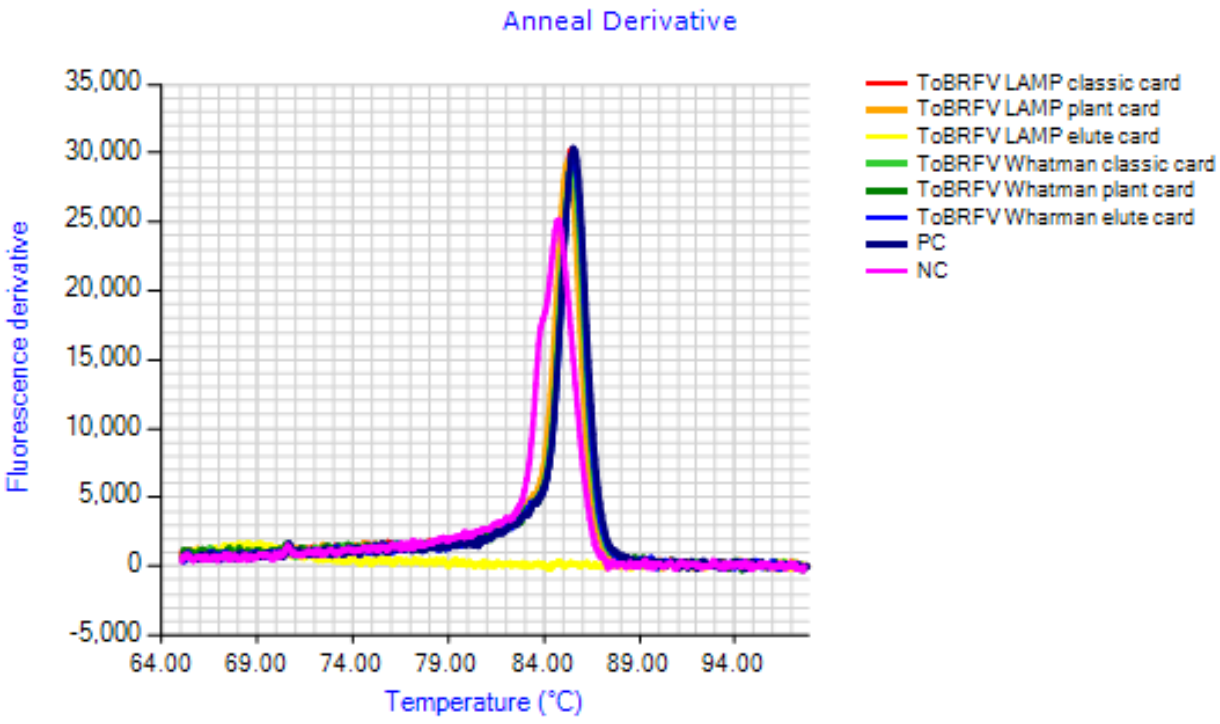
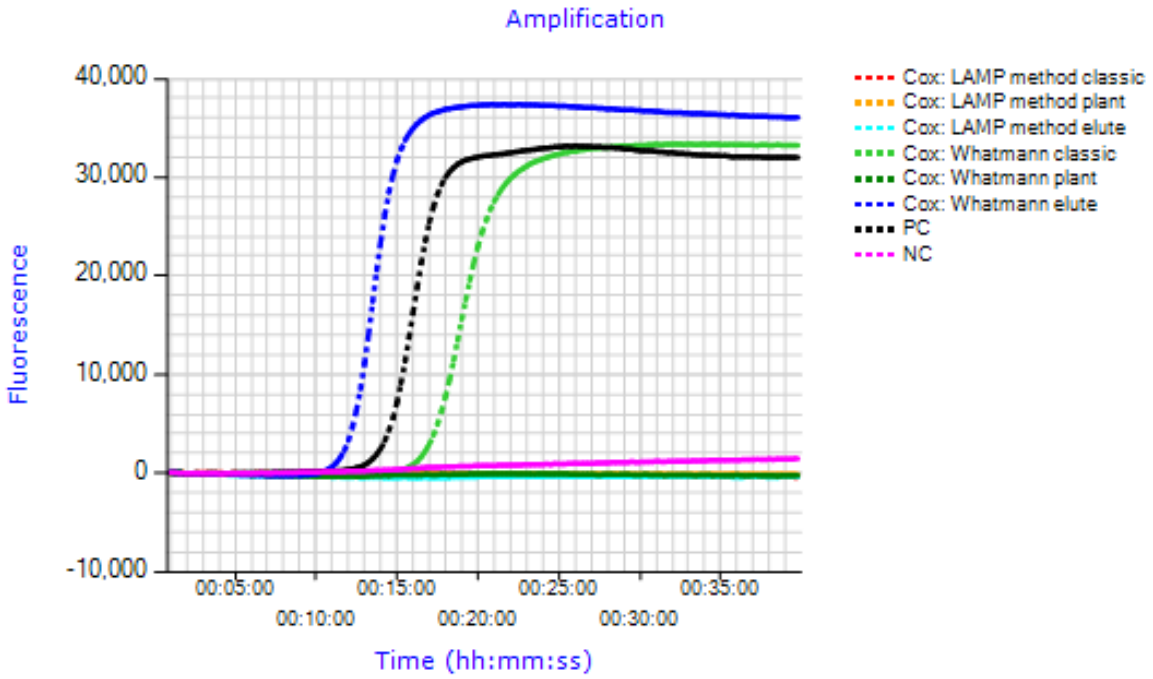


Figure 2-10: Results from a ToBRFV LAMP after extraction from FTA-cards: a) amplification curves, b) melting curves.

a



b

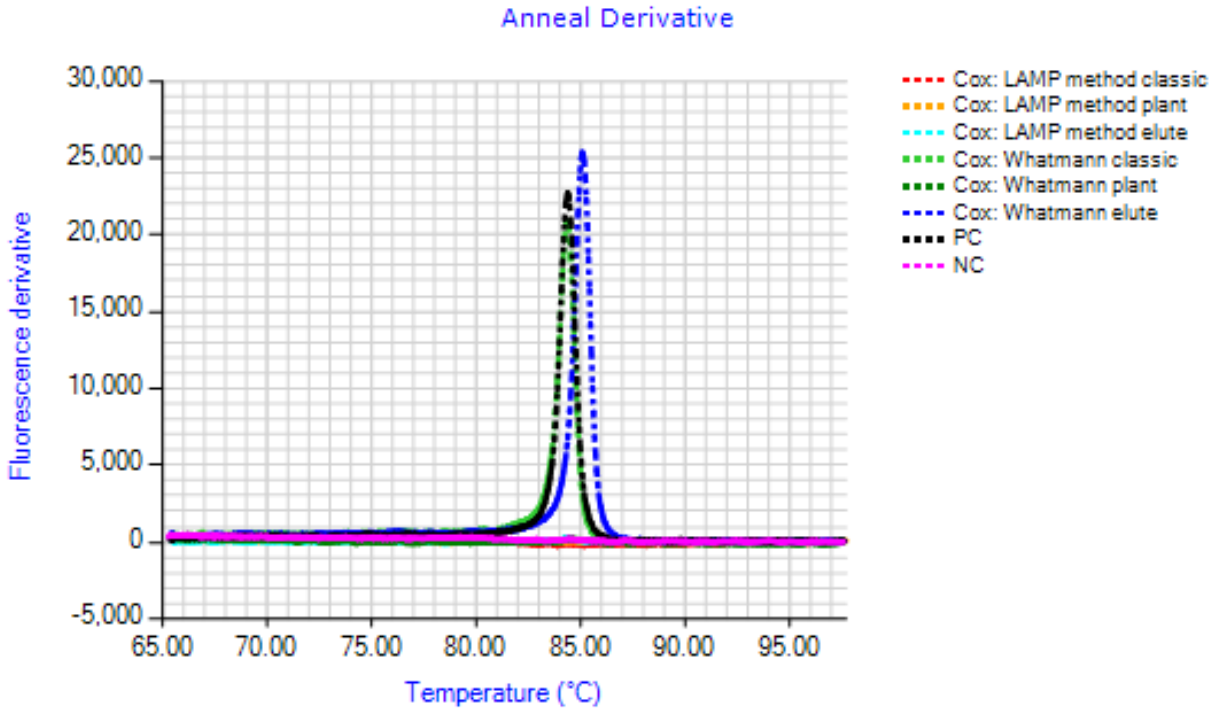


Figure 2-11: Results from a Cox LAMP after extraction from FTA-cards: a) amplification curves, b) melting curves.

2.6 WP2: LAMP

2.6.1 Introduction

For on-site detection of pathogens in the greenhouse or in the field a method is needed that does not require expensive lab equipment and as little preparation as possible. Often PCR or qPCR are used for detection. However, these methods are sensitive for inhibition by plant derived compounds and require an extended DNA extraction protocol and a lab based thermocycler. Isothermal methods, such as LAMP on the other hand, are less sensitive for inhibitors and can be performed at a single temperature (isothermal). These features make the method suitable for the application outside of labs. In addition, LAMP assays can be highly specific for the target DNA or RNA as they consist of 4 to 6 different primers. Since a LAMP run produces a high amount of amplicon the method is also rather sensitive. Still, specificity and sensitivity differ between LAMP assays and are dependent on e.g., the degree of similarity between target and nontarget, the variability within a target-group (e.g., a species) and the region of the genome for which the assay is designed. Therefore, extensive validation of newly developed LAMP assays is needed.

Table 2.6-1: Overview of the different LAMP targets.

Target	**Host plant**
Pospiviroide	Tomato, Pepper, Petunia
Clavibacter michiganensis	Tomato
TMV	Tomato
PepMV	Tomato
ToBRFV	Tomato
TBRV	Tomato
MNSV	Cucumber, melon
TSWV	Petunia
CMV	Petunia
PVY	Petunia
Acidovorax citrulli	Cucumber, melon
Xanthomonas fragariae	Strawberry
Fusarium, Verticillium	Tomato, Pepper, Onion

Pospiviroids

Different pospiviroids cause problems in a wide range of crop species. Thus, it is of importance to be able to detect a range of different pospiviroid species in the host plant of interest. In the present project the host species tomato, pepper and petunia were selected for pospiviroid detection. In a previous project three assays have been developed that together detect the species potato spindle tuber viroid (PSTVd), columnea latent viroid (CLVd), tomato apical stunt viroid (TASVd), pepper chat fruit viroid (PCFVd), tomato chlorotic dwarf viroid (TCDVd), tomato planta macho viroid (TPMVd) and citrus exocortis viroid (CEVd).

Clavibacter michiganensis

The pathovar *C. michagenensis* pv. *michiganensis* as recently been reclassified as one species, *C. michiganensis* (Cm). This pathogen is mostly transmitted via seeds and causes bacterial wilt and canker of tomato. In a previous study an assay for Cm has been developed based on a publication by Yasuhara-Bell et al. (2013).

TMV

Tobacco mosaic virus (TMV) is a member of the genus *Tobamovirus* with a wide host range of at least 199 plant species. The virus is mostly transmitted mechanically via soil, infected tissue, and tools. As all tobamoviruses it contains a single stranded RNA as genetic material with 4 open reading frames (ORFs), 2 for the RNA polymerase, one for the movement protein and one for the coat protein. In this project a

LAMP assay was designed for the movement protein as this region was sufficiently conserved to include all strains.

PepMV

The pepino mosaic virus belongs to the genus *Potexvirus*. It occurs mostly in solanaceous plants and mainly causes problems in tomato. Transmission occurs mechanically, but also via seeds and pollinating insects (Hanssen and Thomma, 2010). The virus has a single stranded RNA genome with five ORFs.

ToBRFV

Tomato brown rugose fruit virus (ToBRFV), a member of the genus *Tobamovirus*, has very recently emerged as a worldwode problem in tomato. Like TMV it contains a single stranded RNA as genetic material with 4 ORFs. In addition, ToBRFV is also mechanically transmitted, though at a low rate seedborne infections can occur. The LAMP assay in this project was designed on the coat protein sequence.

In the project KB 37 Diagnostics the ToBRFV LAMP-assay was modified to a LAMP-Crispr-Cas asay by adding a guide RNA. As this guide RNA detects the LAMP product, this LAMP-CC assay is more specific than a normal LAMP. This assay was developed by Scope Biosciences, who deliver a master mix containing a hot-start enzyme (New England Biolabs), LAMP primers and guide RNA together with the CISPR-Cas enzyme mix.

TBRV

Tomato black ringspot virus (TBRV) belongs to the genus *Nepovirus*. The virus is transmitted via seed or nematode vectors.

MNSV

Melon necrotic spot virus (MNSV) is a member of the genus *Carmovirus* and is mostly found in melon and cucumber. Transmission occurs through soil or water by the chytrid fungus *Olpidium radicale*. The genome consists of a single stranded RNA with five open reading frames. A LAMP assay has been developed earlier by Qiao et al. (2020). However, an in-silico analysis showed that this assay does not cover all strains.

TSWV

Tomato spotted wilt virus (TSWV) belongs to the genus *Tospovirus* and infects both tomato and ornamental plants such as dahlia and petunia. This virus is mainly transmitted by thrips. The genome consists of three RNA segments, the L-, M- and S-segment. The LAMP assay in this project was designed on the S-segment.

CMV

Cucumber mosaic virus (CMV) belongs to the genus *Carmovirus* and is known to infect a wide range of plant species. The virus is mainly transferred by aphids but can also be trasmitted via seed. It has a tri-partite RNA genome with five open reading frames.

PVY

Potato virus Y (PVY) belongs to the genus *Potyvirus* and infects solanaceaous hosts, such as potato and tomato, but also non-solanaceae, such as petunia. Originally, three strains PVY-C, PVY-N and PVY-O were described. However, by now multiple recombinant strains have been identified, the most common being PVY-NTN, PVY-Wi, and PVY-N:O.

Acidovorax citrulli

A. citrulli is a bacterial pathogen which causes bacterial fruit blotch in Cucurbitaceae and is mainly seed borne. In the previous project PPS On-site, a LAMP assay has been evaluated based on the assay by Oya et al. (2008), which was developed for the *hrpG-hrpX* gene spacer region.

Xanthomonas fragariae

X. fragariae is known to cause bacterial angular leaf spot in strawberry. Transmission can occur via infectd maternal plants or rain- and irrigation water (Kim et al., 2016).

Fusarium & Verticillium

Different *Fusarium* and *Verticillium* species can cause wilt symptoms in several important crops and ornamental plants. The most common species causing wilt is *F. oxysporum*, but also the species *F. proliferatum*, *F. solani* and *V. dahliae*. However, the symptoms caused by these pathogens are difficult to distinguish. Therefore, molecular testing is necessary to identify the pathogens responsible for an outbreak.

Multiplex

In some cases, it can be an advantage to be able to detect multiple targets in one sample, for example if the symptoms are not specific for one pathogen. However, due to the high number of primers in a LAMP assay it is often not possible to combine several assays without undesired interactions between the primers. The University of Pennsylvania developed a microchip, in which different LAMP assays can be performed in separate chambers on the same sample. In addition, a pre-amplification step with RPA (recombinase polymerase amplification) can be performed within the chip, which might increase the sensitivity of the assay. In this project, the chip was evaluated for its performance in multiplex LAMP experiments.

Air sampling

The intended air sampling and diagnostics of air samples had to be discontinued due to inavailability of the prototype of equipment by Optigene during the timespan of the project

2.6.2 Methods

For all protocols and samples see Appendix WP. 2.

Pospiviroids

Three LAMP assays (Table 2.6-2) were designed, which together detect the pospiviroids PSTVd, CEVd, PCFVd, TPMVd, TCDVd, TASVD and CLVD.

Table 2.6-2: LAMP primers for pospiviroids.

Set	Primer	Sequence
Set1	PSTVD_A_F3	GAAACCTGGAGCGAACTG
	PSTVD_A_B3	CGGTTCCAAGGGCTAAAC
	PSTVD_A_FIP(F1c+F2)	GGAAGGACACCCGAAGAAAGGGCCGACAGGAGTAATTCC
	PSTVD_A_BIP(B1c+B2)	GCTGTCGCTTCGGCTACTACCCGAAGCAAGTAAGATAGAGAA
	PSTVD_A_LoopF	GGTGAAAACCCTGTTTCGG
	PSTVD_A_LoopB	AACAACTGAAGCTCCCGAG
	TASVd_set3_nr1 F3	CCTGCAGGCATCAAGAAA
	TASVd_set3_nr3 B3	GGAGAGCAACAAAGATAGAGAA
	TASVd_set3_nr3 FIP(F1c+F2)	ACCAGAAGAAAGGAAGGGTGAAGGCTTCGGATCATTCCTG
	TASVd_set3_nr3 BIP(B1c+B2)	TTCCTCTCGCCGGAAGGTAGCTTCAGTTGTTTCCACC
	TASVd_set3_nr3 LoopF	CTGGGATTACTCCTGTCTCAAC
	TASVd_set3_nr3 LoopB	GGAGCTTCTCTCTGGAGACTA
	TASVd_set4N_nr1F3	GAACTTTCTTGAGGTTCCTGT
	TASVd_set4N_nr1B3	GGAGAGCAACAAAGATAGAGAA
	TASVd_set4N_nr1FIP(F1c+F2)	CACGAAGGAGTAGTCCGAAGCGGAGAAGAAGTCCTTCAGG
	TASVd_set4N_nr1BIP(B1c+B2)	TTTCACCCTTCCTTTCTTCGGCGGGTAGTCTCCAGAGAG
	TASVd_set4N_nr1LoopF	CCTCGACTTCCTCCAGGT
	TASVd_set4N_nr1LoopB	CTTCCTCTCGCCTGGAGA
Set2	CLVd_F3	GAGCGGTCTCAGGAGC
	CLVd_FIP_Alt	TCCGGGCGAGGCCGGTAATCCCCGCTGAAACAG
	CLVd_LoopF	GAAGAAAGGAAGGGTGAAAACC
	CLVd_LB	GCCTCAACCTCCTTTTTCT
	CLVd_BIP	GCAGGTTCTGACGCGATAAACACCCTCGCCCG
	CLVd_BIP_ext	GCAGGTTCTGACGCGAGCTTTTCACCCTCGC
	CLVd_B3	CTGCGGTTCCAAGGG
	PCFVd_A_F3	GAAGCAAGCATCTCCTGTT
	PCFVd_A_B3	AGAAGTCGGGTGGAAGAA
	PCFVd_A_FIP(F1c+F2)	CGGTCGACTGAGGAAGGAAACCGTCTTCTGACAGGAGTAATC
	PCFVd_A_BIP(B1c+B2)	CTTCTCGCGCACTGCTGTAAAGCACCTCTGTCAGTTG
	PCFVd_A_LoopF	CCGAAGAAAGGAAGGGTGAA
	PCFVd_A_LoopB	GGCTACTACCCGGTGGATA
Set3	TCDVd_A_F3	CGGAACTAAACTCGTGGT
	TCDVd_A_B3-new	GCGCAAAGGAAGGAAACC
	TCDVd_A_FIP(F1c+F2)	CCTCCGAGCCGCCTATCTTTCCTGTGGTTCACACCTG
	TCDVd_A_BIP(B1c+B2)	AACCTGGAGCGAACTGGCCGGGATTACTCCTGTTTCG
	TCDVd_A_LoopF	TTTCTTTTCTGCACAGGAGGT
	TCDVd_A_LoopB	CAGGGAGCTTGTGGAAGG
	TPMVd_E_F3	CCGCTGAAACAGGGTTTT
	TPMVd_E_B3	GTTCCAGGGTTTTCCACC
	TPMVd_E_FIP(F1c+F2)	AAGCGACAGCGCAAGAGAACACCCTTCCTTTCTTCGG
	TPMVd_E_BIP(B1c+B2)	CGGAGACTACCCGGTGGAAGGAGCCAGCAAGATAGAGA
	TPMVd_E_LoopF	ACCGCAGAGGAAGGAAAC
	TPMVd_E_LoopB	AACTGAAGCTCCCAAGCG
	CEVd_1_ F3	TCTTGAGGTTCCTGTGGT
	CEVd_1_ B3	TAGGGTTCCGAGGGCTTT
	CEVd_1_ LoopF	CTCCTGTTTCTCCGCTGG
	CEVd_LoopB_new	TACTACCCGGTGGATACAA
	CEVd_BIP(B1c+B2)_alt	TCGCCCGGAGCTTCTCTCTTGGGGTTGAAGCTTCAG
	CEVd_2_ FIP(F1c+F2)	GATCGGATGTGGAGCCAGCCTGGAGGAAGTCGAGGTC

Clavibacter michiganensis

The primers were designed on the micA gene and adapted from an earlier design by Yasuhara-Bell et al. (2013) (Table 2.6-3). In addition, an assimilation probe was added to the LoopF primer.

Table 2.6-3: LAMP primers for C. michiganensis sp. michiganensis.

Primer	**Sequences**
Cmm_FIP-CS2	GCGTCGAGCAGCATGTCCCAACACGATGAACGACATCCTC
Cmm_BIP	CGTCCGTCCAGACCCAGATCGCTGGACATGTACGGGCTCA
Cmm_F3	CGACAACAGGAACACAGGT
Cmm_B3-CS1	CCCGCATTCGATGGTGAGC
Cmm_Loop F	TGACCATGACGGGGGTCT
Cmm_Loop F_FAM	/56-FAM/ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGATGACCATGACGGGGGTCT
Universal quencher	TCGGCATCCGCATCCGCATTCGCATCCGGGTCCTCAGCGT/3BHQ_1/

ToBRFV

The LAMP assay was designed on the coat protein and covers the same region as the ISHI-Veg ToBRFV TaqMan (ISHI-Veg, 2019) (Table 2.6-4). The sensitivity was tested with a dilution range of RNA. Specificity was tested on gBlocks and RNA of closely related species. Finally, the LAMP assay was tested on infected plant material (Table 2.6-5). In addition, a colorimetric assay was performed for ToBRFV. The assay was also used in a multiplex with the plant household gene *Cox* (see Appendix),

Table 2.6-4: Selected LAMP primers for ToBRFV.

Primer	**Sequence**
ToBRFV-Y4-F3	GTGGTTTTAAGGTGTATAGGTAC
ToBRFV-4-B3	CTTTCAAATGTGCTCTGATTG
ToBRFV-Y4-FIP-mod	TCGACTTCTATAATCCTAT-AATGCGGTACTAGATCCTC
ToBRFV-Y4-BIP-mod	CGACAACCGCCGAAACGTTA-CAAACCTGTTCCTTTGAC
ToBRFV-Y4-LoopF-mod	GAAAGCTCCTAACAAAGCAGTAACT
ToBRFV-Y4-LoopB-mod	ATGACGCAACGGTGGCTAT

Table 2.6-5: Plant material tested with the ToBRFV LAMP assay.

Code	**Treatment**	**time point of harvest**	**date**	**Sample type**
MT1	mock	7 dpi	16.4.21	ground powder
VT1	ToBRFV	7 dpi	16.4.21	ground powder
MT2	mock	14 dpi	23.4.21	ground powder
VT2	ToBRFV	14 dpi	23.4.21	ground powder
MT3	mock	21 dpi	30.4.21	ground powder
VT3	ToBRFV	21 dpi	30.4.21	ground powder
LP1.1	ToBRFV	26 dpi	5.5.21	leaf punch
LP1.2	ToBRFV	26 dpi	5.5.21	leaf punch
LP2.1	mock	26 dpi	5.5.21	leaf punch
LP2.2	mock	26 dpi	5.5.21	leaf punch

TMV

The TMV primers were designed on the movement protein gene (

Table 2.6-6). An assimilation probe was added to the LoopF primer. The sensivity of the assay was tested on a dilution range of gBlocks. Specificity was tested on both gBlocks from closely related species and RNA spiked to tomato and petunia leaves. For TMV, RNA and infected material were tested. The assay was also tested in a multiplex with Cox.

Table 2.6-6: Selected LAMP primers for TMV.

Primer	**Sequence**
F3-3	GAGTGGAACTTGCCTGAC
B3-3	ACAAACTCCAGAGAAAGCG
LoopF-3	CTCGTCGGCTCTTTCCAT
LoopB-3	GCTATAACCACCCAGGACG
F2-3	GTGTGTCTGGTGGACAAA
B2-3	ACTAAAACTTGCCAGACGTT
F1c-3	GCTGCTGTGTAGTAAGATCCGA
B1c-3	TTTCAGTTCAAGGTCGTTCCCA
LoopF-3-FAM	FAM/ACGCT GAGGA CCCGG ATGCG AATGC GGATG CGGAT GCCGA CTCGTCGGCTCTTTCCAT
Quencher strand	TCGGC ATCCG CATCC GCATT CGCAT CCGGG TCCTC AGCGT/ 3' Black hole quencher 1

TBRV

In accordance with the project participants, it was decided not to develop a LAMP assay for TBRV because most sequences that are available originate from isolates from Poland and Lithuania. These groups differ considerably from each other and it is unknown which group is causing problems in the Netherlands.

MNSV

Primers were designed for a MNSV subgroup from cucumber (Table 2.6-7). Isolates from melon were very diverse and it was not possible to design one or a small number of assays for the detection of all groups. Three different primersets were tested and one was chosen for all further experiments. Sensitivity was tested on a dilution series of gBlocks. Specificity was tested on both gBlocks and RNA of closely related virus species in cucumber. In addition, the assay was tested on RNA and infected leaf material and in a multiplex with *Cox*.

Table 2.6-7: Selected LAMP primers for MNSV.

Primer	**Sequence**
MNSV_I_Dutch_cucumber2_F3	AGAGGGACACACCCAGAC
MNSV_I_Dutch_cucumber2_B3	GCGATCAAACGCACCACTA
MNSV_I_Dutch_cucumber2_FIP	ACGCTAGTTCCAACTCCCCCTAATGACCGAGTTTCCTCAGGA
MNSV_I_Dutch_cucumber2_BIP	CTAACCTTCGGCGCGGTCTAGGTTTAGGGGCAGGCTCCA
MNSV_I_Dutch_cucumber2_LF	TTGCGTGTCTTAATGGGACG
MNSV_I_Dutch_cucumber2_LB	TTGAGCGCGTTTTCTTTGTTG

TSWV

Four different primer sets were tested, and one was chosen for all following experiments (Table 2.6-8). Sensitivity was tested on dilutions of RNA and infected leaf material. Sensitivity was tested on RNA from related species.

Table 2.6-8: Selected LAMP primers for TSWV.

Primer	**Sequentie**
TSWV ID34 F3	ATCAGTGTTGTCTTGGCTA
TSWV ID34 B3	ACTTCCTTTAGCATTAGGATTG
TSWV ID34 FIP	CTAAGGCTTCCCTGGTGTCAATGCAAAATACAAGGACCTC
TSWV ID34 BIP	TTGCACTGTGCTAAAAAGCAAAACTAAGTATAGCAGCATACTCTT
TSWV ID34 LF	ACTTCTTTGGGTCGATCCC
TSWV ID34 LB	GCATTTGAAATGACTGAAGATCAGG

CMV

In accordance with the project participants no LAMP assay was designed for CMV as the virus sequences for the different CMV isolates were very divers and it was not possible to design one or a small number of primer sets that covered all available sequences.

PVY

The primers tested in this study were designed by Treder et al. (2017) and cover the PVY-NTN recombinant, which occurs in petunia (Table 2.6-9). Sensitivity was tested on a dilution series of gBlocks and a dilution series of RNA. Specificity was tested on gBlocks of different PVY strains and closely related species. The assay was tested *on infected plant material.*

Table 2.6-9: PVY-NTN primers by Treder et al. (2017).

Primer	Sequentie
Y4-F3	TGCCAACTGTGATGAATGG
Y4-B3	GTTCGTGATGTGACCTCATAA
Y4-FIP	GCATTCTCAACGATTGGTACGGAGTTTGGGTTATGATG
Y4-BIP	GCAAATCATGGCACATTTCCGTGGCATATATGGTTCCTT
Y4-LF	CAATGGGTATTCGACTTGTTCA
Y4-LB	TCAGATGTTGCAGAAGGGT

Acidovorax citrulli

The assay was designed by Oya et al. (2008) (Table 2.6-10). An assimilation probe was added to the LoopB primer.

Table 2.6-10: Selected LAMP primers for A. citrulli.

Primer	**Sequentie**
Aac-FIP	TACGGCTGTCACAGTCGTAGCTGACTCGCATGATTTCCCCA
Aac-BIP	TTGCACCTCATTGCAAATGCCCCGTCTGGAATGAACTAAGCT
Aac-F3	TTGATTCACCGCCGAACG
Aac-B3	TTACAGACGATAAATGACCCGG
Aac-LoopB	TGAGTGGCGACAGACGCA
Aac-LoopB FAM	/56-FAM/ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGATGAGTGGCGACAGACGCA
Universal quencher	TCGGCATCCGCATCCGCATTCGCATCCGGGTCCTCAGCGT/3BHQ_1/

Xanthomonas fragariae

The *X. fragariae* LAMP assay was developed in collaboration with Fresh Forward. Several primer sets were tested. Two were derived from literature and two were designed on a specific sequence for *X. fragariae* used for TaqMan design by Pooler et al (2016). Primers were tested on a dilution range target gBlocks and target and non-target DNA. Primer set 1 was selected for testing on (infected) strawberry leaf material (Table 2.6-11). The assay was also used in a multiplex with *Cox*.

Table 2.6-11: Selected LAMP primers for X. fragariae.

Primer	**Sequentie**
Xf set1 FIP	TCGGCATACGGCCTTGGAAAAAGAACACCGCAGATATGGCTT
Xf set1 BIP	CGACTGGATGAGTCCGGATTGCTCTTTCATCGTTGGGCTCG
Xf Set1 F3	CGTCCTGCCTTATCCATAGC
Xf set 1 B3	ACTAAGATCCGGTGCCTCTG

Fusarium

The *Fusarium* primer sets were chosen following a literaturestudy on published LAMP assays. All assays were tested on infected plant material.

F. solani

The primers for *F. solani* were designed by Ferdousi et al. (2014) (Table 2.6-12)**.**

Table 2.6-12: Selected LAMP primers by Ferdousi et al. (2014).

Primer	**Sequentie**
Fsol1-FIP	CTTTGTCCAACGTCGCCCGAGTTTTGCGGTTCGACCGTAAT
Fsol1-BIP	AACACCAAACCCTCTTGGCGCAGCGGTTCCTATTGTTGAA
Fsol1-F3	GCTTCTCCCGAGTCCCAA
Fsol1-B3	AGGAACCCTTACCGAGCT
Fsol1-LF	GCATCACGTGGTTCATAACAGACA
Fsol1-LB	GGGGTAAATGCCCCACCAAAAA

Fusarium oxysporum

The primers for *F. oxysporum* were designed by Ghosh et al. (2008) (Table 2.6-13).

Table 2.6-13: Selected LAMP primers by Ghosh et al. (2015).

Primers	**Sequentie**
Foxy2-FIP	CCAGGCGTACTTGAAGGAACCGTCAAGCAGTCACTAACCAT
Foxy2-BIP	GTCAAGCAGTCACTAACCATACGGTGACATAGTAGCGA
Foxy2-F3	ACAACCTCAATGAGTGCG
Foxy2-B3	CATGAGCGACAACATACCA
Foxy2-LF	AGCGTGAGCGTGGTATCAC
Foxy2-LB	ACGGTGACATAGTAGCGA

Fusarium proliferatum

The primers for *F. profileratum* were designed by Wang et al. (2020) (Table 2.6-14).

Table 2.6-14: Selected primers by Wang et al. (2020).

Primers	**Sequentie**
Fpro1-FIP	AAGTTCGAGACTCCTCGCTACTGAGGAAGTAGGATGAGGTATGA
Fpro1-BIP	CTCGGCCTTGAGCTTGTCA-ACAATAGGAAGCCGCTGAG
Fpro1-F3	AGTACCAGTGATCATGTTCTTG
Fpro1-B3	TCCTGTCCACAACCTCAA
Fpro1-LF	TCACCGTCATTGGTATGTTGT
Fpro1-LB	AGGCGTACTTGAAGGAACC

Verticillium dahliae

The primers for *V. dahliae* were designed by Tian et al. (2016) (Table 2.6-15).

Table 2.6-15: Selected primers by Tian et al. (2016).

Primers	**Sequentie**
Vd1-FIP	ACATGGTCAGACCATGGCCG AAAGTCTCATGCCCCCTTCT
Vd1-BIP	CGCCCTCGGCAGTCAAGAT GCTTGGGGCTCAAGGAACG
Vd1-F3	AGTAACCCCCCCCCAAAC
Vd1-B3	TGTCTCGTGTGTGTGTGTG
Vd1-LF	ACCCCAGCACATGATAGACAAA
Vd1-LB	ACCCCTTCACGACAACTGG

The selection of primers from literature testing DNA from the target species and closely related species Finally, the LAMP assays were tested on infected symptomatic plant material for which the identify of the pathogen was unkown (Table 2.6-16). Out of 5 plants different organs from 3 plants with or without symptoms were tested with all LAMP assays.

Table 2.6-16: Infected plant material.

Plant	ID	sample	condition
1	A1	leaf stem	healthy
	A2	leaf	healthy
	B	flower stem	healthy
	C1	leaf stem	diseased
	C2	leaf	diseased
	D	flower stem	diseased
	E	root	healthy
	F	root	diseased
	G	base	
2	A1	leaf stem	healthy
	A2	leaf	healthy
	C1	leaf stem	diseased
	C2	leaf	diseased
	D	flower stem	diseased
	E	root	healthy
	F	root	diseased
	G	base	
3	A1	leaf stem	healthy
	A2	leaf	healthy
	B	flower stem	healthy
	C1	leaf stem	diseased
	C2	leaf	diseased
	D	flower stem	diseased
	E	root	healthy
	F	root	diseased
	G	base	
4	A1	leaf stem	healthy
	A2	leaf	healthy
	B	flower stem	healthy
	C1	leaf stem	diseased
	C2	leaf	diseased
	D	flower stem	diseased
	E	root	healthy
	F	root	diseased
	G	base	
5	A1	leaf stem	healthy
	A2	leaf	healthy
	B	flower stem	healthy
	C1	leaf stem	diseased
	C2	leaf	diseased
	D	flower stem	diseased
	E	root	healthy
	F	root	diseased
	G	base	

The assays were combined with a *Cox* assay as an amplification control. If the melting temperature of both assays was identical an assimilation probe was developed for the target LAMP assay.

RAMP experiments

RPA (recombination polymerase amplification) + LAMP (RAMP) was tested using the developed TMV assay. For the RPA reaction, the F3 and B3 primers of the LAMP assay were used. The RPA assay was stored in the lid of a LAMP reaction tube with the LAMP assay at the bottom. The sample was added to the RPA assay and incubated at 40°C for 15 min. Then the tube was shaken to mix with the LAMP assay and the LAMP was performed in a Genie machine. However, RAMP assays continously showed false positive results and therefore it was decided to only use LAMP in the multiplex chips.

Multiplex chip

Multiplex chips were designed at the University of Pennsylvania and printed by BioNano Technology department of WUR (Figure 2-12). They were washed for 15 min with isopropyl alcohol and were coated with polyethylenglycol for 30 min. 2 µl each of inidividual primer mixes were pippeted into each chamber and dried for two hours. Then the LAMP reaction mix and sample were added to the chip, which was incubated in a custom-made incubation chamber topped with an USB microscope set to acquire a picture every minute (Figure 2-13). Time series fluorescence analysis was done with the Image J software.

The multiplex chip was tested at the University of Pennsylvania for the detection of *Clavibacter michiganensis* pv. *michiganensis*, ToBRFV, TMV and Cox using gBlocks and the primers developed in this project. Cross-contamination, sensistivity and the possibility to detect multiple targets in a background of tomato extract were tested.

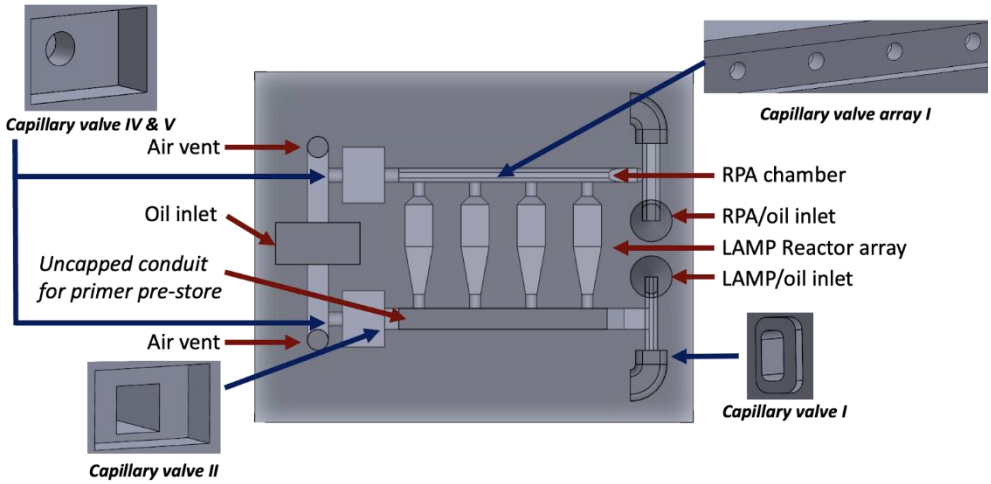


Figure 2-12: Design of the microfluidic chip.

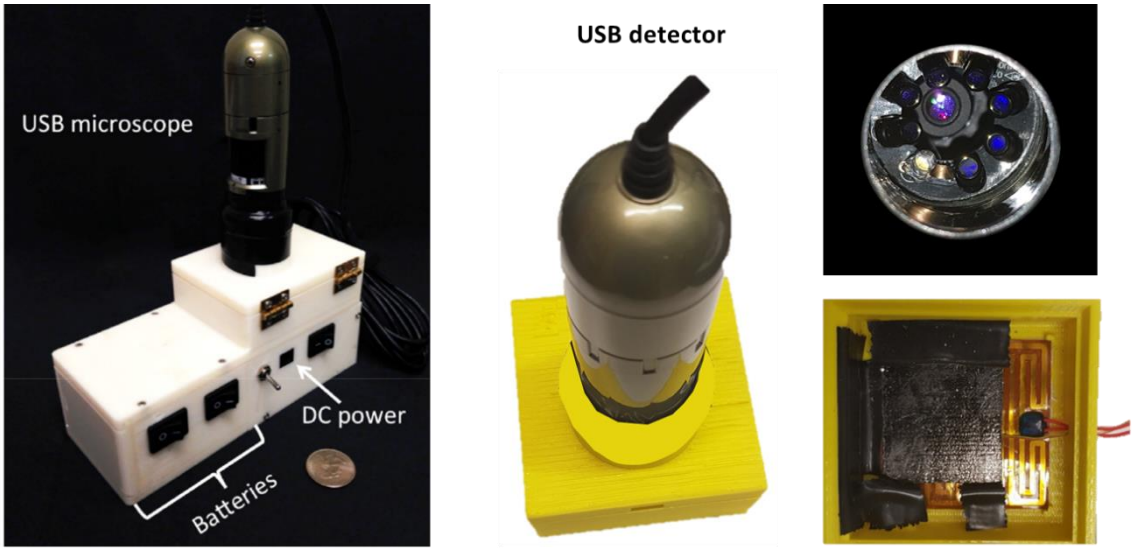


Figure 2-13: Multiplex microchip a) principle, b) example of measurement setup from The University of Pennsylvania.

T65 cup

The T65 cup was developed by BioNano Technology department of WUR to enable on-site execution of LAMP assays without the need for Genie machines or even thermoblocks (unpublished). The T65 cup system consists of an aluminium coffee capsule filled with Rubitherm RT64HC and a 3D printed holder for PCR sized tubes (Figure 2-14). When placed in boiling water that this then removed from heat the polymer RT64HC will melt and remain at a constant temperature of 64°C for approximately 40 min. Thus, a LAMP assay can be carried out with the usual ingredients and quantities within those T65 cups. For detection an end-point colorimetric measurement was chosen. As the ToBRFV assay was already optimized for colorimetric measurement, this assay was used for evaluation of the T65 cup method.

Since the ToBRFV assay was also used in another project, in which a LAMP-Crispr-Cas approach was used for SNP detection among ToBRFV strains, this assay was used in combination with the T65cup. Three samples of plant leaves (MT3, VT3 and RNA extracted from VT3, see ToBRFV assay development) were used.

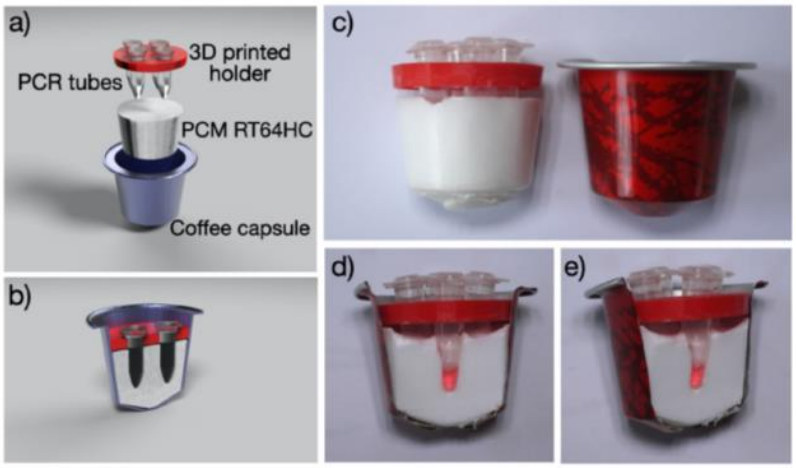


Figure 2-14: Principle of a T65 cup.

2.6.3 Results

Pospiviroids

The results for the pospiviroid assays were presented in the report of PPS On-Site 1-0.

C. michiganensis

Also, the *C. michiganensis* assay development and evaluation was presented in the report of PPS On-site 1-0.

ToBRFV

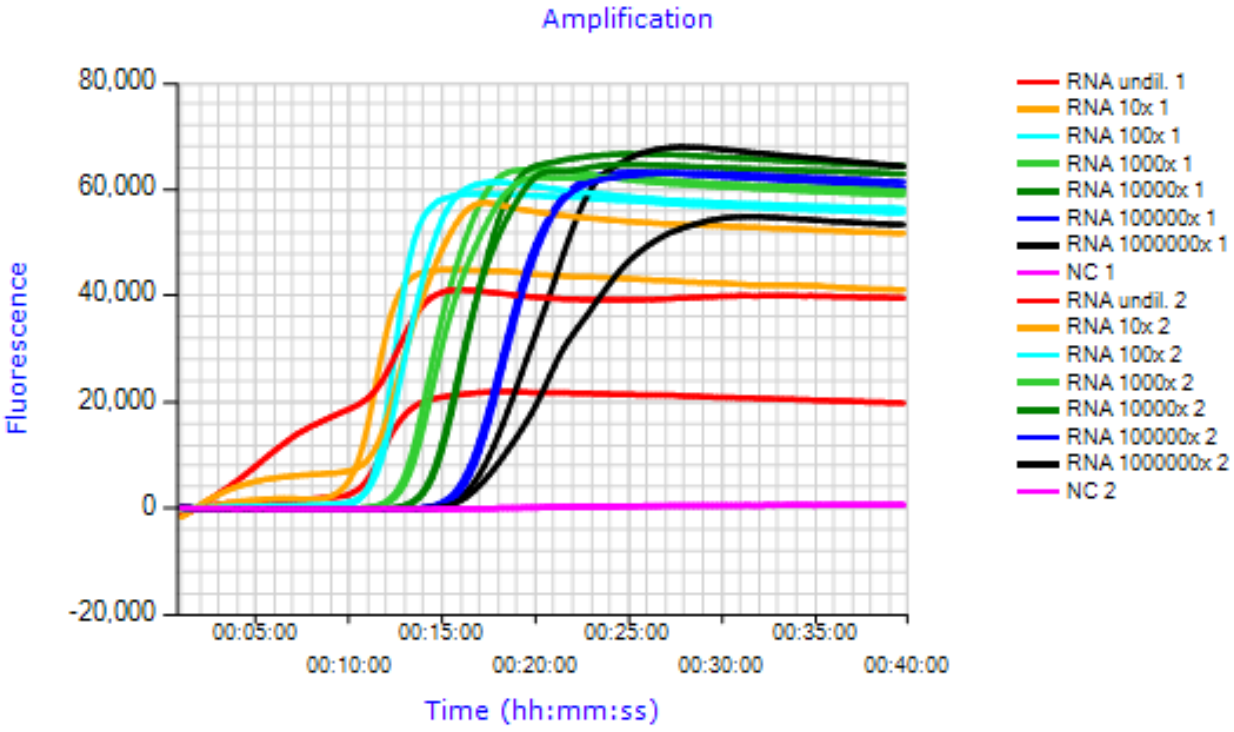
Sensitivity

A 10x serial dilution series was measured in threefold with the developed LAMP assay. A 1000000-fold dilution of RNA isolated from infected material could still be detected (Table 2.6-17, Figure 2-15). Undiluted RNA shows an unusual amplification curve, likely due to inhibition by too high concentrations.

Table 2.6-17: Results of a RNA dilution series measurement.

Target	**Tpos (min) 1**	**Tm (°C) 1**	**Tpos (min) 2**	**Tm (°C) 2**	**Tpos (min) 3**	**Tm (°C) 3**
RNA undiluted	11:45	86.1	12:30	85.9	1:45	85.9
RNA 1:10	11:15	86.3	12:15	86.0	12:15	86.0
RNA 1:100	12:00	86.3	12:15	85.9	12:30	86.0
RNA 1:1000	13:30	86.2	13:45	86.0	-	-
RNA 1:10000	15:15	86.1	15:00	86.0	-	-
RNA 1:100000	17:00	86.1	17:15	85.9	17:30	86.0
RNA 1:1000000	17:45	86.0	17:15	86.0	19:15	86.0
NC	-	-	-	-	-	-

a



b

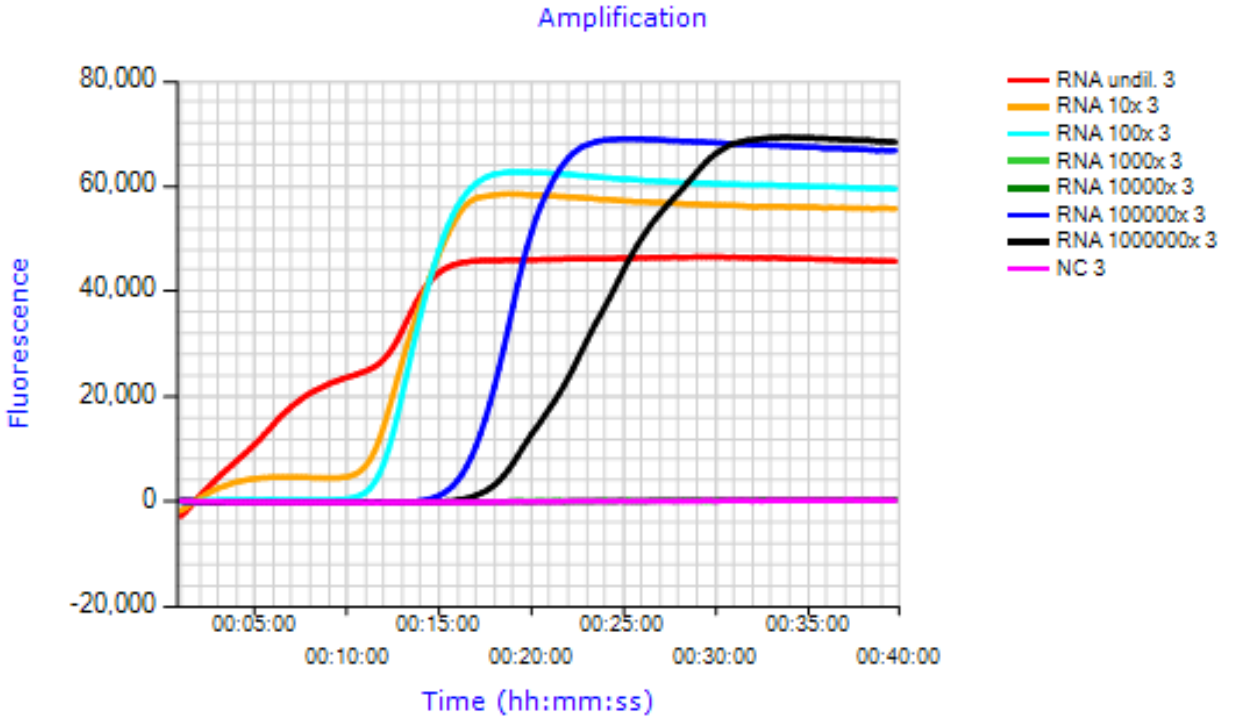


Figure 2-15: RNA dilution series measurement, series a) 1,2, b) 3.

Specificity

The assay was tested on gBlocks from ToBRFV, ReMV, TMV, ToMV, and ToMoMV (Table 2.6-18, Figure 2-16). In addition, RNA of the related species TMV and ToMV and the species PMMoV and TSWV was tested. In one instance there was aspecific amplification of TMV RNA (Table 2.6-19, Figure 2-17). However, threefold repetition of this experiment showed no amplification (Table 2.6-20, Figure 2-18).

Table 2.6-18: Test on gBlocks of non-target species.

Amount of gBlock	**Tpos (min)**	**Tm (°C)**
ReMV gBlock 10^6	-	-
ToBRFV gBlock 1 10^6	12:15	85.6
ToBRFV gBlock 2 10^6	11:45	85.4
TMV gBlock 10^6	-	-
ToMV gBlock 10^6	-	-
ToMMV gBlock 10^6	-	-
NC	-	-

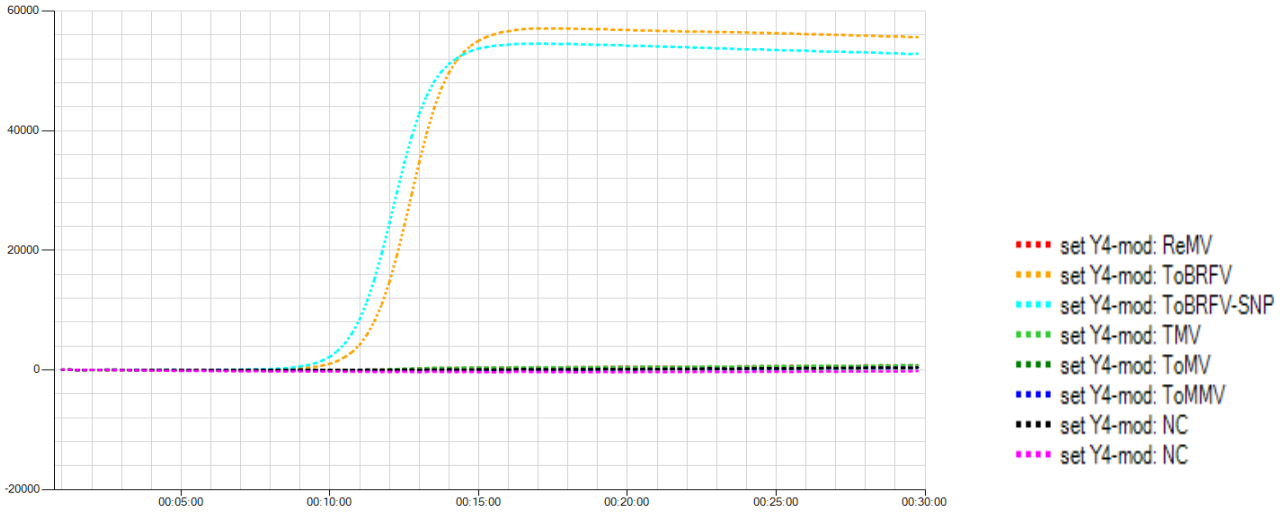


Figure 2-16: Test on gBlocks of non-target species.

Table 2.6-19: Test on non-target RNA.

Target	**Tpos (min)**		
	1	2	3
TMV	-	31:15	-
TMoV	-	-	-
TSWV	-	-	-
PPMoV	-	-	-
PC	24:15	x	x
NC	-	x	x

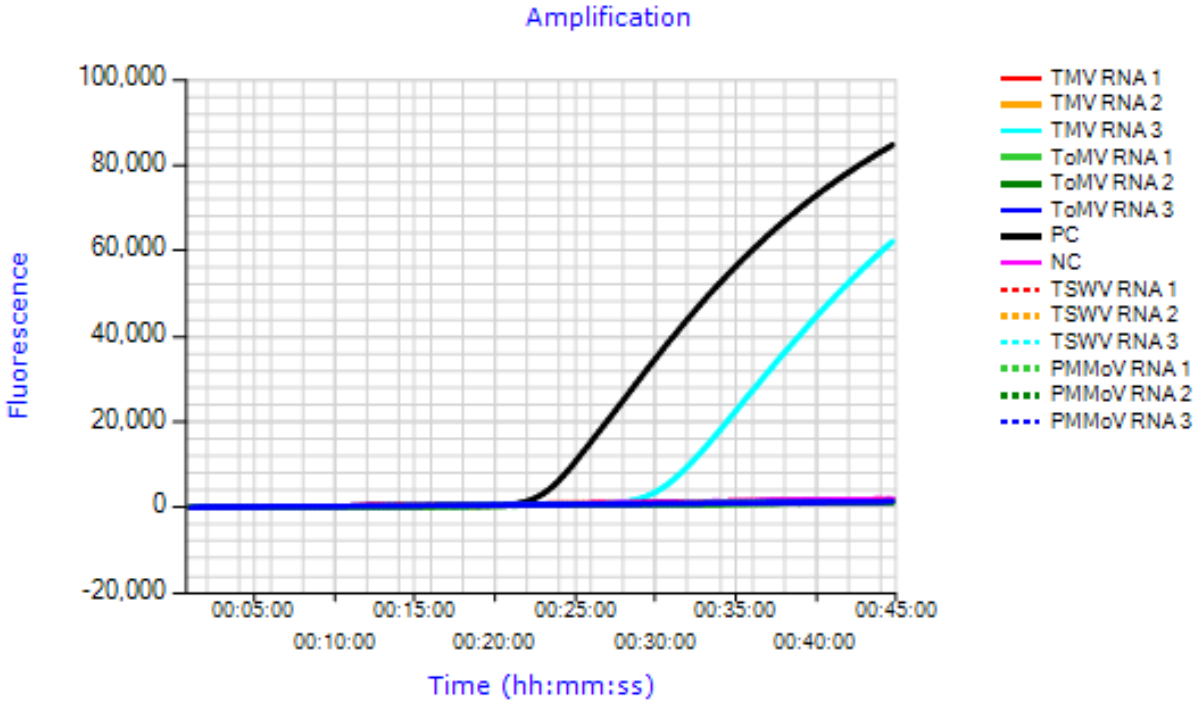


Figure 2-17: Test on non-target RNA.

Table 2.6-20: Test ToBRFV on TMV RNA.

Target	Tpos (min)	Tm (°C)
TMV RNA 1:10	-	-
TMV RNA 1:100	-	-
TMV RNA 1:100	-	-
TMV gBlock	-	-
ToBRFV gBlock	11:30	85.5
NC	-	-

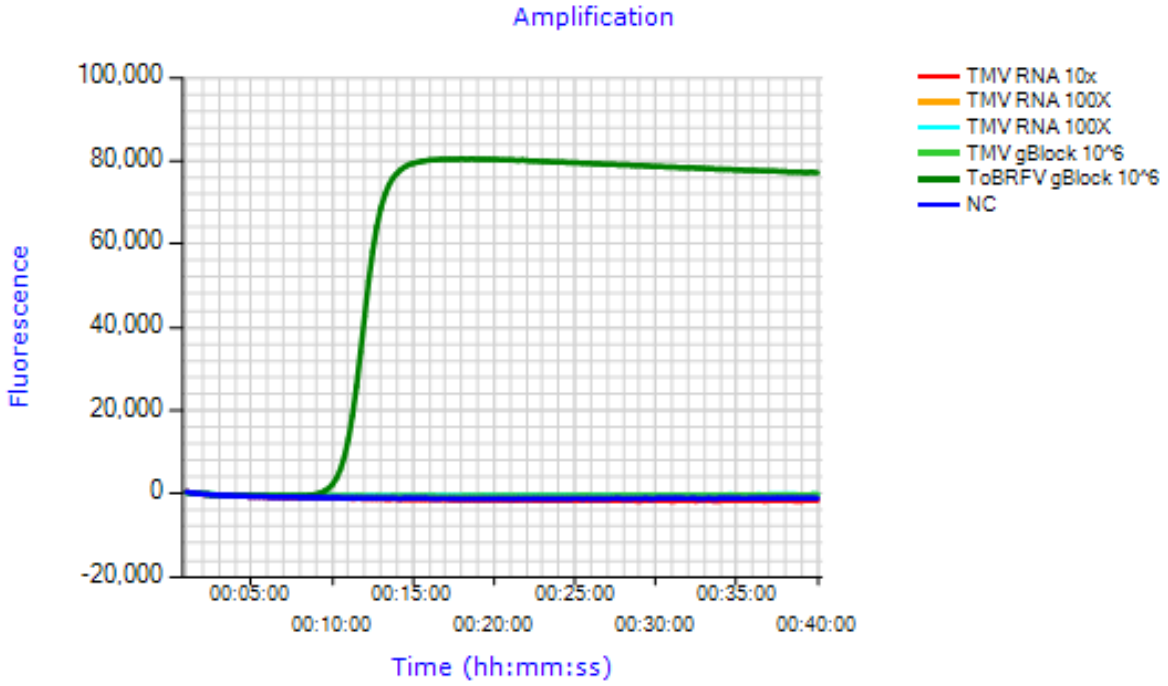


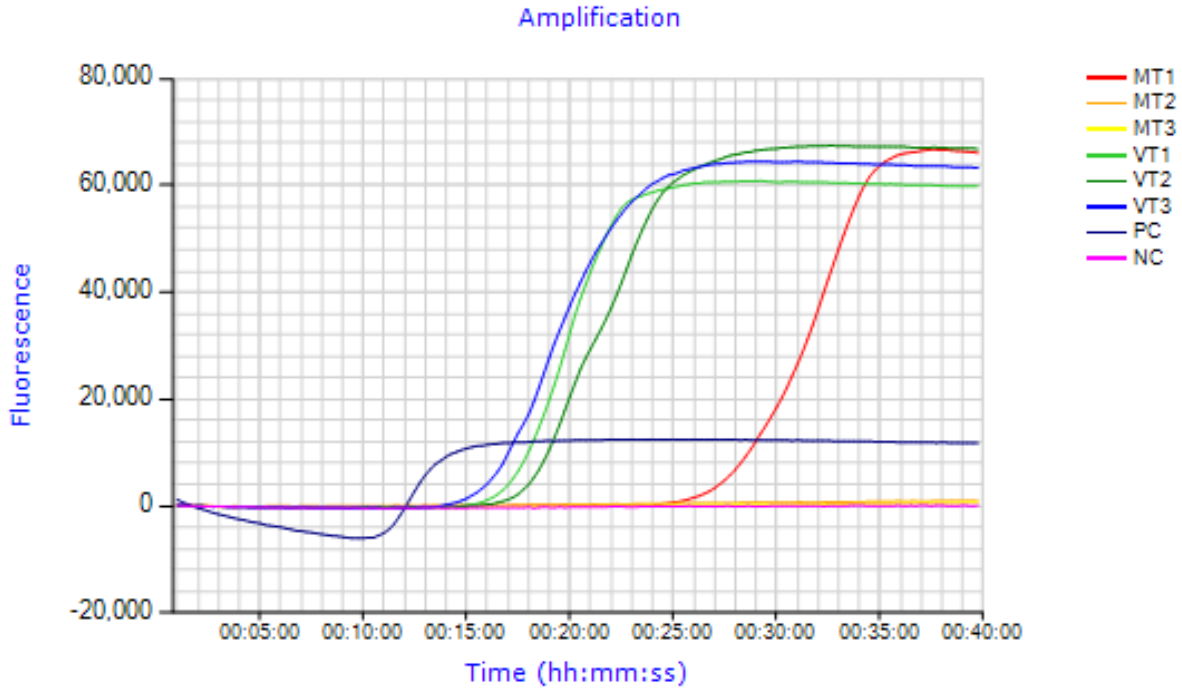
Figure 2-18: Test ToBRFV on TMV RNA.

The assay was tested on samples from tomato plants that had been infected with ToBRFV. On the one hand, these samples were tested with a simple extraction method as described in WP1 and in addition several samples were tested after RNA extraction. For sample codes, see p. 33. The sample MT1 which belongs to the plants inoculated only with water (MT) is positive, which is assumed to be due to contamination during the splitting of the sample for RNA extraction (Table 2.6-21, Figure 2-19). The samples MT2 and MT3, which were not opened, remain negative. All samples of infected material showed positive amplification with both extraction methods.

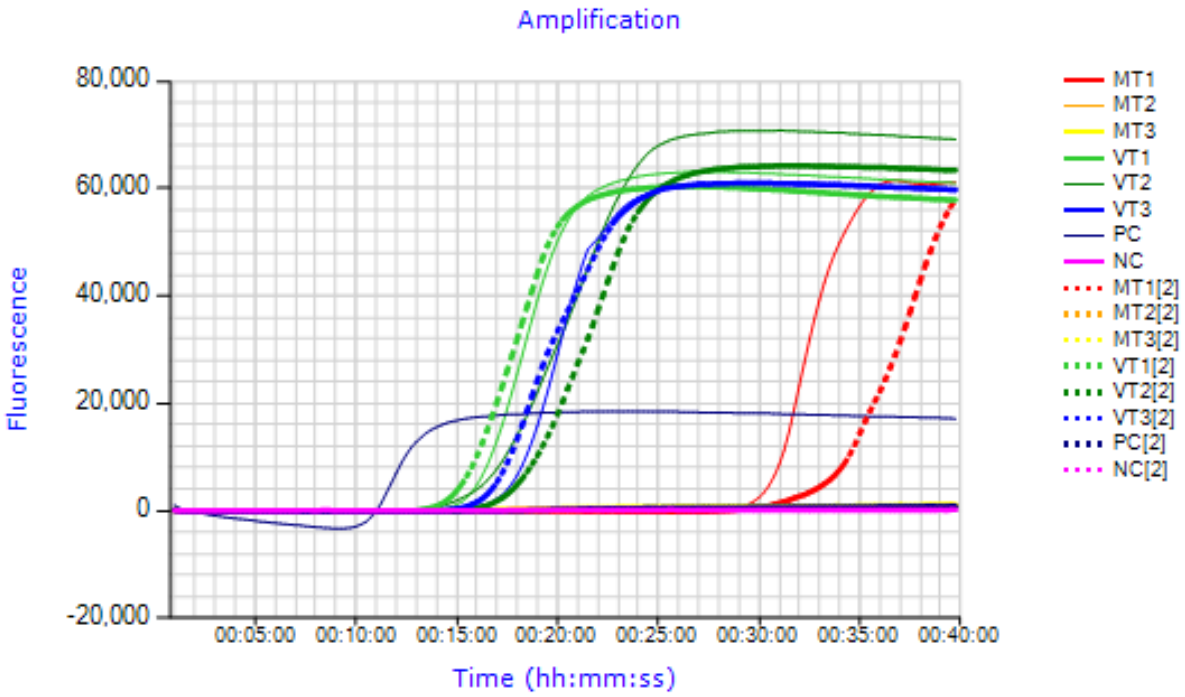
Table 2.6-21: Results of a threefold LAMP test of ToBRFV infected plant material.

	1		**2**		**3**	
Samples	**Tpos (min)**	**Tm (°C)**	**Tpos (min)**	**Tm (°C)**	**Tpos (min)**	**Tm (°C)**
MT1	31:45	85.5	35:00	85.0	28:45	85.2
MT2	-	-	-	-	-	-
MT3	-	-	-	-	-	-
VT1	17:00	85.7	16:15	85.4	18:15	85.4
VT2	17:45	85.4	20:00	85.4	19:15	85.4
VT3	18:45	85.4	17:45	85.3	17:00	85.4
LP1.1	19:30	85.4	20:15	85.4	20:00	85.2
LP1.2	18:45	85.3	20:45	85.2	18:45	85.2
LP2.1	-	-	-	-	-	-
LP2.2	-	-	-	-	-	-
MT1 (RNA)	23:45	85.2	20:30	85.3	20:45	85.2
VT1 (RNA)	12:00	85.5	12:00	85.4	12:00	85.4
VT2 (RNA)	10:15	85.2	10:15	85.3	10:15	85.4
VT3 (RNA)	11:15	85.4	11:15	85.3	11:15	85.3

a


Amplification
Fluorescence
80,000
60,000
40,000
20,000
0
-20,000
00:05:00
00:10:00
00:15:00
00:20:00
00:25:00
00:30:00
00:35:00
00:40:00
Time (hh:mm:ss)
MT1
MT2
MT3
VT1
VT2
VT3
PC
NC

b


Amplification
Fluorescence
80,000
60,000
40,000
20,000
0
-20,000
00:05:00
00:10:00
00:15:00
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00:25:00
00:30:00
00:35:00
00:40:00
Time (hh:mm:ss)
MT1
MT2
MT3
VT1
VT2
VT3
PC
NC
MT1[2]
MT2[2]
MT3[2]
VT1[2]
VT2[2]
VT3[2]
PC[2]
NC[2]

c

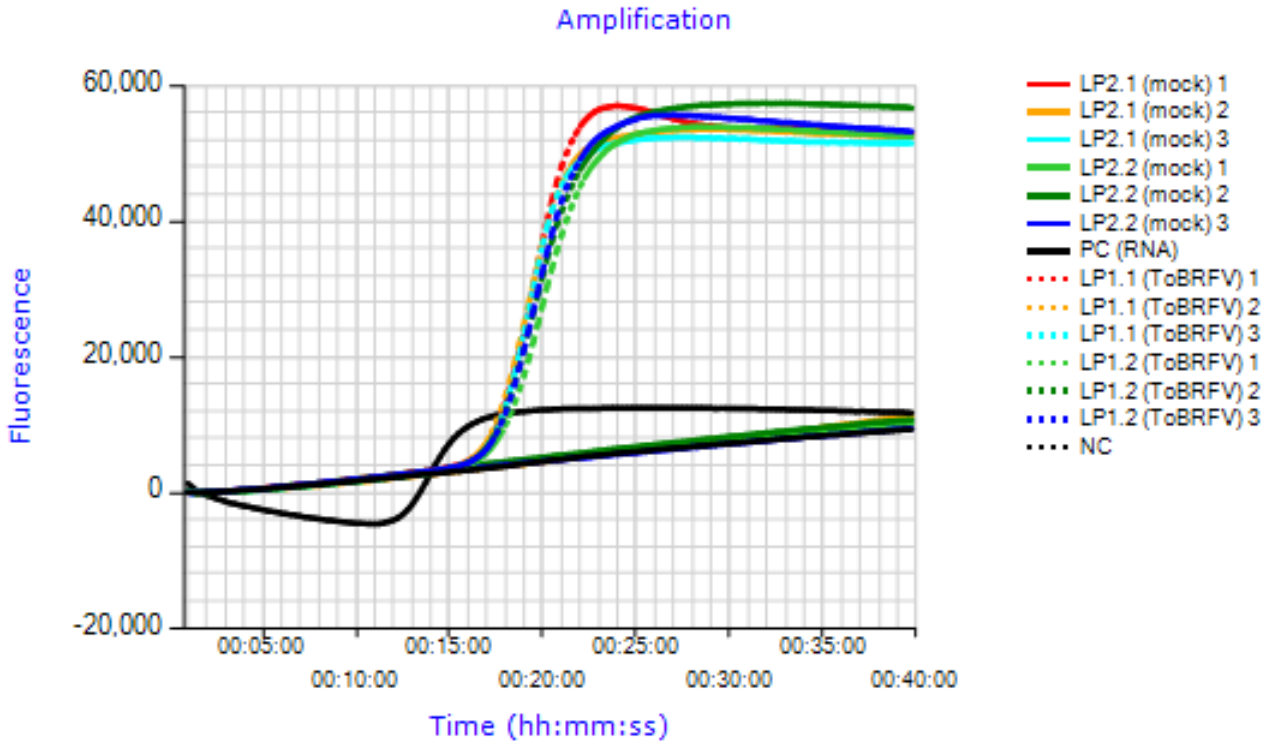
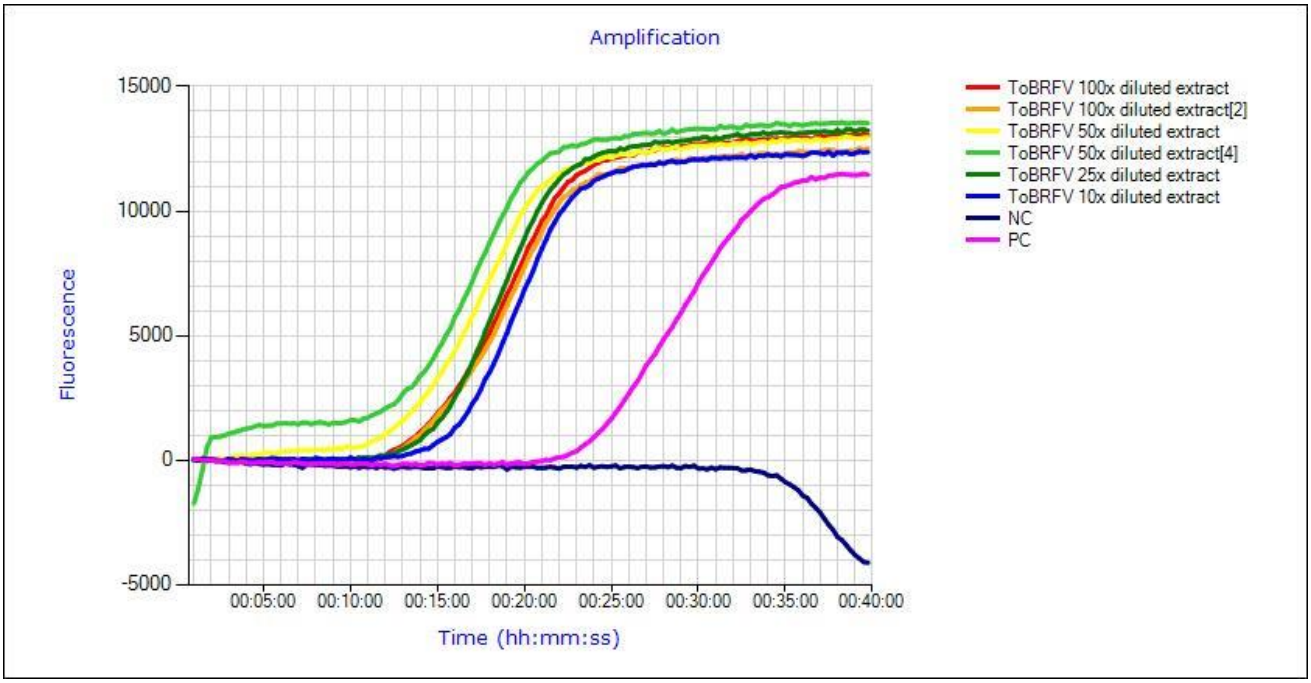


Figure 2-19: Amplification curves of infected material a, b) samples MT1-VT3 in threefold, c) LP samples in threefold.

In addition, a multiplex assay was developed to include the amplification control *Cox*. In infected leaf extract ToBRFV was detected in all samples, whereas *Cox* was only detected in samples not containing ToBRFV (Figure 2-20).

a



b

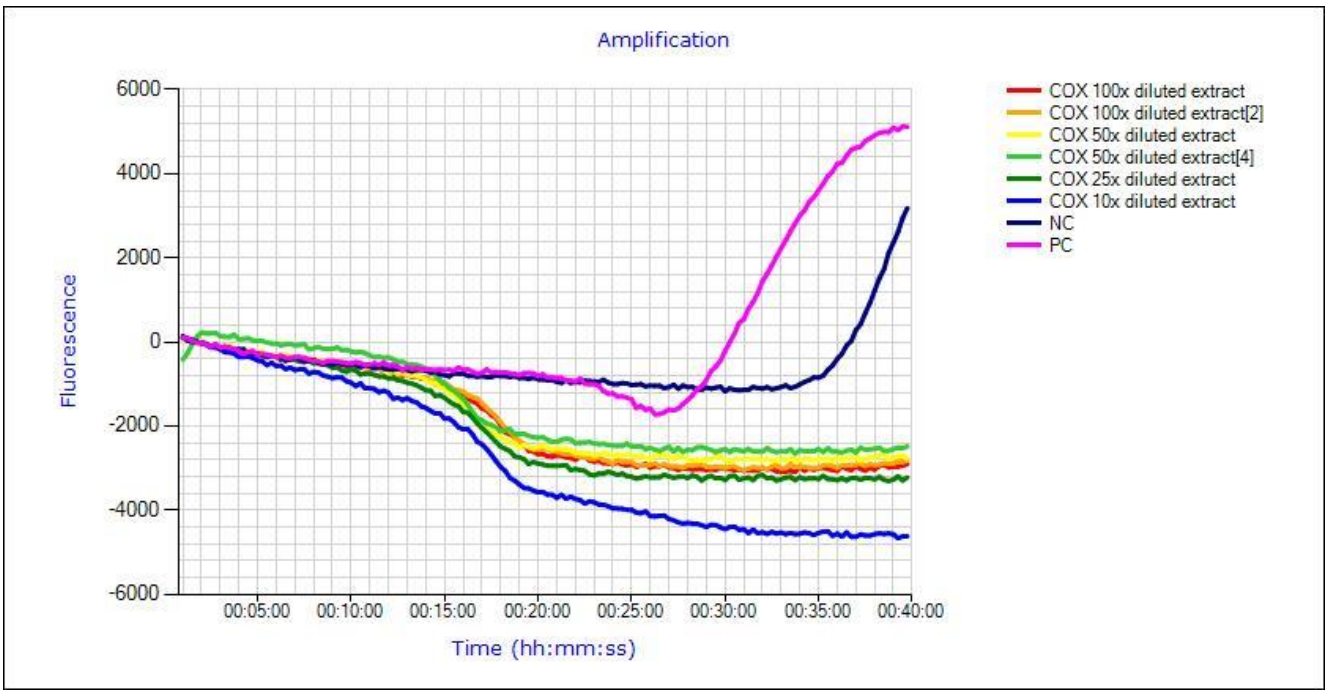


Figure 2-20: a) ToBRFV detection, b) Cox detection in a multiplex assay.

A colorimetric LAMP assay was compared to a real-time LAMP assay. ToBRFV infected tomato extract was used. The extract was diluted in a 10-fold dilution series and the same sample volume was analysed with both assays. In the normal real time LAMP assay 1:1000000 dilution of infected material extract could be detected (Figure 2-21). In the colorimetrix assay the 1:000000 dilution still gave a clearly positve result (Figure 2-22), which was already visible after 40 min. However, the detection is reliable after 60 min.

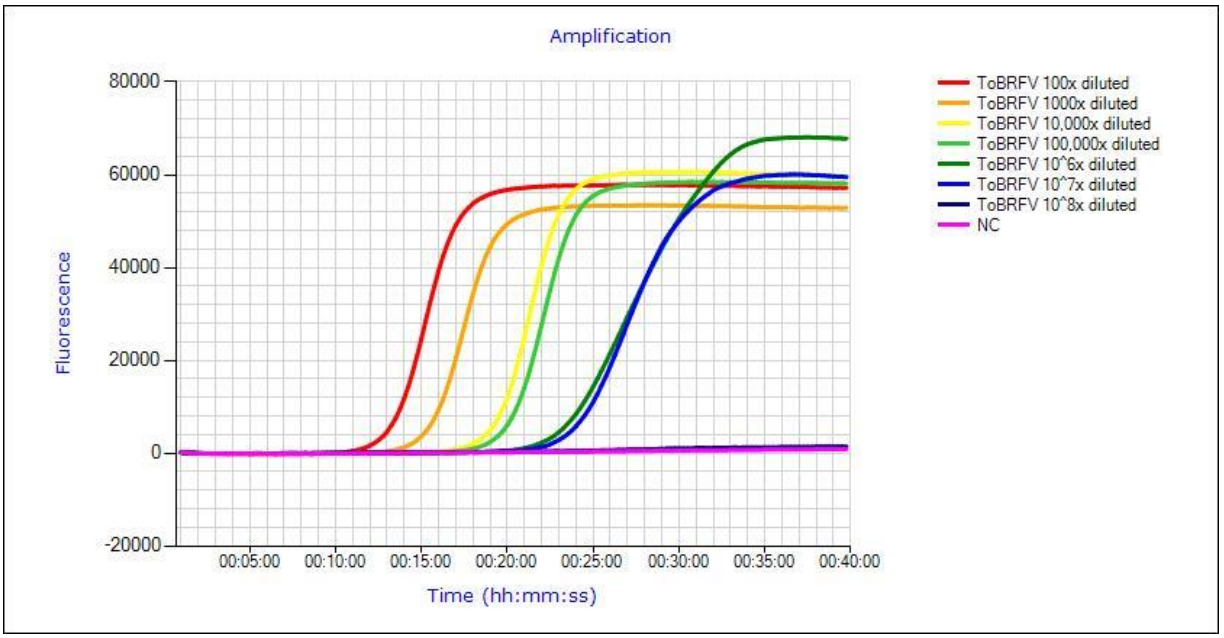


Figure 2-21: Genie LAMP detection of diluted ToBRFV infected material.

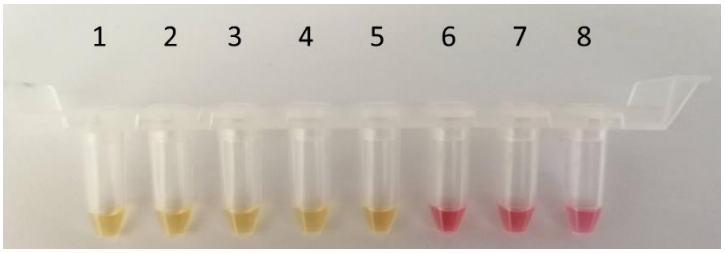


Figure 2-22: Colorimetric LAMP assay of a dilution series of ToBRFV infected material.

TMV

Sensitivity

Sensitivity was tested on a serial dilution of gBlocks. As low as 10^3 copies/µl could be detected (Table 2.6-22, Figure 2-23).

Table 2.6-22: TMV assay tested on a dilution series of gBlocks.

gBlock	**Tpos (min)**	**Tm (C°)**
10^6	05:45	86.1
10^5	06:45	86.1
10^4	07:30	86
10^3	09:30	86
10^2	-	-
10	-	-
1	-	-
NC	-	-
10^6	05:45	85.7
10^5	06:15	85.8
10^4	07:15	85.7
10^3	22:30	85.7
10^2	-	-
10	-	65.4
1	-	-
NC	-	-

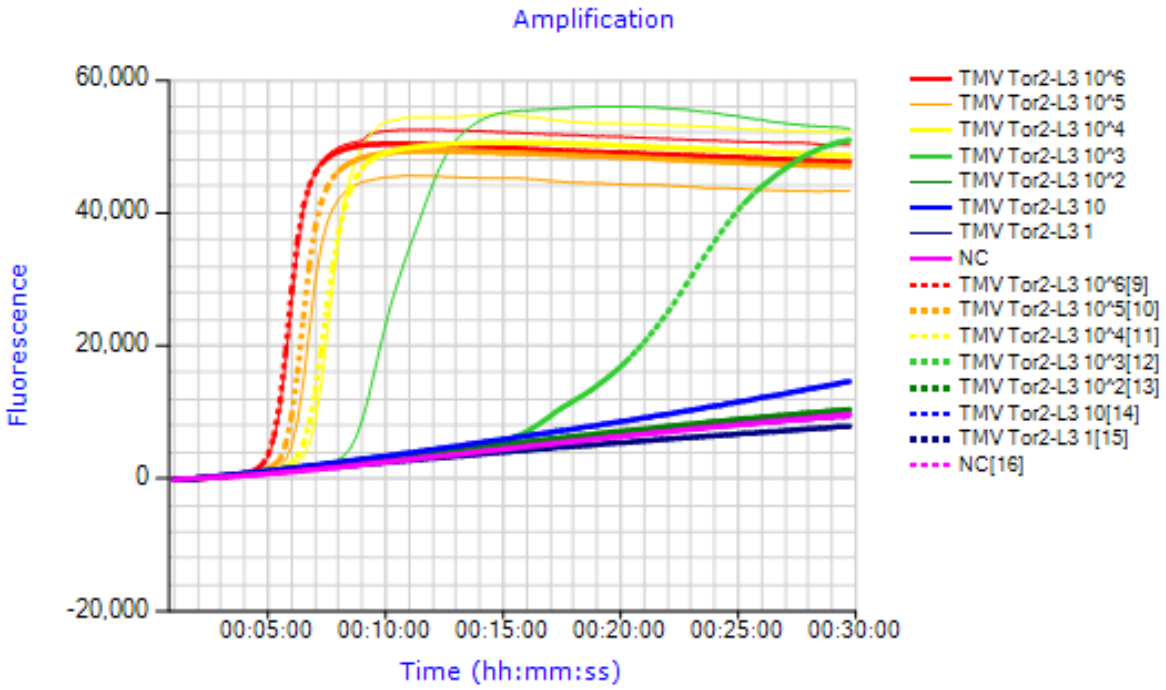


Figure 2-23: TMV LAMP tested on a dilution series of gBlocks.

Specificity

Specificity was tested on gBlocks of the non-targets TMV-Ohio (reclassified as ToMV), TMGMV and ToBRFV. In all cases only TMV was detected (Table 2.6-23, Figure 2-24).

Table 2.6-23: Specificity test on gBlocks.

	Tpos (min)	**Tm (°C)**
ToMV	-	-
TMGMV	-	-
ToBRFV	-	-
TMV-Ohio	-	-
TMV	05:45	85.9
NC	-	-

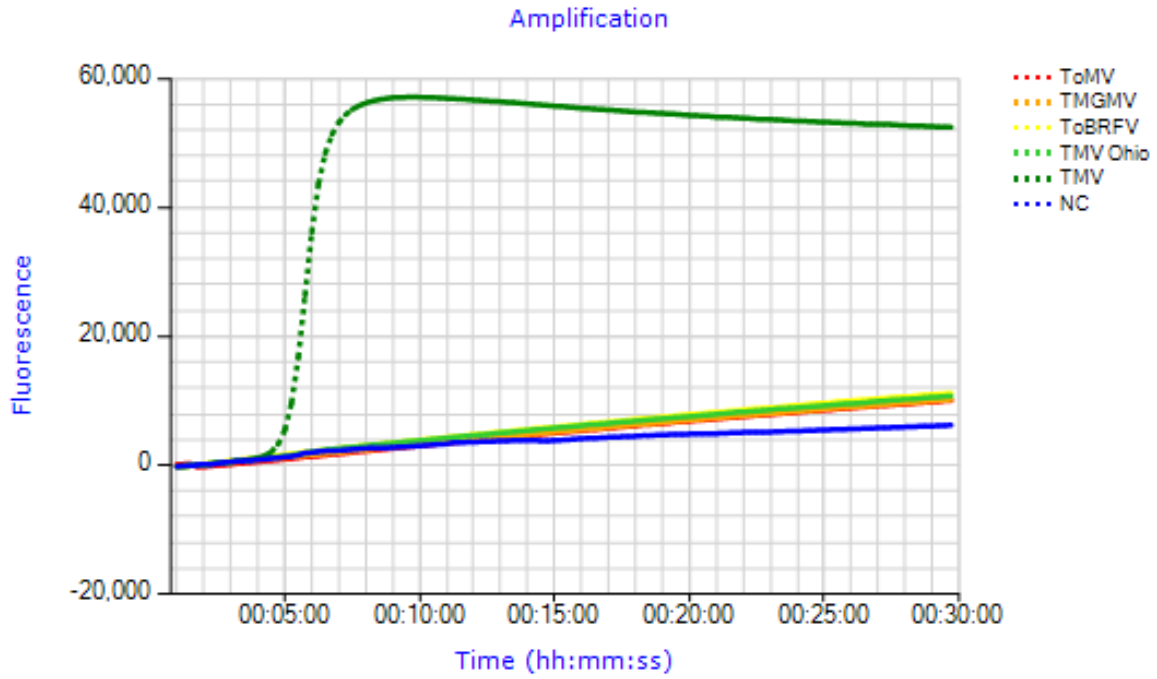


Figure 2-24: Specificity test on non-target gBlocks.

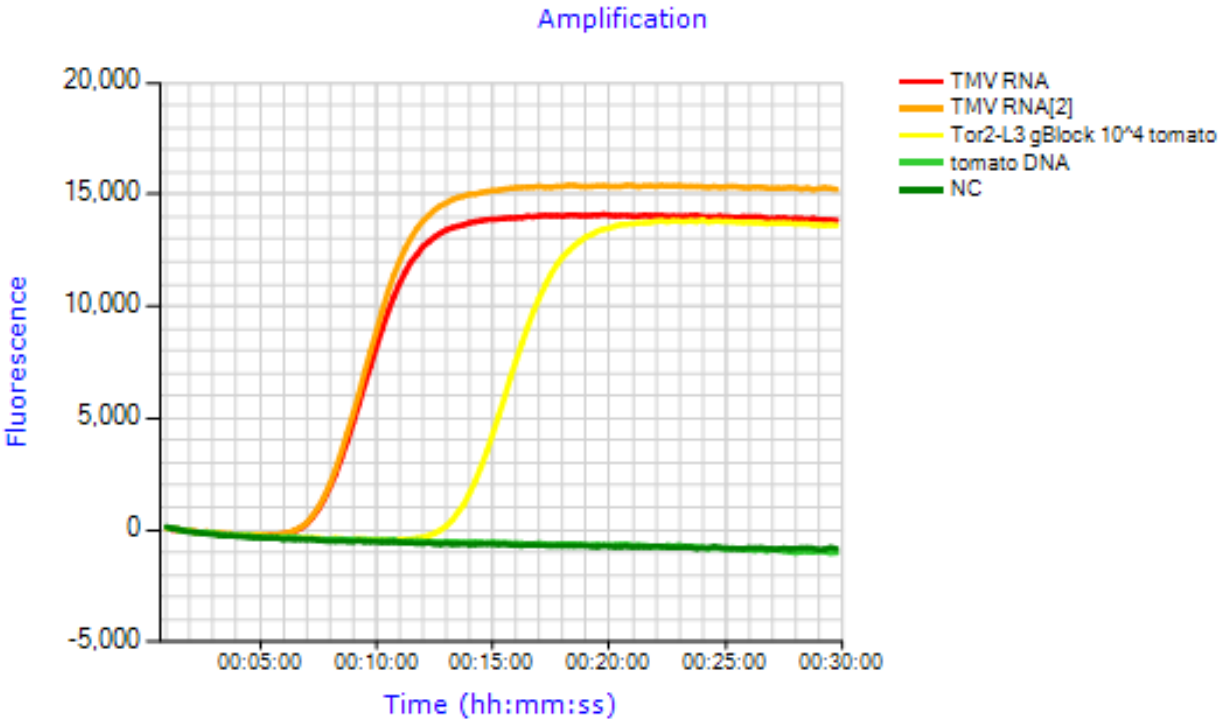
RNA multiplex

The multiplex was carried out on tomato leaf extract spiked with TMV RNA or gBlock. TMV was always detected and the *Cox* control only in the positive control with *Cox* gBlocks (Table 2.6-24, Figure 2-25).

Table 2.6-24: Test on TMV RNA in tomato leaf extract.

	Tpos FAM TMV (min)	**Tpos TR Cox (min)**
TMV RNA 10x dilution	08:45	-
TMV RNA 10x dilution	08:45	-
TMV_Tor2-L3 gBlock 10^4 + tomato leaf extract	15:00	-
Cox	-	25:45
NC	-	24:45

a



b

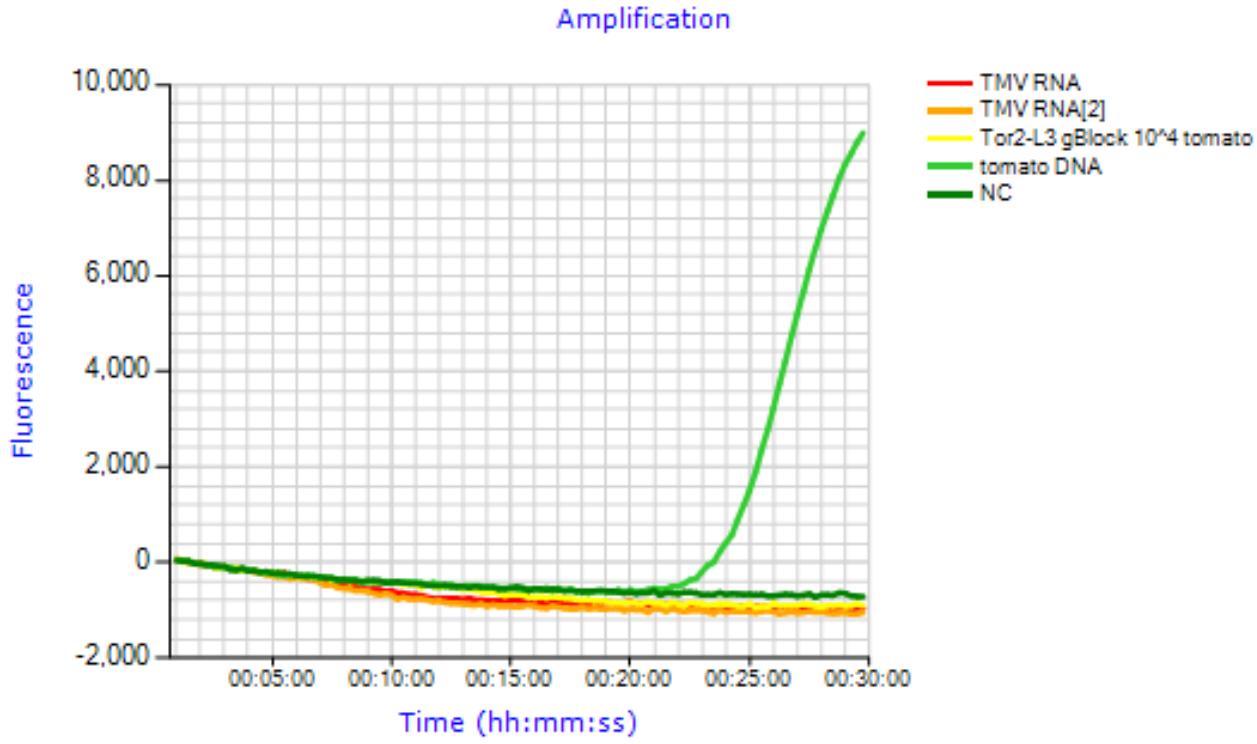


Figure 2-25: Test on TMV RNA in tomato leaf extract. a) Detection of TMV, b) detection of Cox.

Infected leaf material

Only infected petunia leaf was avalailable for this experiment. TMV could be detected in infected material and in the positive control but gave no amplification in the healthy material (Table 2.6-25, Figure 2-26). To further assess specificity the assay was also tested on petunia and tomato leaves spiked with RNA from the non-targets ToMV, ToBRFV, ToMoMV and PPMoV. The LAMP did not show amplification with any of the non-targets (Table 2.6-26, Figure 2-27).

Table 2.6-25: LAMP TMV on infected material.

Sample	**Tpos FAM TMV (min)**	**Tpos TR Cox (min)**
TMV infected leaf	13:30	-
Healthy leaf	-	25:45
TMV RNA 1:10 (1 ul + 4 ul water)	08:30	19:00
tomato DNA (1 ul + 4 ul water)	-	24:30

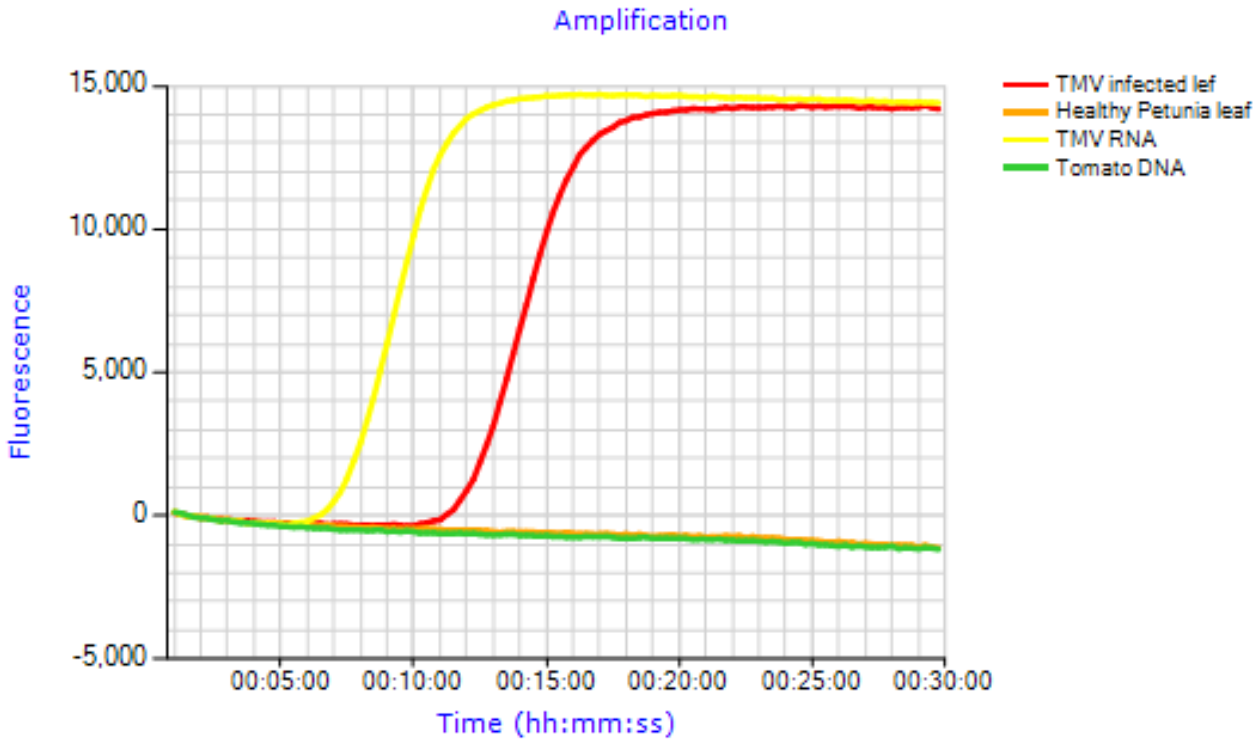
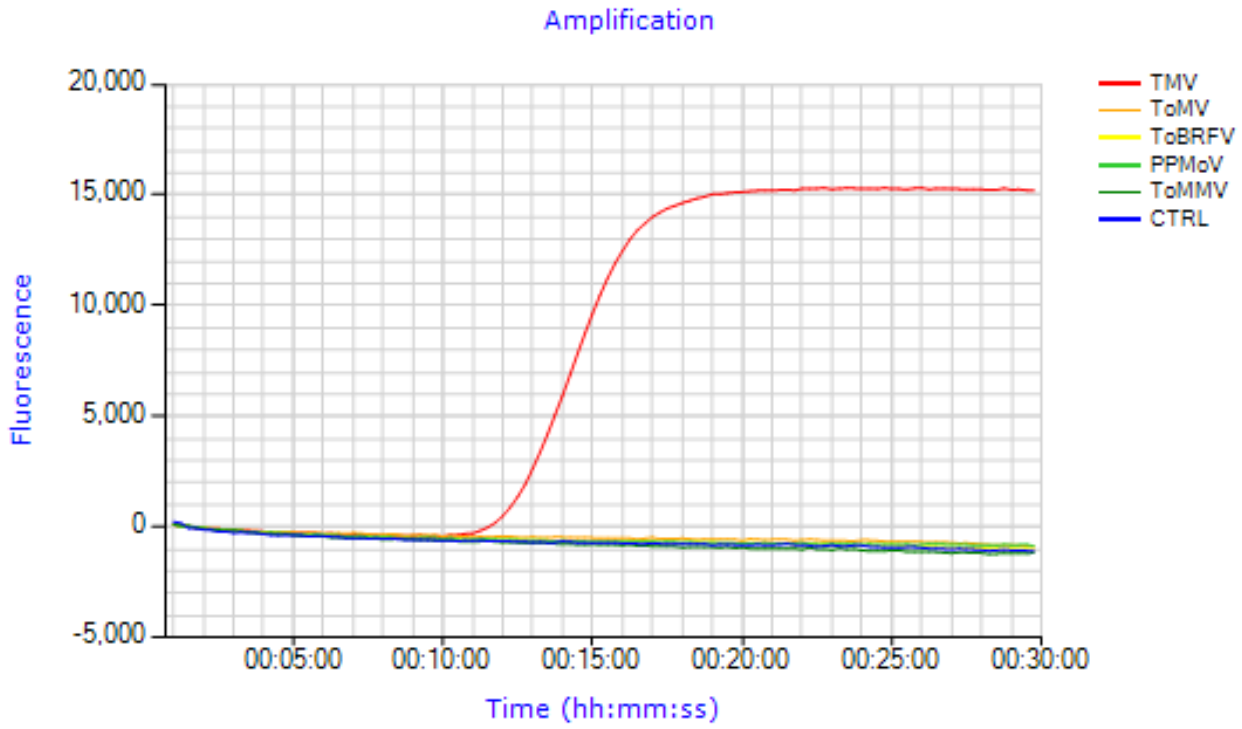


Figure 2-26: LAMP TMV on infected material.

Table 2.6-26: LAMP TMV on leaf material spiked with non-targets.

Sample	Tpos FAM TMV (min)	Tpos TR Cox (min)
TMV Petunia	13:30	-
ToMV Petunia	-	27:45
ToBRFV Petunia	-	27:15
PPMoV Petuna	-	-
ToMMV Petunia	-	27:15
Petunia CTRL	-	27:30
TMV tomato	13:15	-
ToMV tomato	-	25:00
ToBRFV tomato	-	25:30
PPMoV tomato	-	25:45
ToMMV tomato	-	25:00
Tomato CTRL	-	-

a



b

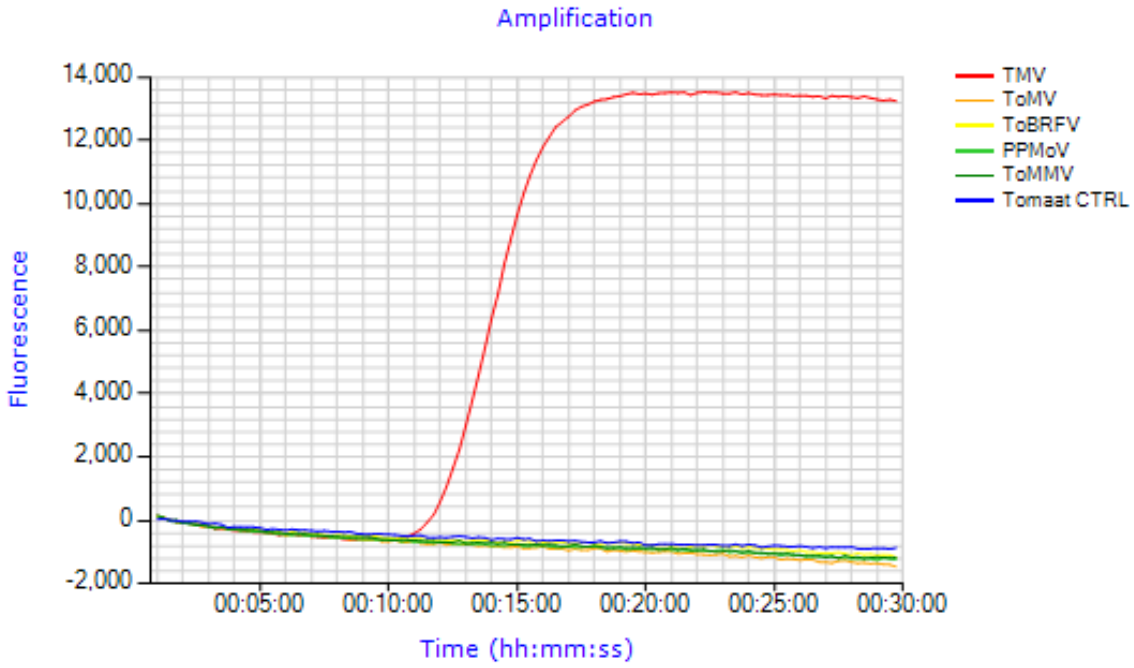


Figure 2-27: LAMP TMV on leaf material from a) petunia and b) tomato spiked with non-target RNA.

MNSV

Sensitivity

In a 10x serial silution series of gBlocks the limit of detection was 10 copies/µl (Table 2.6-27, Figure 2-28).

Table 2.6-27: MNSV LAMP tested on a serial dilution of gBlocks.

		Run1		**Run2**		**Run3**	
Well	Name	**Tpos (min)**	**Tm (°C)**	**Tpos (min)**	**Tm (°C)**	**Tpos (min)**	**Tm (°C)**
1	gBlock 10^6	06:00	86.6	06:00	86.3	06:15	86.8
2	gBlock 10^5	07:00	86.5	07:15	86.4	07:15	86.7
3	gBlock 10^4	08:00	86.5	08:15	86.3	08:15	86.7
4	gBlock 10^3	08:45	86.3	09:15	86.3	09:30	86.7
5	gBlock 10^2	11:00	86.2	10:15	86.4	10:45	86.5
6	gBlock 10^1	19:15	86.2	16:15	86.5	11:45	86.5
7	gBlock 10^0	-	-	-	-	18:30	86.3
8	NTC	-	-	-	-	-	-

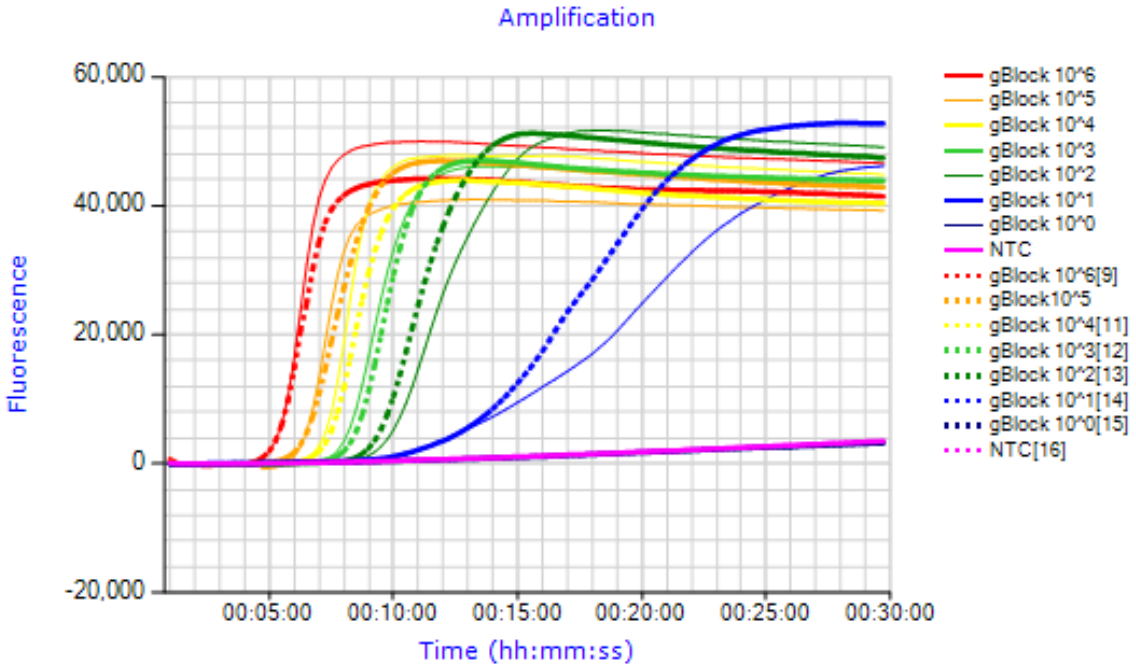


Figure 2-28: MNSV LAMP tested on a gBlock dilution series.

Specificity

Specificity was tested on RNA from the cucumber pathogens CGMMV, CMV and CCNYV. No amplification of the non-target pathogens could be detected (Table 2.6-28, Figure 2-29).

Table 2.6-28: MNSV LAMP test on non-target RNA.

Name	**Tpos (min)**	**Tm (°C)**
MNSV gBlock 10^6	06:15	86.4
MNSV gBlock 10^6	06:30	86.6
CGMMV RNA	-	-
CGMMV RNA	-	-
CMV RNA	-	-
CMV RNA	-	-
NTC	-	-
NTC	-	-

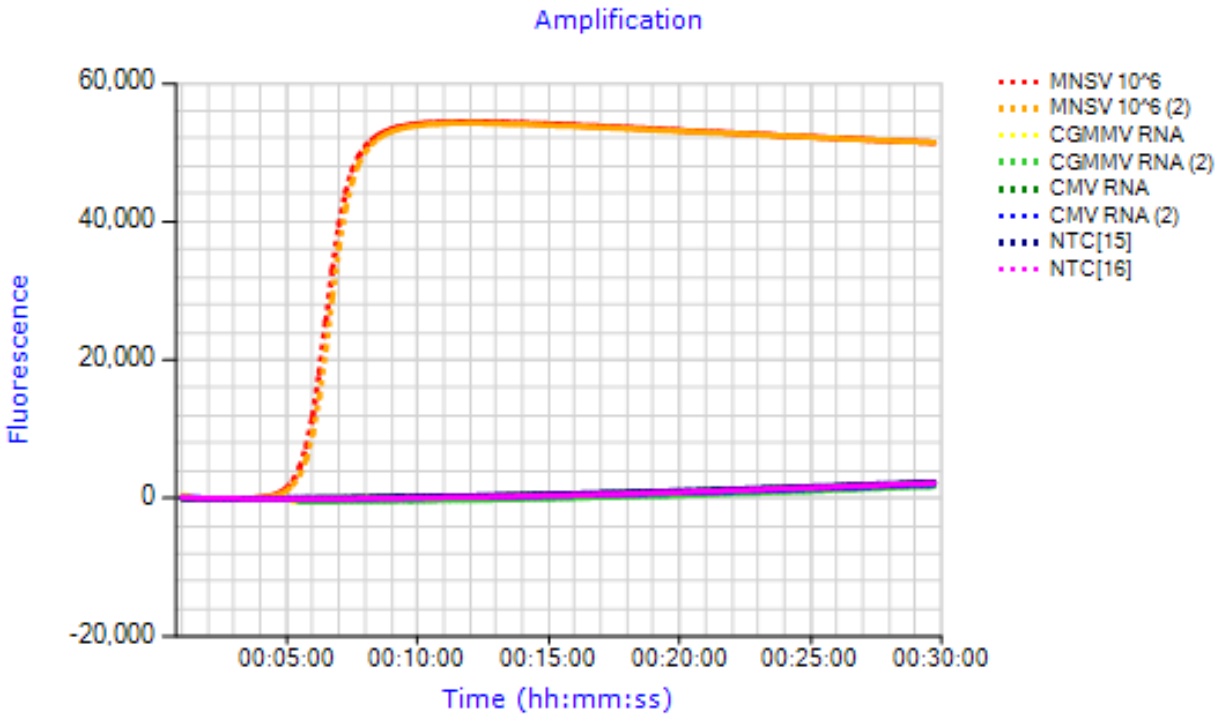


Figure 2-29: MNSV LAMP test on non-target RNA.

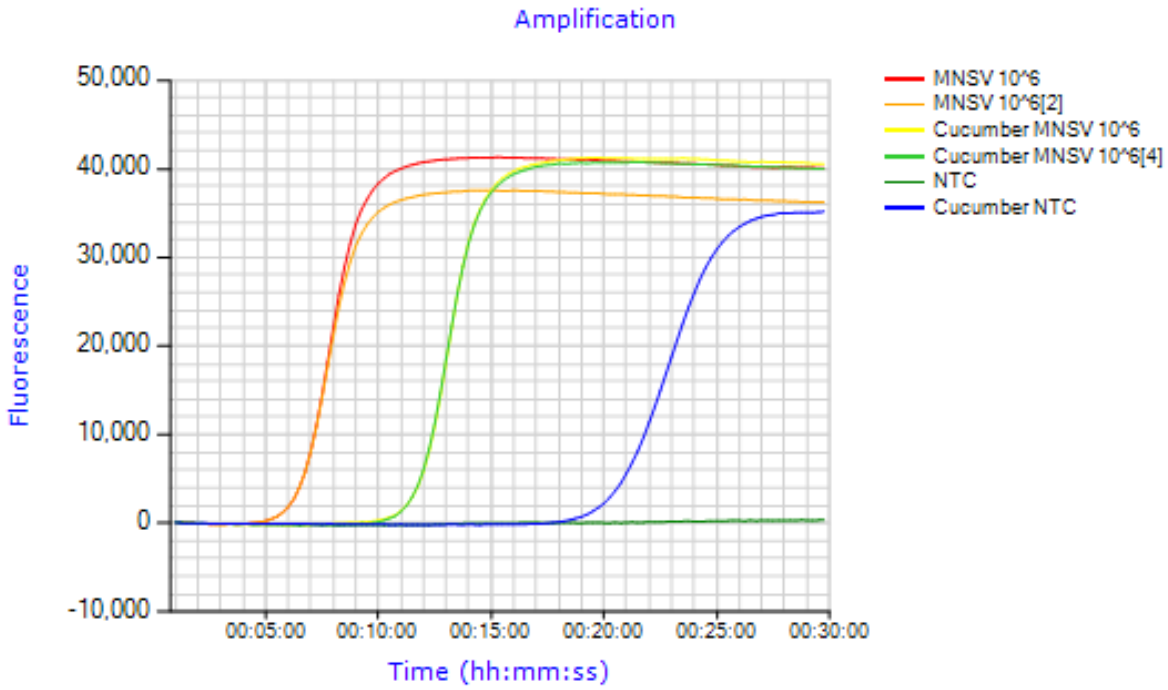
Multiplex

A multiplex LAMP was developed with *Cox* as the amplification control. The *Cox* amplification can be detected by differences in melting temperature. However, this is only the case when the Cox primer mix with amplification probe is used. As expected, amplification of the *Cox* gene was only detected in the healthy cucumber extract (Table 2.6-29, Figure 2-30).

Table 2.6-29: MNSV multiplex test.

Name	**Tpos (min)**	**Tm (°C)**
MNSV gBlock 10^6	07:30	86.7
MNSV gBlock 10^6	07:30	86.7
Cucumber MNSV 10^6	12:45	86.6
Cucumber MNSV 10^6	12:45	86.6
NTC	-	-
Cucumber NTC	22:15	85.3

a



b

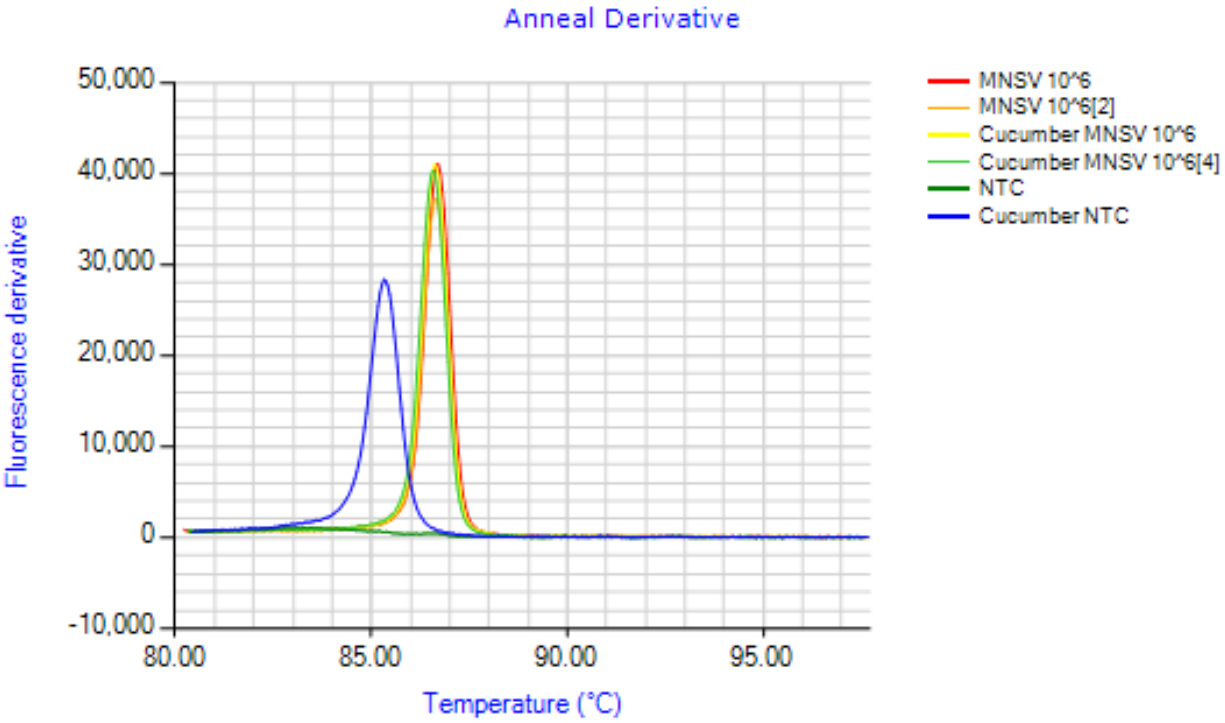


Figure 2-30: MNSV multiplex test a) amplfication curve, b) melting curve.

RNA

The assay was tested on MNSV RNA with positive results (Table 2.6-30, Figure 2-31).

Table 2.6-30: MNSV LAMP test op RNA.

Name	**Tpos (min)**	**Tm (°C)**
MNSV gBlock 10^6 copies	07:00	86.7
MNSV RNA	04:45	86.6
MNSV RNA	04:45	86.6
MNSV RNA	04:45	86.6
NTC	-	-
NTC	-	-

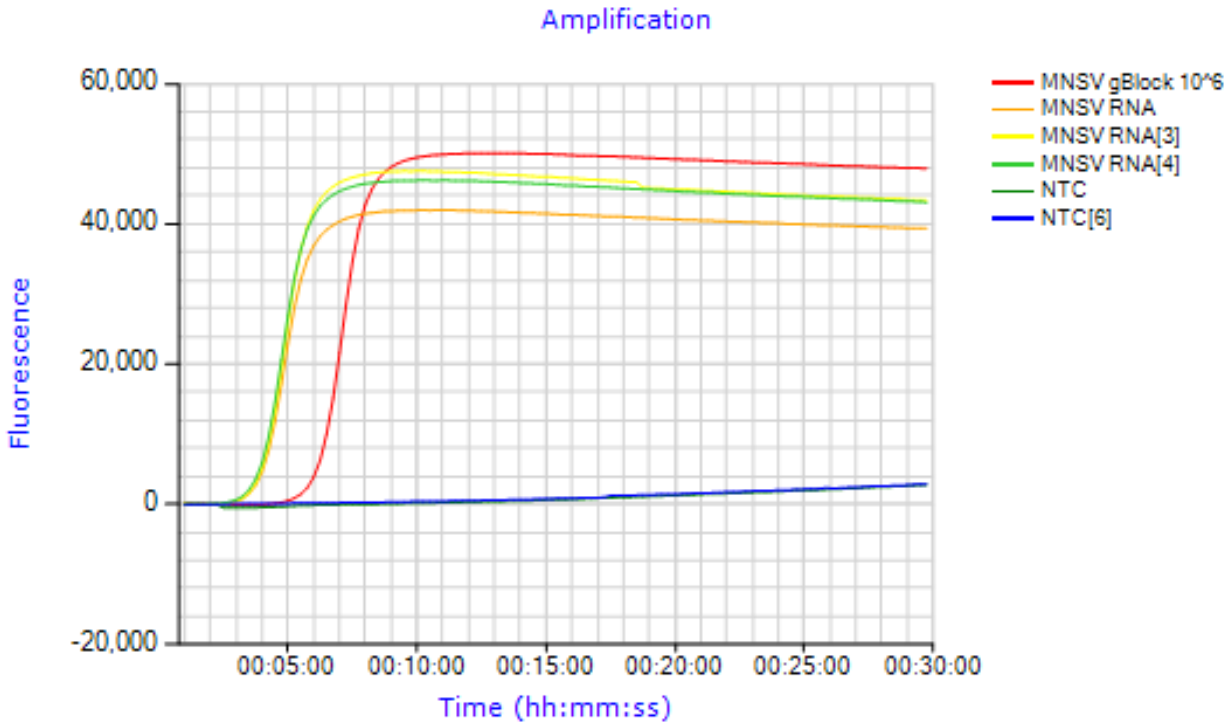


Figure 2-31: MNSV LAMP test op RNA.

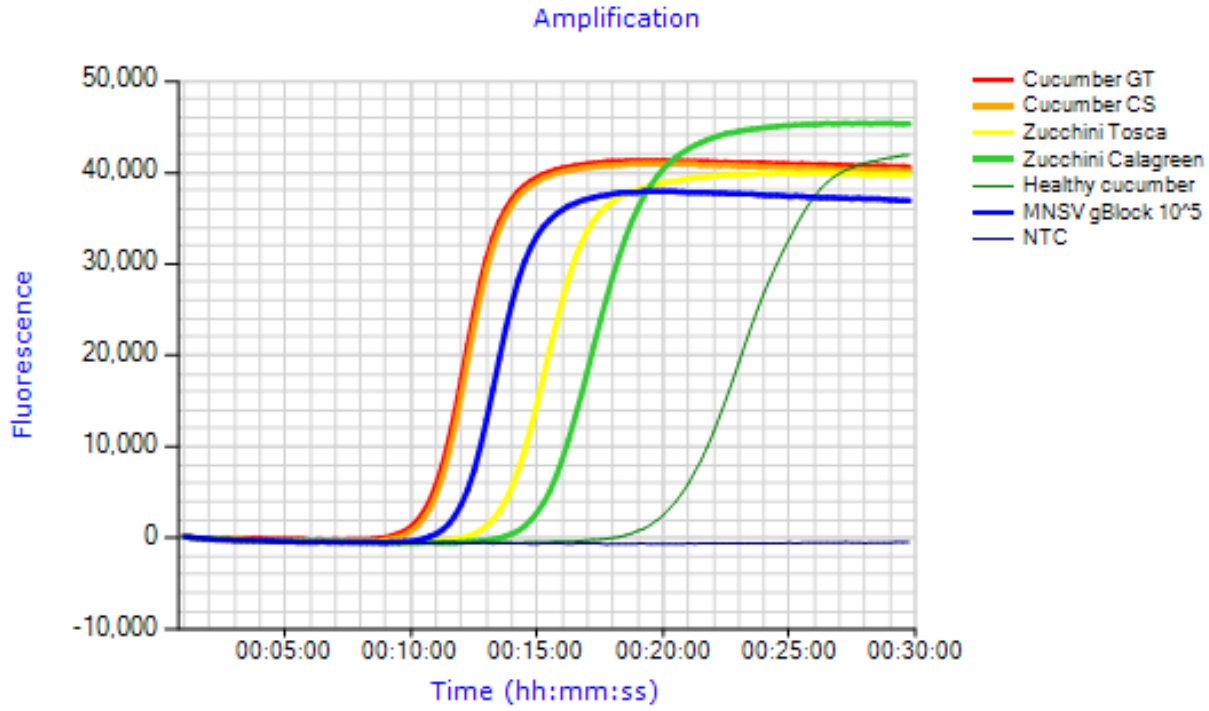
Infected leaf material

The assay was tested on two cucumber cultivars and two zucchini cultivars infected with MNSV. All infected leaves showed a positive amplification with the LAMP assay (Table 2.6-31, Figure 2-32). For the healthy cucumber leaf material only the *Cox* signal appeared.

Table 2.6-31: MNSV LAMP on infected leaf material.

	Sample	**Run 1**		**Run 2**	
Well	**Name**	**Tpos (min)**	**Tm (°C)**	**Tpos (min)**	**Tm (°C)**
1	Cucumber GT	11:45	86.1	12:15	86.2
2	Cucumber CS	12:00	86.2	12:00	86.2
3	Zucchini Tosca	15:00	86.2	15:15	86.2
4	Zucchini Calagreen	16:30	86.3	16:00	86.2
5	Healthy cucumber	22:30	85	21:45	85.1
6	MNSV gBlock 10^5	13:00	86.2	13:15	86.2
7	NTC	-	-	-	-

a



b

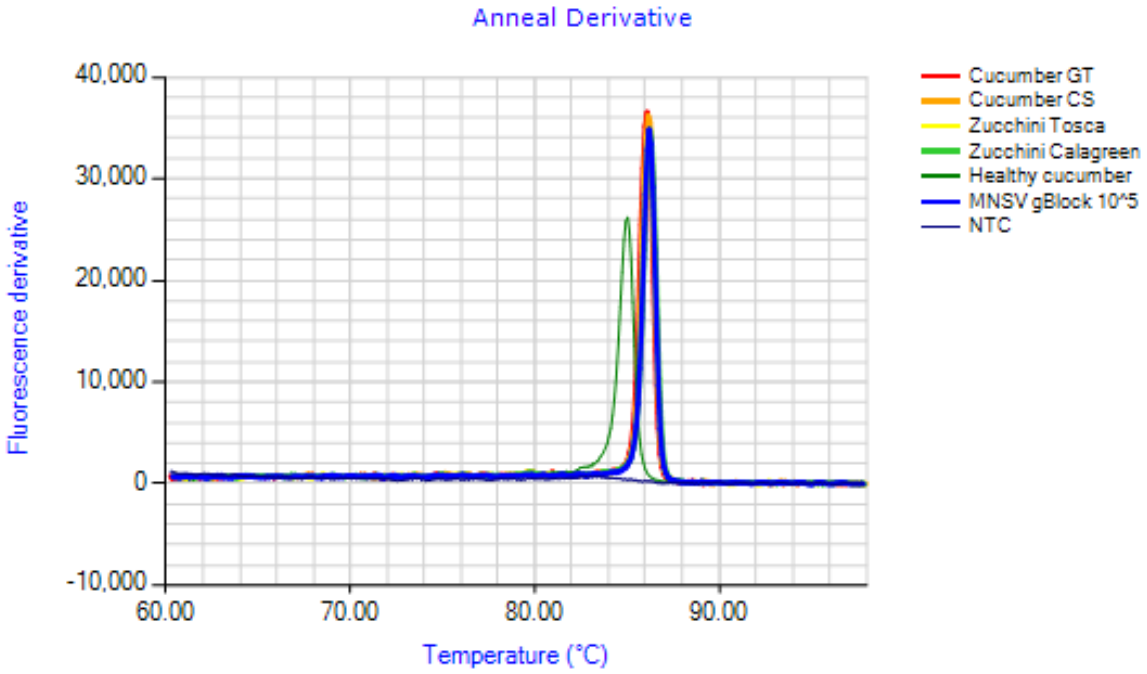


Figure 2-32: MNSV LAMP on infected leaf material, a) amplification curves, b) melting curves.

TSWV

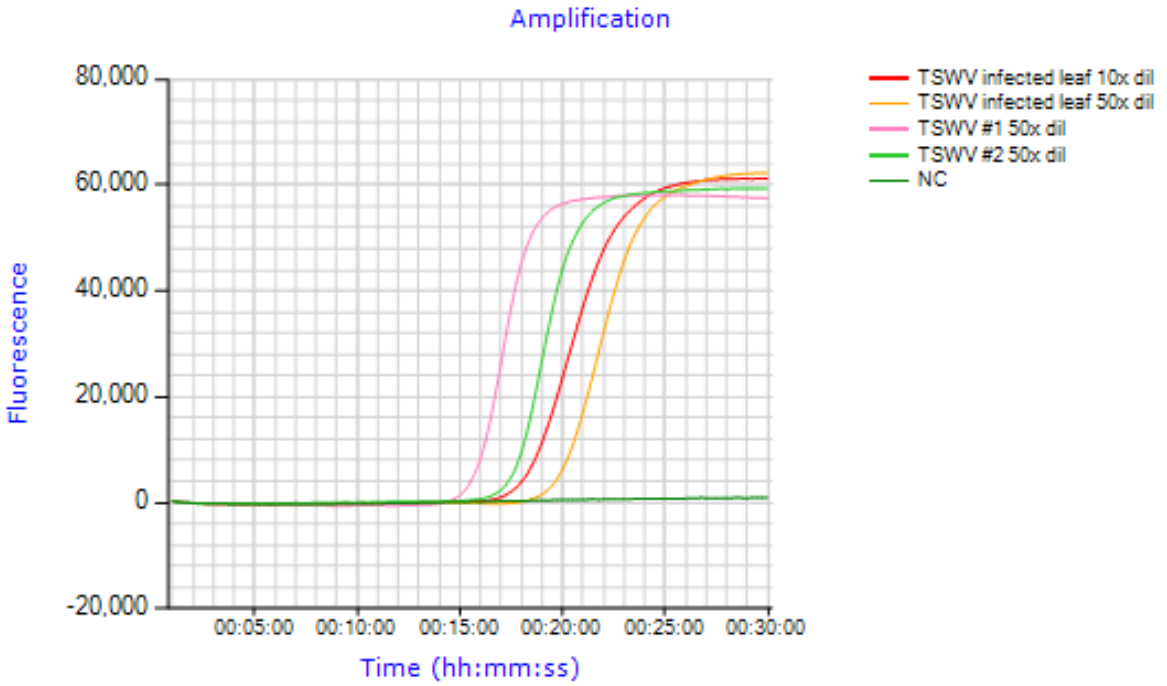
Sensitivity

Sensitivity was tested with a dilution series of gBlocks. However, the gBlock appeared to be unstable and results showed a high variability. Therefore, the evaluation was continued with RNA at a 50x dilution and infected leaf material. Both gave positive amplification (Table 2.6-32, Figure 2-33).

Table 2.6-32: TSWV LAMP on RNA and infected leaf material.

Samples	**Tpos (min)**	**Tm (°C)**
TSWV infected leaf 10x dil	19:45	84.5
TSWV infected leaf 50x dil	21:15	84.5
TSWV #1-1 50x dil	17:00	84.5
TSWV #2-2 50x dil	18:45	84.6

a



b

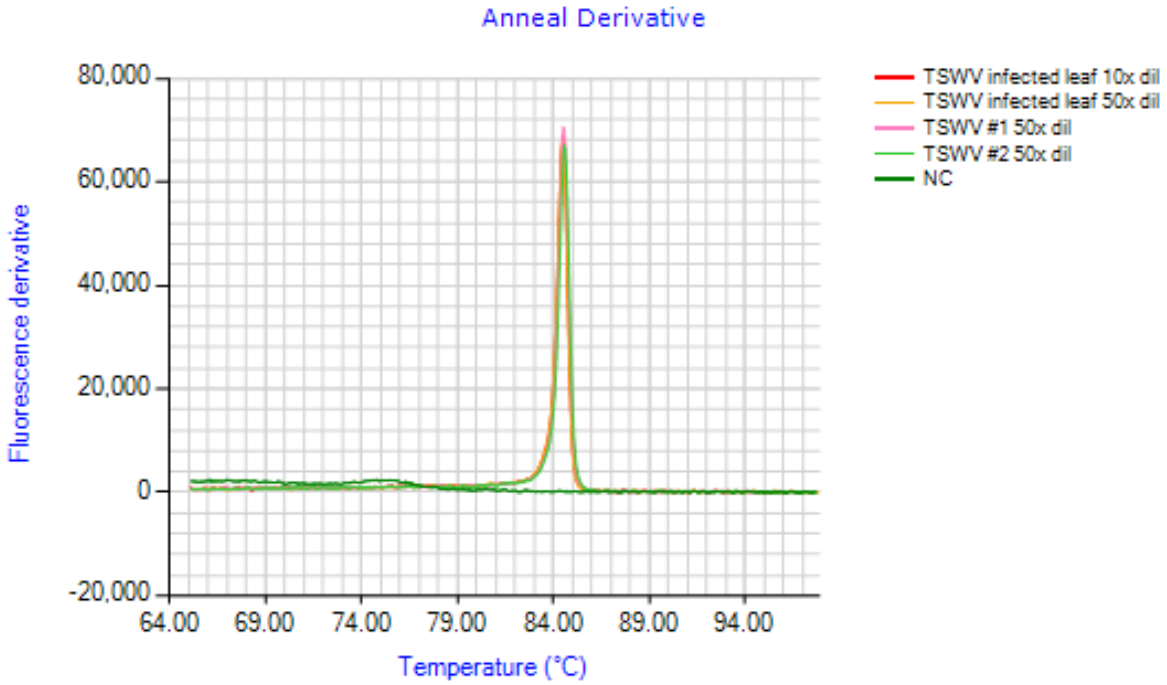


Figure 2-33: TSWV LAMP on RNA and infected leaf material, a) amplification curves, b) melting curves.

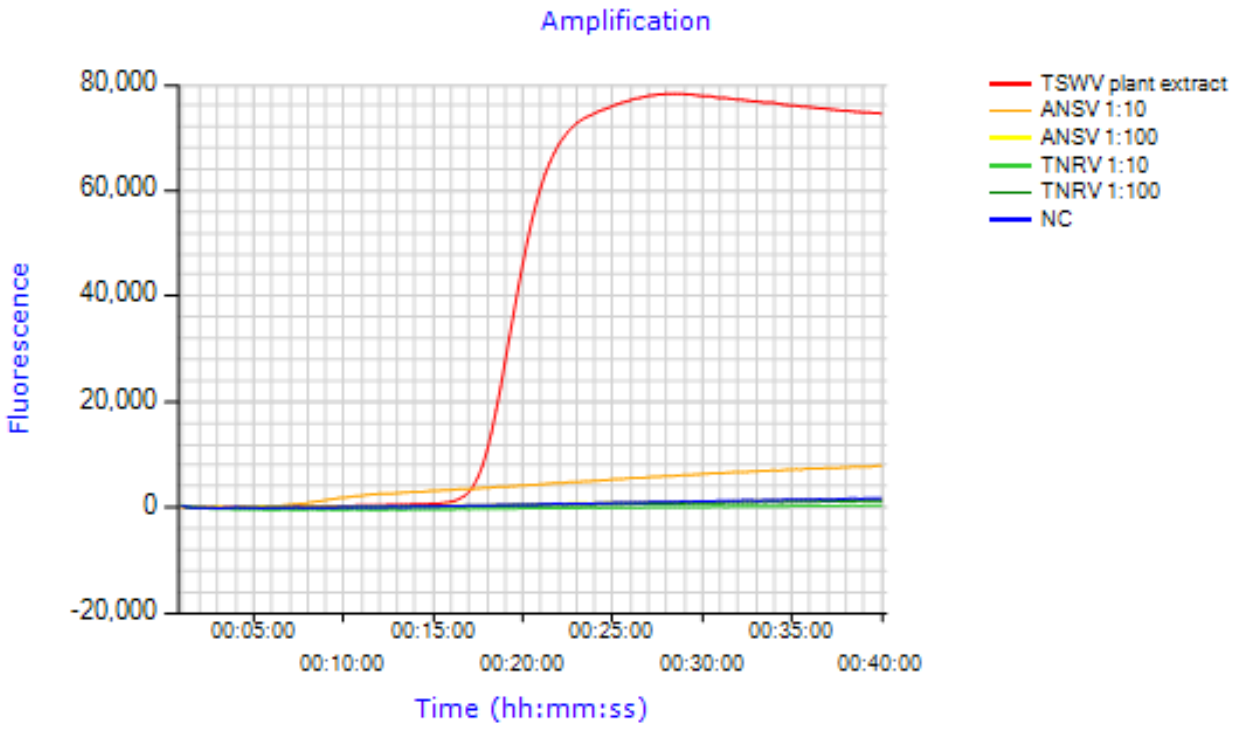
Specificity

Specificity was tested with RNA of two other tospoviruses; alstroemeria necrotic streak virus (ANSV) and tomato necrotic ring virus (TNRV). The assay did not amplifiy these non-targets (Table 2.6-33, Figure 2-34).

Table 2.6-33: TSWV LAMP assay on non-target RNA.

Samples	**Tpos (min)**	**Tm (°C)**
TSWV plant extract 1:50	18:45	84.4
ANSV 1:10	-	-
ANSV 1:100	-	-
TNRV 1:10	-	-
TNRV 1:100	-	-
NC	-	-

a



b

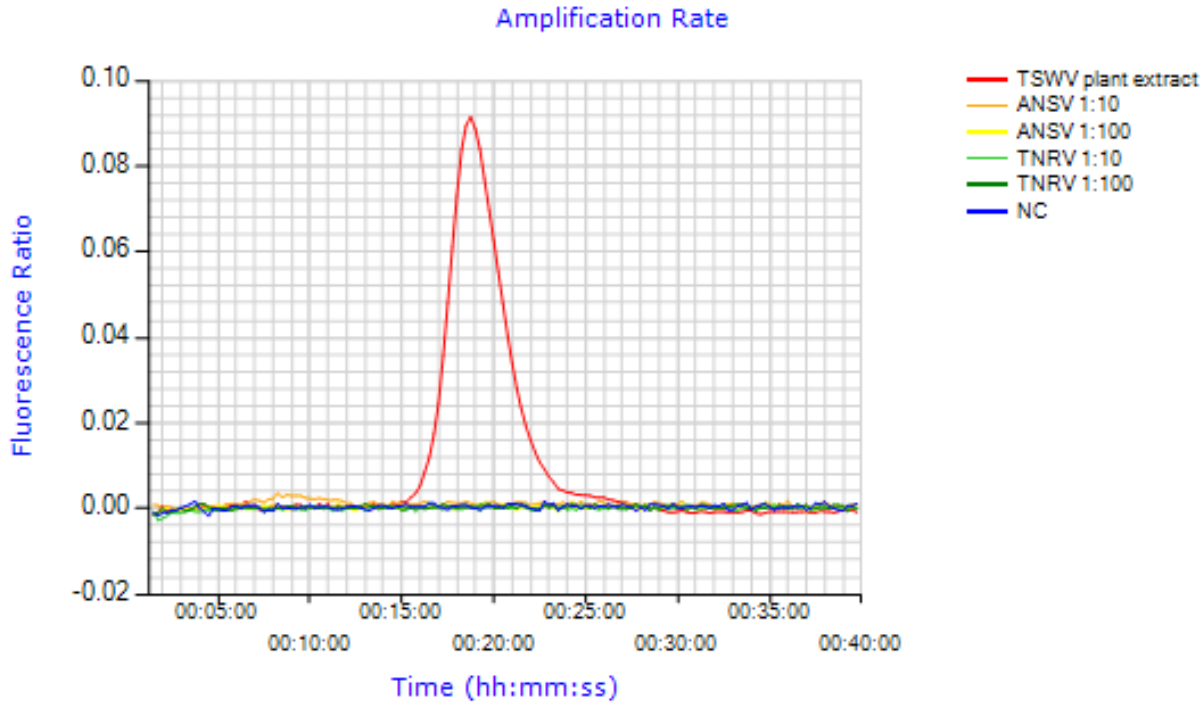


Figure 2-34: TSWV LAMP assay on non-target RNA, a) amplification curves, b) melting curves.

PVY

PVY is a variable species with a number of recombinant strains that differ considerably in their genomic sequence. *In silico* analysis showed that no LAMP assay could cover all known common recombnants. Therefore, an assay for the recombinant PVY-NTN that occurs in Petunia was evaluated.

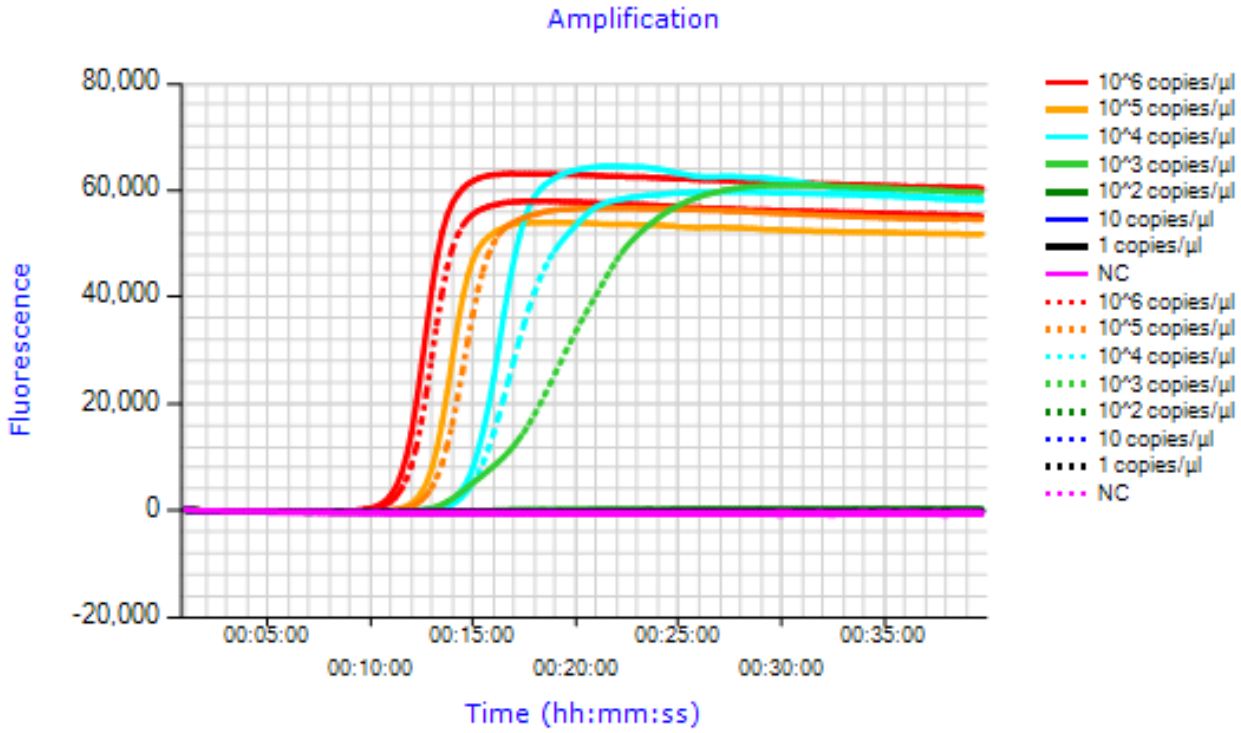
Sensitivity

In a serial dilution of gBlocks concentrations of 10^3-10^4 copies/µl could be detected (Table 2.6-34, Figure 2-35). In addition, a 10-fold serial dilution of PVY RNA isolated from petunia was tested in threefold and once with a new enzyme mix from OptiGene containing RT-polymerase (ISO-001 RT). Dilutions of 1:10000 could still be detected reliably (Table 2.6-35, Figure 2-36). The use of the new enzyme mix resulted in a better dilution series.

Table 2.6-34: PVY LAMP for a serial dilution of gBlocks.

	1		**2**	
copies/µl	**Tpos (min)**	**Tm (°C)**	**Tpos (min)**	**Tm (°C)**
10^6	12:15	84.9	13:30	84.5
10^5	13:30	84.8	14:00	84.6
10^4	15:30	84.8	15:45	84.4
10^3	-	-	14:29	84.5
10^2	-	-	-	-
10^1	-	-	-	-
10^0	-	-	-	-
0	-	-	-	-

a



b

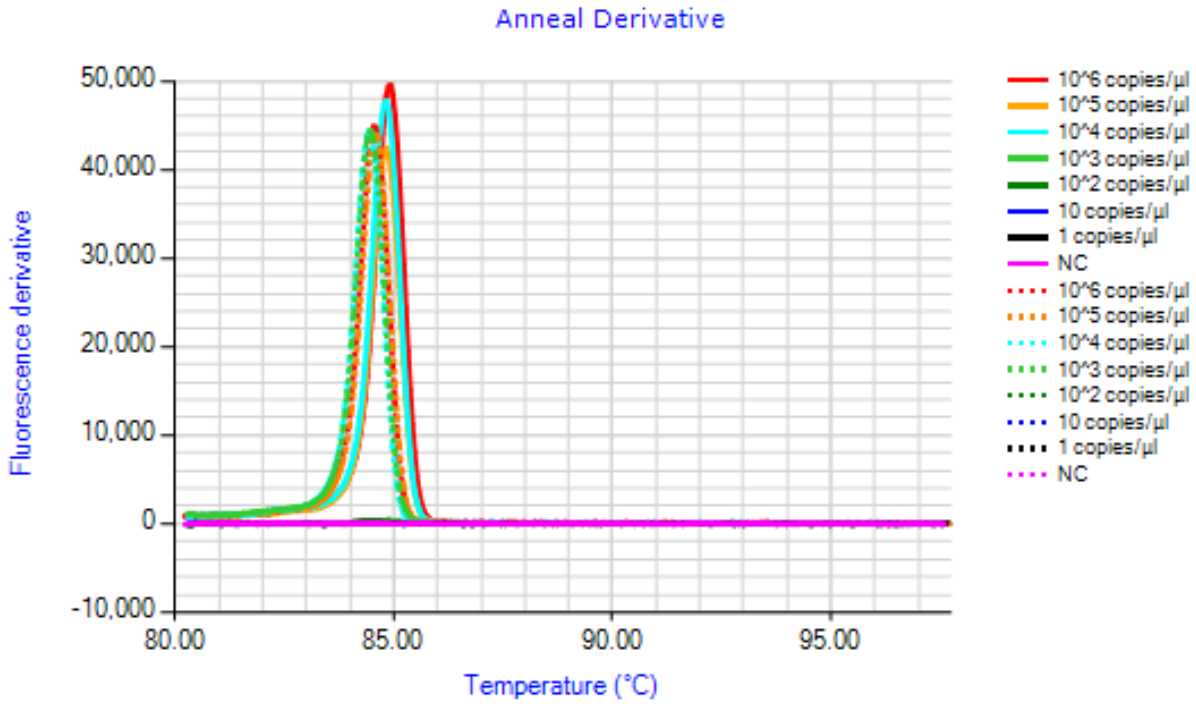
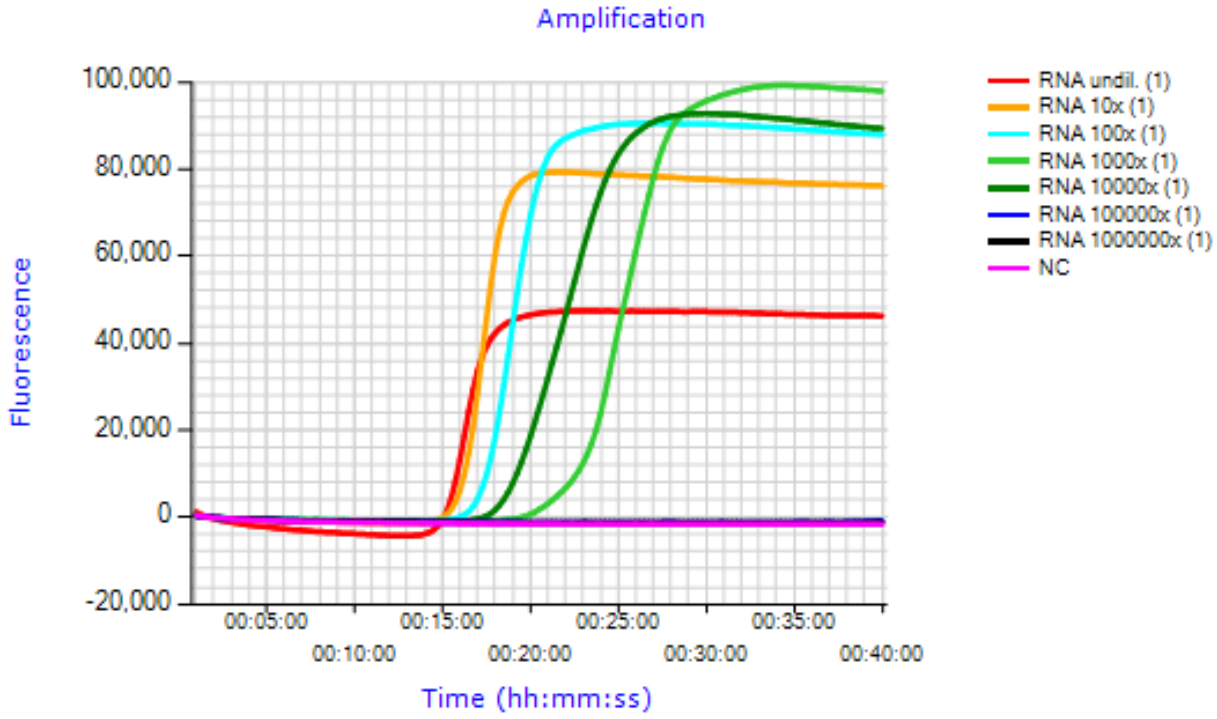


Figure 2-35: PVY LAMP for a serial dilution of gBlocks, a) amplification curves, b) melting curves.

Table 2.6-35: PVY LAMP for a serial dilution of RNA

	1		2		3		ISO-001 RT	
	Tpos (min)	**Tm (°C)**	**Tpos (min)**	**Tm (°C)**	**Tpos (min)**	**Tm (°C)**	**Tpos (min)**	**Tm (°C)**
undiluted	16:00	84.4	16:15	84.6	16:00	84.5	13:45	84.4
10x	17:00	84.5	17:00	84.5	17:00	84.5	14:30	84.4
100x	18:15	84.5	19:00	84.6	-	-	16:30	84.5
1000x	24:15	84.5	18:45	84.4	24:00	84.5	17:45	84.4
10000x	19:45	84.4	-	-	21:15	84.6	21:00	84.5
100000x	-	-	39:45	-	-	-	-	-
1000000x	-	-	-	-	-	-	-	-
0	-	-	-	-	-	-	-	-

a



b

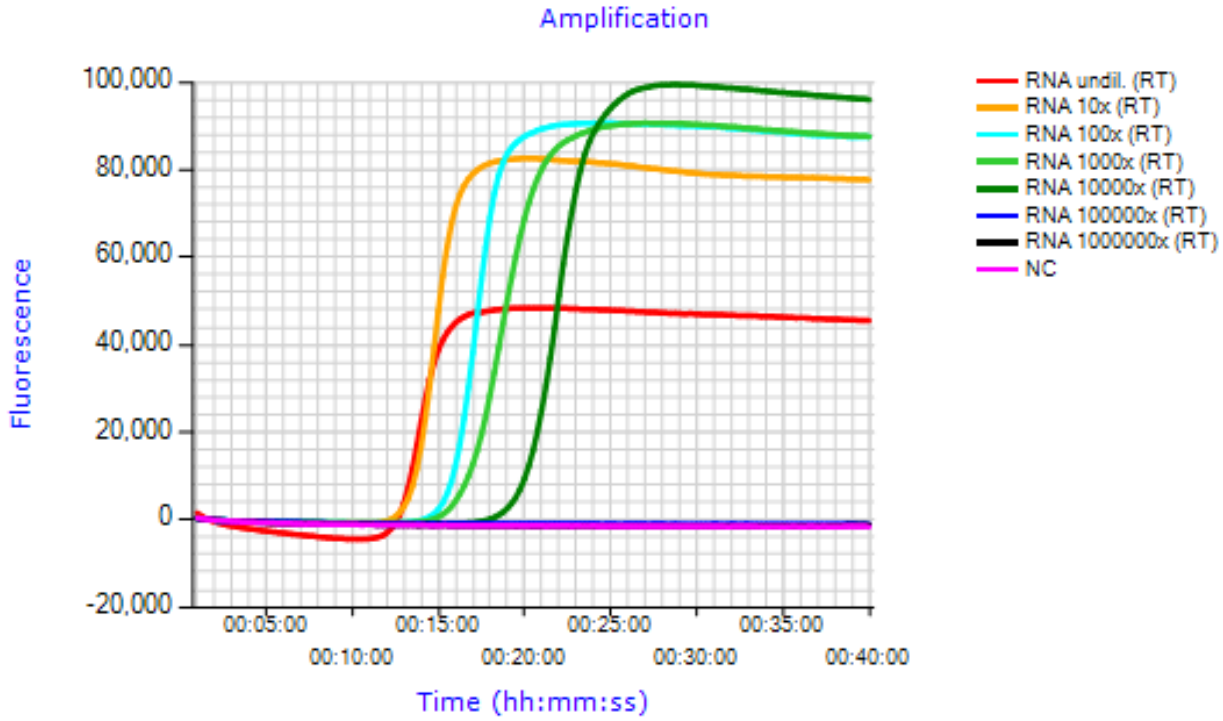


Figure 2-36: PVY LAMP with a) normal enzyme mix and b) ISO-001 RT enzyme mix for a serial dilution of RNA.

Specificity

The LAMP assay was tested on gBlocks of five different PVY strains, the NTN recombinant strain PVY-AJ889866, PVY- JF928458 (PVY-E recombinant), PVY-KC296828 (PVY-N), PVY-MH795859 (PVY-N), PVY-KJ741205 and the non-target species pepino mosaic virus (PepMV), sunflower chlorotic mottle virus (SuCMoV), tobacco necrosis satellite virus (TNSV) and potato viris V (PVV).

Amplification was observed with four of the five PVY strains including the PVY-NTN target strain, but not with PepMV, TNSV and PVV (Table 2.6-36, Figure 2-37). Only with SuCMoV the beginning of amplification can be seen. However, this virus is not known to occur in petunia.

Table 2.6-36: PVY LAMP with PVY gblocks and non-target gBlocks.

	Tpos (min)	**Tm (°C)**
PVY-AJ	09:30	84.8
PVY-JF	23:15	85.1
PVY-KC	-	-
PVY-MH	10:45	84.8
PVY-KJ	15:15	85.2
PepMV	-	-
SuCMoV	37:30	48.4
TNSV	-	-
PVV	-	-
NC	-	-

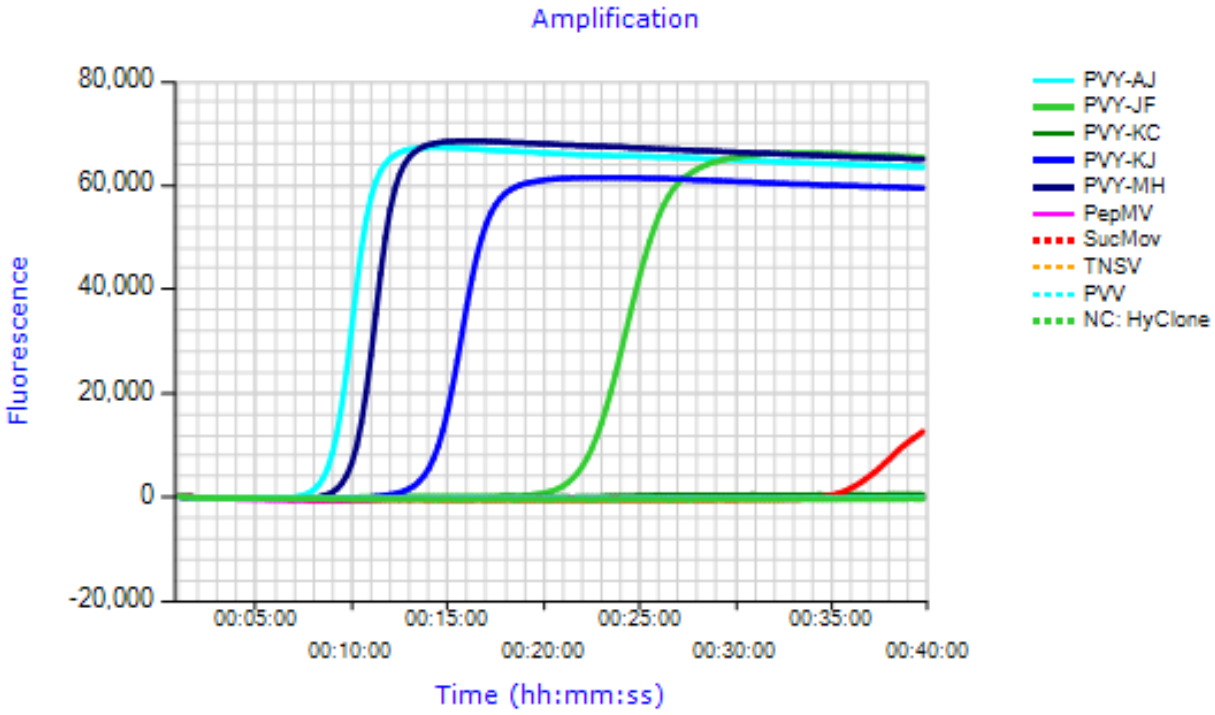


Figure 2-37: PVY LAMP for PVY and non-target species.

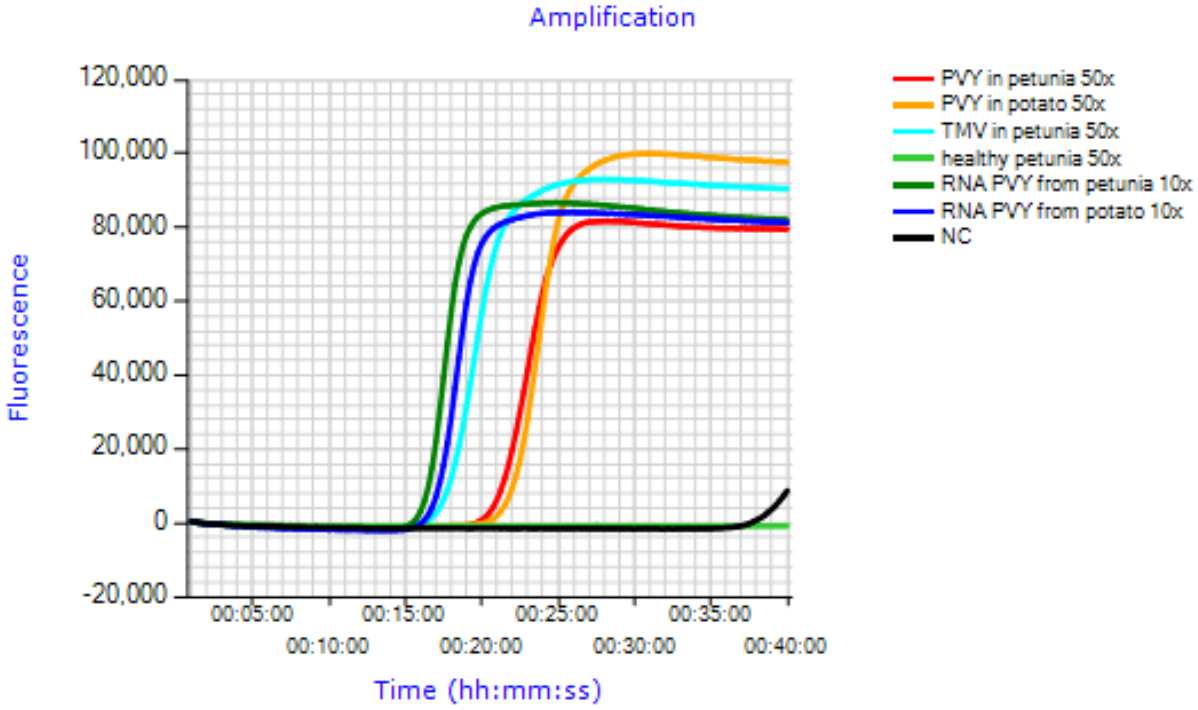
Test on infected material

The LAMP assay was tested on infected petunia and potato leaf and petunia leaf infected with TMV. Amplification could be observed with all samples infected with petunia, but also with the TMV infected sample (Table 2.6-37, Figure 2-38). However, it was confirmed by NAK Tuinbouw that the material likely also contained PVY.

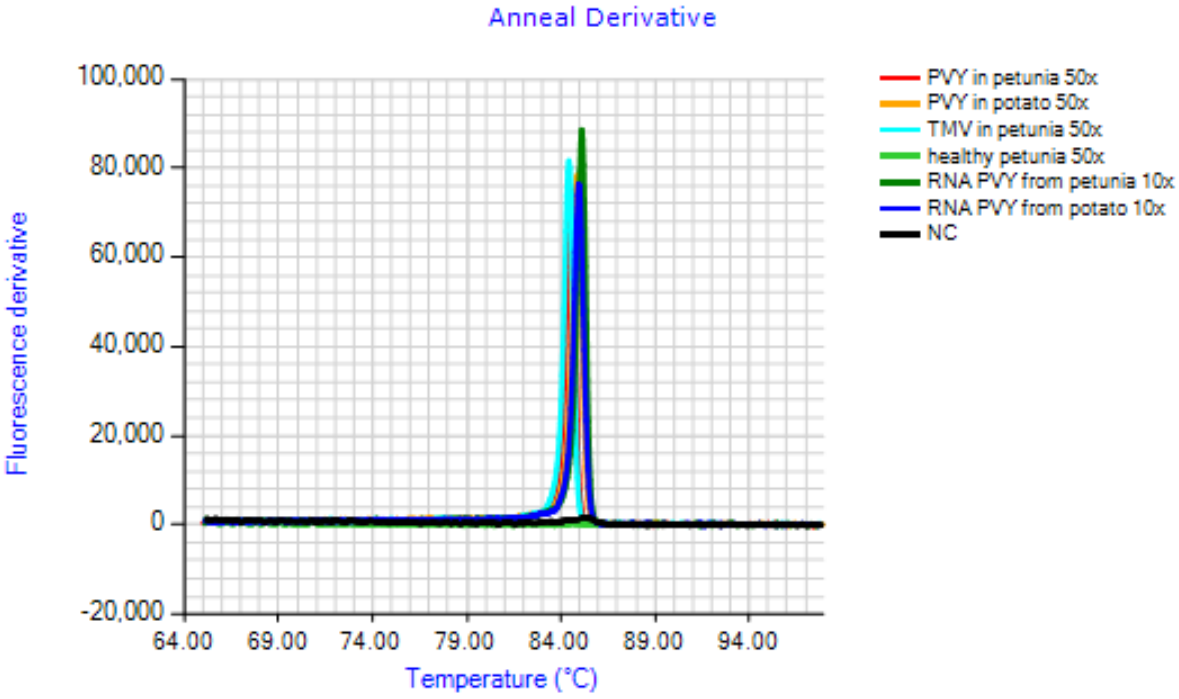
Table 2.6-37: PVY LAMP on infected material with the normal OptiGene enzyme mix (ISO-001) and the new mix (ISO-001 RT).

	ISO-001		**ISO-001 RT**	
	Tpos (min)	**Tm (°C)**	**Tpos (min)**	**Tm (°C)**
PVY in petunia 50x	22:00	84.5	16:15	84.6
PVY in potato 50x	23:00	84.9	16:15	84.8
TMV in petunia 50x	18:30	84.4	15:15	84.4
healthy petunia 50x	-	-	-	-
RNA PVY petunia 10x	17:15	85.1	12:30	84.9
RNA PVY potato 10x	18:00	84.9	13:30	84.9
NC	39:45	-	-	-

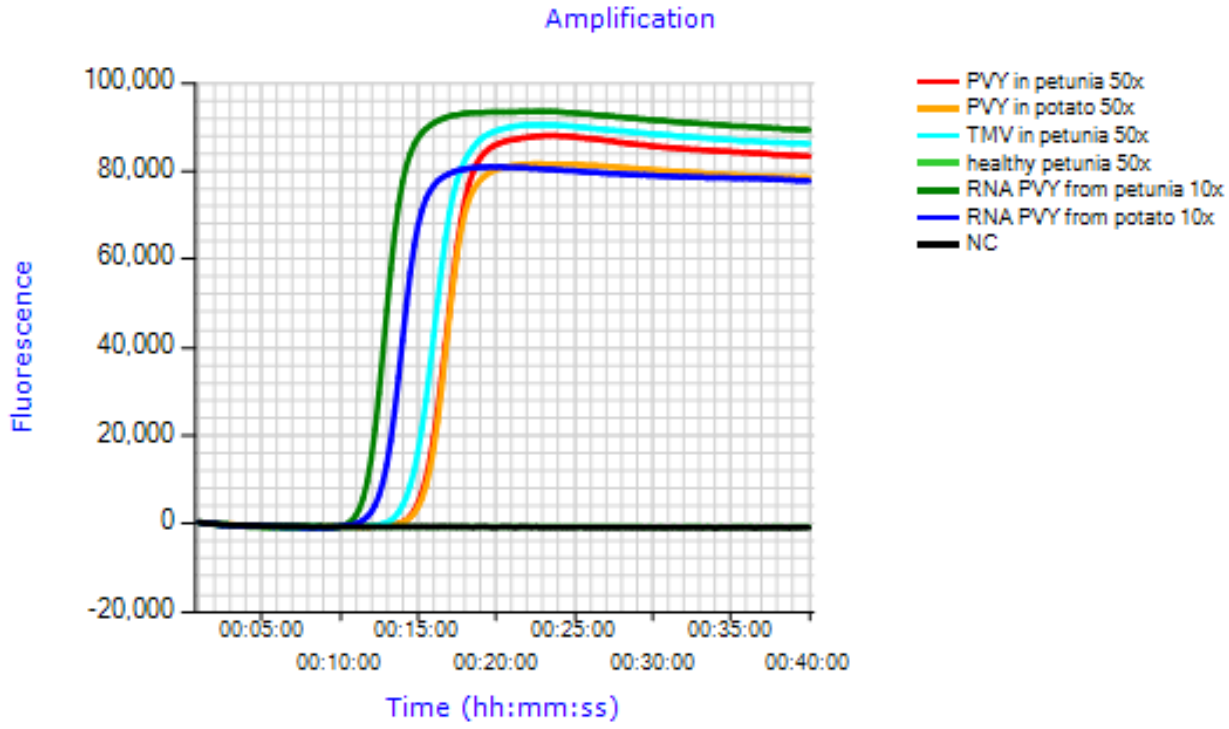
a


Amplification
120,000
100,000
80,000
60,000
40,000
20,000
0
-20,000
Fluorescence
00:05:00
00:10:00
00:15:00
00:20:00
00:25:00
00:30:00
00:35:00
00:40:00
Time (hh:mm:ss)
PVY in petunia 50x
PVY in potato 50x
TMV in petunia 50x
healthy petunia 50x
RNA PVY from petunia 10x
RNA PVY from potato 10x
NC

b


Anneal Derivative
100,000
80,000
60,000
40,000
20,000
0
-20,000
Fluorescence derivative
64.00
69.00
74.00
79.00
84.00
89.00
94.00
Temperature (°C)
PVY in petunia 50x
PVY in potato 50x
TMV in petunia 50x
healthy petunia 50x
RNA PVY from petunia 10x
RNA PVY from potato 10x
NC

c



d

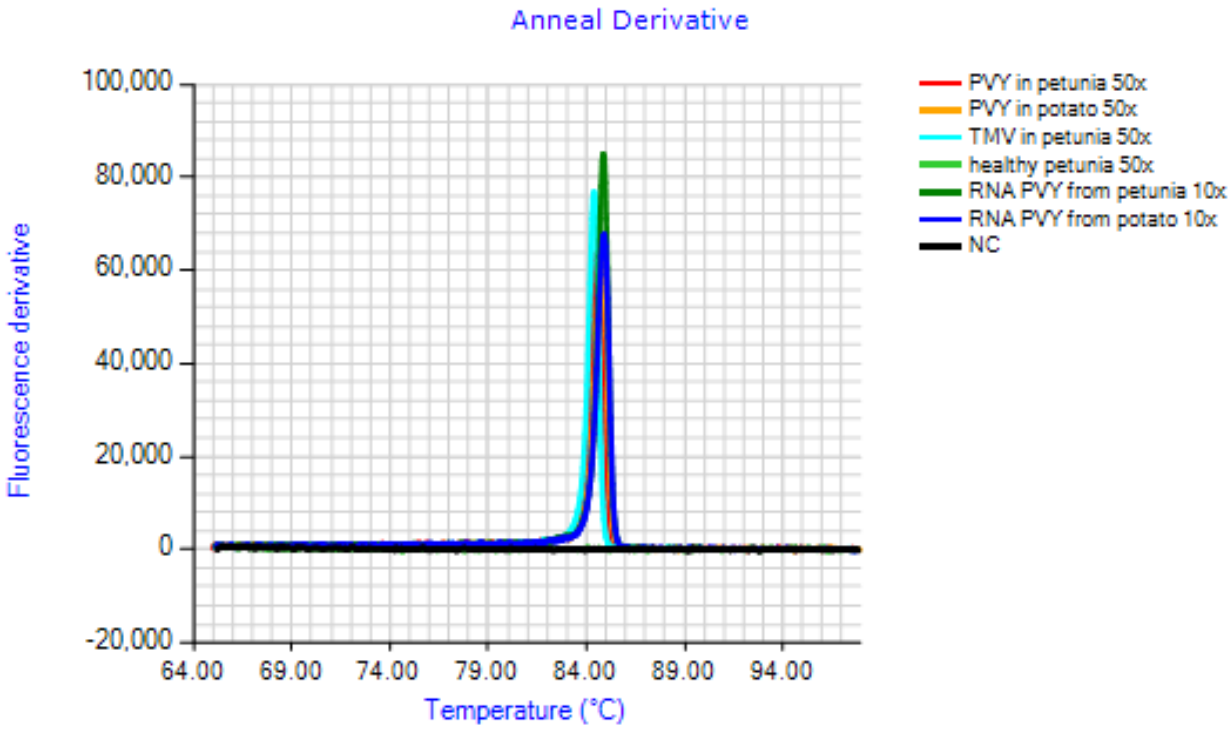


Figure 2-38: PVY LAMP on infected material with a,b) the normal OptiGene enzyme mix and c,d) the new OptiGene enzyme mix (ISO-001 RT)

Acidovorax citrulli

All results can be found in the report of PPS On-Site 1-0.

Xanthomonas fragariae

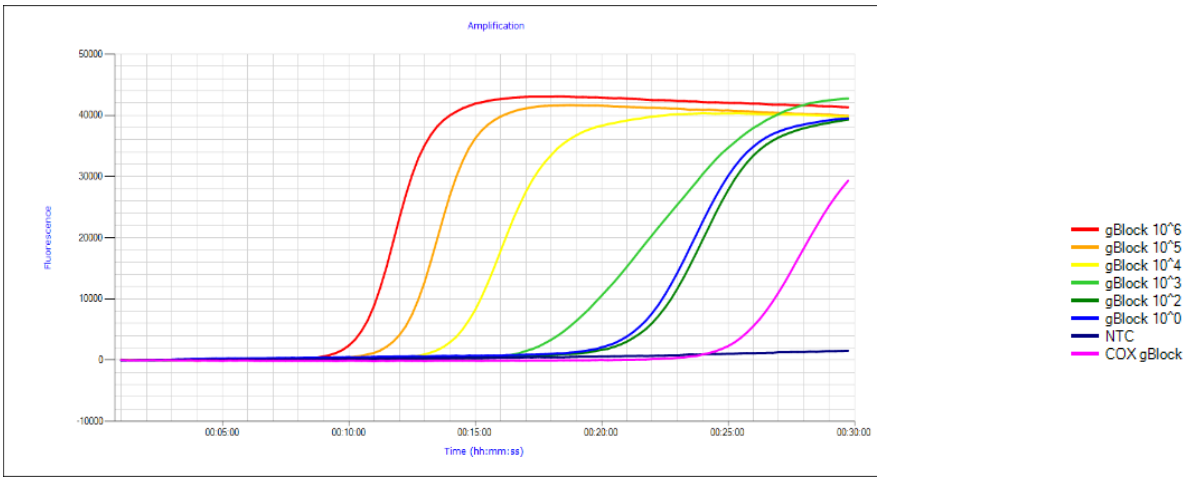
Sensitivity

Sensitivity was assessed with dilution series of gBlocks spiked to strawberry leaf material in an assay with Cox as an amplification control. The gBlocks could be detected at a concentration as low as 10^3 copies/µl (Table 2.6-38, Figure 2-39). At lower dilutions amplification with *Cox* was detected.

Table 2.6-38: LAMP results from strawberry leaf spiked with a dilution series of gBlocks.

XF+ COX	**Tpos (min)**	**Tm (°C)**
10^6	11:30	87.7
10^5	13:15	87.7
10^4	15:45	87.6
10^3	20:00	87.5
10^2	23:30	84.7
10^0	23:15	84.7
NTC	-	-
gBlock Cox	27.3	83.6

a



b

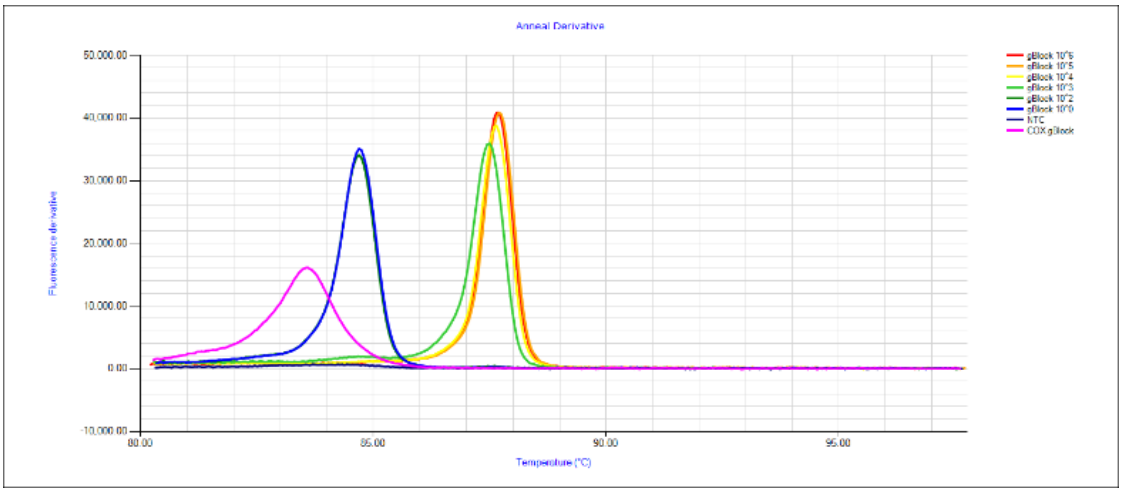


Figure 2-39: X. fragariae LAMP with a dilution series of gBlocks in strawberry leaf material, a) amplification curves, b) melting curves.

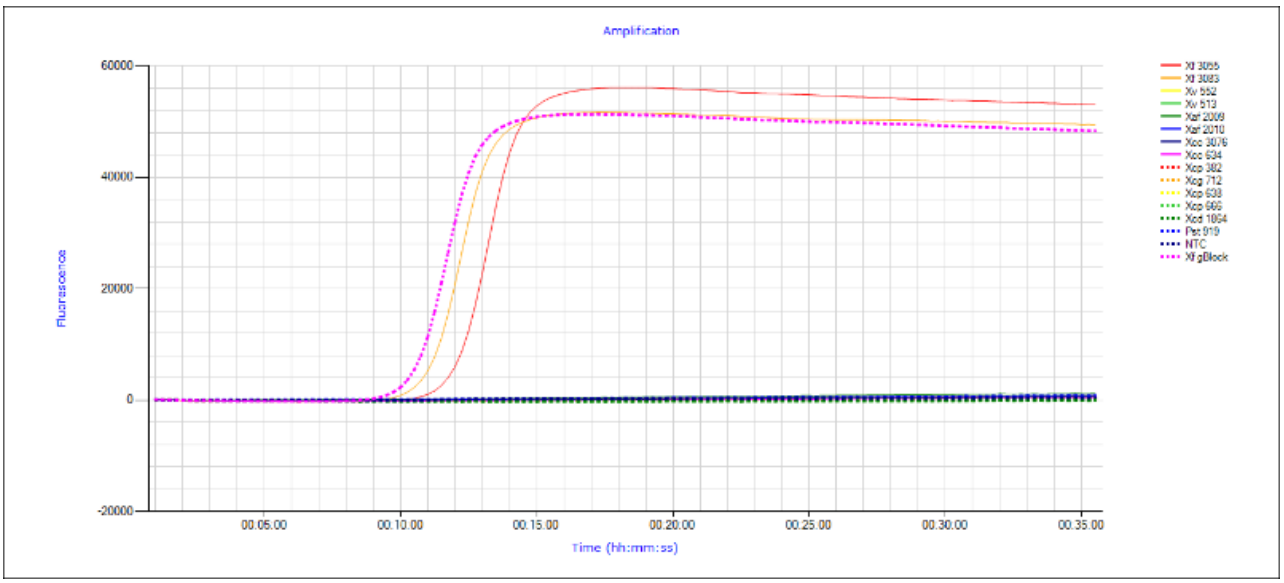
Specificity

Only samples with *X. fragariae* DNA were amplified (Table 2.6-39, Figure 2-40). No false positives occurred.

Table 2.6-39: X. fragariae LAMP with DNA from 2 X. fragariae strains and 12 non-target strains.

Sample	**Tpos (min)**	**Tm (°C)**
X. fragariae 3055	13:00	88.1
X. fragariae 3083	12:00	88.1
gBlock	11:15	87.9

a



b

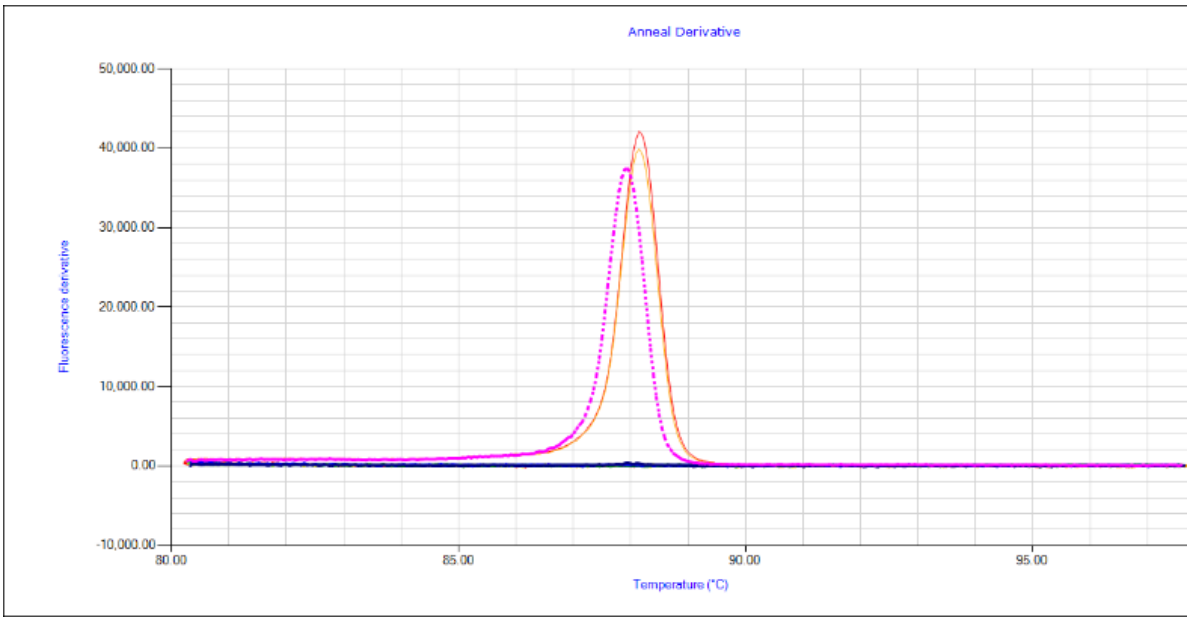


Figure 2-40: X. fragariae LAMP with target and non-target DNA. A) Amplification curves, b) melting curves.

Infected plant material

Strawberry leaves with and without symptoms were tested with the Xf LAMP assay and the Cox assay separately. In three of the four symptomatic leaves *X. fragariae* was detected (Table 2.6-40, Figure 2-41).

Table 2.6-40: X. fragariae LAMP with symptomatic and unsymptomatic strawberry leaves.

	XF primers		**COX primer**	
Name	**Tpos (min)**	**Tm (°C)**	**Tpos (min)**	**Tm (°C)**
leaf no symptoms	-	-	25:45	84.5
leaf no symptoms	-	-		
symptomatic leaf	16:30	87.8	25:15	84.4
symptomatic leaf	11:45	87.8	21:15	84.4
symptomatic leaf			23:15	84.4
symptomatic leaf	14:45	87.7	23:30	84.4
7. NTC	-	-	-	-
8. gBlock Xf	11:15	87.7	-	-
16. gBlock COX	-	-	22:15	84

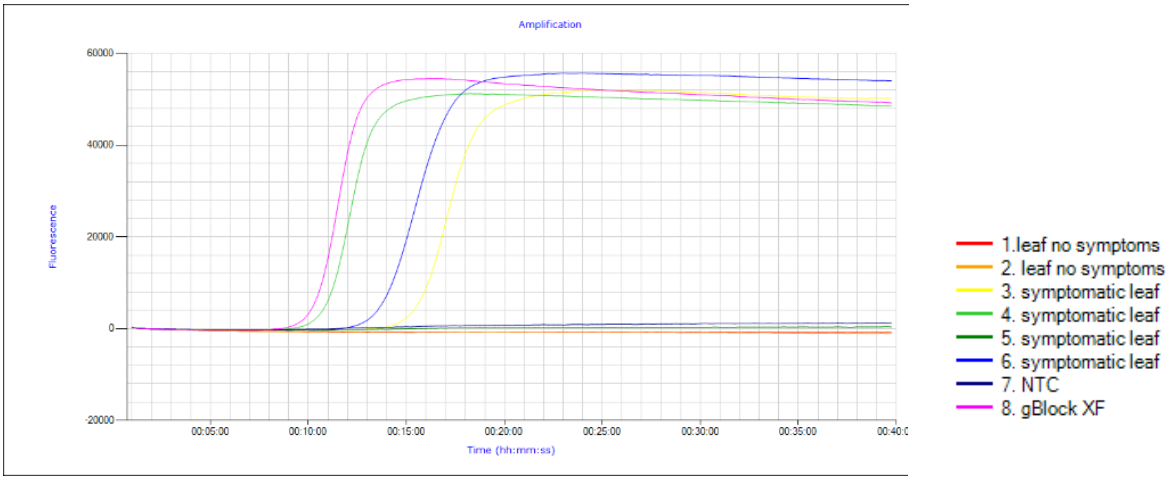


Figure 2-41: X. fragariae LAMP with symptomatic and unsymptomatic strawberry leaves and Xf primers.

Fusarium and *Verticillium*

Primers from literature for *F. oxysporum*, *F. solani* and *F. proliferatum* and *V. dahliae* were evaluated.

Evaluation was performed in infected plant material. Therefore, sensitvity could not be determined.

Fusarium solani

Fusarium solani primers only amplified *F. solani* samples and neither *F. oxysporum* nor *F. proliferatum* (Table 2.6-41, Figure 2-42).

Table 2.6-41: F. solani LAMP with Fusarium targets and non-targets.

Targets	**Tpos (min)**	**Tm (°C)**
F. solani MFO25-1 (phalaenopsis)	19:30	89.5
F. solani MFG11-1 (gerbera)	21:00	89.4
F. oxysporum MFO8-1 (phalaenopsis)	-	-
F. oxysporum MFG1 (gerbera)	-	-
F. oxysporum Ui76.3.1b (ui)	n.t.	n.t.
F. proliferatum MFO20 (phalaenopsis)	-	-
F. proliferatum Ui11.5.2b (ui)	-	-
F. proliferatum MFG10 (gerbera)	n.t.	n.t.
NC: HyClone water	-	-
NC: HyClone water	-	-

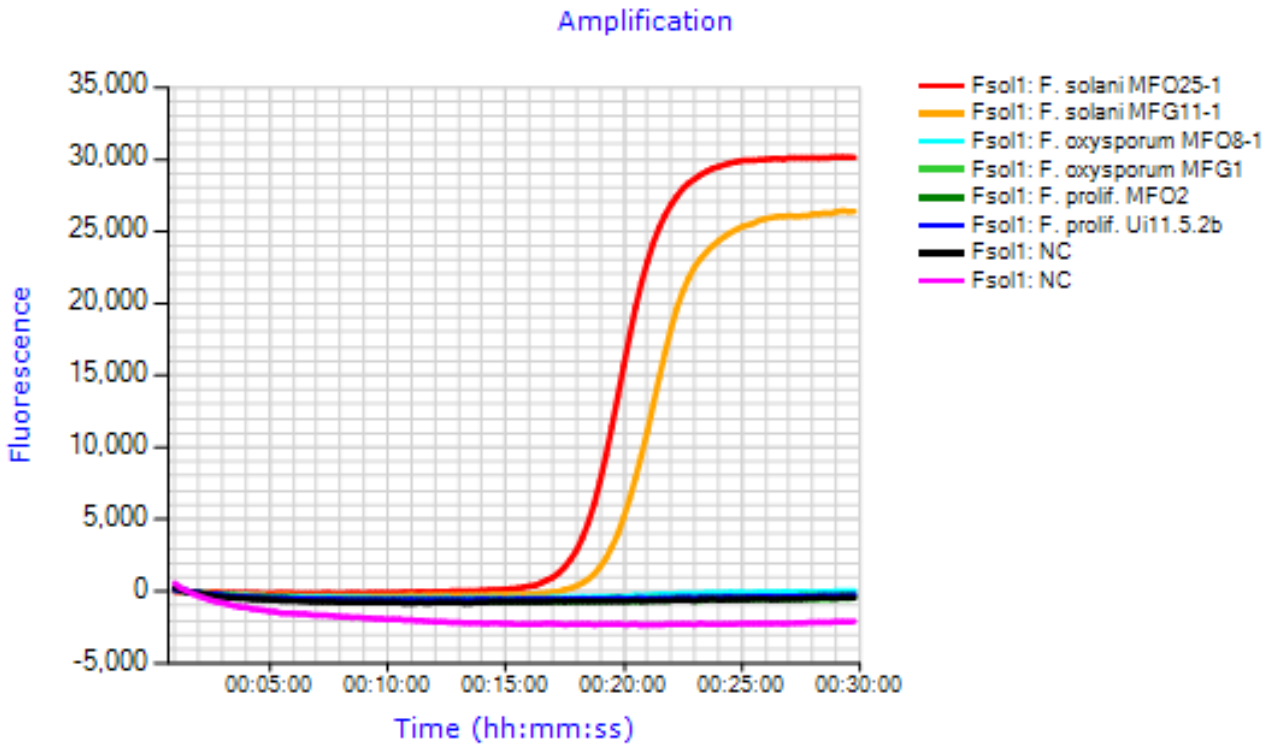


Figure 2-42: F. solani LAMP with Fusarium targets and non-targets.

Fusarium oxysporum

The *Fusarium oxysporum* LAMP only identified the target species (Table 2.6-42, Figure 2-43).

Table 2.6-42: F. oxysporum LAMP with Fusarium targets and non-targets.

Targets	**Tpos (min)**	**Tm (°C)**
F. solani MFO25-1 (phalaenopsis)	-	-
F. solani MFG11-1 (gerbera)	n.t.	n.t.
F. oxysporum MFO8-1 (phalaenopsis)	26:00	88.0
F. oxysporum MFG1 (gerbera)	27:45	88.2
F. oxysporum Ui76.3.1b (ui)	-	-
F. proliferatum MFO20 (phalaenopsis)	-	-
F. proliferatum Ui11.5.2b (ui)	-	-
F. proliferatum MFG10 (gerbera)	n.t.	n.t.
NC: HyClone water	-	-
NC: HyClone water	-	-

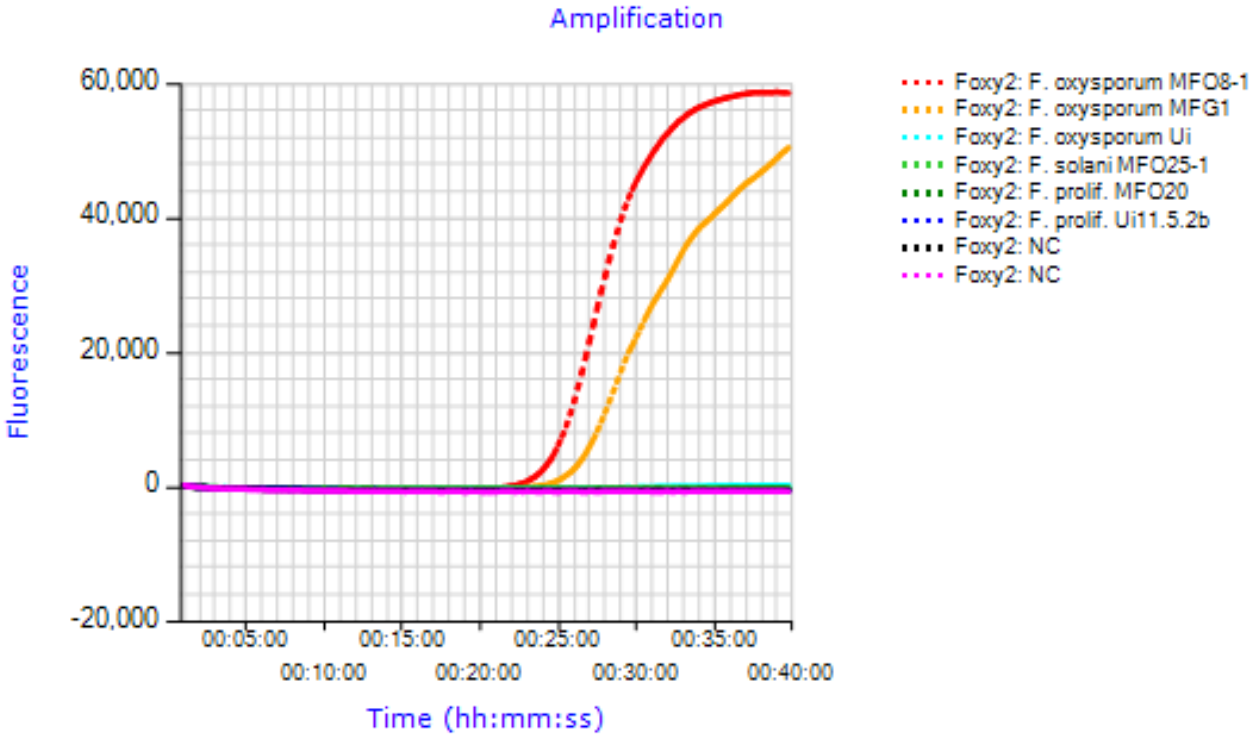


Figure 2-43: F. oxysporum LAMP with Fusarium targets and non-targets.

Fusarium proliferatum

The *F. proliferatum* assay amplified all *F. proliferatum* samples, but also one *F. oxysporum* sample (Table 2.6-43, Figure 2-44).

Table 2.6-43: F. proliferatum LAMP with Fusarium targets and non-targets.

Targets	**Tpos (min)**	**Tm (°C)**
F. solani MFO25-1 (phalaenopsis)	-	-
F. solani MFG11-1 (gerbera)	n.t.	n.t.
F. oxysporum MFO8-1 (phalaenopsis)	13:00	87.9
F. oxysporum MFG1 (gerbera)	-	-
F. oxysporum Ui76.3.1b (ui)	n.t.	n.t.
F. proliferatum MFO20 (phalaenopsis)	11:15	87.9
F. proliferatum Ui11.5.2b (ui)	13:00	88.0
F. proliferatum MFG10 (Gerbera)	11:00	88.1
NC: HyClone water	-	-
NC: HyClone water	-	-

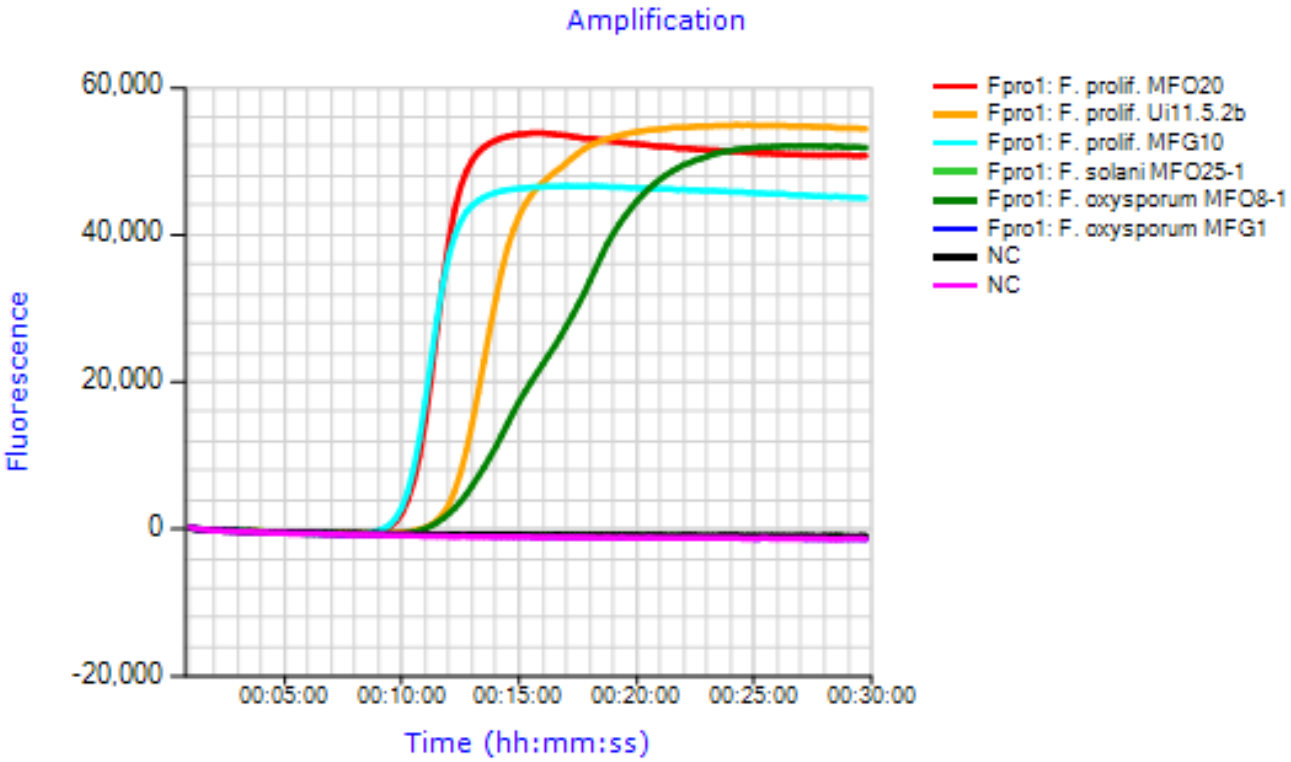


Figure 2-44: F. proliferatum LAMP with Fusarium targets and non-targets.

Verticillium dahliae

The *V. dahliae* assay amplified only *V. dahliae* and none of the Fusarium samples (Table 2.6-44, Figure 2-45).

Table 2.6-44: V. dahliae LAMP with V. dahliae target and Fusarium non-targets.

Targets	**Tpos (min)**	**Tm (°C)**
V. dahliae (duplicate 1)	7:45	90.5
V. dahliae (duplicate 2)	7:30	90.5
F. solani MFO25-1 (phalaenopsis)	-	-
F. solani MFG11-1 (gerbera)	n.t.	n.t.
F. oxysporum MFO8-1 (phalaenopsis)	-	-
F. oxysporum MFG1 (gerbera)	n.t.	n.t.
F. oxysporum Ui76.3.1b (ui)	-	-
F. proliferatum MFO20 (phalaenopsis)	-	-
F. proliferatum Ui11.5.2b (ui)	n.t.	n.t.
F. proliferatum MFG10 (gerbera)	-	-
NC: HyClone water	-	-
NC: HyClone water	n.t.	n.t.

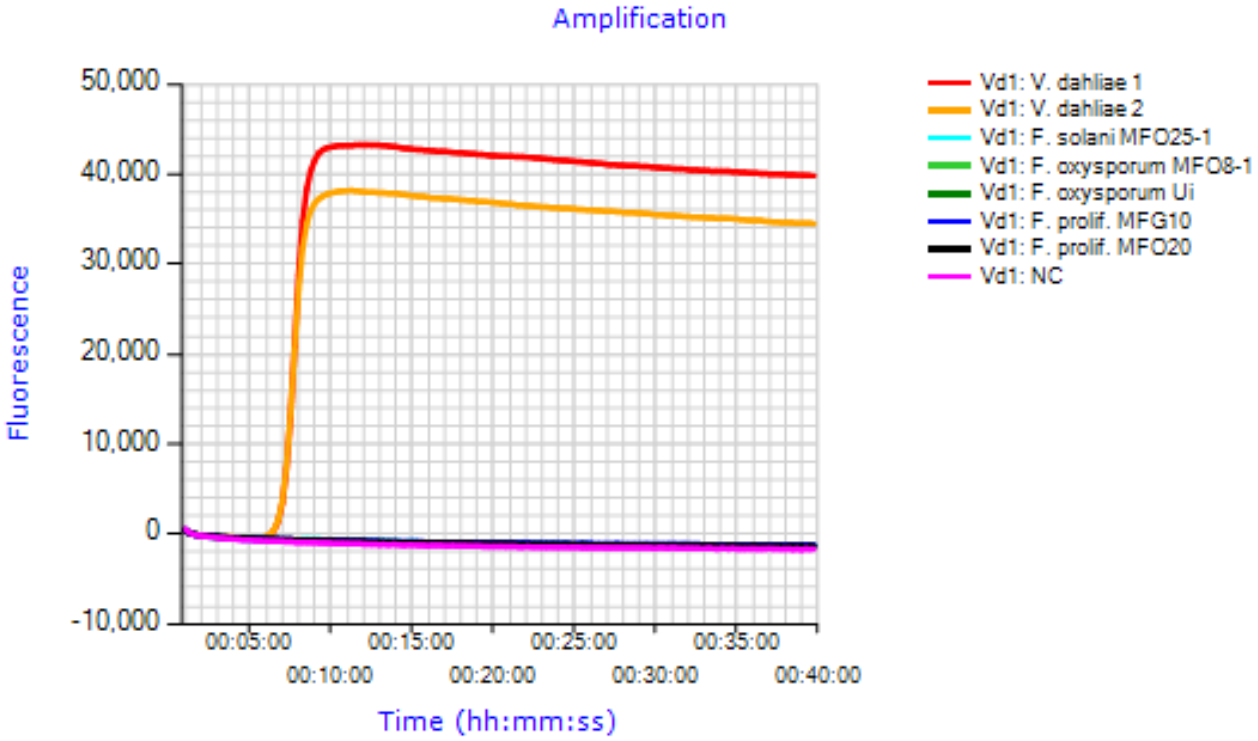


Figure 2-45: V. dahliae LAMP with V. dahliae target and Fusarium non-targets.

Infected plant material

Infected symptomatic gerbera plant material was received from a gerbera grower. The identify of the pathogen was unkown and therefore all *Fusarium* and *Verticillium* LAMP assays were performed.

For all plants TaqMan assays were performed prior to the LAMP assays.

In plant 2, 5 of the 8 tesed samples were positive for *V. dahliae* in the TaqMan assay, but CT values were high indicating low concentration of the pathogen. Two of these samples were also positive with the LAMP assay for *V. dahliae* and one sample that was negative in the TaqMan was positive with LAMP (Table 2.6-45, Figure 2-46). In addition, three samples showed aspecific amplification with the *F. oxysporum* assay with late time of positivity and aspecific melting temperature. On sample showed positive amplification with the *F. solani* primers at almost 40 min.

In plant 3, 6 of 9 samples were positive in the *V. dahliae* TaqMan, again with high Ct values. One of these samples was also positive with the *V. dahliae* LAMP and one sample showed aspecific amplification with the *F. oxysporum* LAMP.

In plant 4, 7 of 9 samples were positive with the *V. dahliae* TaqMan. No positive results were obtained with LAMP.

In plant 5, all samples were positive in the TaqMan for *V. dahliae* and one sample was positive with a very high Ct value for the *F. oxysporum* primers. The three samples with the lowest Ct values were tested with the LAMP assays and all three showed amplification with the V. dahliae LAMP. There was again a specific amplification for *F. oxysporum*.

Table 2.6-45: LAMP results from infected plant material; n.t.=not tested.

		F.oxy		F.sol		F.prol		V.dal 50x diluted		V. dal 10 x diluted	
Plant	**#**	**Tpos (min)**	**Tm (°C)**	**Tpos (min)**	**Tm (°C)**	**Tpos (min)**	**Tm (°C)**	**Tpos (min)**	**Tm (°C)**	**Tpos (min)**	**Tm (°C)**
2	A1	33:45	84.9	39:45	89.9	-	-	-	-	-	-
	A2	-	-	-	-	-	-	-	-	-	-
	C1	-	-	-	-	-	-	-	-	-	-
	C2	-	-	-	-	-	-	-	-	-	-
	D	-	-	-	-	-	-	-	-	24:00	89.7
	E	-	-	-	-	-	-	23:30	90.2	26:00	89.7
	F	38:15	85.9	-	-	-	-	-	-	-	-
	G	38:00	85.8	-	-	-	-	32:15	88.4	32:15	88.4
3	A1	-	-	-	-	-	-	-	-	-	-
	A2	-	-	-	-	-	-	-	-	-	-
	B	-	-	-	-	-	-	-	-	-	-
	C1	-	-	-	-	-	-	-	-	-	-
	C2	-	-	-	-	-	-	-	-	-	-
	D	-	-	-	-	-	-	-	-	22:30	89.9
	E	-	-	-	-	-	-	39:45	-	34:45	83.9
	F	-	-	-	-	-	-	-	-	-	-
	G	22:15	86.1	-	-	-	-	-	-	-	-
5	A1	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	A2	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	B	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	C1	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	C2	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	D	-	-	-	-	-	-	10:00	90.3	9:45	89.8
	E	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	F	29:15	85.6	-	-	-	-	21:00	90.4	11:30	89.9
	G	37:15	85.9	-	-	-	-	-	-	33:45	89.4

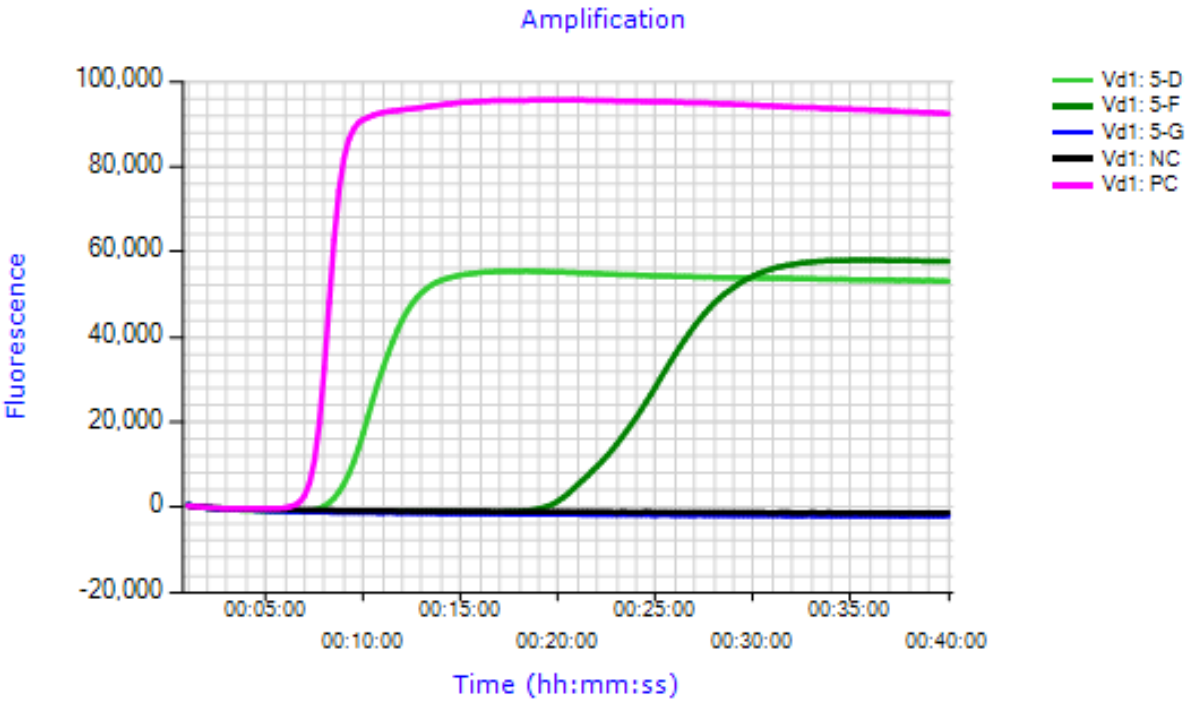


Figure 2-46: LAMP on three samples from plant 5 with the V. dahliae LAMP assay.

Multiplex

The targets Cmm, TMV and ToBRFV were chosen for the multiplex assay.

The tested microchip enables an RPA amplification step prior to the LAMP reaction. Therefore RPA-LAMP assays were performed first in a Genie machine (Figure 2-47).

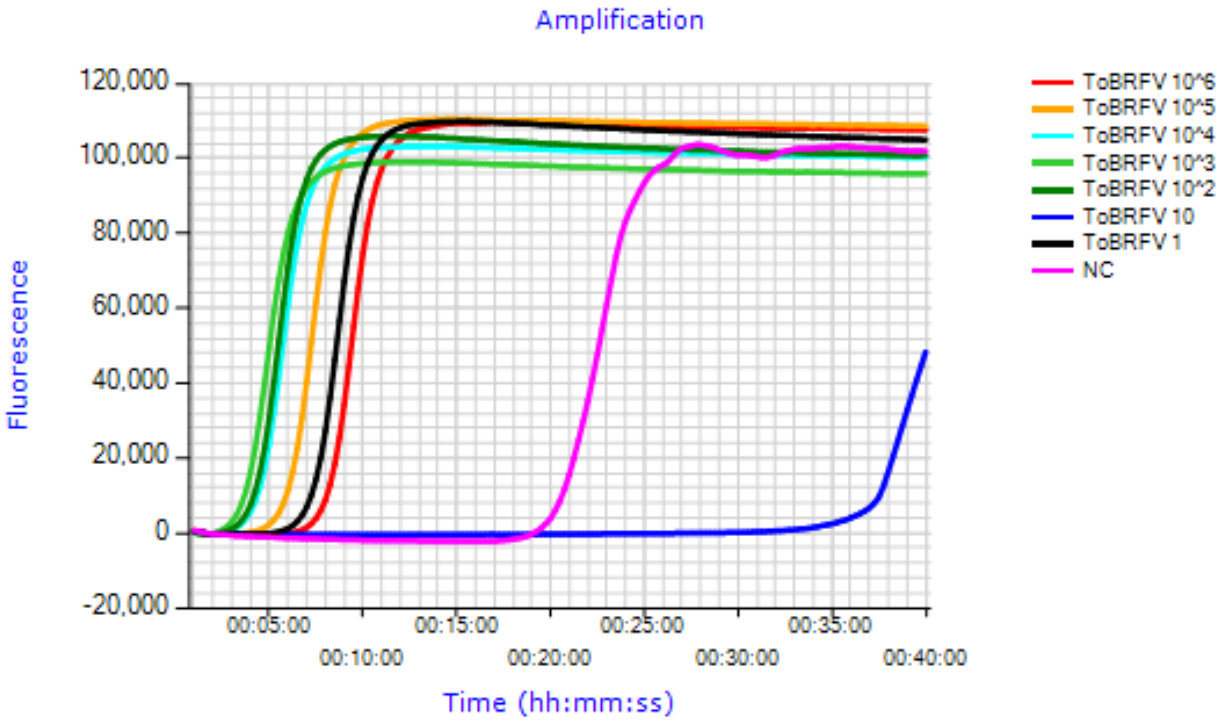
Figure 2-47: *Genie II machine.*

The results showed that using RPA usually results in false positive reactions in the negative control (Table 2.6-46, Figure 2-48), which is not the case for LAMP without the RPA step. The same results were observed by researchers at the University of Pennsylvania.

Table 2.6-46: Results of a RAMP and LAMP assay on a dilution series of ToBRFV gBlocks

	RAMP		**LAMP**	
	Tpos (min)	**Tm (°C)**	**Tpos (min)**	**Tm (°C)**
ToBRFV gBlock 10^6	09:00	85,8	12:15	85,4
ToBRFV gBlock 10^5	07:00	85,7	14:15	85,4
ToBRFV gBlock 10^4	05:15	85,8	16:00	85,6
ToBRFV gBlock 10^3	04:45	85.7	17:00	85.6
ToBRFV gBlock 10^2	05:15	85.7	-	-
ToBRFV gBlock 10^1	38:15:00	84.8	-	-
ToBRFV 1	08:15	85.7	-	-
0	21:30	84.9	-	-

a



b

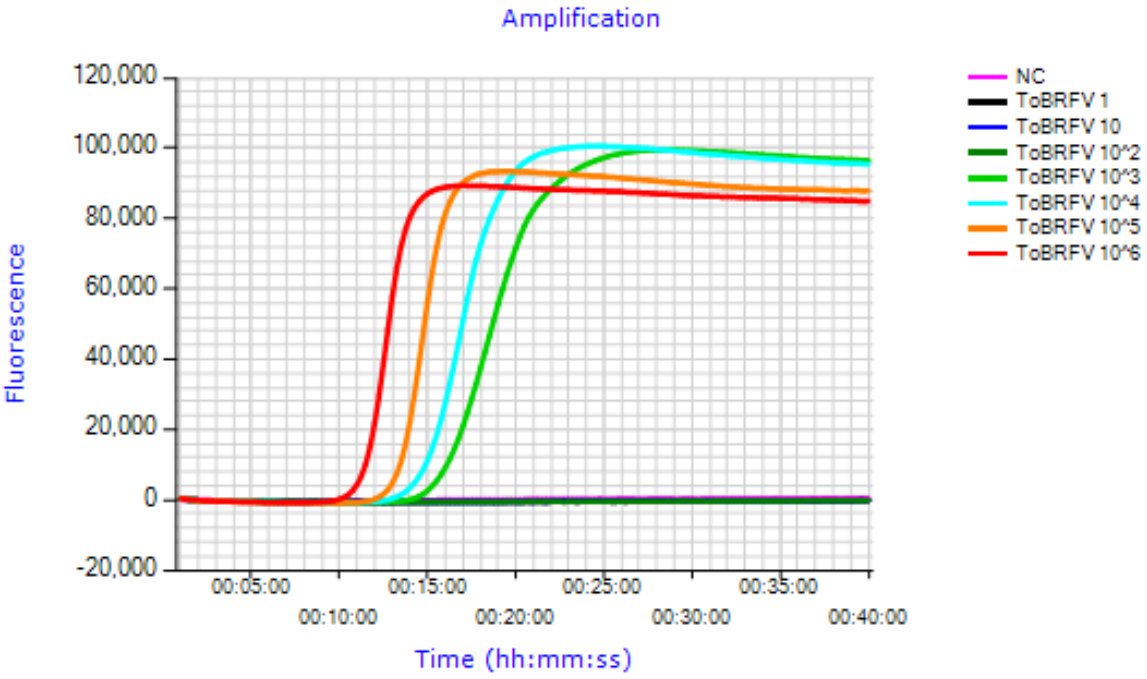


Figure 2-48: Results of a a) RAMP and b) LAMP assay on a dilution series of ToBRFV gBlocks.

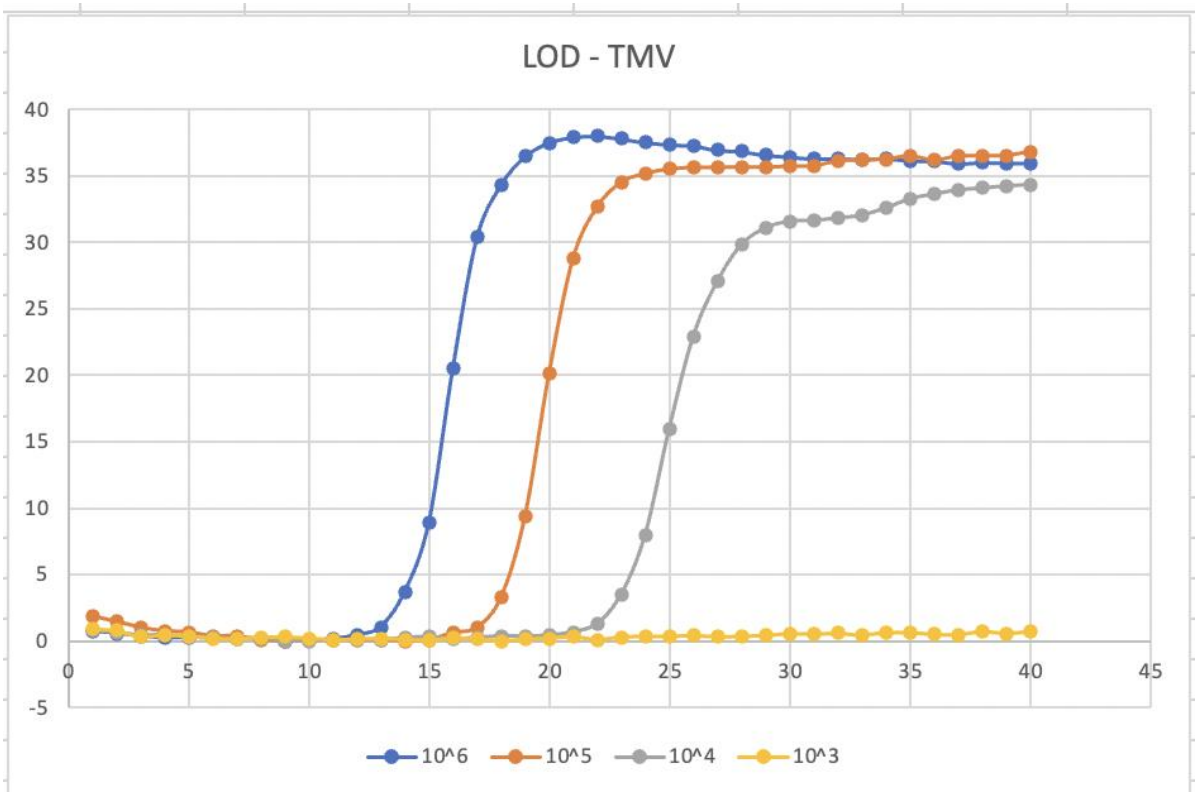
The multiplex chip experiments were mostly carried out at the University of Pennsylvania by Huiwen Bai.

During testing of the LAMP assays it was discovered that the Cmm assay needs an assimilation probe to work properly. However, fluorescence detected by the assimilation probe cannot be measured in combination with the chip. Therefore, only TMV and ToBRFV were used for chip experiments.

Sensitivity

For both the TMV and the ToBRFV assay the detection limit in the chip was 10^4 copies/µl, which is slighlty higher than the detection limit in a LAMP performed in the Genie (Figure 2-49, Figure 2-50).

a


LOD - TMV
40
35
30
25
20
15
10
5
0
-5
0
5
10
15
20
25
30
35
40
45
10^6
10^5
10^4
10^3

b

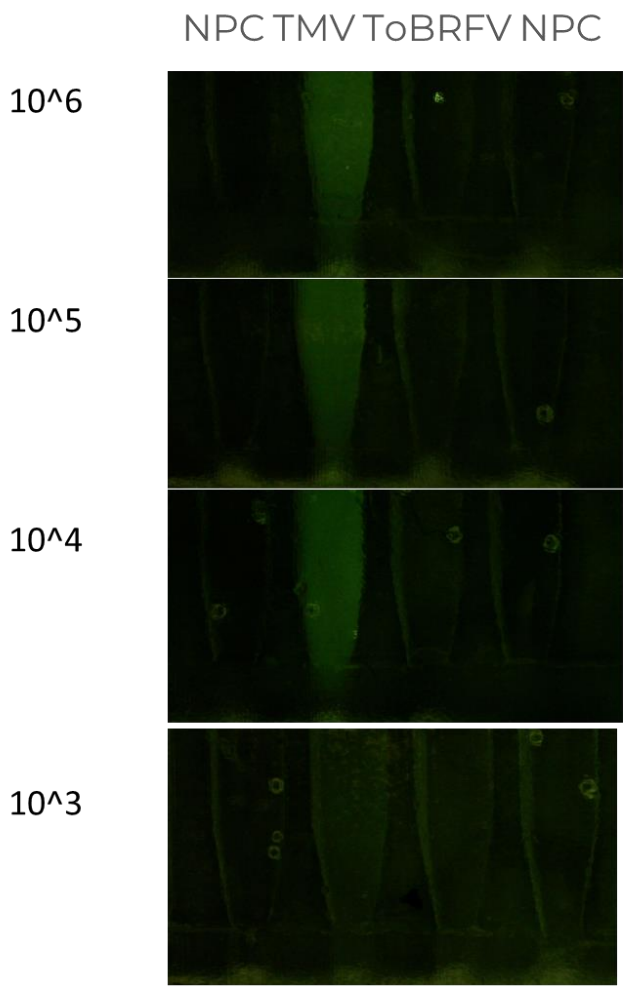
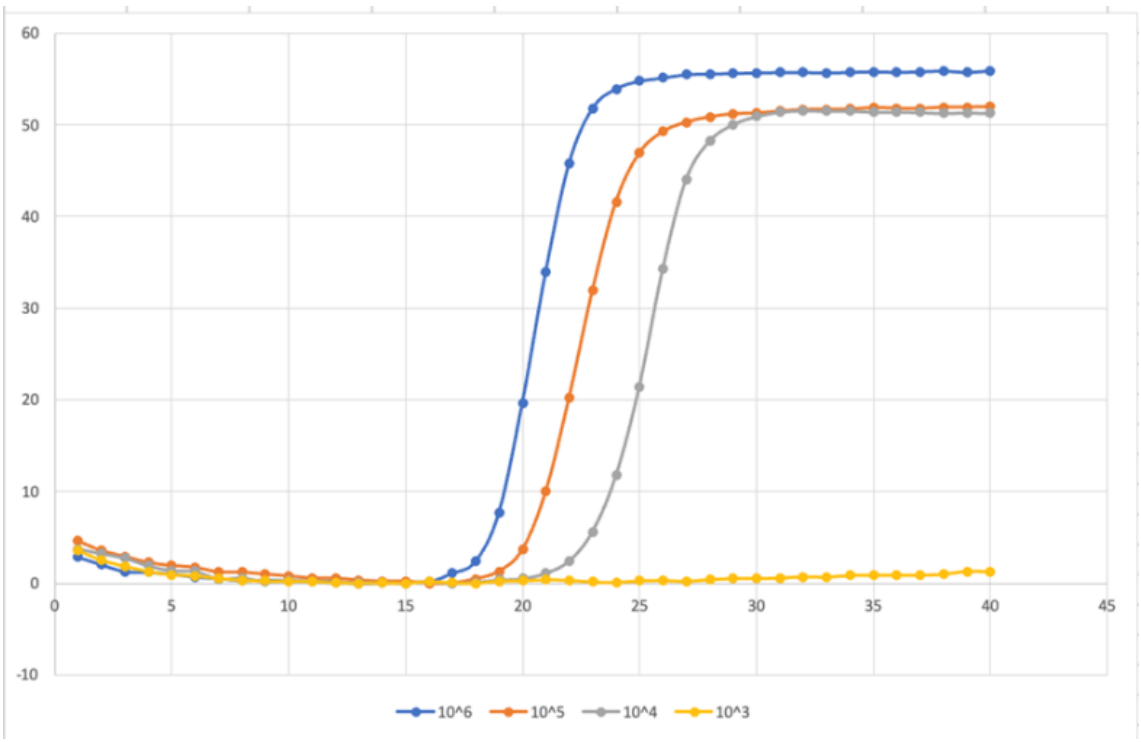


Figure 2-49: TMV gBlock detection in multiplex chips; a) real-time fluorescence measurement, b) end-point picture of the four chips.

a



b

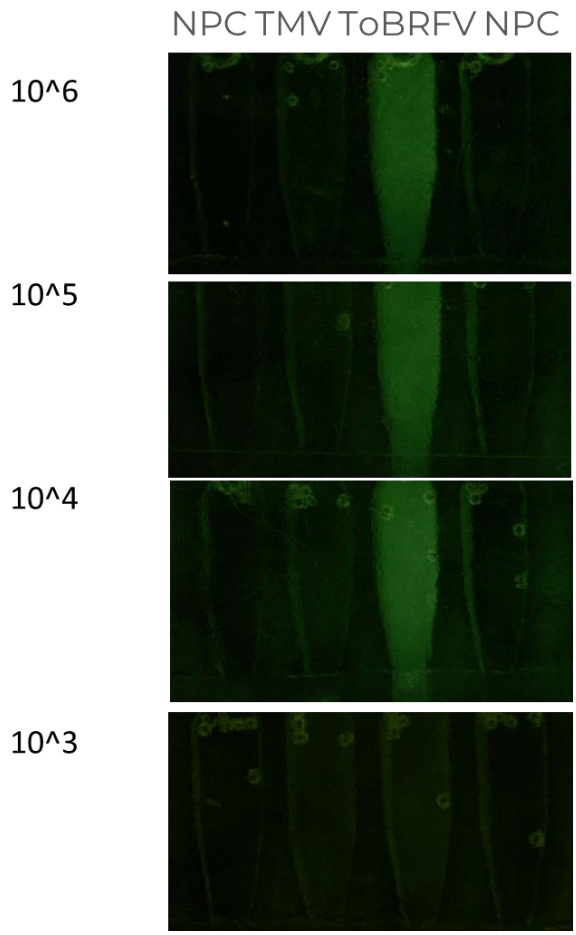
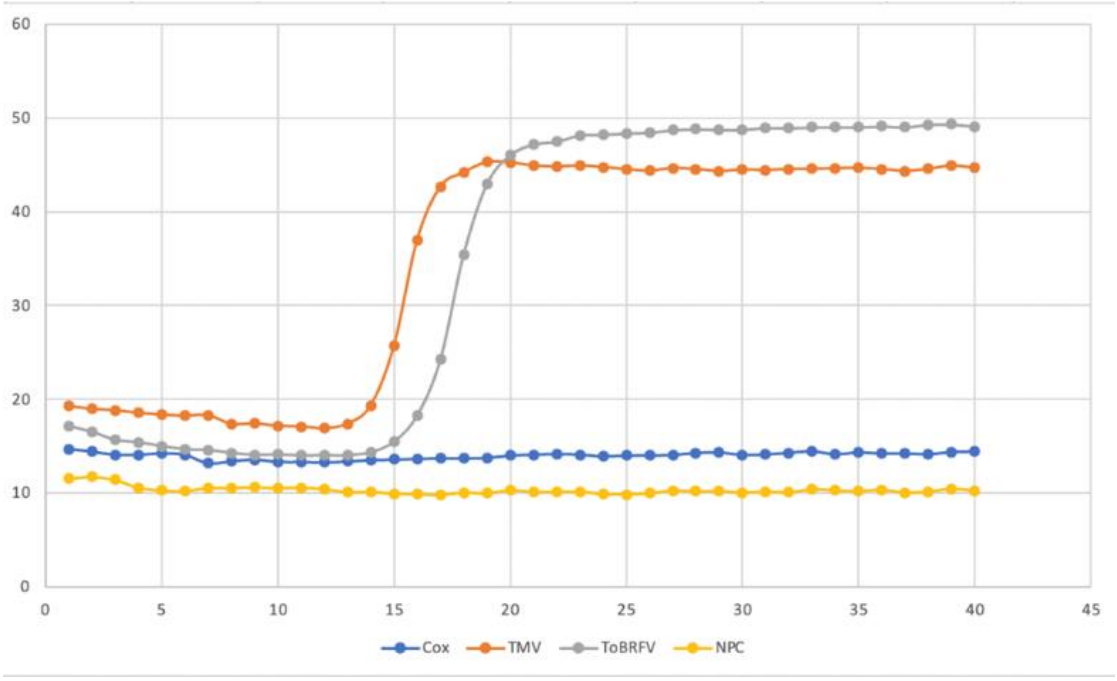


Figure 2-50: ToBRFV gBlock detection in multiplex chips; a) real-time fluorescence measurement, b) end-point picture of the four chips.

Multiplex detection

Addition of both TMV and ToBRFV gBlocks to the chips resulted in detection of both targets in the respective chambers (Figure 2-51). When the gBlocks were added to a background of tomato extract, also the *Cox* amplification control could be detected (Figure 2-52). In addition, the two targets were added to tomato extract at different concentrations (TMV 10^6 copies/µl and ToBRFV 104 copies/µl). Still, both targets and the Cox control could be detected (Figure 2-53).

a



b

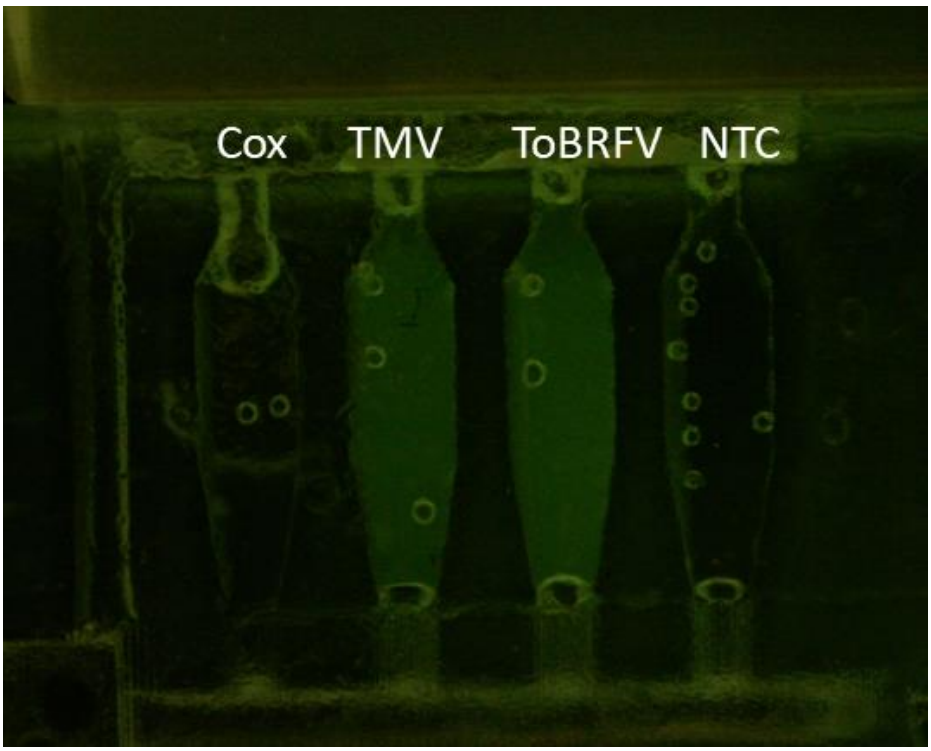
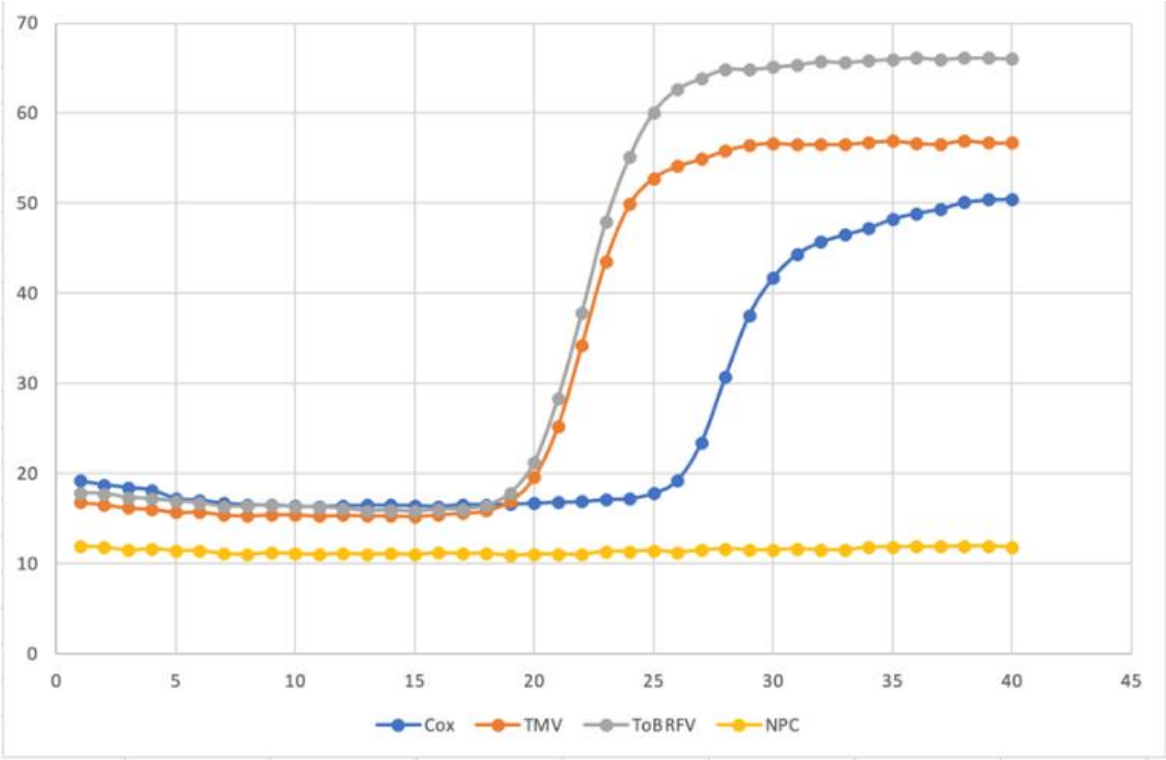


Figure 2-51: Multiplex detection of TMV and ToBRFV gBlocks; a) real-time fluorescence measurement, b) end-point picture of the four chips.

a



b

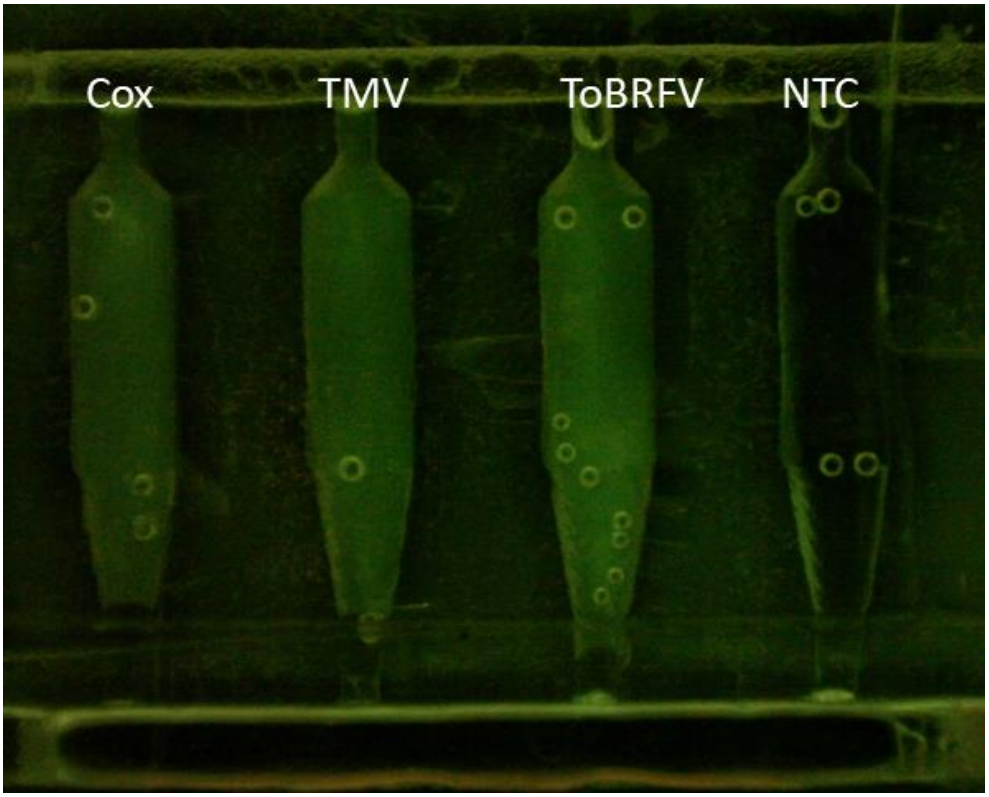
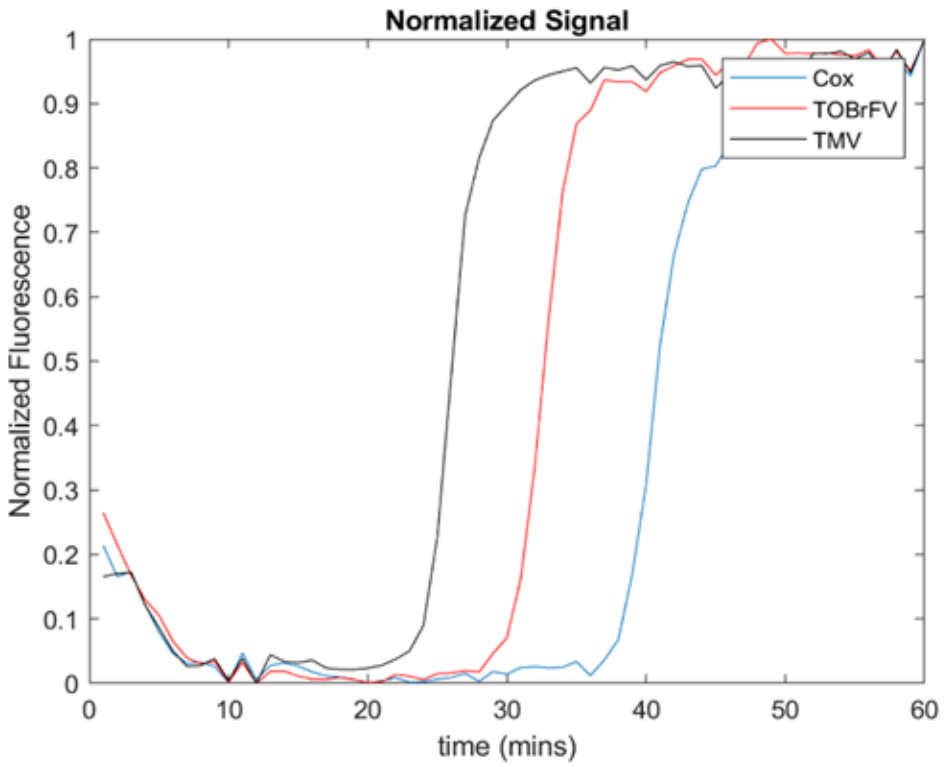


Figure 2-52: Multiplex detection of TMV and ToBRFV gBlocks in tomato extract; a) real-time fluorescence measurement, b) end-point picture of the four chips.

a



b

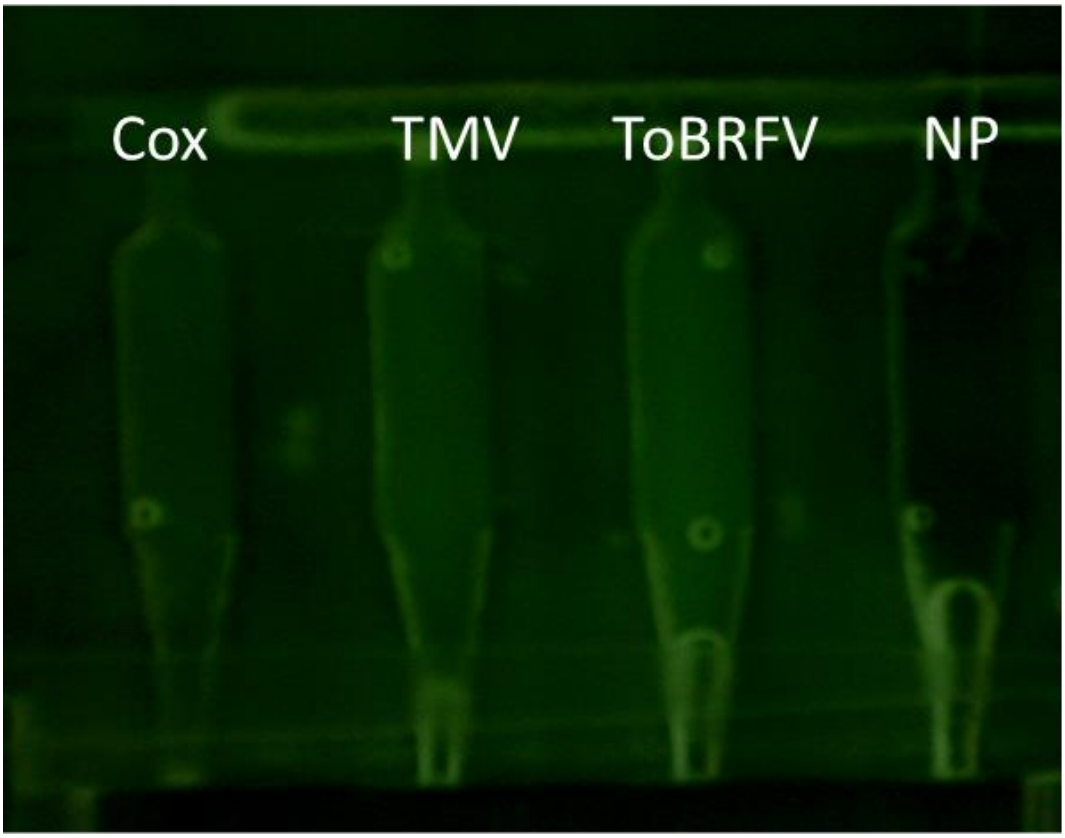


Figure 2-53: Multiplex detection of TMV (10^6 copies/µl) and ToBRFV (10^4 copies/µl) gBlocks in tomato extract; a) real-time fluorescence measurement, b) end-point picture of the four chips.

Multiplex chip experiments (WUR)

Testing the multiplex chip at WUR was met with several issues. A custom-built set-up for chip incubation and fluorescence measurement had to be acquired before the beginning of the experiments. Loading of the chips with both primers and reaction mix is complex due to the small size of the openings and the formation of air bubbles that can impede the measurability of results. Practice is needed to correctly load a chip and the device should be moved as little as possible after loading. Initial tests showed not only excessive bubble formation, but also a high background fluorescence of the chip material and the USB camera losing focus during the incubation time. Therefore, no results could be obtained during these tests. Discussion of the issues with researchers from the University of Pennsylvania and the BioNanotechnology department of WUR eventually led to a testing of chips using a colorimetric LAMP assay instead of fluorescence detection, in both the current incubation set-up and a PCR heating plate. In this experiment the chip was loaded with the primermixes for TMV, Cox and ToBRFV, leaving the last chamber empty as a positive control. ToBRV gBlocks were added together with the reaction mix. While in the custom-built incubator all samples were seemingly positive (yellow) and reaction mix escaped the chambers (Figure 2-54), the reactions on the PCR heating plate yielded positive results for ToBRFV only as expected and there was no displacement of reaction mix (Figure 2-55). This strongly indicates that the temperature of the custom-built incubator was too high at the position of the multiplex chip, leading to change in color of the mix and overflow from the chambers. However, the PCR plate heater does not allow for real-time fluorescent measurement as the USB camera cannot be mounted on the lid.

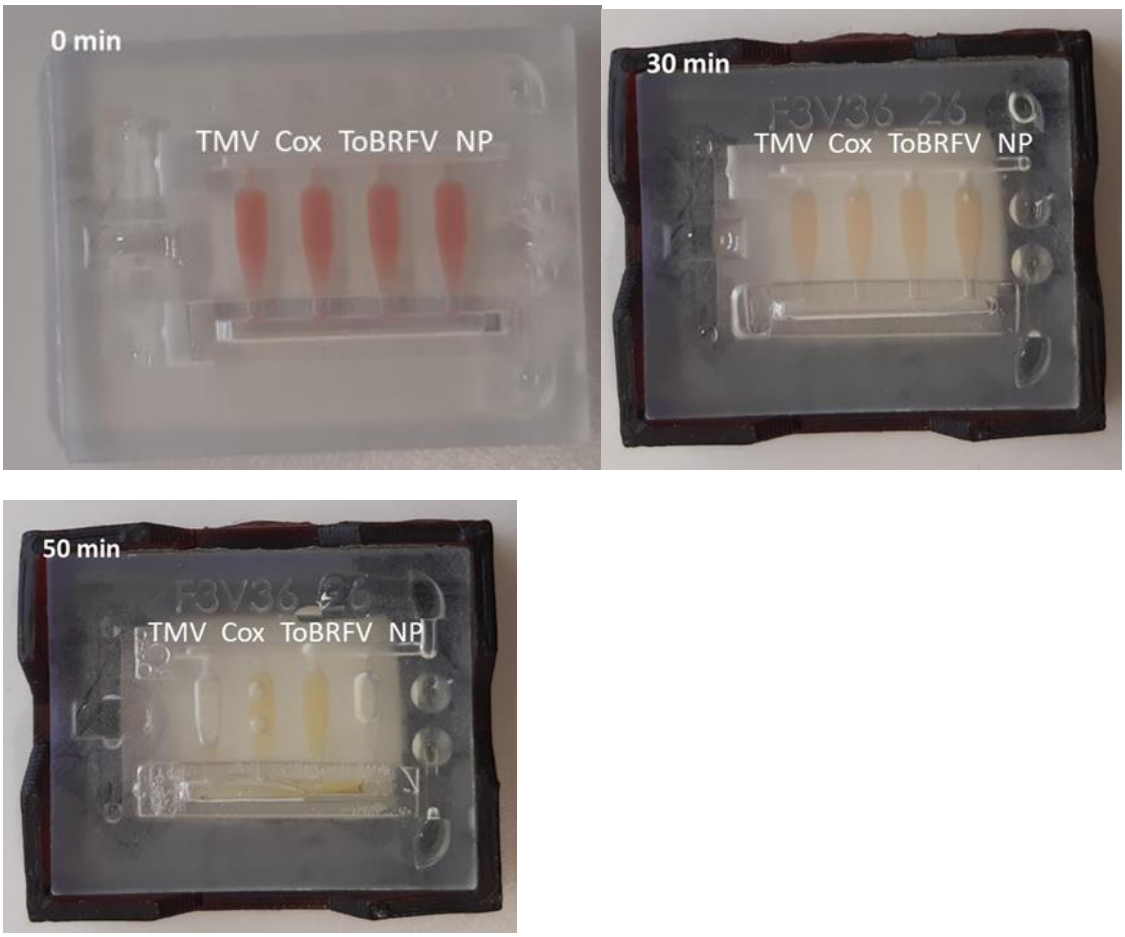


Figure 2-54: Colorimetric LAMP assay in a multiplex chip loaded with primers for TMV, Cox and ToBRFV and a non-primer control (NP) incubated for 50 min in a custom-built incubator.

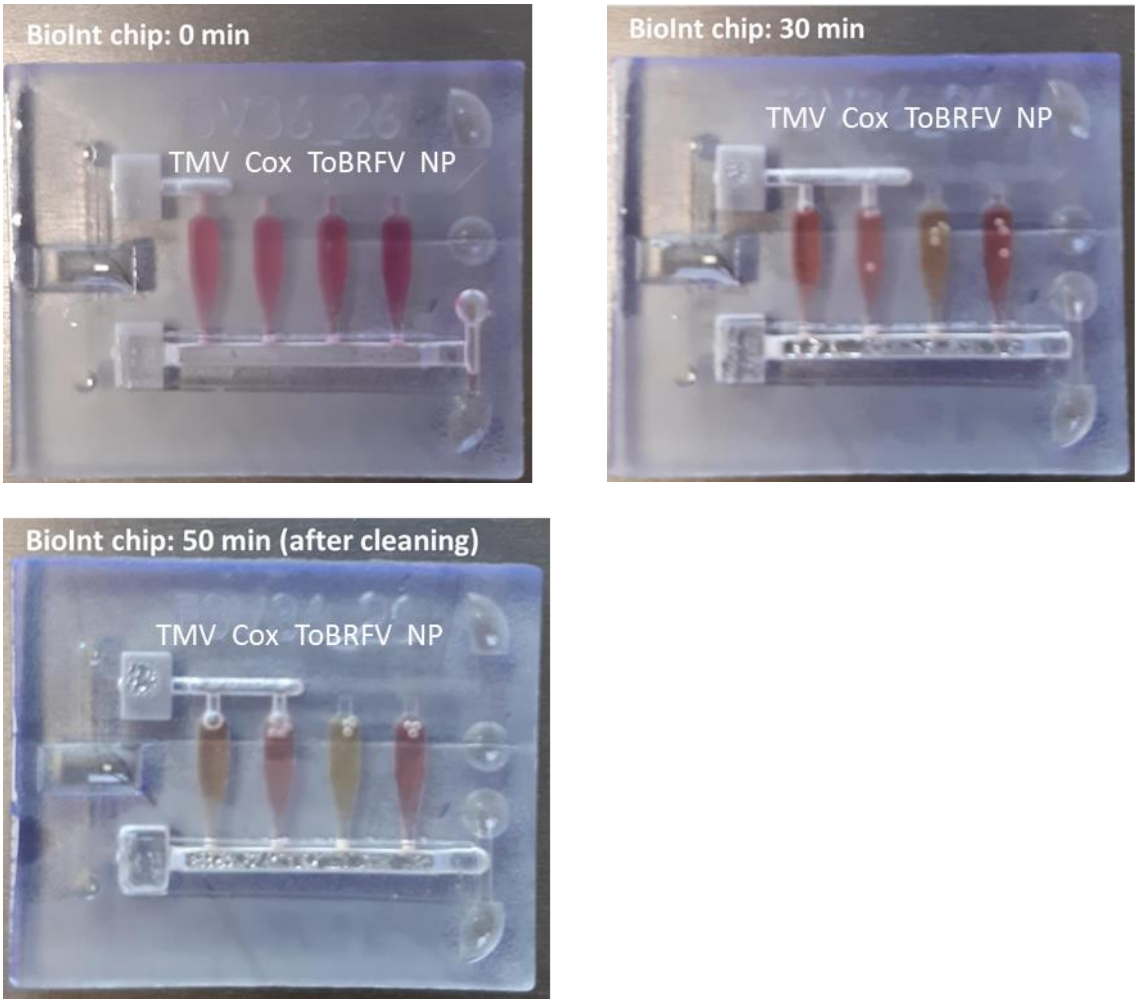


Figure 2-55: Colorimetric LAMP assay in in a multiplex chip loaded with primers for TMV, Cox and ToBRFV and a non-primer control (NP) incubated for 50 min on a PCR heating plate.

T65 cup

The T65 cup developed by BioNano Technologies (WUR) allows for isothermal amplification using only simple equipment.

The colorimetric assay showed no amplification (pink color) in the mock-inoculated sample MT3, while for the positive control all three samples changed in color (Figure 2-56). For the VT3 samples from infected plants the color change was clear in two of the three replicates, but more ambiguous in one sample.

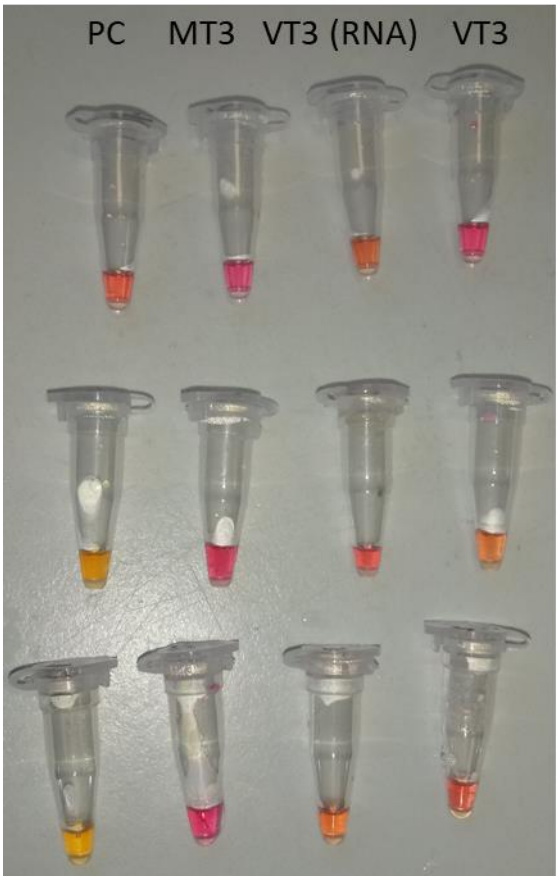


Figure 2-56: T65cup assay with ToBRFV infected leaf material.

2.6.4 Conclusions

1. The **ToBRFV** LAMP assay is highly sensitive and able to detect ToBRFV infection in symptomatic plant material, even after dilution (detection limit as measured is a 1:1000000 dilution of RNA extracted from plant material). It is also specific and does not lead to amplification of closely related species. In addition, the ToBRFV LAMP can be used in a multiplex with a *Cox* assay as an amplification control.
   A colorimetric assay can be used instead of normal detection by fluorescence measurement, but this approach is less sensitive, more time consuming and sometimes relies on interpretation by the tester. However, colorimetric detection requires less equipment and is therefore more suitable for on-site detection. Further evaluation of colorimetric detection is required to increase reliability and sensitivity of the method. Also, or other simple read-out methods such as lateral-flow devices and fluorescence measurement by mobile-phone should be investigated.
2. The **TMV** LAMP assay is sensitive (detection limit: 10^3 copies of gBlocks / µl) as well as specific and suitable for the detection of the virus in tomato as well as petunia plant material. Material from other plant species was not tested, but it can be assumed that detection will work as well. Also, the TMV LAMP can be employed in a multiplex with the *Cox* gene.
3. The **MNSV** LAMP assay for MNSV strains in cucumber is highly sensitive with a limit of detection of 10 copies gBlocks/µl. It is also specific as no amplification occured with RNA from related viruses present in cucumber. It is suitable for detection in infected and symptomatic cucumber leaf material. The assay can also be combined with the *Cox* assay in a multiplex.
   This assay was specifically developed for MNSV in cucumber for which the genomic sequence was available at the time of primer design. It was not tested for MNSV strains present in melon.
4. The **TSWV** LAMP assay is sufficiently sensitive to detect RNA isolated from infected plants at a 1:50 dilution and related viruses were not amplified. The assay also performed well on infected leaf material.
5. The LAMP assay for **PVY** in Petunia is highly sensitive with a limit of detection of a 1:10000 dilution of RNA isolated from infected plant material. The assays also show a weak detection for SucMoV, but this virus is not present in petunia and should therefore not interfere with the specificity of the assay. The assay was shown to be able to detect PVY in infected petuna leaf material.
6. The LAMP assay for ***X. fragariae*** in strawberry is sensitive (detection limit: 10^3 copies of gBlocks/µl) and specific for this one species. The assay can detect infection in symptomatic leaf material and can be combined with the *Cox* assay in a multiplex.
7. For each of the 4 ***Fusarium*** and ***Verticillium*** targets, LAMP assays from literature were identified. For *F. solani*, *F. oxysporum* and *V. dahliae* the assays were specific for the target species. Only the *F. proliferatum* assay shows cross-reaction with *F. oxysporum*. Nevertheless, it proved to be the best assay currently available from literature.
   Testing the assays on infected plant material shows that the LAMP assay is slightly less sensitive than the TaqMan assay. For most samples, TaqMan detection was just above limit of detection (usually a CT of 40). In most of these samples *V. dahliae* could not be detected with LAMP assays. In the case of higher concentrations, however, detection suceeded.
8. The **multiplex microchip** for the simultaneous detection of three pathogens in one sample was shown to enable the detection ToBRFV and TMV simultaneously. It requires only simple equipment such as a self-built incubator and a USB camera. However, correct usage of the chip is complex and requires experience, suggesting that the chip needs to be further developed for increased user-friendliness.
9. The **T65 cup** assay represents a simple method to conduct a LAMP assay only using boiling water and can therefore potentially be used in almost all settings. However, simple measurement of a fluorescence signal with e.g., a smartphone camera is not possible yet due to the low sensitivity of these cameras. Colorimetric evaluation is possible, but the color change is often not unambigous. Simple measurement techniques need to be developed further to allow easy and unrestricted use of this method.

2.7 WP3: MinION seq

2.7.1 Introduction

Nanopore sequencing

Since 2005 Oxford Nanopore Technologies (ONT) has developed the so called Nanopore sequencing mehod. This technique uses flow cells that contain a large amount of very small pores in an electro resistant membrane. When a DNA or RNA molecule passes through a pore every passing base creates a distinct disruption in the electric signal in the nanopore (raw data, Figure 2-57). This signal can be decoded (basecalled, Figure 2-57) into the original sequence. In order to let a DNA or RNA molecule attach to a pore, adapters have to be ligated to the molecules during sample preparation.

This sequencing techniques has several advantages over earlier developed next generation sequencing techniques as for instance Illumina - or PacBio sequencing. First, it can be used for real-time sequencing as data becomes available and can be analysed during the sequencing process. Second, nanopore sequencing does not only produce a large amount of sequencing data (~20 GB per run) but can also generate very long sequencing reads with up to 2 Mb in length. Third, the MinION sequencing machine is a small portable device that can be directly connected to a laptop for further sequencing analysis. Moreover, sequencing throughput can be upscaled with machines such as the GridION and PromethION, which allow the use of several flow cells at once. In addition, the Flongle flow cells have become available. These flow cells come at a lower price than regular flow cells with a lower number of pores and are for one-time use only. This makes them perfect for diagnostic applications, which usually require a relatively small amount of data and where cross-contamination between sequencing runs needs to be avoided.

Currently, sequencing error rates are still higher in ONT sequencing compared to other sequencing techniques. However, accuracy is steadily increasing and is expected to be soon equal to other techniques. Due to its portability and real-time data output, Nanopore sequencing is sometimes referred to as an on-site sequencing technique. Indeed, for some applications that require only minimal sample preparation, ONT sequencing can be used in non-lab environments and produce results within a few hours. However, for the detection of plant diseases many sample processing steps are required, which require specific equipment and trained personnel. Therefore, Nanopore sequencing for the detection of plant diseases has to be conducted in a laboratory and often takes several days. Still, ONT sequencing has a much shorter runtime than other currently used techniques.

Amplicon and multiplex sequencing

For detection and identification purposes two strategies can be used. First, all DNA or RNA in the sample can be directly sequenced without a priori amplification. For RNA sequencing often a cDNA synthesis step is added. The advantage of direct sequencing is that all organisms in a sample can be detected including unexpected species. It also omits the amplifciation step, thereby shortening preparation time. On the other hand, some species might not be detected due to low concentrations. The second strategy is amplicon sequencing, which involves the generation of amplicons by PCR. Amplicon sequencing is frequently used in combination with sequencing techniques such as Illumina. Usually, a short piece of DNA or RNA is amplified, which is suitable for the identification of the target species. For example, for bacteria parts of the 16S rRNA gene are used, which allow the differentiation of bacterial genera and in some cases species. While Illumina sequencing is restricted in amplicon length to approximately 300 bp, with ONT sequencing much longer amplicons can be sequenced, potentially allowing for a higher resolution and differentiation of species.

Tobamoviruses

The genome of a virus from the genus *Tobamovirus* consists of a single-stranded RNA molecule of a length of approx. 6400 nt with 4 known open reading frames (ORFs): 2 encoding the RNA polymerase, one encoding a movement protein, and one for the coat protein (Pagan et al. 2010). The genus contains 22 species, which can be separated into 3 subgroups. Subgroup I mainly infects solanaceous hosts, subgroup II infects cucurbits and legumes and subgroup III is known two infect brassicas and asterids. In greenhouses, subgroup I is a serious problem in crops like tomato and paprika. Although detection assays are available for several separate species, it is often unknown which species causes the observed symptoms. In this case it is advantageous to be able to test for a range of species. Correct identification

can be important for tracing disease origins and spread and employing correct control measures. It is also possible that crops are infected with several pathogen species at the same time.

To be able to detect a number of different tobamoviruses, i.e., cucumber green mottle mosaic virus (CGMMV), pepper mild mottle virus (PMMoV), tobacco mosaic virus (TMV), tomato brown rugose fruit virus (ToBRFV), tomato mosaic virus (ToMV) and tomato mottle mosaic virus (ToMMV), we aimed at developing a detection method based on ONT amplicon sequencing. In this method, sequences of the six species are specifically amplified in a multiplex PCR assay. These amplicons are then sequenced by ONT sequencing. Consequentially, the species or combination of species causing symptoms can be identified within a relatively short timeframe.

Multiplex assays

Multiplex PCR assays are defined as assays that detect multiple genes or species using different primers pairs (usually two primers per gene/species) in one assay. This approach allows the amplification of several targets without the need to choose one a priori but also represents some unique challenges. First, every primer pair must be specific for the respective target and not show aspecific amplification of non-target genes or species. Second, primers should not interact with each other as this lowers efficiency and can create artifacts. These two points alone are a challenge as primers need to be scanned for interactions with each other and both targets and non-targets. As the number of assays in a multiplex increases the number of possible interactions increases exponentially. Third, all primer pairs should amplify their target at a similar efficiency, for differences in efficiency would lead to one amplicon being greatly overrepresented hindering detection of underrepresented amplicons. Finally, all primers need to be able to perform well under similar conditions with respect to temperature and salinity. Therefore, primer design for multiplex assays is impossible to perform manually and needs dedicated software.

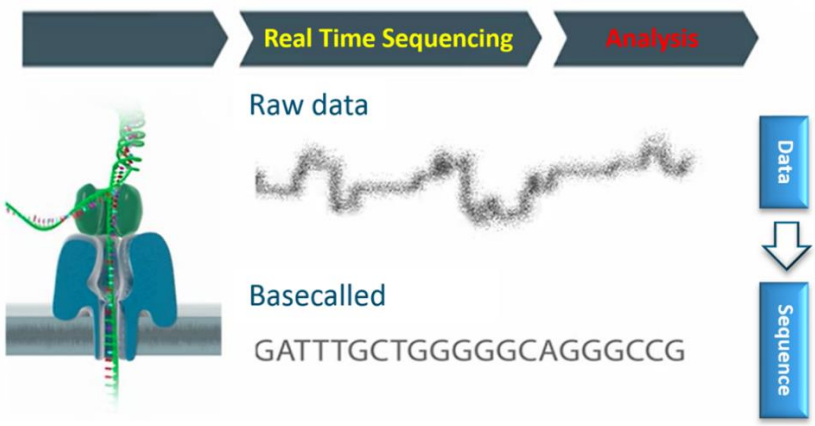


Figure 2-57: Principle of sequencing by nanopores (Oxford Nanopores).

Nematodes

Soil contains a multitude of nematodes belonging to different feeding groups, such as bacterivores, fungivores, omnivores and different kinds of plant parasitic nematodes. Identification of plant parasitic nematodes is currently done by microscopy approaches, which is time intensive and requires training in nematode identification. So far, sequencing approaches to determine the nematode community, as is common for bacteria and fungi, have not yet successfully been established. First, amplicon sequencing is needed for identification of soil nematodes due to their relatively low concentrations in samples. Consequentially, it is challenging to identify a region in the genome that is conserved enough to design primers for all nematode species and variable enough to distinguish between species. Second, so far there are no large database with nematode sequences. The availability of such a database is essential for correct identification.

However, the application of ONT sequencing has created new possibilities. As nanopore sequencing generates longer reads, amplicon sizes can be increased. These longer amplicons enable a better taxonomic resolution. In addition, the sequencing database has constantly increased in the recent years.

In this project, the possibility of determining nematode community composition in soil with ONT sequencing was tested.

2.7.1.1 Methods

Multiplex primer design

In order to overcome the challenges of multiplex primer design, the software "PanelPlex Consensus" developed by DNA Software was used in combination with the software MultiPick. PanelPlex consensus allows highly specific primer design as the user specifies an inclusivity list including all target sequences that need to be detected (e.g., all full genome sequences of TMV). Next the user specifies all non-target species in the exclusivity list (e.g., all full genome sequences of related viruses) and a background list which in this case consisted of the full tomato genome, which should not be detected by the assay. Subsequently, the software produces a number of possible primers that fulfill all criteria of inclusivity and exclusivity. During this process, a thermoblast algorithm is used. Thus, sequences are not only matched on similarity, but also on their themodynamic properties. This procedure was followed for all six tobamoviruses resulting in 10 primer pairs per species (20 for TMV as 2 primer pairs are needed for the detection of all strains within the species) (Figure 2-58a).

In the following, the software MultiPick was used to generate combinations of the primer pairs for the single targets, tacking into account interactions between the primers and amplification efficiency. While PanelPlex consensus is a browser-based software that can be used freely after purchasing the license, MultiPick is not yet released. Therefore, the obtained primers were sent to DNA Software to perform the MultiPick step. This resulted in 7 different sets. Each set contained one primer pair for each target (2 primer pairs for TMV) (Figure 2-58b).

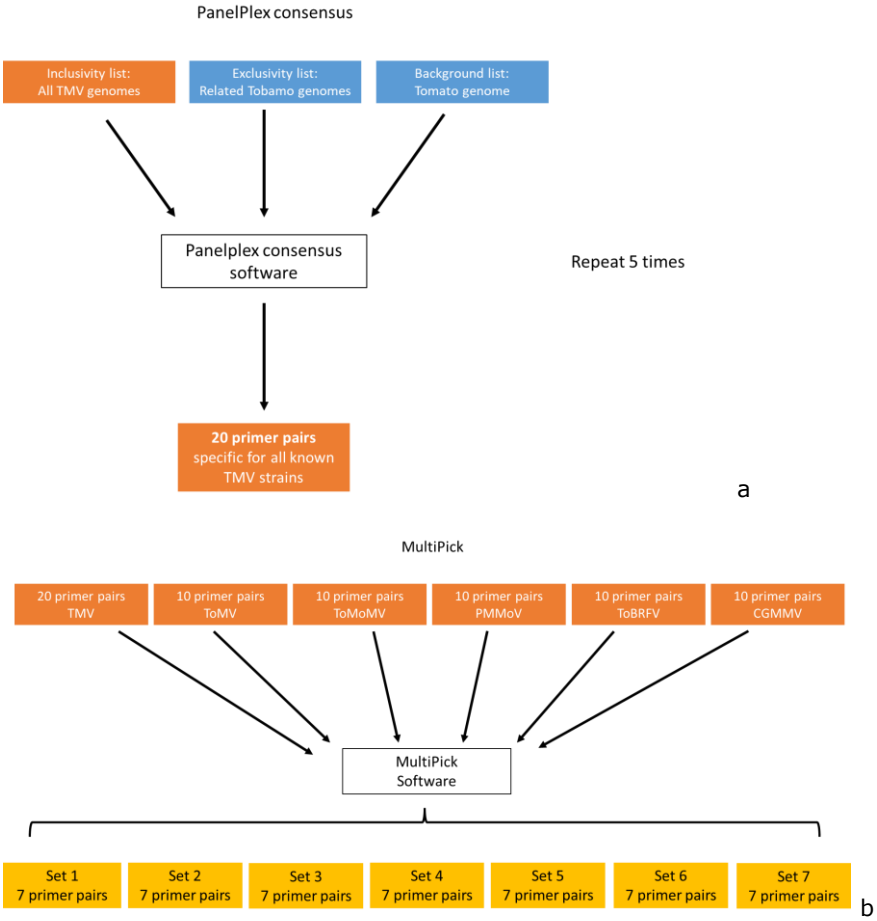


Figure 2-58: Schematic depiction of the primer design process using a) PanelPlex consensus followed by b) MultiPick.

Primer testing

All 7 primer sets generated by MultiPick contained in total 13 different primer pairs (reflecting overlap between the sets). Set2 was omitted because *in silico* evaluation showed mismatches with some of the targets. The remaining 6 primer sets were first tested separately for amplification of the target using RNA from the six viruses as an input. The amplicons thus generated were used for further sequencing experiments. For this, amplicon concentration was measured and diluted to equal concentrations.

For choosing the best primer set, all sets were tested on a mix of the amplicons generated in the previous step. Amplicons mixes of a concentration of 10^4 copies/µl and 10^5 copies/µl were amplified with each primer mix with tailed primers (tails for Nanopore sequencing). In addition, tomato extract with no addition of amplicons was used as a negative control. Amplicons were purified, barcoded and further prepared according to ONT protocols. Six samples were sequenced on a flongle flow cell at the same time. Analysis of the generated reads showed that primer set 3 and 4 generated the most even distribution of reads from the six targets. The other sets generated only very low reads of PMMoV. Therefore, the primersets 3 and 4 were chosen for further experiments.

To test inclusivity of the primers a number of gBlocks was ordered for the respective target sequence with different numbers of SNPs. These were tested by PCR only. All primers produced amplicons with their respective target gBlocks.

Multiplex testing

The performance of the 2 primer mixes were tested on mixes of target amplicons, simulating the event of an infection with several viruses. For this experiment three amplicons were mixed, one at a concentration of 10^3, one at 10^4 and one at 10^5. The expectation is to be able to detect all targets, even if some are present at much lower amounts then others. The amplicon mixes were amplified with tailed primers. Sample prep, barcoding and sequencing were done according to protocol. Sequencing analysis was done with the MinKNOW software on the used MinION device (Mk1C) using the amplicon sequences as reference. Analysis was also attempted with the (What's in my pot) WIMP workflow at the ONT cloud based EPI2ME analysis platform. However, in this analysis many non-target organisms were detected, which is due to a poorly curated reference database.

Based on the results, primerset 3 was selected for all further experiments.

RNA testing

RNA of the six viruses was obtained from Naktuinbouw, except for ToBRFV, which was taken from our own collection. All single RNA's and 6 mixes of two (see Appendix) were tested. First a RT-PCR was done with the tailed primers in order to obtain amplicons. Samples were prepared and sequenced according to protocol. Analysis was done both with MinKNOW and Decona (https://github.com/Saskia-Oosterbroek/decona). See Figure 2-59 for the scheme of a typical sequencing workflow including the estimated time for each step.

FTA card samples

For this experiment extracts from FTA-cards with CGMMV, PMMoV, TMV and ToMV (received from EWS) were used. In addition, in the same run RNA samples from CGMMV, PMMoV, TMV ToMV, ToMMV, ToBRFV and mixes of TMV with ToMV and of ToMMV with ToBRFV (received from NAKTuinbouw) were used. Amplicons were generated with primer mix 3. Analysis was done with MinKNOW.

Sequencing analysis

There are several options for sequencing analysis. The first step is basecalling. In this project, basecalling was performed on the Mk1c with MinKNOW, the software installed on the MinION Mk1C device, using the high accurarcy basecalling model. Alternatively, the Nanopore software guppy can be used for basecalling on a computer. If barcodes are used, debarcoding has to be done to separate samples. Debarcoding (i.e., demultiplexing) can be done during basecalling with MinKNOW or guppy. Debarcoding can also be done after basecalling using guppy. In this study, the reads were required to have barcodes on both ends and excluded reads with barcodes in the middle.

For identification of reads alignments were done using several options. MinKNOW has an alignment option where a custom reference can be provided. However, the resulting alignment files have to be exported and

inspected with a third-party software. In this study CLC Genomic Workbench was used for inspecting alignments. Other options for alignments are performed on the Linux command line with the advantage that all software is open access. Nanopore provides EPI2ME Labs workflows (https://labs.epi2me.io/wfindex/) including the wf-alignment workflow. Another option is decona, an open-source pipeline for the Linux command line and employs several programs dedicated to Nanopore sequencing data (https://github.com/Saskia-Oosterbroek/decona). Optionally, also demultiplexing can be done using decona. Finally, data analysis can be performed in the cloud using the what's in my pot (WIMP) pipeline from Nanopore. However, this analysis uses the NCBI database as a reference. Due to poor curation of some sequences at NCBI usually a number of false positive results is obtained. Therefore, this option was not used in the present study.

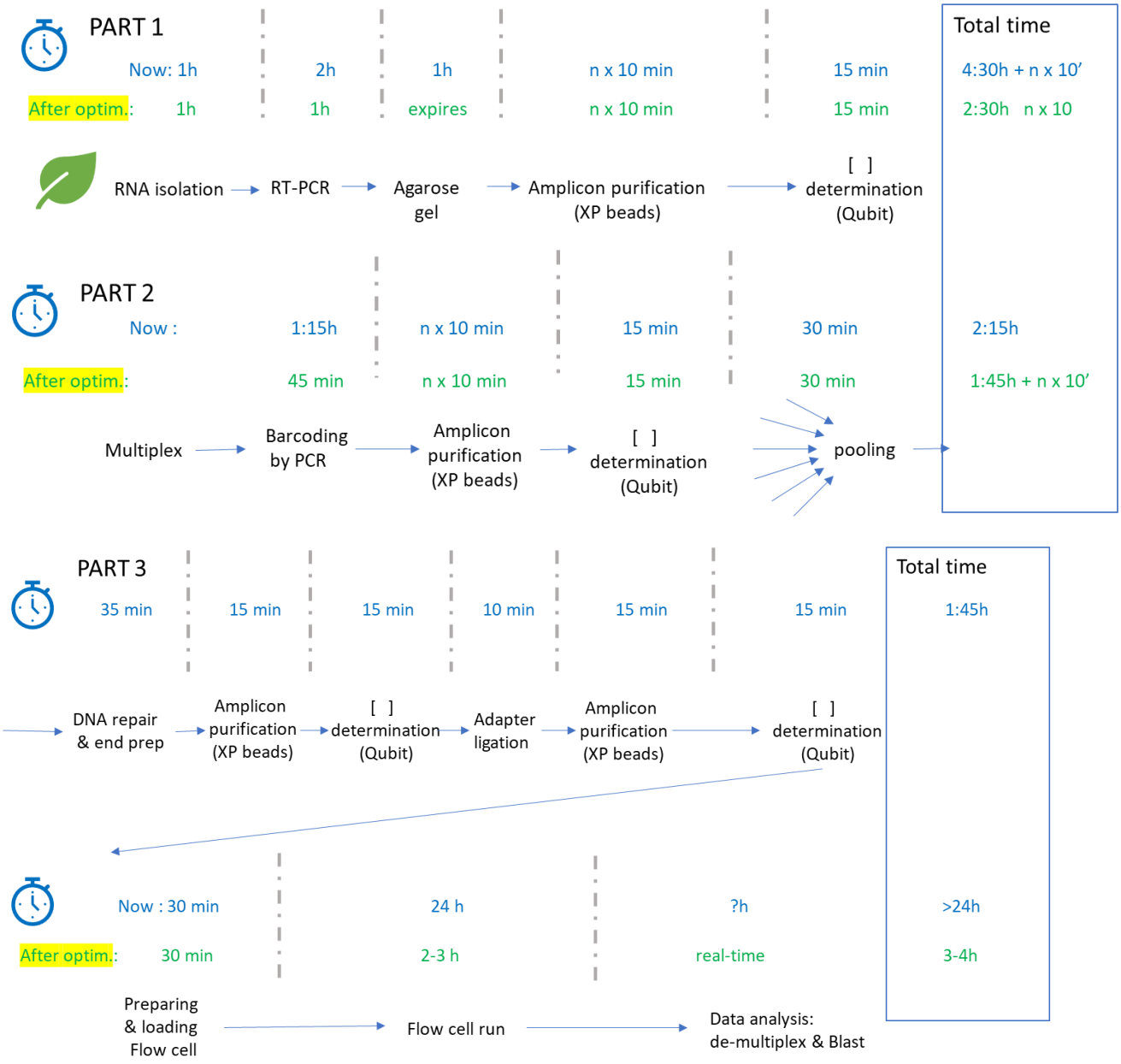


Figure 2-59: Schematic workflow of MinION sequencing from RNA extracton to data analysis.

Direct cDNA sequencing

In order to test if direct cDNA sequencing is an alternative for amplicons sequencing the ToMMV RNA sample provided from Naktuinbouw was selected. cDNA was generated using random primers as described in Liefting et al. (2021). Sample preparation was done as described in the Appendix. The complete workflow

had a length of approximately 6.5 h. Sequence analysis was done by genome assembly from basecalled fastq-data using Flye with the following parameters:

```
--nano-raw <input folder> --out-dir <output directory> --asm-coverage 50 --genome-size 8k --scaffold
```

Nematode sequencing

For benchmarking of the method, a dilution series of a pure suspension of the nematode *Pratylenchus penetrans* was obtained from WUR Field Crops in Lelystad (Table 2.7-1). In addition, three nematode suspensions from field samples were obtained (Table 2.7-2).

Table 2.7-1: Pure nematode suspensions obtained from WUR Field Crops.

No.	Species	#Nematodes	Volume (ml)
1	*P. penetrans*	3000	25
2	*P. penetrans*	3000	25
3	*P. penetrans*	3000	25
4	*P. penetrans*	1000	8
5	*P. penetrans*	1000	8
6	*P. penetrans*	1000	8
7	*P. penetrans*	300	2.5
8	*P. penetrans*	300	2.5
9	*P. penetrans*	300	2.5
10	*P. penetrans*	30	1
11	*P. penetrans*	30	1
12	*P. penetrans*	30	1

Table 2.7-2: Nematode suspesnsions from field samples from WUR Field Crops.

No.	Species	#Nematodes	Volume (ml)
1	NR2	200 pp	11
2	NR12	70 pp	20
3	NR18	253 pp	28

After centrifugation and concentration, DNA was extracted from the suspensions using the Qiagen DNeasy Blood & Tissue kit. A TaqMan real-time PCR was carried out specific for *P. penetrans* (Dauphinais et al., 2018) (Table 2.7-3).

Table 2.7-3: Primers and probe of a TaqMan assay for P. penetrans.

Primer/Probe	Sequence	Dye
Fw	GAGACTTTCGAGAAGGCGATATG	
Rv	AGGACCGAATTGGCAGAAG	
Probe	CCCGGATTGGAGGAATGTTGTTCGT	FAM

For amplicon sequencing primers were obtained from the Nematology group of WUR, amplyfing the 18S rRNA sequence (~1800 bp). Unfortunately, the primer sequences are not public available yet. However, no amplification was observed for the three field samples from WUR field crops. Therefore, mixes were created of amplicons from *P. penetrans* and the nematode *Globodera pallida* in different ratios (see Appendix). These mixes were barcoded and sequenced.

Basecalling was done in MinKNOW on the Mk1C device. Demultiplexing was done with the gupy barcoder requiring barcodes on one side only as requiring barcodes on both sides led to a lot of unclassified reads. Sequence analysis was done with decona with a minimal sequence length of 1700 bp, a quality score of 9 and a cluster size of 50. The SILVA_138.1_SSURef_tax_silva database was used for classification.

2.7.1.2 Results

Primer testing

The 6 primersets that were judged suitable after *in silico* testing were tested on amplicons generated from RNA in singleplex assays. Sequencing of the generated amplicons showed that the number of reads differed strongly between the six targets for most primersets (Table 2.7-4). For all sets except 3 and 4 hardly any reads were obtained for PMMoV. Therefore, these two sets were chosen for further experiments.

Table 2.7-4: Number (#) and percentage (%) of reads obtained with the six different primer sets for the different amplicon targets in singleplex assays. Added targets for each barcode are marked grey. Added targets which were detected in low amounts are written in red. Analysis was done with MinKNOW.

Barcode	1		2		3		4		5		6		unclassified	
Primerset	Set1		Set3		Set4		Set5		Set6		Set7		unclassified	
	#	%	#	%	#	%	#	%	#	%	#	%	#	%
no	3,550	4.6	22,773	13.2	6,008	10.1	4,434	2.1	4,105	3.4	12,227	4.3	49,655	11.3
CGMMV_1_3_4_5_6_7	3,641	4.7	5,260	3.0	6,885	11.6	14,943	7.0	5,715	4.7	12,218	4.3	18,346	4.2
ToMV_1_3	25,358	32.7	33,525	19.4	5	0.0	6	0.0	5	0.0	9	0.0	37,586	8.6
ToMV_4_5_6_7	1,337	1.7	946	0.5	13,518	22.8	94,396	44.5	48,767	40.1	95,681	33.7	108,240	24.7
PMMoV_1_5_6_7	868	1.1	0	0.0	0	0.0	1,994	0.9	937	0.8	2,135	0.8	2,725	0.6
PPMoV_3_4	12	0.0	22,430	13.0	4,583	7.7	17	0.0	2	0.0	17	0.0	7,261	1.7
ToMoV_6	5	0.0	4	0.0	1	0.0	1	0.0	7,710	6.3	3	0.0	4,705	1.1
ToMoV_1_3_4_5_7	9,326	12.0	16,941	9.8	3,308	5.6	27,794	13.1	20	0.0	31,552	11.1	40,457	9.2
TMV_4	27	0.0	6	0.0	13,808	23.3	12	0.0	1	0.0	11	0.0	7,179	1.6
TMV_11_31_51_61_71	5,515	7.1	10,135	5.9	6	0.0	26,463	12.5	9,994	8.2	34,596	12.2	37,440	8.5
TMV_41	13	0.0	6	0.0	4,645	7.8	20	0.0	7	0.0	15	0.0	3,159	0.7
TMV_1_3_5_6_7	10,199	13.1	24,035	13.9	21	0.0	37,375	17.6	17,160	14.1	38,616	13.6	54,100	12.3
ToBRFV_1_3_4_6_7	17,751	22.9	37,031	21.4	6,421	10.8	46	0.0	27,326	22.4	57,201	20.1	65,559	14.9
ToBRFV_5	2	0.0	0	0.0	0	0	4,588	2.2	1	0.0	1	0.0	2,303	0.5
total	77,604	100	173,092	100	59,209	100	212,089	100	121,750	100	284,282	100	438,715	100
% per barcode	5.7		12.7		4.3		15.5		8.9		20.8		32.1	

Testing with gBlocks

Several different gBlocks were used per target to represent strains with mismatchs with the primers. After PCR the amplicons were analysed on an agarose gel. All target gBlocks were amplified with the appropriate primers irrespective of mismatches (Figure 2-60, Table 2.7-5). Even for TMV, which is a highly variable species the primers could amplify all gBlocks (data not shown).

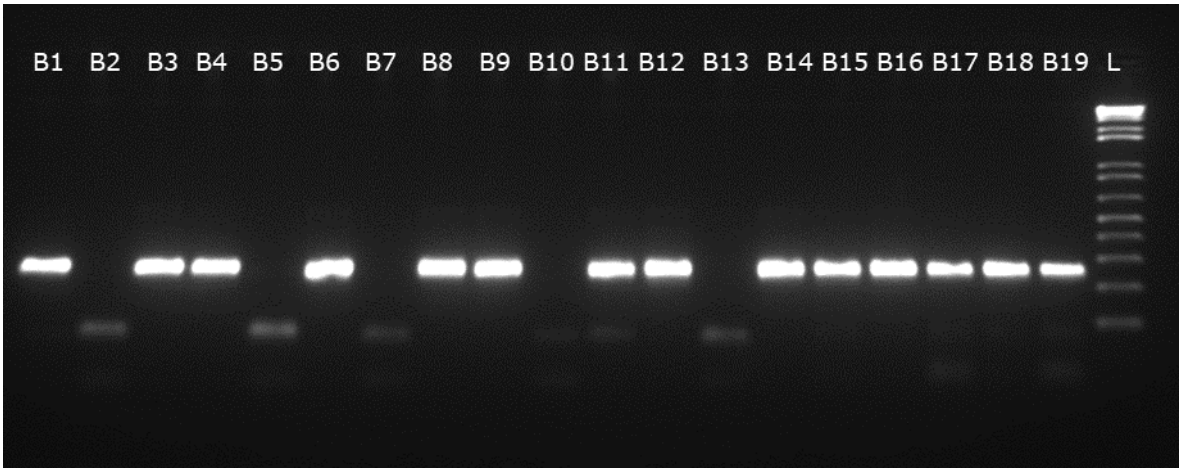


Figure 2-60: Amplicons created from gBlocks. For used gBlock and primer pair see Table 57; L=1Kb Plus DNA ladder.

Table 2.7-5: Used primers and gBlocks for generation of amplicons.

Number	gBlock	primerset	primerpair	amplicon expected
B1	ToMV_2	3	ToMV	yes
B2	ToMV_2	4	ToMV	no
B3	PMMoV_1	3/4	PMMoV	yes
B4	ToMV_1	3	ToMV	yes
B5	ToMV_1	4	ToMV	no
B6	PMMoV_2	3/4	PMMoV	yes
B7	ToMV_3	3	ToMV	no
B8	ToMV_3	4	ToMV	yes
B9	PMMoV_3	3/4	PMMoV	yes
B10	ToMV_4	3	ToMV	no
B11	ToMV_4	4	ToMV	yes
B12	ToMoV_1	3/4	ToMoV	yes
B13	ToMV_5	3	ToMV	no
B14	ToMV_5	4	ToMV	yes
B15	ToMoV_2	3/4	ToMoV	yes
B16	CGMMV_1	3/4	CGMMV	yes
B17	ToBRFV_1	3/4	CGMMV	yes
B18	CGMMV_2	3/4	CGMMV	yes
B19	ToBRFV_2	3/4	CGMMV	yes

Multiplex

The sets 3 and 4 were tested on mixes of 3 amplicons in 3 different concentrations (10^3, 10^4, and 10^5 copies/µl). For both primer sets all added targets could be detected with a concentration a low as 10^3 (Table 2.7-6). However, with both primer sets also a low number of reads for the other tobamoviruses could be found. This might be due to low level contaminations as a consequence of handling high concentrations of the amplicon targets in close proximity. Alternatively, the primers are not entirely specific i.e., can create and amplicon from another of the target species. This should not lead to detection if only the expected amplicons are used as a reference. However, it is possible that the amplicon itself is not discriminating enough leading to misidentification. Blasting primers shows the possibility of unspecific primer binding. Nevertheless, the primers were created using Thermoblast, which does not only take into account sequence compatibility. Therefore, primer blast cannot be used as an evaluation.

Still, in all cases the added target was recovered at much higher numbers than the other targets that were not added to the reaction.

Based on the results of this experiment, the primer set 3 was selected, although both primer sets show good results.

Table 2.7-6: Number of reads obtained with the primer sets 3 (barcode 01-06) and 4 (barcode 07-12) for mixes of three targets at different concentrations. Added targets for each barcode are marked grey. Analysis was done with MinKNOW.

barcode	TMV	CGMMV	ToBRFV	ToMV	PPMoV	ToMMV		unclassified
BC01	2	31,486	29,490	2	1	464		6,414
BC02	3	3,906	3,633	4	5	129,304		1,160
BC03	7	23	37,406	0	1	661		4,354
BC04	4,854	17	7	580	133,460	3		635
BC05	280	3	6	87,023	14,413	1		219
BC06	141,921	0	1	29,007	2,131	1		496
BC07	0	74,905	8,996	0	2	172		5,844
BC08	1	26,425	1,448	12	6	107,689		2,231
BC09	5	149	59,062	1	8	1,182		5,054
BC10	12,308	7	5	90	60,898	1		477
BC11	3,158	1	3	90,462	27,775	0		391
BC12	39,420	0	1	556	73	0		401
unclassified	12,184	4,664	14,836	17,964	19,415	21,363		5,482

RNA testing

Testing RNA samples of the six targets showed that all are detected by amplicon sequencing (Table 2.7-7). Especially when only one target is present detection is unambigous. Certain combinations of targets can lead to one being severely underrepresented, as for example in a combination of TMV and ToBRFV, where for ToBRFV (BC12) only a very low number of reads could be detected. As this problem was not observed previously this might be due to less efficient cDNA synthesis. In addition, there was a background of detected virus species that were not present in the assay. This could again be due to contamination. And again, it is also possible that the primers are not entirely specific i.e., can create and amplicon from another of the target species, which is then misidentified due to this amplicon not being discriminating enough. To investigate the latter possibility amplicons were generated from all six targets with all primers in singleplex. It was found that especially the TMV, ToMV and ToMMV primers also amplified most other targets and also the ToBRFV primers amplified TMV (Table 2.7-8). This shows that both aspecific amplification coupled with misidentification and contamination are possible. Still, contamination is more probable since in sequencing analysis all viruses present in the background showed a high similarity with the reference sequence. In addition, this experiment shows that contrary to expectations the primers designed by PanelPlex consensus were not specific.

Table 2.7-7: Number of reads sequenced from RNA amplicon sequencing of single viruses or mixes of the RNA of two; species expected to be detected are emphasized in grey.

		Identified species							
barcode	sample	CGMMV	PPMoV	TMV_1	TMV	ToMoMV	ToMV	ToBRFV	unclassified
BC01	CGMMV	**44,926**	37	-	90	152	112	79	**5,212**
BC02	PMMoV	66	**52,862**	-	174	307	220	117	**31,312**
BC03	TMV	36	2	-	**43,352**	94	74	45	201
BC04	ToMMV	25	9	-	88	**112,514**	129	88	236
BC05	ToMV	11	7	-	48	144	**81,732**	43	148
BC06	ToBRFV	7	9	-	111	219	145	**106,736**	296
BC07	CGMMV + ToBRFV	**26,859**	37	-	71	139	100	**28,745**	**2,573**
BC08	PMMoV+ ToMV	89	**61,837**	-	177	408	**46,100**	152	**35,274**
BC09	TMV + ToMMV	4	6	-	**6,294**	**44,067**	62	32	108
BC10	CGMMV + ToMV	**438**	3	-	40	88	**47,492**	37	148
BC11	PMMoV + ToMMV	2	**210**	-	47	**64,089**	74	39	282
BC12	TMV + ToBRFV	3	5	-	**60,193**	130	110	**64**	300
unclassified	unclassified	4,693	2,999	-	6,320	11,754	9,834	6,294	5,479

*Table 2.7-8: Amplification of all six tobamo viruses with all primer sets. *= amplification, but amplicon with wrong size; species expected to be detected are emphasized in grey.*

RT-PCR								
		target						
strip#	primerset	CGMMV	PMMoV	TMV	ToBRFV	ToMMV	ToMV	MQ
1	CGMMV	+	-	*	*	*	-	-
2	PMMoV	-	+	-	-	-	-	-
3	TMV	-	-	+	+	+	+	-
4	TMV-2	-	+	+	+	+	-	-
5	ToBRFV	-	-	+	+	-	-	-
6	ToMMV	*	*	*	*	+	*	-
7	ToMV	-	+	+	+	+	+	-

FTA-cards

Amplicon analysis on an agarose gel after cDNA synthesis showed that CGMMV samples produced only little amplicon (Figure 2-61). Also, the ToBRFV sample showed a weak band, indicating that the sample concentration was low.

After sequencing, different options for debarcoding were tested. Requiring reads having barcodes on both ends and excluding reads with barcodes in the middle (i.e., potential chimeras) decreased the number of classified reads. However, these options are still recommended for increasing overall quality of debarcoding. Increasing or decreasing the minimum barcode score respectively decreased or increased the number of classified reads, but dit not change the relative number of reads obtained for each target. Therefore, here we present the reads per barcode with a minimum score of 60 (default).

In almost all samples the results were according to expectations (Table 2.7-9). However, the ToMV FTA card sample appears to contain a large amount of TMV as well. Also, ToMV was found in the ToMMV RNA sample and in the ToMMV+ToBRFV mix sample. Other Tobamovirus species were found in samples they were not expected to be present, but this was restricted to a few reads. Moreover, ToBRFV could be detected in the ToBRFV-only sample, but not in the mix with ToMMV. This might be due to a low concentration in the ToBRFV sample and is confirmed by the low amount of cDNA amplicon produced. It is likely that during cDNA synthesis in the mix, ToBRFV cDNA synthesis was probably less efficient and the resulting low amount of amplicons was not detected at the resulting sequencing depth.

These results indicate that while MinION amplicon-sequencing can confirm that a target is present (i.e., has a high number of reads), the presence of other targets cannot be excluded. The detection of a low number of reasds might originate from mispriming, contamination during sample preparation, but also indicate the actual presence of a target.Moreover, the absence of reads of a target could also be due to a low concentration resulting in inefficient cDNA synthesis.

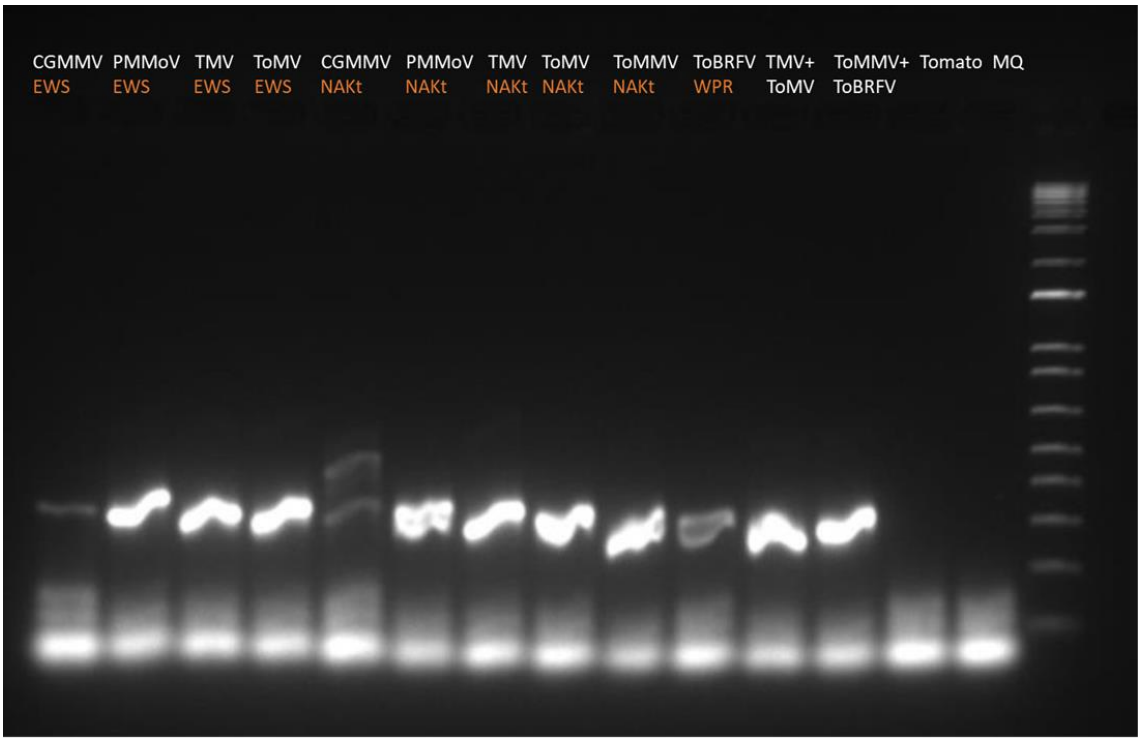


Figure 2-61: Amplicons generated by cDNA synthesis from FTA-card samples from EWS and RNA samples from NAKt and WPR.

Table 2.7-9: Number of reads generated by amplicon sequencing from FTA card extracts or purified RNA of single viruses or mixes of the RNA of two; species expected to be detected are emphasized in grey.

						Identified species		
barcode	**sample**	**CGMMV**	**ToMV**	**PPMoV**	**ToMMV**	**TMV**	**ToBRFV**	**unidentified**
BC01	CGMMV EWS	**17697**	-	-	-	63	-	200
BC02	PMMoV EWS	-	1	**43821**	5	2		46
BC03	TMV EWS	-	-	-	-	**16583**	1	11
BC04	ToMV EWS		**1037**	4	4	56912	15	29
BC05	CGMMV NAKt	**29515**	-	-	2	-	-	4794
BC06	PMMoV Nakt	-	-	**47813**	1	-	1	113
BC07	TMV Nakt	-	1	-	4	**30304**	-	75
BC08	ToMV NAKt	-	**26136**	-	-	1	-	42
BC09	ToMMV NAKt	1	53	-	**18792**	1	-	62
BC10	ToBRFV WPR	-	6	-	5	2	**16524**	54
BC11	TMV+ToMV	-	**4074**	-	1	**29792**	-	57
BC12	ToMMV+ ToBRFV	-	24	-	**12237**	1	-	16

Sequencing analysis

Overall, there are only negligible differences between basecalling and demultiplexing options.

For basecalling with guppy on a linux machine, the following command was used.

```
guppy_basecaller --input_path <folder containing fast5 files> --save_path <output folder> --flowcell FLO-FLG001 --kit SQK-LSK109 --num_callers 4 --cpu_threads_per_caller 4
```

For subsequent demultiplexing the following command was used.

```
guppy_barcoder --input_path <folder containing FASTQ and/or FASTA files> --save_path <output folder> --config configuration.cfg --barcode_kits <kit name>
```

For identification of reads, alignments using MinKNOW are the quickest option, as identification can take place almost real-time during the sequencing run. However, for retrieving the number of reads per target, alignments still have to be reviewed with a software such as CLC Genomic Workbench. The alignment is always performed with (undisclosed) default parameters.

The Nanopore command line workflow wf-alignment does not allow an adjustment of parameters eithers. However, in contrast to MinKNOW, the output consists of two Excel files with a summary of the number of reads per target and alignment files. The following command was used for alignment by wf-alignment.

```
nextflow run epi2me-labs/wf-alignment --fastq <input folder> --out_dir <output directory> --references <path to references>
```

The decona command line workflow allows to adjust several parameters i.e., the minimum sequence length, the maximum sequence length, the quality score of the sequences, the cluster percentage and the cluster size. In addition, it offers Medaka polishing. In this study decona was run with the following options.

```
decona -f -l 250 -q 10 -c 0.80 -n 50 -M -B <References.fasta>
```

The reference file consisted of the amplicon sequences of each Tobamo target. As strains can show sequence variation several sequences per target were included, all originating from the list that was used for primer design. The number of different references was determined by the variability of each target and the number of available sequences.

With the same reference, the three different approaches produced slightly different results in the analysis of FTA card samples (

Table 2.7-10). The wf-alignment workflow showed different instances of misindentification. The decona pipeline aligned less reads than the other two methods, which is likely due to quality and length filtering. However, this pipeline identified the least false positives.

Table 2.7-10: Number of reads generated by amplicon sequencing from FTA card extracts or purified RNA of single viruses or mixes of the RNA of two, analysed with three different methods (i.e., MinKNOW, wf-alignment, decona); species with less than 10 reads were omitted; species expected to be detected are emphasized in grey.

Barcode	sample	Analyse	CGMMV	PPMoV	TMV	ToBRFV	ToMMV	ToMV
BC01	CGMMV EWS	MinKNOW	17697	-	63	-	-	-
		wf-alignment	17757	-	65	-	-	-
		decona	16197	-	59	-	-	-
BC02	PMMoV EWS	MinKNOW	-	43821	-	-	-	-
		wf-alignment	-	43802	-	-	-	-
		decona	-	40247	-	-	-	-
BC03	TMV EWS	MinKNOW	-	-	16583	-	-	-
		wf-alignment	-	-	16570	-	-	-
		decona	-	-	15257	-	-	-
BC04	ToMV EWS	MinKNOW	-	-	56912	15	-	1037
		wf-alignment	-	-	56905	15	-	1037
		decona	-	-	53576	-	-	938

BC05	CGMMV NAKt	MinKNOW	29515	-	-	-	-	-
		wf-alignment	33649	-	-	-	-	-
		decona	31091	-	-	-	-	-
BC06	PMMoV Nakt	MinKNOW	-	47813	-	-	-	-
		wf-alignment	-	47778	-	-	-	-
		decona	-	43621	-	-	-	-
BC07	TMV Nakt	MinKNOW	-	-	30304	-	-	-
		wf-alignment	-	-	28584	1764	-	-
		decona	-	-	28207	-	-	-
BC08	ToMV NAKt	MinKNOW	-	-	-	-	-	26136
		wf-alignment	-	-	-	-	-	26139
		decona	-	-	-	-	-	23628
BC09	ToMMV NAKt	MinKNOW	-	-	-	-	18792	53
		wf-alignment	-	-	-	-	18795	59
		decona	-	-	-	-	17352	-
BC10	ToBRFV WPR	MinKNOW	-	-	-	16524	-	-
		wf-alignment	-	6118	-	10407	-	-
		decona	-	-	-	14910	-	-
BC11	TMV+ToMV	MinKNOW	-	-	29792	-	-	4074
		wf-alignment	-	-	28020	1788	-	4074
		decona	-	-	27537	-	-	3689
BC12	ToMMV+ToBRFV	MinKNOW	-	-	-	-	12237	24
		wf-alignment	-	-	-	-	12237	25
		decona	-	-	-	-	11178	-

Direct cDNA sequencing

Within 20 hours of Flongle run time 878.1 K reads were generated and 562 K reads were succesfully basecalled. The two contigs resulting from assembly with Flye were both identified as ToMMV by Blast (Table 2.7-11).

Table 2.7-11: Blast results for 2 contigs after assembly of ToMMV sequencing data by Flye.

Query	Identity (%)	Length	Accession	Description
Contig 1	99.53	3852	OK334224	Tomato mottle mosaic virus isolate ToMMV_83, complete genome
Contig 2	99.63	1337	KT810183	Tomato mottle mosaic virus isolate NY-13, omplete genome

Nematode sequencing

For the *P. penetrans* TaqMan also the supenatant of the spinned-down nematode suspension with 3000 nematodes was used as nematodes could still be seen in the supernatant. The results show clear differences between the suspensions with the highest CT for the samples with the lowest concentrations and the lowest CT for samples with the highest concentrations of *P. penetrans* (Figure 2-62).

P. penetrans could also be detected in the three field samples. Still, PCR for the 18S rRNA gene failed to generate amplicons from these samples.

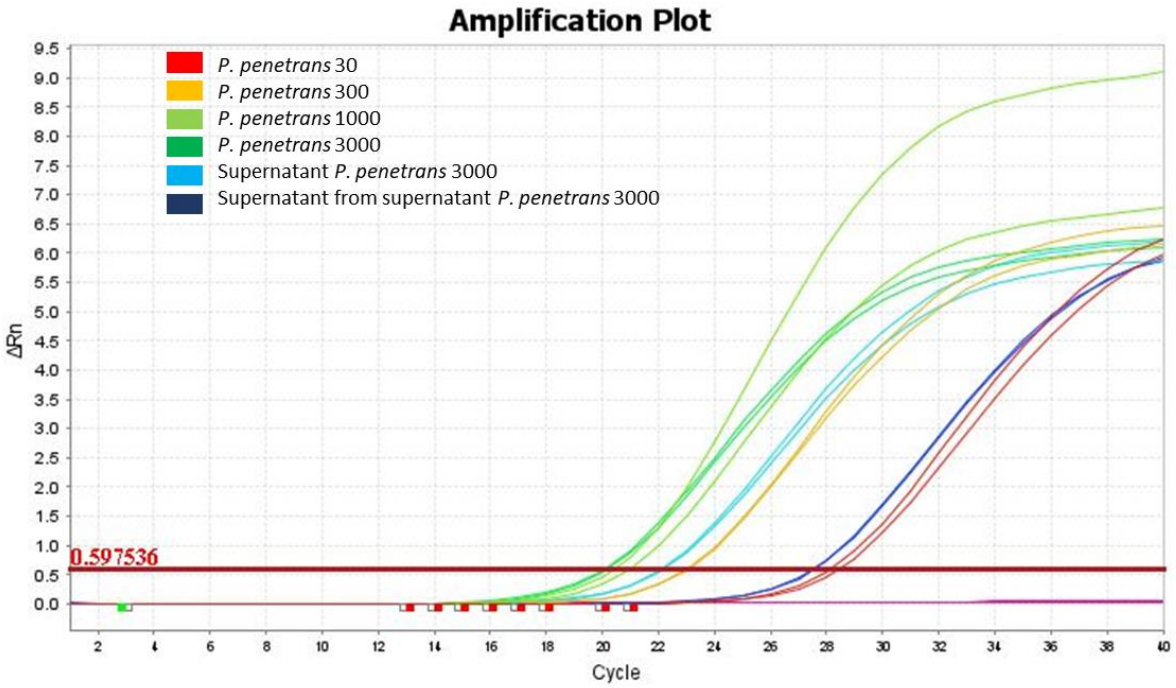


Figure 2-62: Amplification curves of a TaqMan assay for a dilution series of P. penetrans.

Sequence reads were usually identified to species level with both *P. penetrans* and *P. convallariae* being identified in the genus *Pratylenchus* and both *G. pallida* and *G. ellingtonae* being identified in the genus *Globodera*. This might be caused by the high similarity between 18S rRNA gene sequences of the species. The reads for the different species were summed for *Pratylenchus* and *Globodera* respectively (Table 2.7-12).

The sequencing results revealed as expected a decreasing number of reads of *Pratylenchus* with decreasing amplicon input from barcode 7 to barcode 12 and an increasing number of *Globodera*. In addition, high numbers of reads for the species *Chiloplacus propinquus* were found, which are most likely originating from the *P. penetrans* samples. Also the genera *Acrobeloides* and *Merlinius* and a numer of reads only classified as Nematoda were found. Blasting of representative sequences revealed that the 18S rRNA sequences are very similar to *Chiloplacus* and might therefore rather belong to this genus. These results indicate that the *Pratylenchus* culture was highly contaminated with *Chiloplacus propinquus*. Also reads identified as *Rotylenchus* could belong to Globodera.

When summing Pratylenchus and Chiloplacus reads and adding the reads from the genera Acrobeloides, Merlinius and the unidentified nematodes and adding Rotylenchus to Globodera, the read ratios between Pratylenchus and Globodera are very similar to what would be expected from the input ratios (

Table *2.7-13*).

In addition, reads of the fungus *Plectosphaerella* sp. were found in samples containing *Globodera* amplicons suggesting a contamination of the used *Globodera* DNA.

Table 2.7-12: Number of reads generated by amplicon sequencing in the six samples.

Barcode	Expected species	*Pratylenchus*	*Globodera*	*Chiloplacus*	*Acrobeloides*	*Pseudoacrobeles*	*Geocenamus*	*Rotylenchus*	*Merlinius*	Nematoda	*Plecto-sphaerella*
BC07	100% *Pratylenchus* 0% *Globodera*	3375	0	6451	1205	236	96	0	0	620	0
BC08	99% *Pratylenchus* 1% *Globodera*	2263	259	4661	1215	0	170	0	0	332	0
BC09	90% *Pratylenchus* 10% *Globodera*	2562	1211	4534	1231	0	0	83	89	274	0
BC10	10% *Pratylenchus* 90% *Globodera*	336	2184	476	0	0	0	0	0	52	180
BC11	1% *Pratylenchus* 99% *Globodera*	150	25984	161	0	0	0	0	0	0	1362
BC12	0% *Pratylenchus* 100% *Globodera*	0	18275	0	0	0	0	0	0	0	771

Table 2.7-13: Summed number of reads belonging to the genera Pratylenchus and Globodera and percentages of reads.

Barcode	Expected species	*Pratylenchus* reads	*Globodera* reads	Percentage *Pratylenchus*	Percentage *Globodera*
BC07	100% *Pratylenchus* 0% *Globodera*	11887		100	0
BC08	99% *Pratylenchus* 1% *Globodera*	8471	259	97.03	2.97
BC09	90% *Pratylenchus* 10% *Globodera*	8690	1294	87.04	12.96
BC10	10% *Pratylenchus* 90% *Globodera*	864	2184	28.35	71.65
BC11	1% *Pratylenchus* 99% *Globodera*	311	25984	1.18	98.82
BC12	0% *Pratylenchus* 100% *Globodera*		18275	0	100

Conclusions

The goal of this project was to assess if ONT sequencing is suitable for the detection of plant pathogens and pests. Recent studies have shown that this sequencing method can be used for the identification of unknown pathogens and for studying pathogens populations (Della Bartola et al., 2020; Liefting et al., 2021). However, little is known about the merit of specific detection of one or several targets using ONT sequencing. In this study we used sets of specific primer pairs for the simultaneous detection of 6 tobamoviruses.

Our study showed that it is possible to detect multiple plant viruses by subsequent amplicon generation and ONT sequencing. Still, both advantages and distinct diadvantages were discovered in the durination of the project. One advantage of ONT sequencing compared to other sequencing techniques was the short time from sampling until the results were available, as sequencing could be done in house whitout the need to send samples to a sequencing facility. On the other hand, sample preparation was work-intensive and required both trained personnel and specialized equipiment. Unlike detection methods such as LAMP, amplicon generation by PCR required more complicated extraction methods. In addition, both amplicon generation and library preparation were time-consuming. Therefore, we conclude that ONT sequencing for plant pathogens it is not yet fully suitable for on-site applications, although new developments will possibly simplify sample preparation in the near future.

An advantage of sequencing compared to other detection methods is the ability to detect all potential pathogens and even unexpected threats in a sample. This is usually achieved by direct sequencing of the total generic material in a sample or by sequencing amplicons generated with primers that are generic for a group of viruses. However, the latter was not possible for tobamoviruses due to the high sequence variation between species. Therefore, a multiplex assay consisting of six primer sets was designed in this study. The PanelPlex consensus software, that we tested in this study, was shown not to be able to generate sufficiently specific primers and led to highly skewed resullts. At the time of this study, we were not aware of any other software claiming to be able to design these highly complex assays. Possibly, in the future, more powerful algorithms can be used to produce large and specific multiplex assays. In addition, sequence variation within the amplicons should be taken into account.

For targets with a high concentration, such as tobamoviruses direct sequencing of total RNA should be considered. Liefting et al. (2021) demonstrated that also viruses without PolyA tails can be detected using random hexamers for cDNA generation. Also in this project ToMMV was identified using direct cDNA sequencing. In contrast to amplicon sequencing the analysis yielded no other viruses than ToMMV. This supports th assumption that the misidentifications in amplicon sequencing were artefacts potentially produced by insufficient specificity of the amplicons. To circumvent this problem only the amplicon sequences could be included in the reference database and analysis parameters could be made more stringent. However, this has also been found to lead to the exclusion of species that were actually present. In this respect, whole cDNA sequencing appears to be more accurate in this study. Moreover, obtaining the complete genomic sequences of viruses enables researchers to assess the emergence of new varieties.

Amplicon generation remains the method of choice if the concentration of the pathogen or pest is expected to be low. Therefore, for the assessment of the soil nematode community general primers were chosen that are able to generate amplicons from all nematode species which can subsequently be identified by sequencing. Nanopore sequencing is suitable for sequencing long amplicons which allow for a higher resolution compared to short amplicon sequencing techniques, potentially allowing for identification to species level. For unknown reasons, no amplicons could be generated from field sample communities. This might have been due to inibitors present in these samples. However, sequencing of mixed amplicons from the nematodes Pratylenchus penetrans and *Globodera pallida* with general 18S rRNA gene primers resulted in a good resprentation of the input ratios of the respective amplicons. This suggests that sequencing itself does not lead to a strong bias against one of the two species. Still, amplicon generation might introduce biases as this was not investigated in this study.

Several other nematode species were identified in the samples. While one of them was a real contamination of the *Pratylenchus* culture, most were likely the result of a misidentification due to a high similarity between species and even genera. These results indicate that even the sequencing of long amplicons might not be sufficient to distinguish species, at least with respect to the 18S rRNA gene sequence. Currently, reference databases are only available for few sequences such as the small and large subunit of the rRNA gene, which might not give sufficient resolution. Increasing sequencing effor is likely to increase the number of available loci for species identification, such as the whole ITS and the COI sequences, which

might enable unambiguous species identification (Kawanobe et al., 2021). It must be noted though, that misidentification only represented a small number of reads and that most reads were correctly identified to species level, which is a marked improvement compared to earlier studies using short sequence read techniques (Kenmotsu et al., 2020).

We conclude that Nanopore sequencing has a high potential for enabling the detection of pathogens and pests in complex communities. The approach of using highly specific primers for amplicon sequencing in a multiplex approach, however, is not suitable for on-site detection in practice. For nematodes, the use of general primers for long-read sequencing has high potential and needs to be further evaluated.

3 Summary

Increased globalization and international trade favour the spread of plant pathogens and pests, which can form potential threats for crop production and food safety. In order to control and prevent spread of these pathogens and pests, early detection is essential. Ideally, detection methods should fast and should be suitable to be performed by personnel at border controls, at growers and farmers directly on-site. In the project PPS "On-site plantpathogen detection and barcode sequencing for improving plant health and phytosanitary control" three important topics for on-site detection were investigated. In workpackage 1 extraction methods were optimized for fast and simple DNA or RNA extraction. In the workpackage 2 a number of LAMP assays were developed for the detection of specific plant diseases and the possibilities of a LAMP multiplex were investigated. In workpackage 3 ONT sequencing was used to detect and identify tobamoviruses and plant parasitic nematodes.

In the first workpackage methods were optimized to rapidly extract DNA or RNA from samples that is then directly suitable for testing. Here we found that simple PEG-buffer is sufficient for extraction from leaf material of a variety of plant species. The extract can, after dilution, directly be used for detection by LAMP. In addition, we could show that FTA-cards can be used to take samples from diseased plant material, which can then be transported and used for detection in a remote laboratory. Thus, it is possible to sample even in places without detection facilities without the problem of sample degradation.

In the second workpackage, LAMP assays were developed for a variety of plant pathogens and tested for sensitivity and specificity. These LAMP assays were verified to be able to detect the pathogens TMV, ToBRFV, MNSV, TSWV, PVY, *X. fragariae, F. solani, F. oxysporum, F. proliferatum* and *V. dahliae* in symptomatic plant material. A multiplex assay on a microchip for pathogens in tomato was tested and showed the ability to detect at least two targets in the same sample. However, this device is not yet suitable for on-site use and requires further developments. The T65cup assay for performing LAMPs in a waterbath with simple equipment was found to be promising. Still read-out methods such as colorimetric detection or fluorescence measurement by mobile phone need to be evaluated further.

In the third workpackage tobamoviruses were detected by multiplex amplicon sequencing with the ONT method. While it was possible to detect all target viruses, this approach was not optimal because of a lack of specificity of primers and amplicons and differences in amplification efficiency. For tobamoviruses direct sequencing of the complete RNA after cDNA synthesis is advisable. In addition, two nematode species in different ratios were detected using sequencing sequencing of amplicons generated by general primers. This approach shows the possibility for identification at species level and should be further tested and developed. Sequencing could be an alternative for microscopic identification in the future.

Deliverables

- Protocols of simple extraction methods from plant leaf material
- Ten evaluated LAMP assays for specific plant pathogens
- Knowledge on the use of a multiplex microchip for multiplex LAMP detection
- Knowledge on the use of Nanopore sequencing (MinION) for the detection of plant pathogens and pests including primers and protocols
- Three LAMP workshops at grower cooperations, one workshop at Dümmen Orange and one at East West Seed.
- Workshop On-site detection at Plantgezondheidsevent Bleiswijk, 13 October 2022

- Nanopore movie by WUR
- Final report

Acknowledgements

The project committee for their contributions in the various meetings during the course of the project.

Stichting Programmafonds Glastuinbouw, Topsector T&U and the private parties for their cash and in-kind contributions.

4 Appendices

4.1 WP1: DNA/RNA extraction

Comparison of extraction buffers

Extraction buffer comparison was done for ToBRFV in tomato and *Acidovorax citrulli* in melon. For ToBRFV healthy and infected leaf material were used. Samples were either 100% infected material, 10% infected material with 90% healthy material or 100% healthy material. Buffers tested were:

- OptiGene PEG lysis buffer (+40% chelex)
- Self-made PEG buffer (+40% chelex)
- Quickextract buffer (Epicentre) (+40% chelex)
- USEB buffer (contains 5% chelex)

Methods

- Fill extraction tubes with a metal ball and the respective buffer
- Add 120 mg plant material
- shake for 1 min and incubate for 20 min at room temperature
- dilute 50x in OptiGene dilution buffer

LAMP

On all samples both a LAMP assay for ToBRFV and for Cox were performed.

Reaction mix ToBRFV

Ingredient	µl/reaction
ISO-DR001 + RT + 50%	20
ToBRFV primer mix	1
target	5

Reaction mix Cox

Ingredient	µl/reaction
ISO-DR001 + RT + 50%	20
Cox primer mix	1
target	5

Program: 40 min at 65°C, melting curve 98°C-60°C

For *A. citrulli* the same protocol was followed.

Reaction mix A. citrulli

Ingredient	µl/reaction
ISO-DR001 + RT + 50%	20
A.citrulli primer mix	1
target	5

Comparison of PEG buffers

Extraction of DNA from leaf material with the OptiGene Lysis buffer and self-made PEG buffer was compared.

PEG buffer

- 60% PEG (200)
- 20mM KOH
- pH 13.3-13.5

Leaf material

Beetroot
Broccoli
Carrot
Dahlia
Green beans
Kale
Leek
Oxheart cabbage
Savoy cabbage
Spinach
Strawberry
Sweet William

Methods

- fill extraction tubes with 1 metal ball, 1 ml extraction buffer and 40% chelex
- add 125 mg plant leaf material to each tube
- shake for 1 min and incubate for 20 min at room temperature
- dilute 50x in OptiGene dilution buffer

LAMP

LAMP was performed with the *Cox* assay.

Reaction mix

Ingredient	µl/reaction
ISO-001	15
COX primer mix	1
Hyclone water	4
Total	20
Target	5

Program: 40 min at 65°C, melting curve 98°C-60°C

FTA cards

Standard LAMP protocol

Elution

- Take 3 punches per smaple with a 3 mm punch and put in a sterile 1.5 ml tube
- Add 100 µl OptiGene lysis buffer and incubate at room temperature for 20 min
- Dilute 10x in OptiGene dilution buffer

Whatman protocol

Elution

- See Whatman FTA Elue protocol, in short:
- Take 3 punches per sample with 3 mm punch and put in a sterile 1.5 ml tube
- Wash by adding 500 µl sterile water and pulse vortex 3 times for in total 5 s
- Remove the wash water with a pipette and squeeze punches to remove as much water as possible.
- Repeat
- Add 100 µl sterile water and pulse vortex the tube for 5 s.

- Heat the tube in a heating block at 95- 100 °C for 15-30 min
- Remove from the heating block and vortex approx. 60 times.
- Briefly centrifuge
- Remove the punches from the eluant with a sterile pipette and discard.
- The samples can be stored at -20°C

Preparation for LAMP

- For samples in which a high amount of target is expected, dilute 50x in sterile water
- Use 1 µl sample for LAMP

4.2 WP2: LAMP

Cox

Primer sequences

Primer	**Sequence**
Cox_sol_F3	TATGGGAGCCGTTTTTGC
Cox_sol_B3	AACTGCTAAGRGCATTCC
Cox_sol_FIP	ATGGATTTGRCCTAAAGTTTCAGGGCAGGATTTCACTATTGGGT
Cox_sol_BIP	TGCATTTCTTAGGGCTTTCGGATCCRGCGTAAGCATCTG
Cox_sol_LoopF	ATGTCCGACCAAAGATTTTACC
Cox_sol_LoopB	GTATGCCACGTCGCATTCC

Primer mix

Primer	**Concentration (µM)**	**µl/reaction**
Cox_sol_F3	0.8	0.2
Cox_sol_B3	0.8	0.2
Cox_sol_FIP	0.2	0.05
Cox_sol_BIP	0.2	0.05
Cox_sol_LoopF	0.4	0.1
Cox_sol_LoopB	0.4	0.1
HyClone water		0.3

Clavibacter michiganensis

Primer mix

Primer	**Sequence**	**Concentration (µM)**	**µl/reaction**
Cm_FIP-CS2	GCGTCGAGCAGCATGTCCCAACACGATGAACGACATCCTC	1.6	0.4
Cm_BIP	CGTCCGTCCAGACCCAGATCGCTGGACATGTACGGGCTCA	1.6	0.4
Cm_F3	CGACAACAGGAACACAGGT	0.2	0.05
Cm_B3-CS1	CCCGCATTCGATGGTGAGC	0.2	0.05
Cmm_Loop F	TGACCATGACGGGGGTCT	0.8	0.2
Cm_Loop F	/56-FAM/ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCG ATGACCATGACGGGGGTCT	0.08	0.02
Quencher probe	TCGGCATCCGCATCCGCATTCGCATCCGGGTCCTCAGCGT/3BHQ_1/	0.16	0.04
HyClone Water		2	

Reaction mix

Ingredient	µl/reaction
ISO-001	15
primer mix	2
HyClone water	7
sample	1
total volume	25

ToBRFV

In the project KB 37 Diagnostics the ToBRFV LAMP-assay was modified to a LAMP-Crispr-Cas (LAMP-CC) asay by adding a guide RNA. As this guide RNA detects the LAMP product, this LAMP-CC assay is more specific than a normal LAMP. This assay was developed by ScopeBioscience, who deliver a master mix containing a warm-start enzyme (New England Biolabs) and primers and guide RNA.

The sensitivity and specificity testing on RNA was performed using the BioScope master mix, while earlier experiments on specificty with gBlocks and the test on infected plant material were performed with the normal ToBRFV LAMP assay.

Primer mix

Primer	Concentration (µM)	µl/reaction
F3	0.2	0.05
B3	0.2	0.05
FIP	0.8	0.2
BIP	0.8	0.2
LoopF	0.4	0.1
LoopB	0.4	0.1
HyClone water		1.3

Reaction mix normal LAMP assay

Ingredient	µl/reaction
ISO-001	15
primer mix	2
HyClone water	6
sample	2
total	25

Reaction mix LAMP-CC

Ingredient	µl/reaction
Scope ToBRFV mastermix	15
HyClone water	4
sample	1
total volume	20

Sensitvity testing

Samples: ToBRFV RNA isolated from 2x 50 m infected leaf material 10x dilution series

Strip layout	
1	0x diluted
2	10x diluted
3	100x diluted
4	1000x diluted
5	10000x diuted
6	100000x diluted
7	1000000x diluted
8	NC

Reaction program: 65 °C 45 minutes

Specificity

Samples: gBlocks at a concentration of 10^6 copies/µl

Striplayout	
1	ReMV
2	ToBRFV
3	ToBRFV_SNP
4	TMV
5	ToMV
6	ToMMV
7	NC
8	NC

Reaction program: 65 °C 30 minutes, meltcurve 98°C-60°C

Samples: RNA samples from Naktuinbouw

Striplayout		
	A	B
1	TMV	TSWV
2	TMV	TSWV
3	TMV	TSWV
4	ToMV	PMMoV
5	ToMV	PMMoV
6	ToMV	PMMoV
7	PC	
8	NC	

Infected plant material

All samples were extracted with a simple extraction procedure. The samples VT1, VT2, VT3 and MT1 were split and also extracted with an extraction kit.

Simple extraction

- for the punches: add 3 punches to an extraction tube
- for the powder: transfer approximately 100 mg to an extraction tube
- add 1 ml PEG lysis buffer (OptiGene) + 40% chelex to all tubes
- shake the tubes by hand for 1 minute
- incubate for 20 minutes at RT
- dilute the extracts 50x in OptiGene dilution buffer
- use 1 or 5 µl for the different analysis methods (5 µl was used for both LAMP and LAMP-CC)

RNeasy plant mini kit

The kit was used according to manufacturers instructions with the following adjustments.

- 2 min incubation at 56°C after adding 450 µl RLT buffer to the tomato leaf powder and vortexing

Reaction program: 65 °C 30 minutes, meltcuve 98°C-60°C

Multiplex assay

Reaction mix

Ingredient	**Label**	**µl/reaction**
Master mix ISO-001nd		15
Primer mix (ToBRFV)	FAM	2
Primer mix (COX)	Texas Red	2
Reverse Transcriptase		0.25
Hyclone water		0.75
Total		**20**
Target		5

Colorimetric assay

Reaction mix

LAMP reaction mix WarmStart	**1x (µl)**
NEB Warm-start colorimetric LAMP master mix	12.5
Primer mix (ToBRFV)	2.5
Hyclone water	5
Total	**20**
Target	5

Samples: Serial dilution of ToBRFV RNA

Striplayout	
1	ToBRFV 100x diluted
2	ToBRFV 1000x diluted
3	ToBRFV 10,000x diluted
4	ToBRFV 100,000x diluted
5	ToBRFV 10^6 x diluted
6	ToBRFV 10^7 x diluted
7	ToBRFV 10^8 x diluted
8	NC

Program: 65 °C 40 minutes

TMV

Primer mix

primer	concentration	µl/reaction
F3	0.2 µM	0.05
B3	0.2 µM	0.05
FIP	0.8 µM	0.2
BIP	0.8 µM	0.2
LoopF	0.4 µM	0.1
LoopB	0.4 µM	0.1
HyClone water		1.3
total		2

Primer mix multiplex

primer	concentration	µl/reaction
F3	0.2	0.05
B3	0.2	0.05
FIP	0.8	0.2
BIP	0.8	0.2
LoopF	0.4	0.1
LoopB	0.4	0.1
LoopF probe	0.04	0.01
Quencher	0.08	0.02
HyClone water	-	0.27
total vol.	-	1

Reaction mix singleplex

primer	µl/reaction
ISO_001	15
PM	2
MQ	7
gBlock	1
total	25

Reaction mix multiplex

Ingredient	µl/reaction
Isothermal master mix, no dye (ISO-001, OptiGene)	15
primer mix target	1
primer mix Cox	1
HyClone water	3
sample (diluted cell suspension with plant DNA background)	5
total volume	25

Sensitivity

Samples: 10-fold filution series of a TMV gBlock

Striplayout	
1	10^6
2	10^5
3	10^4
4	10^3
5	10^2
6	10
7	1
8	NC

Reaction program: 65 °C 40 minutes, meltcuve 98°C-60°C

Specificity

Samples: gBlocks of closely related non targets at a concentration of 10^6 copies/µl

Striplayout	
1	ToMV
2	TMGMV
3	ToBRFV
4	TMV-Ohio
5	TMV_HE818457 Gblock
6	MQ

Reaction program: 65 °C 40 minutes, meltcurve 98°C-60°C

RNA multiplex

Samples: TMV RNA from Naktuinbouw diluted 1:10 (due to small volume) and TMV gBlocks at a concentration of 10^4 copies/µl

Strip Layout	
1	TMV RNA 1:10
2	TMV RNA 1:10
3	TMV_Tor2-L3 Gblock 10^4 + tomaat
4	Cox
5	NC

Program: 65 °C 40 minutes

Infected leaf material

Samples: petunia leaf infected with TMV (from NAK Tuinbouw), healthy petunia leaf, TMV RNA 1:10 diluted, tomato DNA

Strip layout	
1	TMV infected leaf
2	Healthy leaf
3	TMV RNA 1:10
4	tomato DNA

Samples for specificity assay: Petunia leaf infected wih TMV, petunia leaves spiked with: ToMV RNA, ToBRFV extract, PMMoV RNA, ToMMV RNA; tomato leaves spiked with: ToMV RNA, ToBRFV extract, PMMoV RNA, ToMMV RNA.

Extraction and spiking

- add 900 ul PEG lysis buffer (Optigene) with 0.4 g Chelex-100 to a 5 ml extraction tube with a metal ball
- add 125 mg leaf material and 50 ul of 1:10 diluted RNA (except for TMV extract where 50 ul extract was used)
- shake the tube for 1 min
- incubate at room temperature for 20 min
- mix briefly and wait for Chelex to settle
- dilute extract 50 times with 1 ml dilution buffer and 20 ul extract

Strip layout	
1	Petunia leaf extract TMV infected
2	Petunia leaf extract spiked with ToMV
3	Petunia leaf extract spiked with ToBRFV
4	Petunia leaf extract spiked with PPMoV
5	Petunia leaf extract spiked with ToMMV
6	Petunia leaf extract CTRL
1	Tomato leaf extract gespiked with TMV
2	Tomato leaf extract spiked with ToMV
3	Tomato leaf extract spiked with ToBRFV
4	Tomatot leaf extract spiked with PPMoV
5	Tomato leaf extract spiked with ToMoV
6	Tomato leaf extract CTRL

Program: 65 °C 40 minutes

MNSV

Primer mix

primer	**concentration**	**µl/reaction**
F3	0.2 µM	0.05
B3	0.2 µM	0.05
FIP	0.8 µM	0.2
BIP	0.8 µM	0.2
LoopF	0.4 µM	0.1
LoopB	0.4 µM	0.1
HyClone water		1.3
total		2

Reaction mix

Ingredient	**µl/reaction**
ISO_001	15
PM	2
HyClone water	7
gBlock	1
total	25

Reaction mix multiplex

Ingredient	**µl/reaction**
Isothermal master mix, no dye (ISO-001, OptiGene)	15
primer mix target	1
primer mix Cox	1
HyClone water	7
sample (diluted cell suspension with plant DNA background)	1
total volume	25

Sensitivity

Samples: 10-fold serial dilution of MNSV gBlocks

Strip layout	
1	MNSV gBlock 10^6
2	MNSV gBlock 10^5
3	MNSV gBlock 10^4
4	MNSV gBlock 10^3
5	MNSV gBlock 10^2
6	MNSV gBlock 10^1
7	MNSV gBlock 1
8	NTC

Reaction program: 65 °C 40 minutes, meltcurve 98°C-60°C

Specificity

Samples: MNSV gBlock at a concentration of 10^6 copies/µl, CGMMV RNA and CMV RNA

Strip layout	
1	MNSV gBlock 10^6
2	MNSV gBlock 10^6
3	CGMMV RNA
4	CGMMV RNA
5	CMV RNA
6	CMV RNA
7	NTC
8	NTC

Multiplex

Samples: MNSV gBlocks at a concentration of 10^6 copies/µl, cucumber spiked with MNSV gBlocks at a concentration of 10^6 gBlocks

Extraction: see TMV

Strip layout		
1	MNSV gBlock 10^6	
2	MNSV gBlock 10^6	
3	Cucumber fruit spiked with MNSV gBlock	
4	Cucumber fruit spiked with MNSV gBlock	
5	NTC	
6	Cucumber NTC	

RNA

Samples: MNSV gBlocks at a concentration of 10^6 copies/µl, MNSV RNA isolated from infected plant material 1:10 diluted

Strip layout	
1	MNSV gblock
2	MNSV RNA
3	MNSV RNA
4	MNSV RNA
5	NTC
6	NTC

Infected leaf material

Samples: Leaves from two cultivars of cucumber (gele tros and chinese slangen) and two cultivars of zucchini (Tosca and Cala Green) infected with MNSV. Healthy cucumber leaf material was used as a negative control.

Leaf extraction

- add 900 ul PEG lysis buffer (Optigene) with 0.4 g Chelex-100 to a 5 ml extraction tube with a metal ball
- add 125 mg leaf material
- shake the tube for 1 min
- incubate at room temperature for 20 min
- mix briefly and wait for Chelex to settle
- dilute extract 50 times with dilution buffer

Strip layout	
1	Cucumber gele tros (GT)
2	Cucumber chinese slangen (CS)
3	Zucchini Tosca
4	Zucchini Cala Green
5	Healthy cucumber
6	MNSV gBlock 10^6
7	NTC

TSWV

Primer mix

primer	concentration	µl/reaction
F3	0.2	0.05
B3	0.2	0.05
FIP	0.8	0.2
BIP	0.8	0.2
LF	0.4	0.1
LB	0.4	0.1
		1.3

Reaction mix

Ingredient	µl/reaction
ISO-001	15
primer mix	2
water	7
sample	1
total vol.	25

RNA and infected leaves

Samples: TSWV infected leaves at a 10x and 50x dilution, TSWV RNA at a 1:50 dilution

Leaf extraction

- add 900 ul PEG lysis buffer (Optigene) with 0.4 g Chelex-100 to a 5 ml extraction tube with a metal ball
- add 15 mg leaf material
- shake the tube for 1 min
- incubate at room temperature for 20 min
- mix briefly and wait for Chelex to settle
- dilute extract 10 times and 50 times with dilution buffer

Strip layout	
1	TSWV infected leaf 10x dil
2	TSWV infected leaf 50x dil
3	TSWV #1-1 50x dil
4	TSWV #2-2 50x dil
5	NC

Specificity

Samples: TSWV infected leaf extract, ANSV (alstomeria necrotic streak virus) RNA, TNRV (tomato necrotic ring virus) RNA

Strip layout	
1	TSWV plant extract 1:50
2	ANSV 1:10
3	ANSV 1:100
4	TNRV 1:10
5	TNRV 1:100
6	NC

PVY

Primer mix

primer	concentration	µl/reaction
F3	0.2 µM	0.05
B3	0.2 µM	0.05
FIP	0.8 µM	0.2
BIP	0.8 µM	0.2
LoopF	0.4 µM	0.1
LoopB	0.4 µM	0.1
HyClone water		1.3
total		2

Reaction mix

Ingredient	µl/reaction
ISO-001	15
PM	2
HyClone water	7
gBlock	1
total	25

Sensitivity gBlocks

Samples: PVY-NTN gBlock dilution series

Strip layout	
1	10^6
2	10^5
3	10^4
4	10^3
5	10^2
6	10
7	1
8	0

Sensitivity RNA

Samples: diution series of RNA from infeted Petunia material (from Naktuinbouw).

Strip layout	
1	0x
2	10x
3	100x
4	1000x
5	10000x
6	100000x
7	1000000x
8	0

Specificity

Samples: gBlocks from different PVY variants: PVY-AJ889866, PVY-JF928458, PVY-KC296828, PVY-KJ741205, PVY-MH795859, gBlocks from PepMoV, SuCMoV, TNSV, PVV at a concentration of 10^6 copies/µl.

Striplayout	
1	PVY-AJ
2	PVY-JF
3	PVY-KC
4	PVY-MH
5	PVY-AJ
6	PepMoV
7	SucMoV
8	NC

Striplayout	
1	PVY-KJ
2	TNSV
3	PVV
4	NC

Infected leaf material

Samples: petunia leaf infected with PVY, potato leaf infected with PVY, petunia leaf infected with TMV, healthy petuna leaf, and PVY RNA at a 1:10 dilution (all from NAKTuinbouw).

Leaf extraction

- add 900 ul PEG lysis buffer (Optigene) with 0.4 g Chelex-100 to a 5 ml extraction tube with a metal ball
- weigh and add leaf material (if not sufficient for 125 mg)
- shake the tube for 1 min
- incubate at room temperature for 20 min
- mix briefly and wait for Chelex to settle
- dilute extract 50 times with dilution buffer

samples	weight
PVY in petunia	110 mg
PVY in potato	50 mg
TMV in petunia	45 mg
healthy petunia	44 mg

Strip layout	
1	PVY in petunia
2	PVY in potato
3	TMV in petunia
4	healthy petunia
5	RNA PVY petunia
6	RNA PVY potato
7	NC

Xanthomonas fragariae

Primer mix

primer	concentration	µl/reaction
F3	0.4 µM	0.1
B3	0.4 µM	0.1
FIP	1.6 µM	0.4
BIP	1.6 µM	0.4
HyClone		1
total		2

Reaction mix

Reaction mix	1
ISO_001	15
HyClone water	1
PM COX	2
PM XF	2
sample	5
Total	25

Sensitivity

Samples: dilution series of gBlocks of X. fragariae spiked to healthy strawberry leaf material

Striplayout	
1	10^6
2	10^5
3	10^4
4	10^3
5	10^2
6	10
7	NTC
8	gBlock XF

Specificity

Samples: DNA samples from 2 isolates of X. fragariae and 12 closely related non-target strains.

Strip layout	
1	*X. fragariae* 3055
2	*X. fragariae* 3083
3	*X. vesicatoria* 552
4	*X. vesicatoria* 513
5	*X. arboricola* pv. *fragariae* 2009
6	*X. arboricola* pv. *fragariae* 2010
7	*X. campestris* pv. *campestris*
8	*X. campestris* pv. *campestris* 634
1	*X. campestris* pv. *phaseoli* 382
2	*X. campestris* pv. *graminis* 712
3	*X. campestris* pv. *populi* 638
4	*X. campestris* pv. *phaseoli* 666
5	*X. campestris* pv. *diefenbachia* 1864
6	*Pseudomonas syringae* pv. *tomato* 919
7	NTC
8	*X. fragariae* gBlock

Infected leaf material

Samples: 2 symptomatic and 4 non-symptomatic strawberry leaves were tested.

Leaf extraction

- add 900 ul PEG lysis buffer (Optigene) with 0.4 g Chelex-100 to a 5 ml extraction tube with a metal ball
- add 125 mg leaf material
- shake the tube for 1 min
- incubate at room temperature for 20 min
- mix briefly and wait for Chelex to settle
- dilute extract 50 times with dilution buffer

Striplayout		Symptoms
1	Leaf 1	no
2	Leaf 2	no
3	Leaf 3	yes
4	Leaf 4	yes
5	Leaf 5	yes
6	Leaf 6	yes
7	NTC	
8	gBlock XF	

Fusarium and *Verticillium*

Primer mix

primer	**concentration**	**µl/reaction**
F3	0.2	0.05
B3	0.2	0.05
FIP	0.8	0.2
BIP	0.8	0.2
LoopF	0.4	0.1
LoopB	0.4	0.1
HyClone water		0.3

Reaction mix

Ingredient	**µl/reaction**
ISO-001	15
primer mix	1
HyClone water	8
sample	1
total volume	25

Fusarium solani

Samples: DNA samples of *F. solani* (MFO25-1, from phalaenopsis), *F. solani* (MFG11-1 from gerbera), *F. oxysporum* (MFO8-1 from phalaenopsis), *F. oxysporum* (MFG1 from gerbera), *F. proliferatum* (MFO20 from phalaenopsis), *F. proliferatum* (Ui11.5.2b from onion)

Reaction program: 65°C for 30 min, default melting curve

Fusarium oxysporum

Samples: DNA samples of *F. oxysporum* (MFO8-1 from phalaenopsis), *F. oxysporum* (MFG1 from gerbera), *F. oxysporum* (Ui76.3.1b from onion), *F. solani* (MFO25-1, from phalaenopsis), *F. proliferatum* (MFO20 from phalaenopsis), *F. proliferatum* (Ui11.5.2b from onion)

Reaction program: 65°C for 30 min, default melting curve

Fusarium proliferatum

Samples: DNA samples of *F. proliferatum* (MFO20 from phalaenopsis), *F. proliferatum* (Ui11.5.2b from onion), *F. proliferatum* (MFG10 from gerbera), *F. solani* (MFO25-1, from phalaenopsis), *F. oxysporum* (MFO8-1 from phalaenopsis), *F. oxysporum* (MFG1 from gerbera)

Reaction program: 65°C for 30 min, default melting curve

Verticillium dahliae

Samples: DNA samples of *Verticillium dahliae*, *F. solani* (MFO25-1, from phalaenopsis), *F. oxysporum* (MFO8-1 from phalaenopsis), *F. oxysporum* (Ui76.3.1b from onion), *F. proliferatum* (MFO20 from phalaenopsis), *F. proliferatum* (MFG10 from gerbera)

Reaction program: 65°C for 30 min, default melting curve

Infected material

Samples: 5 gerbera plants from split into leaves, stems and roots with or without symptoms

Plant	**#**	**sample**	**condition**
1	A1	leaf stem	healthy
	A2	leaf	healthy
	B	flower stem	healthy
	C1	leaf stem	diseased
	C2	leaf	diseased
	D	flower stem	diseased
	E	root	healthy
	F	root	diseased
	G	base	
2	A1	leaf stem	healthy
	A2	leaf	healthy
	C1	leaf stem	diseased
	C2	leaf	diseased
	D	flower stem	diseased
	E	root	healthy
	F	root	diseased
	G	base	
3	A1	leaf stem	healthy
	A2	leaf	healthy
	B	flower stem	healthy
	C1	leaf stem	diseased
	C2	leaf	diseased
	D	flower stem	diseased
	E	root	healthy
	F	root	diseased
	G	base	
4	A1	leaf stem	healthy
	A2	leaf	healthy
	B	flower stem	healthy
	C1	leaf stem	diseased
	C2	leaf	diseased
	D	flower stem	diseased

	E	root	healthy
	F	root	diseased
	G	base	
5	A1	leaf stem	healthy
	A2	leaf	healthy
	B	flower stem	healthy
	C1	leaf stem	diseased
	C2	leaf	diseased
	D	flower stem	diseased
	E	root	healthy
	F	root	diseased
	G	base	

Samples for LAMP extraction

plant 2
A1
A2
B
C1
C2
D
E
F
G
plant 3
A1
A2
B
C1
C2
D
E
F
G
plant 5
D
F
G

Leaf extraction

- add 900 ul PEG lysis buffer (Optigene) with 0.4 g Chelex-100 to a 5 ml extraction tube with a metal ball
- add 125 mg leaf material
- shake the tube for 1 min
- incubate at room temperature for 20 min
- mix briefly and wait for Chelex to settle
- dilute extract 50 times with dilution buffer

TaqMan results

				TaqMan			
Plant	**#**	**sample**	**condition**	Foxy (Ct)	Fsol (Ct)	Fprol (Ct)	Vdal (Ct)
1	A1	leaf stem	healthy	undet.	undet.	undet.	undet.
	A2	leaf	healthy	undet.	undet.	undet.	undet.
	B	flower stem	healthy	undet.	undet.	undet.	undet.
	C1	leaf stem	diseased	undet.	undet.	undet.	undet.
	C2	leaf	diseased	undet.	undet.	undet.	undet.
	D	flower stem	diseased	undet.	undet.	undet.	undet.
	E	root	healthy	undet.	undet.	undet.	undet.
	F	root	diseased	undet.	undet.	undet.	undet.
	G	base		undet.	undet.	undet.	undet.

				TaqMan			
Plant	**#**	**sample**	**condition**	Foxy (Ct)	Fsol (Ct)	Fprol (Ct)	Vdal (Ct)
2	A1	leaf stem	healthy	undet.	undet.	undet.	32.5
	A2	leaf	healthy	undet.	undet.	undet.	undet.
	C1	leaf stem	diseased	undet.	undet.	undet.	30.3
	C2	leaf	diseased	undet.	undet.	undet.	undet.
	D	flower stem	diseased	undet.	undet.	undet.	undet.
	E	root	healthy	undet.	undet.	undet.	31.2
	F	root	diseased	undet.	undet.	undet.	36.8
	G	base		undet.	undet.	undet.	31.4

				TaqMan			
Plant	**#**	**sample**	**condition**	Foxy (Ct)	Fsol (Ct)	Fprol (Ct)	Vdal (Ct)
3	A1	leaf stem	healthy	undet.	undet.	undet.	undet.
	A2	leaf	healthy	undet.	undet.	undet.	undet.
	B	flower stem	healthy	undet.	undet.	undet.	37
	C1	leaf stem	diseased	undet.	undet.	undet.	30.5
	C2	leaf	diseased	undet.	undet.	undet.	31.6
	D	flower stem	diseased	undet.	undet.	undet.	29
	E	root	healthy	undet.	undet.	undet.	undet.
	F	root	diseased	undet.	undet.	undet.	37.2
	G	base		undet.	undet.	undet.	29.7

				TaqMan			
Plant	**#**	**sample**	**condition**	Foxy (Ct)	Fsol (Ct)	Fprol (Ct)	Vdal (Ct)
4	A1	leaf stem	healthy	undet.	undet.	undet.	29.8
	A2	leaf	healthy	undet.	undet.	undet.	undet.
	B	flower stem	healthy	undet.	undet.	undet.	34.5
	C1	leaf stem	diseased	undet.	undet.	undet.	27.4
	C2	leaf	diseased	undet.	undet.	undet.	30.3
	D	flower stem	diseased	undet.	undet.	undet.	26.4
	E	root	healthy	undet.	undet.	undet.	undet.
	F	root	diseased	undet.	undet.	undet.	28.8
	G	base		undet.	undet.	undet.	31.3

				TaqMan			
Plant	**#**	**sample**	**condition**	Foxy (Ct)	Fsol (Ct)	Fprol (Ct)	Vdal (Ct)
5	A1	leaf stem	healthy	undet.	undet.	undet.	28
	A2	leaf	healthy	undet.	undet.	undet.	34.8
	B	flower stem	healthy	undet.	undet.	undet.	31.7
	C1	leaf stem	diseased	undet.	undet.	undet.	29.2
	C2	leaf	diseased	undet.	undet.	undet.	32.4
	D	flower stem	diseased	undet.	undet.	undet.	21.6
	E	root	healthy	undet.	undet.	undet.	31.3
	F	root	diseased	34.4	undet.	undet.	27.6
	G	base		undet.	undet.	undet.	28.2

Multiplex assay

Multiplex chip loading

- Add 1uL LAMP primer mix to chambers through the uncapped conduit
- Dry 1 h at room temperature
- Seal lower channel with piece of plate seal. Make sure the 4 "channels/tubes" are not completely covered with tape.
- Load LAMP reaction mix (enzyme + sample + water) through the LAMP inlet (on the right side of the chip, the lower round inlet)
- Make sure all 4 channels are filled. If filled, remove the mix by pipetting out of the lower channel via the LAMP inlet. Clean with tissue to make sure all reaction mix is gone.
- Seal all inlets with mineral oil starting wih the left oil inlet followed by the RPA and LAMP inlets. Be carefull with the amount of oil, don't overflow the chambers too much
- Incubate the chip at 65°C for 50 min on a heating plate, taking a picture every minute with a DinoLite protable microscope

Ingredient	**µl/reaction**
ISO-001	12
PM	1
HyClone water	6.2
sample	0.8
total	20

Sensitivity

Samples: 10-fold serial dilution series of TMV gBlocks, 10-fold serial dilution series of ToBRFV gBlocks

TMV dilution series (copies/µl)	ToBRRFV dilution series (copies/µl)
10^6	10^6
10^5	10^5
10^4	10^4
10^3	10^3

Chip layout

Chamber 1	Chamber 2	Chamber 3	Chamber 4
NPC (no primers)	TMV primer mix	ToBRFV primer mix	Cox primer mix

Multiplex detection

Samples: TMV and ToBRFV gBlocks at a concentration of 10^6 copies/µl mixed in 1:1 ratio

Chip layout

Chamber 1	Chamber 2	Chamber 3	Chamber 4
Cox primer mix	TMV primer mix	ToBRFV primer mix	NPC

Multiplex detection in tomato extract

Samples: TMV and ToBRFV gBlocks respectively mixed with tomato extract to a concentration of 10^6 copies/µl

Chip layout

Chamber 1	Chamber 2	Chamber 3	Chamber 4
Cox primer mix	TMV primer mix	ToBRFV primer mix	NPC

Multiplex detection with different concentrations

Samples: TMV gBlocks at a concentration of 10^6 copies/µl and ToBRFV gBlocks at a concentration of 10^4 copies/µl in tomato extract.

Chip layout

Chamber 1	Chamber 2	Chamber 3	Chamber 4
Cox primer mix	TMV primer mix	ToBRFV primer mix	NPC

T65 cup

For primer- and reaction mix see ToBRFV colorimetric assays. The samples used were the infected plant sampeles used for the evaluation of the ToBRFV assays: MT3, VT3 and RNA extracted from VT3. Three replicates were prepared in standard PCR tubes and inserted into 3 T65 cups with 4 tubes per cup. The cups were placed into l of boiling water and the water was removed from heat. After 45 minutes the cups were removed from the water bath and color change was evaluated.

4.3 WP3 MinION sequencing

Tobamovirus sequencing

Amplicon generation from RNA

Amplicons were generated from target RNA with each of the respective primer sets. The Promega Access RT-PCR System was used. Reaction mixes of 25 µl contained 1 ul (10 µM) of each primer, 5 µl 5x AMV/Tfl 5X Reaction Buffer, 1 µl MgSO4 (25mM), 0.5 µl Tfl DNA polymerase, 0.5 µl AMV reverse transcriptase, 0.5 µl dNTP (10mM each dNTP), 6 µl RNase-free water and 1 µl target RNA. The reaction contained the following steps: 45 min at 45°C, 2 min at 94°C, 40 cycles of 30 s at 94°C, 30 s at 60°C and 1 min at 68°C, followed by 7 min at 68°C. Per amplicon 1 µl with the addition of 1 µl GelRed solution and 4 µl RNAse-free water was loaded on a 2% agarose gel.

PCR amplifciation protocol

Optimization of the amplification step resulted in the following protocol for DNA targets (i.e., amplicons generated from RNA):

- PCR master mix: 5 µl 5x GoTaq buffer, 1 µl dNTP 5x, 2.5 µl primer mix (3 µM), 0.125 µl GoTaq polymerase, 15.375 µl RNase free water
- To 24 µl of master mix 1 µl of the sample is added
- PCR reaction conditions: 2 min at 94°C followed by 40 cycles of 30 s at 94°C, 30 s of 60°C, and 30 s at 72°C followed by 7 min at 72°C
- The expected amplicon has a size of 250 bp

Optimization of the amplification step from RNA resulted in the following protocol for RNA targets:

- 1 µl of RNA was denatured in a PCR thermocycler together with 4 µl RNAse free water for 3 min at 60°C and cooled with ice.
- RT-PCR master mix: 5 µl 5x AMV/Tfl 5X Reaction Buffer, 0.5 µl dNTP (10 µM), 1 µl MgSO4 (25 mM), 0.5 µl AMV reversde transcriptase, 0.5 µl Tfl DNA polymerase, 2.5 µl primer mix (3 µM) and 10 µl RNase free water
- PCR reaction conditions: 15 min at 45°C, 2 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 60°C and 30 s at 68°C, followed by 7 min at 68°C

Primer sets 3 and 4

Set3

Primer name	Sequence	Tail
CGMMV_fw	CTGTTCGCTCGTTGGATCATCAAGACAGA	TTTCTGTTGGTGCTGATATTGC
CGMMV_rev	CTACCTTTGAACAAGTGCTGCGTATAA	ACTTGCCTGTCGCTCTATCTTC
PMMoV_fw_2	CGAGGTCGATGTTGTTGAAACTCGCAGAACA	TTTCTGTTGGTGCTGATATTGC
PMMoV_rev_2	CGAGGTCGATGTTGTTGAAACTCGCAGAACA	ACTTGCCTGTCGCTCTATCTTC
TMV_fw	GCTTGCAAAGTTTCGATCTCGAACCGGAAAAA	TTTCTGTTGGTGCTGATATTGC
TMV_rev	ATTGGGTCTGCCCACGCTGATGACA	ACTTGCCTGTCGCTCTATCTTC
TMV_fw_2	TTCCCGGATGTACAGCATTCAGCAAA	TTTCTGTTGGTGCTGATATTGC
TMV_rev_2	CCAACTGTGTGTGATACGCACAATTGTTCAA	ACTTGCCTGTCGCTCTATCTTC
ToBRFV_fw	TTGGCCGGTCTGTCTGGAGACCAACCAGA	TTTCTGTTGGTGCTGATATTGC
ToBRFV_rev	ACGGGTTTCGAGATCTATAGCAGCTGTA	ACTTGCCTGTCGCTCTATCTTC
ToMMV_fw_t	CCATGGATGTGTTGGAGTTGGATATTTCCAAA	TTTCTGTTGGTGCTGATATTGC
ToMMV_rev_t	TCGAGGCTAGGCACGCGGCTATAATA	ACTTGCCTGTCGCTCTATCTTC
ToMV_fw	ATTTAATGCTAGGGACCGCAGGCCTAAA	TTTCTGTTGGTGCTGATATTGC
ToMV_rev	ACGTATGCTCGCCCTTTGAACAGATGA	ACTTGCCTGTCGCTCTATCTTC

Set 4

Primer name	Sequence	Tail
CGMMV_fw	CTGTTCGCTCGTTGGATCATCAAGACAGA	TTTCTGTTGGTGCTGATATTGC
CGMMV_rev	CTACCTTTGAACAAGTGCTGCGTATAA	ACTTGCCTGTCGCTCTATCTTC
PMMoV_fw_2	CGAGGTCGATGTTGTTGAAACTCGCAGAACA	TTTCTGTTGGTGCTGATATTGC
PMMoV_rev_2	CGAGGTCGATGTTGTTGAAACTCGCAGAACA	ACTTGCCTGTCGCTCTATCTTC
TMV_fw_3	CTTATCAGAGTGGCAGGCGACGCATTAGA	TTTCTGTTGGTGCTGATATTGC

TMV_rev_3	GCTGCTGTCATTGGGTCGACTTCCAAA	ACTTGCCTGTCGCTCTATCTTC
TMV_fw_4	AAGACCGCCCCGGCTGGTTCGTTTGTTTA	TTTCTGTTGGTGCTGATATTGC
TMV_rev_4	CCCTTAAGAAGGTTGACCTCAGACAATGA	ACTTGCCTGTCGCTCTATCTTC
ToBRFV_fw	TTGGCCGGTCTGTCTGGAGACCAACCAGA	TTTCTGTTGGTGCTGATATTGC
ToBRFV_rev	ACGGGTTTCGAGATCTATAGCAGCTGTA	ACTTGCCTGTCGCTCTATCTTC
ToMMV_fw_t	CCATGGATGTGTTGGAGTTGGATATTTCCAAA	TTTCTGTTGGTGCTGATATTGC
ToMMV_rev_t	TCGAGGCTAGGCACGCGGCTATAATA	ACTTGCCTGTCGCTCTATCTTC
ToMV_fw_2	CGGTTAGACTCGCAAAGTTTCGAACCAAA	TTTCTGTTGGTGCTGATATTGC
ToMV_rev_2	ACAATTCTACAGGGTCAGCCCATACAGATGA	ACTTGCCTGTCGCTCTATCTTC

MinION Sample preparation

Amplicon purification

- Materials
  - Agencourt AMPure XP beads (Beckman Coulter, Cat. no. A63880)
  - Magnetic rack
  - Ethanol (70% fresh prepared) = 500 µl per sample
  - Nuclease free water
  - DNA Lobind Eppendorf tubes (EP0030108051)
  - Hulamixer
- Methods
  - Homogenize the AMpure bead solution by vortexing
  - Transfer PCR product (24 µl) to a clean Lobind tube
  - add bead solution (24 µl) to the 24µl PCR product (ratio 1:1) and mix by flicking the tube
  - Incubate at room temperature for 5 minutes on the Hulamixer
  - Spin tube down in a mini centrifuge
  - Place tube on a magnetic rack and wait until beads have settled on the side of tube (~1-2 minutes)
  - Carefully discard the supernatant ~ 48 µl (keep tube on the magnetic rack)
  - Wash beads by adding 200 µl fresh Ethanol (70%) along the opposite side of the beads
  - Wait 30 s and discard the ethanol
  - Repeat the two previous washing steps
  - Spin tube down in a mini centrifuge and place it back on the magnetic rack to remove residual ethanol
  - Let beads air dry for a maximum of 1 minute and remove tube from the magnetic rack
  - Elute the purified DNA by adding 40 µl of nuclease free water and mix by flicking the tube
  - Incubate at room temperature for 5 minutes on the Hulamixer
  - Spin down tube in a mini centrifuge and place it on the magnetic rack (~1-2 minutes)
  - Pipette off the supernatant to a fresh low DNA bind tube when the solution is clear and discard the beads
- DNA Concentration is determined with the BR dsDNA Qubit kit
- 2 µl is used for the measurement.
- fmol is calculated with: NEBioCalculator (NEBioCalculator)
- ~20 ng is loaded on a 2% agarose gel (0.5xTBE) to evaluate purification

Barcoding

- Materials
  - LongAmp Taq 2X Master Mix (e.g. NEB M0287)
  - Nuclease-free water (e.g. ThermoFisher, cat # AM9937)
  - 1.5 ml Eppendorf DNA LoBind tubes (EP0030108051)
  - PCR Barcoding Expansion kit EXP-PBC001
- Methods
  - Transfer 100 fmol DNA per sample to a 1.5 ml Eppendorf DNA LoBind tube
  - Adjust the volume to 24 µl with nuclease-free water
  - Mix thoroughly by flicking the tube to avoid unwanted shearing
  - Spin down briefly in a microfuge

- Set up a barcoding PCR reaction as follows for each pool: 1 µl PCR barcode (one per sample), 24 µl diluted first round PCR product, 25 µl LongAmp Taq 2x master mix
- Mix gently by flicking the tube, and spin down
- Amplify using the following cycling conditions: 3 min at 95°C followed by 15 cycles of 15 s at 95°C, 15 s at 62°C and 30 s at 65°C followed by 5 min at 65°C.

Second purification

- Materials
  - Agencourt AMPure XP beads (Beckman Coulter, Cat. no. A63880)
  - Magnetic rack
  - Ethanol (70% fresh prepared) = 400µl per sample
  - Nuclease free water
  - 1.5 ml Eppendorf DNA LoBind tubes (EP0030108051)
  - Hulamixer
- Methods
  - Homogenize the AMpure XP bead solution by vortexing
  - Transfer barcoded PCR product (50 µl) to a clean Lobind tube 1.5 ml
  - Add bead solution (50 µl) to the 50 µl PCR product (ratio 1:1) and mix by flicking the tube
  - Incubate at room temperature for 5 minutes on the Hulamixer
  - Spin tube down in a mini centrifuge
  - Place tube on a magnetic rack and wait until beads have settled on the side of tube (~1-2 minutes
  - Carefully discard the supernatant ~ 100µl (keep tube on the magnetic rack)
  - Wash beads by adding 400 µl fresh Ethanol (70%) along the opposite side of the beads
  - Wait 30 s and discard the ethanol
  - Repeat the two previous washing steps
  - Spin tube down in a mini centrifuge and place it back on the magnetic rack to remove residual ethanol
  - Let beads air dry for a maximum of 1 minute and remove tube from the magnetic rack
  - Elute the purified DNA by adding 25 µl of nuclease free water and mix by flicking the tube
  - Incubate at room temperature for 5 minutes on the Hulamixer
  - Spin down tube in a mini centrifuge and place it on the magnetic rack (~1-2 minutes)
  - Pipette off the supernatant to a fresh low DNA bind tube when the solution is clear and discard the beads
  - Quantify the barcoded library using Qubit BR dsDNA kit
  - 2ul of the purified barcoded amplicon are measured
  - pool all barcoded amplicons in the desired ratios.
  - Prepare 0.5 µg of pooled barcoded libraries in 23.5 µl Nuclease-free water.
  - For a Flongle flow cell run a total of 500 ng DNA is needed.

DNA repair and end-prep

- Materials:
  - Agencourt AMPure XP beads
  - NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (cat # E7180S). Alternatively, you can use the three NEBNext® products below:
  - NEBNext FFPE Repair Mix (M6630)
  - NEBNext Ultra II End repair/dA-tailing Module (E7546)
  - NEBNext Quick Ligation Module (E6056)
- Methods
  - Thaw DNA CS (DCS) at RT, spin down, mix by pipetting, and place on ice.
  - Prepare the NEBNext FFPE DNA Repair Mix and NEBNext Ultra II End repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice.
  - Prepare the DNA in Nuclease-free water Transfer 500 ng genomic DNA into a 1.5 ml Eppendorf DNA LoBind tube
  - Adjust the volume to 25 µl with Nuclease-free water
  - Mix thoroughly by flicking the tube to avoid unwanted shearing
  - Spin down briefly in a microfuge

- In a 0.2 ml thin-walled PCR tube mix the following: 0.5 µl DNA CS, 23.5 µl DNA, 1.75 µl NEBNext FFPE DNA Repair Buffer, 1 µl NEBNext FFPE DNA Repair Mix, 1.75 µl Ultra II End-prep reaction buffer, and 1.5 µl Ultra II End-prep enzyme mix
- Mix gently by flicking the tube, and spin down
- Using a thermal cycler, incubate at 20°C for 5 mins and 65°C for 5 mins.

Third purification

- Materials
  - Agencourt AMPure XP beads (Beckman Coulter, Cat. no. A63880) Lot no. 18957500
  - Magnetic rack
  - Ethanol (70% fresh prepared) = 400µl per sample
  - Nuclease free water
  - 1.5 ml Eppendorf DNA LoBind tubes (EP0030108051)
  - Hulamixer
- Methods
  - Homogenize the AMpure XP bead solution by vortexing
  - Transfer the sample to a clean LoBind tube 1.5 ml
  - Add bead solution (30 µl) to the sample and mix by flicking the tube
  - Incubate at room temperature for 5 minutes on the Hulamixer
  - Spin tube down in a mini centrifuge and pellet on a magnet until eluate is clear and colourless
  - Carefully discard the supernatant ~ 100µl (keep tube on the magnetic rack)
  - Wash beads by adding 200 µl fresh Ethanol (70%) along the opposite side of the beads
  - Wait 30 s and discard the ethanol
  - Repeat the two previous washing steps
  - Spin tube down in a mini centrifuge and place it back on the magnetic rack to remove residual ethanol
  - Let beads air dry for a maximum of 1 minute and remove tube from the magnetic rack
  - Elute the purified DNA by adding 31 µl of nuclease free water and mix by flicking the tube
  - Incubate at room temperature for 2 minutes
  - place it on the magnetic rack (~1-2 minutes)
  - Pipette off the supernatant to a fresh low DNA bind tube when the solution is clear and discard the beads

Adapter ligation and clean-up

- Materials:
  - Ligation Sequencing Kit (SQK-LSK109)
  - Flow Cell Priming Kit (EXP-FLP002)
- Methods
  - Spin down the Adapter Mix (AMX) and Quick T4 Ligase, and place on ice.
  - Thaw Ligation Buffer (LNB) at RT, spin down and mix by pipetting.
  - Due to viscosity, vortexing this buffer is ineffective.
  - Place on ice immediately after thawing and mixing.
  - Thaw the Elution Buffer (EB) at RT, mix by vortexing, spin down and place on ice.
  - thaw one tube of Short Fragment Buffer (SFB) at RT, mix by vortexing, spin down and place on ice.
  - in a 1.5 ml Eppendorf DNA LoBind tube, mix in the following order: 30 µl DNA sample from the previous step, 12.5 µl Ligation buffer (LNB), 5 µl NEBNext Quick T4 DNA Ligase, 2.5 µl Adapter Mix (AMX)
  - Mix gently by flicking the tube, and spin down.
  - Incubate the reaction for 10 minutes at RT.
  - Resuspend the AMPure XP beads by vortexing.
  - Add 40 µl of resuspended AMPure XP beads to the reaction and mix by flicking the tube. Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.
  - Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.
  - Wash the beads by adding 250 µl Short Fragment Buffer (SFB).

- Flick the beads to resuspend, spin down, then return the tube to the magnetic rack and allow the beads to pellet.
- Remove the supernatant using a pipette and discard.
- Repeat the previous step.
- Spin down and place the tube back on the magnet.
- Pipette off any residual supernatant.
- Allow to dry for 1 min but do not dry the pellet to the point of cracking.
- Remove the tube from the magnetic rack and resuspend the pellet in 7 µl Elution Buffer (EB).
- Spin down and incubate for 10 minutes at RT.
- Pellet the beads on a magnet until the eluate is clear and colourless.
- emove and retain 7 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.
- Quantify 1 µl of eluted sample using the Qubit HS DNA kit and Qubit fluorometer.
- The prepared library is used for loading into the flow cell. 100 fmol can be loaded without issue.

Flow cell loading

- Materials
  - Ligation Sequencing Kit (SQK-LSK109)
  - Flow Cell Priming Kit (EXP-FLP002)
  - Flongle Sequencing Expansion
- Methods
  - Thaw the Sequencing Buffer II (SBII), Loading Beads II (LBII) and Flush Buffer (FB) from the Flongle Sequencing Expansion and Flush Tether (FLT) from your sequencing kit at RT.
  - Mix the Sequencing Buffer II (SBII), Flush Buffer (FB) and Flush Tether (FLT) tubes by vortexing and spin down at RT.
  - Place the Flongle adapter into the MinION
  - Place the flow cell into the Flongle adapter and press the flow cell down until you hear a click.
  - In a fresh 1.5 ml Eppendorf DNA LoBind tube, mix 117 µl of Flush Buffer (FB) with 3 µl of Flush Tether (FLT) and mix by pipetting.
  - Peel back the seal tab from the Flongle flow cell, up to a point where the sample port is exposed.
  - To prime your flow cell with the mix of Flush Buffer (FB) and Flush Tether (FLT) that was prepared earlier, ensure that there is no air gap in the sample port or the pipette tip.
  - Place the P200 pipette tip inside the sample port and slowly dispense the priming fluid into the Flongle flow cell.
  - To avoid flushing the flow cell too vigorously, load the priming mix by twisting the pipette plunger down.
  - Vortex the vial of Loading Beads II (LBII).
  - Note that the beads settle quickly, so immediately prepare the sequencing mix in a fresh 1.5 ml Eppendorf DNA LoBind tube for loading the Flongle, as follows: 15 µl Sequencing buffer II (SB II), 10 µl Loading beads II (LB II) mixed immediately before use, 5 µl DNA library (diluted in EB)
  - To add the sequencing mix to the flow cell, ensure that there is no air gap in the sample port or the pipette tip
  - Place the P100 tip inside the sample port and slowly dispense the sequencing mix into the flow cell by twisting the pipette plunger down.
  - Seal the Flongle flow cell using the adhesive on the seal tab.

Run settings

- Minimal Q score: 9
- Min barcode score: 60
- demultiplexing and alignment with 1 amplicon sequence for every target in combination with primer set 3

Data analysis

The raw sequencing data is basecalled directly on the Mk1C using high accuracy basecalling. Also debarcoding is done immediately during the sequencing run. The presence of the barcode on both ends of the sequence is required for binning.

MinKNOW

For data analysis directly during the run in the MinKNOW software a custom refrence database was specified containing the expected amplicon sequences in a fasta file. Basecalled and debarcoded reads are aligned to the reference. The advantage of this method is that results can be obtained during the run. However, only few analysis parameters can be adjusted

Decona

As an alternative method, sequences were aligned with the decona pipeline (https://github.com/Saskia-Oosterbroek/decona). The pipeline was run with the following parameters: minimal read length of 250 nt, quality score 10, clustering percentage 80% clustersize 10 and BLAST against the custom reference database also used for MinKnow analysis. The advantage of this method is the possibility to adjust parameters like clustersize and clustering percentage. However, this method has to be carried out after the sequencing run is finished.

Amplicon mixes

Mixes of 3 amplicons that were tested with the primer sets 3 and 4.

PCR #	Bar-code	run #	primer mix + tails	amplicon					
				Ampli-con 1	Ampli-con 2	Ampli-con 4+5	Ampli-con 8	Ampli-con 10	Ampli-con 12
1	BC01	1	3	1E+05			1E+04	1E+03	
2	BC02	1	3	1E+04			1E+03	1E+05	
3	BC03	1	3	1E+03			1E+05	1E+04	
4	BC04	1	3		1E+05	1E+04			1E+03
5	BC05	1	3		1E+04	1E+03			1E+05
6	BC06	1	3		1E+03	1E+05			1E+04
				Ampli-con 1	Ampli-con 3	Ampli-con 6+7	Ampli-con 8	Ampli-con 10	Ampli-con 13
7	BC07	2	4	1E+05			1E+04	1E+03	
8	BC08	2	4	1E+04			1E+03	1E+05	
9	BC09	2	4	1E+03			1E+05	1E+04	
10	BC10	2	4		1E+05	1E+04			1E+03
11	BC11	2	4		1E+04	1E+03			1E+05
12	BC12	2	4		1E+03	1E+05			1E+04

gBlocks
The following gBlocks were tested to assess inclusivity. In every gBlock two target sequences were ligated as the sequences are 250 nt long and the maximum length of a gBlock is 500 nt. For TMV always target sequences from the first and second primer pair were ligated.

Name	Sequence	Primers	SNPsse

		et 1/2	q1/seq2
TMV_gBlock1	**TAATACGACTCACTATAG**GCTTGCAAAGTTTCGATCTCGAACCGGAAAAAAGGGAAAATTAGTAGTAGTGATCGGTCAGTGCCGAACAAGAACTATAGAAATGTTAAGGATTTTGGAGAAATGAGTTTTAAAAAGAATAATTTAATCGATGATGATTCAGAGACTACTGTCGCCGAATCGGATTCGTTTTAAATATGTCTTACAGTATCACTACTCCATCTCAGTTCGTGTTCTTGTCATCAGCGTGGGCCGACCCAATTTTCCGGATGTGCAACACTCCGCGAATCTTATGTGGAATTTTGAAGCAAAACTGTTTAAAAAACAGTATGGATACTTTTGCGGAAGGTATGTAATACATCACGACAGAGGATGCATTGTGTATTACGATCCCCTAAAGTTGATCTCGAAACTTGGTGCTAAACACATCAAGGATTGGGAACACTTGGAGGAGTTCAGAAGGTCTCTTTGTTGAACAATTGTGCGTATTACACACAGTTGG	3	1/3
TMV_gBlock2	**TAATACGACTCACTATAG**GCTTGCAAAGTTTCGATCTCGAACCGGGAAAAAGGGAAAGTTAGTAGTAGTGATCGGTCAATGCCGAACAAGAACTATAGAAATGTTAAGGATTTTGGAGGAATGAGTTTTAAAAAGAATAATTTAATCGATGATGATTCGGAGACTACTGTCGCCGAATCGGATTCGTTTTAAATATGTCTTACAGTATCACTACTCCATCTCAGTTTGTGTTCTTGTCATCAGCGTGGGCTGACCCAATTTTCCAGATGTGCAACACTCCGCGAATCTTATGTGGAATTTTGAAGCAAAACTGTTTAAAAAACAGTATGGATACTTTTGCGGAAGGTATGTAATACATCACGATAGAGGATGCATTGTGTATTACGATCCCCTAAAGTTAATCTCGAAACTTGGTGCTAAACACATCAAGGATTGGGAACACTTGGAGGAGTTCAGAAGGTCTCTTTGTTGAACAATTGTGCGTATTACACACAGTTGG	3	2/8
TMV_gBlock3	**TAATACGACTCACTATAGG**CTTGCAAAGTTTCGATCTCGGACCGGAAAAAAGGGAAAAATAGTAGTAGTGATCGGTCAGTGCCGAACAAGAACTATAGAAATGTTAAGGATTTTGGAGGAATGAGTTTAAAAAAGAATAATTTAATCGATGATGATTCGGAGGCTACTGTCGCCGAATCGGATTCGTTTTAAATATGTCTTACAGTATCACTACTCCATCTCAGTTCGTGTTCTTGTCATCAGCGTGGGCCGACCCAATTTTCCGGATGTGCAACACTCCGCGAATCTTATGTGGAATTTTGAAGCAAAACTGTTTAAAAAACAGTATGGATACTTTTGCGGAAGATATGTAATACATCACGACAGAGGATGCATTGCGTATTACGATCCCCTAAAGTTGATCTCGAAACTTGGTGCTAAACACATCAAGGATTGGGAACACTTGGAGGAGTTCAGAAGGTCTCTTTGTTGAACAATTGTGCGTATTACACACAGTTGG	3	2/7
TMV_gBlock4	TAATACGACTCACTATAGGCTTGCAAAGTTTCGATCTCGAACCGGAAAAAGGGAAAAATAGTAGTAGTGATCGGTCGCAGCCGAACAAGAACTATGGAAATGTTAAAGATTTTGGAGGAATGAGTTTTAAAAAGAATAATTTAATCGATGATGATTTGGAGACTTCTGTCGCCGAATCGGATTCGTTTTAAATATGTCTTACAATATTACTACTCCATCTCAGTTCGTGTTCTTGTCATCAGCGTGGGCTGACCCGATTTTCCGGATGTGCAACATTCCGCGAATCTTATGTGGAATTTTGAGGCAAAACTGTTTAAAAAACAGTATGGATACTTTTGCGGAAGGTATGTAATACATCACGACAGAGGATGCATTGTGTATTACGATCCCCTGAAGTTGATCTCGAAACTTGGTGCTAAACACATCAAGGATTGGGAACACTTGGAGGAGTTCAGAAGGTCTCTTTGTTTTGAACAATTGTGCGTATTACACACAGTTGG	3	2/6
TMV_gBlock5	TAATACGACTCACTATAGACTTGCTAAGTTCCGATCTCGAACTGGAAAAAAGGGAAAAGTAATAATAATATTCGGTCGGTGCCGAACAGGAACCATAGAAATGGCAGAAATTTTAAAGAGGTGGTTGAAAAGAATAATTTAATCGGAGATGATTCGGAGACTTCAGTCGCCGAGTCGGATTCGTTATAAATATGTCTTACACAATTACAACTCCATCTCAGTTCGTGTTCTTGTCATCAGCATGGGCCGATCCAACTTCCCGGATGTACAGCATTCAGCAAATCTCATGTGGAACTTTGAAGCAAAACTGTTCAAAAAACAGTATGGTTACTTCTGCGGAAGGTATGTGATACATCATGATAGAGGCTGTATAGTATATTATGATCCTCTAAAGTTAATCTCGAAACTCGGTGCTAAACACATCAAGGATTGGGAGCACTTAGAGGAGTTCAGAAGATCTCTTTGTTGAACAATTGTGCGTATCACACACAGTTGG	3	6/0
TMV_gBlock6	TAATACGACTCACTATAGCTTATCAGAGTGGCAGGCGACGCATTAGAGATCTATATGTGACTTTCCACGACAGATTAGTGACCGAGTATAAATCCTCTGTGGACATGCCTGCGCTTGACATTAGGAAGAGGATGGAAGAAACGGAAGTGATGTACAATGCACTCTCAGAGTTATCGGTGTTAAGGGAGTCTGACAAATTCGATGTTGATGTTTTTTCCCAGATGTGCAAATCTTTGGAAGTCGACCCAATGACAGCAGCAAGACCGCCCCTCCAGGTTCGTTTGTTTATAAAAGTCTGGTGAAGTATTTGTCTGATAAAGTTCTTTTTAGAAGTTTGTTTATAGATGGCTCTAGTTGTTAAAGGGAAAGTGAACATCAATGAGTTTATCGACCTGACAAAAATGGAAAAGATCTTACCGTCGATGTTTACTCCAGTGAAGAGTGTCATGTGTTCCAAAGTTGATAAAGTTATCATTGTCAGAGGTGAACCTTCTTAAAGG	4	0/5
TMV_gBlock7	TAATACGACTCACTATAGCTTATCAGAGTGGCAGGTGACGCATTAGAGATCTATATGTGACTTTCCACGACAGATTAGTGACTGAGTACAAGGCCTCTGTGGACATGCCTGCGCTTGACATTAGGAAGAAGATGGAAGAAACGGAAGTGATGTACAATGCACTTTCGGAGTTATCGGTGTTAAGGGAGTCTGACAAATTCGATGTTGATGTTTTTTCCCAGATGTGCCAATCTTTGGAAGTTGACCCGATGACAGCAGCAAGACCGCCCCTCCAGGTTCGTTTGTTTATAAAAGTCTGGTGAAGTATTTGTCTGATAAAGTTCTTTTTAGAAGTTTGTTTATAGATGGCTCTAGTTGTTAAAGGAAAAGTGAATATCAATGAGTTTATCGACCTGACAAAAATGGAGAAGATCTTGCCGTCGATGTTTACC	4	3/5

	CCTGTAAAGAGTGTCATGTGTTCCAAAGTTGATAAACATGTCATTGTCAGAGGTAAACCTTC TTAAAGG		
TMV_g Block8	TAATACGACTCACTATAGCTTATCAGCGTGGCAGGTGACGCATTGGAGATCTATATGTGAC TTTCCACGACAGATTAGTGACTGAGTACAAGGCCTCAGTGGACATGCCTGCACTTGACATT AGGAAGAAGATGGAAGAAACGGAAGTGATGTACAATGCACTTTCGGAGTTATCGGTGTTAA GGGAGTCTGACAAATTCGATGTTGGTGTTTTTTCCCAGATGTGCCAATCTTTGGAAGTTGAC CCAATGACAGCAGCAAGACCGCCCCTCCAGGTTCGTTTGTTTATAAAAGTCTGGTGAAGTA TTTGTCTGACAAAGTTCTTTTTAGAAGTTTGTTTATAGATGGCTCTAGTTGTTAAAGGAAAA GTGAATATCAATGAGTTTATCGACCTGACAAAAATGGAGAAGATCTTACCGTCGATGTTTAC CCCTGTAAAGAGTGTCATGTGTTCCAAAGTTGATAACATGTCATTGTCAGAGGTAAACCTTC TTAAAGG	4	4 / 6
TMV_g Block9	TAATACGACTCACTATAGCTTATCAGAGTGGCAGGTGATGCATTAGAGATCTATATGTGACT TTCCACGACAGATTAGTGACTGAGTACAAGGCCTCTGTGGACATGCCTGCGCTTGACATTA GGAAGAAGATGGAAGAAACGGAAGTGATGTACAATGCACTTTCGGAGTTATCGGTGTTAA GGGAGTCTGACAAATTCGATGTTGATGTTTTTTCCCAGATGTGCCAATCTTTGGAAGTTGAC CCAATGACTGCAGCAAGACCGCCCCTCCAGGTTCGTTTGTTTATAAAAGTCTGGTGAAGTA TTTGTCTGATAAAGTTCTTTTTAGAAGTTTGTTTATAGATGGCTCTAGTTGTTAAAGGAAAAG TGAATATCAATGAGTTTATCGACCTGACGAAAATGGAGAAGATCTTACCGTCGATGTTTACC CCTGTGAAGAGTGTCATGTGTTCCAAAGTTGATAAACATGTCATTGTCAGAGGTAAACCTTC TTAAAGG	4	5 / 5
TMV_g Block10	TAATACGACTCACTATAGCTGATTAAAGTGGCGGGTGATGCATTAGAGATTTATATGTAACT TTCCACGACAGGTTAGTAACCGAATACAAAGCCTCAGTTGATATGCCAGCGCTTGACATCA GGAAAAGAATGGAAGAAACAGAGGTGATGTACAACGCGCTTTCGGAACTGTCGGTGCTTA AGGATTCTGACAAATTTGATGTTGATGTTTTTTCCCAGATGTGCCAATCACTGGAGGTGGAC CCAATGACTGCAGCAAAGACCGCCCCGGCTGGTTCGTTTGTTTATAAGAGTTTGGTGAAGT ATCTGTCCGATAAAGTGCTTTTTAGAAGCTTGTTCATAGATGGCTCTAGTTGTTAAAGGGAA AGTGAACATCAATGAGTTTATTGACTTGACCAAAACGGAGAAATTTTTACCGTCGATGTTTA CTCCGGTCAAAAGTGTGATGTGTTCAAAGGTCGACAAGCATTCATTGTCTGAGGTCAACCT TCTTAAGGG	4	1 1 / 0
ToMV_g Block2_ PMMoV _gBlock 1	TAATACGACTCACTATAGATTTAATGCTAGGGACCGCAGGCCTAAAAGTAGTAAGCGAAGA ACAGACGCTTATTGCAACCAAAGCCTACCCAGAATTCCAAATTACATTCTACAACACGCAGA ACGCTGTGCATTCCCTTGCAGGCGGTCTCCGATCATTAGAATTGGAATATCTGATGATGCA AATTCCCTACGGATCATTGACATATGATATCGGAGGTAATTTTGCATCTCATCTGTTCAAAG GGCGAGCATACGTCGAGGTCGATGTTGTTGAAACTCGCAGAACAATATTACCTTCTTCTTG AATCAGAAGTACGAGGGGCAAGTGATGTGCGCGTCAAGCGTTACACGTTCTGTATCGCAT GAAGTTATTCAAGGTGCAGCAGTAATGAATCCAGTGTCTAAACCACTCAAAGGGAAGGTGA TTACATTCACTCAATCTGACAAATCATTGCTGCTCTCAAGGGGTTACGAAGATGTGCACACC GTTCATGAGG	3 / 3 , 4	0 / 0
ToMV_g Block1_ PPMoV_ gBlock2	TAATACGACTCACTATAGATTTAATGCTAGGGACCGCAGGCCTAAAAGTAGTAAGCGAAGA ACAGACGCTTATTGCAACCAAAGCCTACCCAGAATTCCAAATTACGTTCTACAACACGCAGA ATGCTGTGCATTCCCTTGCAGGCGGTCTCCGATCATTAGAATTGGAATATCTGATGATGCA AATTCCCTACGGATCATTGACATATGATATCGGAGGTAATTTTGCATCTCGTCTGTTCAAAG GGCGAGCATACGTCCGAGGTCGATGCTGTTGAAACTCGCAGAACATATCACCTTCTTCTTG AATCAGAAGTACGAAGGGCAAGTTATGTGCACATCAAGTGTTACACGCTCGGTGTCACACG AGGTCATCCAAGGTGCAGCGGTAATGAATCCAGTGTCTAAACCACTTAAAGGGAAGGTGAT TACATTCACTCAGTCAGACAAGTCGTTGCTGCTCTCGAGGGGTTACGAAGATGTGCATACC GTTCATGAGG	3 / 3 , 4	1 / 2
ToMV_g Blocks3 _PPMoV _gBlock 3	TAATACGACTCACTATAGCGGTTAGACTCGCAAAGTTTCGAACCAAAAAAATAATAATAATT TGGGTAAGGGGCGTTCAGGCGGAAGGCCTAAACCAAAAAGTTTTGATGAAGTTGAAAAAG AGTTTGATAATTTGATTGAAGATGAAGCCGAGACGTCGGTCGCGGATTCTGATTCGTATTA AATATGTCTTACTCAATCACTTCTCCATCGCAATTTGTGTTTTTGTCATCTGTATGGGCTGAC CCTGTAGAATTGTCGAAGTCGATGCAGTTGAAACTCGCAGAACAATATTACCTTCTTCTTGA ATCAGAAGTACGAAGGGCAAGTTATGTGCACATCAAGTGTTACACGCTCGGTGTCACACGA GGTCATTCAAGGTGCAGCGGTAATGAATCCAGTGTCTAAACCACTTAAAGGGAAGGTGATT ACATTCACTCAGTCAGACAAGTCATTGCTGCTCTCGAGGGGTTACGAAGATGTGCATACCG TTCATGAGG	4 / 3 , 4	0 / 4
ToMV_g Blocks4 _ToMV V_gBlo ck1	TAATACGACTCACTATAGCGGTTAGACTCGCAAAGTTTCGAACCAAAAAAATAATAATAATT TAGGTAAGGGGCGTTCAGGCGGAAGGCCTAAACCAAAAAGTTTTGATGAAGTTGAAAAAG AGTTTGATAATTTGATTGAAGATGAAGCCGAGACGTCGGTCGCGGATTCTGATTCGTATTA AATATGTCTTACTCAATCACTTCTCCATCGCAATTTGTGTTTTTGTCATCTGTATGGGCTGAC CCTATAGAATTGTCCATGGATGTGTTGGAGTTGGATATTTCCAAATGAGTTTCACTGCGCAG TAGAGTATGAAATCTGGAGAAGACTGGGTCTTGAAGATTTTCTGGCAGAAGTGTGGAAACA AGGGCACAGAAAAACCACTCTTAAAGATTACACAGCTGGTATAAAAACATGTTTATGGTACC AGAGAAAGAGTGGTGATGTTACAACTTTCATAGGAAATACTGTTATTATAGCCGCGTGCCT AGCCTCGA	4 / 3 , 4	1 / 0
ToMV_g Blocks5 _ToMM	TAATACGACTCACTATAGCGGTTAGACTCGCAAAGTTTCGAACCAGAAAAATAATAATAATT TAGGTAAGGGGCGTTCAGGCGGAAGGCCTAAACCAAAAAGTTTTGATGAAGTTGAAAAAG AGTTTGATAATTTGATTGAAGATGAAGCCGAGACGTCGGTCGCGGATTCTGATTCGTATTA AATATGTCTTACTCAATCACTTCTCCATCGCAATTTGTGTTTTTGTCGTCTGTATGGGCTGAC	4 / 3	3 / 2

V_gBlock2	CCTATAGAATTGTCCATGGATGTGTTGGAGTTGGATATTTCCAAATGAGTTTCACTGCGCAG TAGAGTATGAAATCTGGAGAAGACTGGGTCTAGAAGATTTTCTGGCAGAAGTGTGGAAACA AGGGCATAGGAAAACCACTCTTAAAGATTACACAGCTGGTATAAAAACATGTTTATGGTACC AGAGAAAGAGTGGTGATGTTACAACTTTCATAGGAAATACTGTCATTATCGCCGCGTGCCT AGCCTCAA	3,4	
CGMMV_gBlock1_ToBRFV_gBlock1	TAATACGACTCACTATAGCTGTTCGCTCGTTGGATCATCAAGACAGATTTTCTCGTGTGGTC AGCACAGAGCACACCAGGCTTGTAACTGACGCGTATCCGGAGTTTTCGATTAGCTTTACCG CCACCAAGAACTCTGTACACTCCCTTGCGGGTGGTCTGAGGCTTCTTGAATTGGAATATAT GATGATGCAGGTGCCCTACGGCTCACCTTGTTATGACATCGGCGGTAACTATACGCAGCAC TTGTTCAAAGGTAGTTGGCCGGTCTGTCTGGAGACCAACCAGAGGAACGAGGAAATAGTG TCATTAGAGCAATTCCACATGGCAACGGCTAGTTCGTTAATTCGGAAACAGATGAGTTCGA TTGTGTACACGGGCCCCATTAAAGTTCAGCAAATGAAAAACTTTATTGATAGCCTGGTAGCA TCACTCTCTGCTGCGGTGTCGAACCTAGTCAAGATCCTAAAGGATACAGCTGCTATAGATC TCGAAACCCGT	3,4	1/0
CGMMV_gBlock2_ToBRFV_gBlock2	TAATACGACTCACTATAGCTGTTCGCTCGTTGGATCATCAAGATAGATTTTCTCGTGTGGTC AGCACAGAGCACACCAGGCTTGTAACTGATGCGTATCCGGAGTTTTCGATTAGCTTTACCG CCACTAAGAACTCTGTACACTCCCTTGCGGGTGGTCTGAGGCTTCTTGAATTGGAATATAT GATGATGCAAGTGCCCTACGGCTCACCTTGTTATGACATCGGCGGTAACTATACGCAGCAC TTGTTCAAAGGTAGTTGGCCGGTCTGTCTGGCGACCAACCAGAGGAACGAGGAAATAGAG TCATTAGAGCAATTCCACATGGCAACGGCTAGTTCGTTAATTCGGAAACAGATGAGTTCGA TTGTGTACACGGGCCCCATTAAAGTTCAGCAAATGAAAAACTTTATTGATAGCCTGGTAGCA TCACTCTCTGCTGCGGTGTCGAACCTAGTCAAGATCCTAAAGGATACAGCTGCTATAGATC TCGAAACCCGT	3,4	2/1

RNA samples for sequencing with primer set 3

No.	**Virus**	**Origin**
1	CGMMV	Naktuinbouw
2	PMMoV	Naktuinbouw
3	TMV	Naktuinbouw
4	ToMMV	Naktuinbouw
5	ToMV	Naktuinbouw
6	ToBRFV	WPR
7	Mix CGMMV+ToBRFV	
8	Mix PMMoV+ ToMV	
9	Mix TMV+ ToMMV	
10	Mix CGMMV+ToMV	
11	Mix PMMoV+ToMMV	
12	Mix TMV+ ToBRFV	

FTA card samples

FTA card samples were provided by East-West seed and used for WP1, 2 and 3. The samples TMV, ToMV, PPMoV and CGMMV were used for MinIon sequencing. Extracts were prepared as described in WP1 and used as input for the cDNA synthesis.

Material	Origin	RNA/FTA
CGMMV dried	EWS	FTA
PMMoV	EWS	FTA
TMV	EWS	FTA
ToMV	EWS	FTA
CGMMV	NAKt	RNA
PMMoV	NAKt	RNA
TMV	NAKt	RNA
ToMMV	NAKt	RNA
ToBRFV	WPR	RNA
TMV+ToMV	NAKt	RNA
ToMMV+ToBRFV	NAKt/WPR	RNA

Protocol for direct cDNA sequencing

The protocol is based on Liefting et al. (2021).

Material

- RapidOut DNA Removal kit (Thermo Scientifc)
- QIAseq FastSelect -rRNA Plant kit (Qiagen)

- 5X First-Strand buffer supplied with SuperScript III Reverse Transcriptase (Thermo Scientific)
- Agencourt AMPure XP beads (Beckman Coulter)
- Maxima H Minus Double Strand cDNA synthesis kit (Thermo Scientific)
- Amplicon purification kit (Roche)
- NEBNext Ultra II End Repair/dA-Tailing Module (New England Biolabs)
- NEB Blunt/TA Ligase Master Mix (New England Biolabs)
- ONT Direct cDNA Sequencing kit (SQK-DCS109)
- ONT Flow Cell Priming kit (EXP-FLP002)
- ONT Flongle Sequencing Expansion kit (EXP-FSE001)
- Qubit RNA HS Assay kit (Thermo Scientific)
- Qubit 1x dsDNA HS Assay kit (Thermo Scientific)
- Ethanol
- Isopropanol
- Nuclease free water

Methods

- Host ribodepletion
    - Add components as listed into a 0.2 ml PCR tuber on ice: 1 µg RNA (DNA removed), 8 µl 5X First-Strand Buffer, 1 µl QIAseq FastSelect-rRNA Plant, Nuclease free water to 40 µl
    - Place tubes in a thermocycler with the following program: 2 min at 75°C, 2 min at 70°C, 2 min at 65°C, 2 min at 60°C, 2 min at 55°C, 2 min at 37°C and 2 min at 25°C.
    - Transfer the sample to a 1.5 ml LoBind tube and add 32 µl of resuspended AMPure XP beads; mix by pipetting up and down at leas 10 times, spin down.
    - Incubate at room temperature for 5 min.
    - Add 200 µl of freshly prepared 80% ethanol to the tube while in the magnetic rack. Wait for 30 s, then carefully remove and discard the ethanol.
    - Repeat the previous step for a total of 2 washing steps. Completely remove all traces of ethanol after the second wash.
    - Air dry the beads for 5 min while the tube is in the magnetic rack. Do not over dry.
    - Elute the RNA from the beads with 15 µl of nuclease-free water. Pipette mix 10 times and spin down.
    - Incubate at room temperature for 2 min.
    - Place the tube in a magnetic separation rack until the solution is clear.
    - Remove 14 µl of the supernatant into a new PCR tube and place on ice.
    - Quantitate the ribodepleted RNA using a Qubit fluorometer.
- Double-stranded cDNA synthesis
    - Add the following components to a 0.2 ml PCR tube on ice: 13 µl Ribosomal RNA depleted RNA, 1 µl Random hexamer 100 µM from the Maxima kit.
    - Mix gently by pipetting, spin down and incubate at 65°C for 5 min. Chill on ice, spin down again and place on ice.
    - Add the following components: 5 µl 4x First-Strand Reaction Mix, 1 µl First-Strand Enzyme Mix.
    - Mix gently by pipetting, spin down and incubate at 25°C for 10 min, followed by 50°C for 30 min.
    - Terminate the reaction by heating at 85°C for 5 min, then place on ice.
    - Continue immediately with the second-strand synthesis reaction. Second-strand cDNA synthesis.
    - Add the components listed in Table 5 in the indicated order to give a total volume of 100 µL.
    - The sample was divided over 2 tubes.
    - Second strand cDNA reaction mix: 20 µl First-Strand cDNA Synthesis Reaction Mix, 55 µl Nuclease free water, 20 µl 5X Second-Strand Reaction Mix, 5 µl Second-Strand Enzyme Mix.
    - Mix by pipetting and spin down.
    - Incubate at 16 ◦C for 60 min.
    - Stop the reaction by adding 6 µl of 0.5 M EDTA, pH 8.0 and mix gently.

    - Remove residual RNA by adding 10 µl of RNase I to the second-strand synthesis reaction tube and incubate at room temperature for 5 min. Double-stranded cDNA purification using the Roche amplicon purification kit.
    - To 116 µl add 580 µl Binding buffer and mix well
    - Insert one High Pure Filter Tube into one Collection Tube.
    - Transfer the sample from step 1 using a pipette to the upper reservoir of the Filter Tube.
    - Centrifuge 30 s at 13000g in a standard table top centrifuge at RT.
    - Disconnect the Filter Tube, and discard the flow through solution.
    - Reconnect the Filter Tube to the same Collection Tube.
    - Add 500 µl Wash Buffer to the upper reservoir.
    - Centrifuge 30 s at 13000g.
    - Discard the flow through solution.
    - Recombine the Filter Tube with the same Collection Tube.
    - Add 200 µl Wash Buffer.
    - Centrifuge 30 s at 13000g.
    - Discard the flow through solution and Collection Tube.
    - Centrifuge the empty purification column for an additional 2 min at 13000g to completely remove any residual wash buffer.
    - Transfer the purification column to a new 1.5 ml DNA LoBind tube.
    - Add 30 µl of Elution Buffer (prewarmed to 65°C) to the centre of the column membrane and incubate for 1 min.
    - Centrifuge at 13000g for 1 min.
    - Pipette the eluate to the centre of the same column membrane and incubate for 1 min.
    - Centrifuge at 13000g for 1 min. Discard the purification column.
    - Quantitate the cDNA using a Qubit fluorometer. 2 ul was used for the measurement.
- End-prep
    - Perform end repair and dA-tailing of cDNA mixing the following components in a 0.2 ml PCR tube: 25 µl Purified double-stranded cDNA sample, 3.5 µl Ultra II End-Prep Reaction Buffer and 1.5 µl Ultra II End-Prep Enzyme Mix
    - Mix gently by pipetting and spin down.
    - Incubate at 20°C for 5 min and 65°C for 5 min.
    - Transfer the sample to a new 1.5 ml LoBind tube, add 30 µl of resuspended AMPure XP beads to the end-prep reaction and mix by pipetting.
    - Incubate on a Hula mixer at room temperature for 5 min.
    - Spin down the sample and place the tube in a magnetic separation rack. After the solution is clear, slowly remove and discard the supernatant, being careful not to disturb the beads.
    - Add 200 µl of freshly prepared 70% ethanol to the tube while in the magnetic rack. Carefully remove and discard the ethanol.
    - Repeat the previous step for a total of two washing steps.
    - Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for ~30 sec, but do not dry the pellet to the point of cracking.
    - Remove the tube from the magnetic rack and resuspend the pellet in 24 µl of nuclease free water. Incubate at room temperature for 2 min.
    - Place the tube in a magnetic separation rack until the solution is clear.
    - Remove 22.5 µl of the supernatant into a new 1.5 ml LoBind tube.
- Adapter Ligation
    - Perform adapter ligation of the end-prepped cDNA by assembling the following reaction mix: 22.5 µl End-prepped cDNA, 2.5 µl Adapter Mix (AMX) and 25 µl Blunt/TA Ligation Master Mix
    - Mix gently by flicking the tube and spin down.
    - Incubate at room temperature for 10 min.
    - Add 20 µl of resuspended AMPure XP beads to the adapter ligation mix and mix by pipetting.
    - Incubate on a Hula mixer at room temperature for 5 min. (f) Place the tube in a magnetic separation rack until the solution is clear and pipette off the supernatant.
    - Add 100 µL of wash buffer (WSB) to the beads and resuspend by pipetting to remove free adapter. Return the tube to the magnetic separation rack, allow the beads to pellet and pipette off the supernatant.
    - Repeat the previous step.

    - Spin down and place the tube in the magnetic separation rack. Pipette off residual supernatant.
    - Remove the tube from the magnetic separation rack and dry the pellet for 30 s. Resuspend the beads in 7 µl of Elution Buffer (EB).
    - Incubate on a Hula mixer at room temperature for 10 min.
    - Pellet the beads on a magnet until the eluate is clear.
    - Remove and retain 7 µl of eluate into a clean 1.5 ml LowBind tube.
    - Quantitate 1 µl of eluted cDNA using a Qubit fluorometer.
    - Store the library on ice until ready to load the Flongle flow cell.
- Priming and loading the flongle flow cell
    - Perform a Flongle flow cell check with the MinKNOW software. Keep the flow cell in the MinION device for priming and loading of the flow cell.
    - In a new 1.5 ml LoBind tube, mix 117 µl of Flush Buffer (FB) with 3 µl of Flush Tether (FLT) and mix by pipetting. Use this mix to prime the Flongle flow cell according to the protocol on the Oxford Nanopore website.
    - Prepare the sequencing mix in a new 1.5 ml LowBind tube: 14 µl Sequencing buffer II (SQBII), 10 µl Loading Beads II (LBII) and 6 µl cDNA library
    - Immediately load the sequencing mix into the Flongle flow cell according to the protocol on the ONT website.
    - Run the Flongle flowcell for 30h or stop when the sequencing speed and quality of the reads drops below the Quality score of 7 or when no pores are sequencing anymore.

Nematode sequencing

DNA extraction from nematode suspensions in water (Qiagen DNeasy Blood & Tissue kit)

- Centrifuge suspensions fro 30 min at 500 rpm
- Carefully remove supernatant
    - If there are still nematodes present in the supernatant centrifuge again for 10 min at 1000 rpm and carry forward as an additional sample
- Resuspend the pellet and transfer to a 2 ml Eppendorf tube
- Centrifuge for 5 min at 1000 rpm
- Carefully remove the supernatant
- Add a pinch of silicon carbide beads to the samples
- Vortex samples for 1 min
- Add 20 µl proteinase K and 180 µl ATL buffer to wach sample
- Incubate overnight at 56°C and 2000 rpm
- Transfer the samples to a new 2 ml tube avoiding carry-over of the silicon beads
- Add 200 µl ethanol (96-100%), mix by vortexing
- Pipet the samples into a DNeasy Mini spin column placed in a 2 ml collection tube
- Centrifuge at ≥6000xg for 1 min, discard the flow-through and the collection tube
- Place the spin column in a new 2 ml collection tube, add 500 µl buffer AW1
- Centrifure at ≥6000xg for 1 min, discard the flow-through
- Add 500 µl buffer AW2 and centrifuge for 3 min at 20,000xg
- Place the spin-column in a new 2 ml collection tube, centrifuge for 1 min at 20,000xg
- Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube
- Elute the DNA by adding 100 µl buffer AE to the center of the spin column membrane
- Incubate at room temperature for 1 min, centrifuge for 1 min at ≥6000xg

Nematode sequencing

P. penetrans TaqMan

Reaction mix

Component	Volume/sample (µl)
PerfeCTa qPCR ToughMix Low RIX (2x)	10
Forward primer (10 µM)	0.5
Reverse primer (10 µM)	0.5
Probe (5 µM)	1

RNAse free water	7
Total volume	19
Sample volume	1

Reaction program

Temperature	Time	Cycles
95°C	5 min	
95°C	30 s	40 x
62°C	30 s	40 x

Amplification

The amplification was carried out with primers obtained from the Nematology Chair group and can therefore not be disclosed in this report.

Reaction mix

Component	Volume/sample (µl)
Q5 Reaction buffer (5x)	10
Forward primer (10 µM)	2.5
Reverse primer (10 µM)	2.5
dNTPs (5 mM)	2
Q5 HF DNA polymerase	0.5
RNAse free water	27.5
Target	5
Total	50

Reaction program

Temperature	Time	Cycles
98°C	30 s	
98°C	10 s	30 x
58°C	30 s	30 x
72°C	1 min	30x
72°C	2 min	

Protocol for sequencing

Material

- Qubit dsDNA HS assay kit
- NEB Q5 Hot Start High Fidelity
- NEB LongAmp Taq 2X Master Mix
- Agencourt AMPure XP beads
- NEBNext Ultra II End repair/dA-tailing Module
- NEBNext FFPE DNA Repair Mix
- NEBNext Quick Ligation Module
- SQK-LSK109 Ligation sequencing kit
- EXP-FLP002 Flow cell priming kit
- EXP-FSE001 Flongle expansion kit
- EXP-PBC001 Barcoding expansion 1-12
- 70% Ethanol
- Eppendorf Lobind tubes

Methods

- Amplicon purification
    - Homogenize the AMPure bead solution by vortexing.
    - Transfer 80 µl PCR product to a clean 1.5 ml Lobind tube .
    - Add 144 µl bead solution to the 80 µl PCR product (ratio 1.8) and mix by flicking the tube.
    - Incubate at room temperature for 2 min.
    - Spin tube down in a mini centrifuge.
    - Place tube on a magnetic rack and wait until beads have settled on the side of tube (2 minutes).
    - Carefully discard the supernatant ~140 µl (keep tube on the magnetic rack).
    - Wash beads by adding 400 µL fresh Ethanol (70%) along the opposite side of the beads.
    - Wait 30 s and discard the ethanol.
    - Repeat the two previous washing steps.
    - Spin tube down in a mini centrifuge and place it back on the magnetic rack to remove residual ethanol.
    - Let beads air dry for a maximum of 2 min and remove tube from the magnetic rack.
    - Elute the purified DNA by adding 55 µl of nuclease free water and mix by flicking the tube.
    - Incubate at room temperature for 2 min.
    - Spin down tube in a mini centrifuge and place it on the magnetic rack (2 min).
    - Pipette off the supernatant to a fresh tube when the solution is clear and discard the beads.
    - Measure amplicons concentration in a Qubit fluorometer and pool amplicons in the intended ratios.

Mixes of *P. penetrans* en *G. pallida* amplicons for sequencing

Barcode	Percentage of *P. penetrans* DNA	Percentage of *G. pallida* DNA
BC07	100%	0%
BC08	99%	1%
BC09	90%	10%
BC10	10%	90%
BC11	1%	99%
BC12	0%	100%

- Barcoding PCR
    - Mix 200 fmol of PCR product (24 µl) with 1 µl PCR barcode and 25 µl LongAmp Taq 2x Master mix.
    - Spin down briefly.
    - Carry out the barcoing PCR at 95°C for 3 min, followed by 15 cylces of 15 s at 95°C, 15 s at 62°C and 2 min at 65°C followed by 5 min at 65°C.
- Amplicon purification
    - Homogenize the AMPure bead solution by vortexing.
    - Transfer 50 µl PCR product to a clean Lobind tube 1.5 ml.
    - Add 90 µL bead solution to the 50 µL PCR product (ratio 1.8) and mix by flicking the tube.
    - Incubate at room temperature for 2 minutes.
    - Spin tube down in a mini centrifuge.
    - Place tube on a magnetic rack and wait until beads have settled on the side of tube (2 minutes).
    - Carefully discard the supernatant ~140 µl (keep tube on the magnetic rack).
    - Wash beads by adding 400 µL fresh ethanol (70%) along the opposite side of the beads.
    - Wait 30 s and discard the ethanol.
    - Repeat the two previous washing steps.
    - Spin tube down in a mini centrifuge and place it back on the magnetic rack to remove residual ethanol.
    - Let beads air dry for a maximum of 2 minute and remove tube from the magnetic rack.
    - Elute the purified DNA by adding 35 µl of nuclease free water and mix by flicking the tube.

    - Incubate at room temperature for 2 minutes.
    - Spin down tube in a mini centrifuge and place it on the magnetic rack (2 minutes).
    - Pipette off the supernatant to a fresh tube when the solution is clear and discard the beads.
    - Measure the DNA sample concentration using a Qubit fluorometer with the Qubit dsDNA BR assay kit using 2 µl of sample.
    - Pool equimolar amounts of each barcoded sample into a 1.5 ml Lobind tube.
    - Prepare 1 µg of pooled barcoded libraries in 47 µl nuclease free water.
- DNA repair and End-prep
    - Prepare the NEBNext FFPE DNA Repair Mix and NEBNext Ultra II End Repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice.
    - Mix the following in a 0.2 ml thin-walled PCR tube: 1 µl DNA CS, 47 µl DNA, 3.5 µl NEBNext FFPE DNA Repair buffer, 2 µl NEBNext DNA Repair mix, 3.5 µl Ultra II End-prep reaction buffer, 3 µl Ultra II End-prep enzyme mix.
    - Incubate at 20°C for 5 min and at 65°C for 5 min.
- End-prep and clean-up
    - Resuspend the AMPure XP beads by vortexing.
    - Transfer the sample (60 µl) to a clean 1.5 ml Eppendorf DNA LoBind tube.
    - Add 60 µl of resuspended AMPure XP beads to the end-prep reaction and mix by flicking the tube
    - Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.
    - Prepare 500 µl of fresh 70% ethanol in Nuclease-free water.
    - Spin down the sample and pellet on a magnet until eluate is clear and colourless (~1-2 minutes). Keep the tube on the magnet, and pipette off the supernatant.
    - Keep the tube on the magnet and wash the beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet.
    - Remove the ethanol using a pipette and discard.
    - Repeat the previous step.
    - Spin down and place the tube back on the magnet. Pipette off any residual ethanol.
    - Allow to dry for 1 minute. Do not overdry the pellet.
    - Remove the tube from the magnetic rack and resuspend the pellet in 61 µl Nuclease-free water.
    - Spin down and incubate for 2 minutes at RT.
    - Pellet the beads on a magnet until the eluate is clear and colourless (~1-2 minutes).
    - Remove and retain 61 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.
- Ligation of Sequencing adapters
    - *Spin down* the Adapter Mix (AMX) and Quick T4 Ligase, and place on ice.
    - Thaw Ligation Buffer (LNB) at *room temperature*, *spin down* and mix by pipetting. Due to viscosity, vortexing this buffer is ineffective. Place on ice immediately after thawing and mixing.
    - Thaw the Elution Buffer (EB) at *room temperature*, mix by vortexing, *spin down* and place on ice.
    - To retain DNA fragments of all sizes, thaw one tube of Short Fragment Buffer (SFB) at *room temperature*, mix by vortexing, *spin down* and place on ice.
    - In a 1.5 ml Eppendorf DNA LoBind tube, mix in the following order: 60 µl DNA sample, 25 µl ligatio buffer (LNB), 10 µl NEBNext Quick T4 DNA Ligase and 5 µl Adapter mix (AMX).
    - Incubate for 10 min at room temperature
- Clean-up
    - Resuspend the AMPure XP beads by vortexing.
    - Add 40 µl of resuspended AMPure XP beads to the reaction (100 µl) and mix by pipetting.
    - Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.
    - Spin down the sample and place on magnetic rack, allow beads to pellet and pipette off supernatant.
    - Wash the beads by adding 250 µl Short Fragment Buffer (SFB).
    - Important: To remove the adapters, flick the beads and resuspend thoroughly, spin down, then return the tube to the magnetic rack and allow the beads to pellet.
    - Remove the supernatant using a pipette and discard.
    - Repeat the previous step.
    - Spin down and place the tube back on the magnet. Pipette off any residual supernatant.

    - Allow to dry for 1 minute. Do not overdry the pellet.
    - Remove the tube from the magnetic rack and resuspend the pellet in 15 µl Elution Buffer (EB).
    - Spin down and incubate for 10 minutes at room temperature. For high molecular weight DNA, incubating at 37°C can improve the recovery of long fragments.
    - Pellet the beads on a magnet until the eluate is clear and colourless (~1-2 minutes).
    - Remove and retain 15 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.
    - Measure the DNA sample concentration using a Qubit fluorometer with the Qubit dsDNA HS assay kit using 1 µL sample.
- Prime cell priming and loading
    - Perform a Flongle flow cell check with the MinKNOW software. Keep the flow cell in the MinION device for priming and loading of the flow cell.
    - Mix the Sequencing Buffer II (SBII), Flush Buffer (FB) and Flush Tether (FLT) tubes by vortexing and spin down at RT.
    - In a fresh 1.5 ml Eppendorf DNA LoBind tube, mix 117 µl of Flush Buffer (FB) with 3 µl of Flush Tether (FLT) and mix by pipetting.
    - Peel back the seal tab from the Flongle flow cell, up to a point where the sample port is exposed.
    - Before priming the Flongle flow cell with the mix of Flush Buffer (FB) and Flush Tether (FLT) ensure that there is no air gap in the sample port or in the pipette tip.
    - Place the P200 pipette tip inside the sample port and slowly dispense the priming fluid into the Flongle flow cell.
    - To avoid flushing the flow cell too vigorously, load the priming mix by twisting the pipette plunger down.
    - Prepare the sequencing mix containing 15 µl Sequencing buffer II (SBII), 10 µl Loading beads (LBII) and 5 µl DNA library (50 fmol is recommended)
    - To add the sequencing mix to the flow cell, ensure that there is no air gap in the sample port or the pipette tip.
    - Place the P100 tip inside the sample port and slowly dispense the sequencing mix into the flow cell by twisting the pipette plunger down.
    - Seal the Flongle flow cell using the adhesive on the seal tab.
    - Replace the sequencing platform lid.
    - Run for 24 h .

4.4 References

Dauphinais, N., Vandal, M., Gagnon, A.-È., Bélair, G., Véronneau, P.-Y., Mimee, B., 2018. Development and application of a multiplex qPCR method for the simultaneous detection and quantification of Pratylenchus alleni and P. penetrans in Quebec, Canada. Plant Dis. 102, 970-976.
Della Bartola, M., Byrne, S., Mullins, E., 2020. Characterization of potato virus Y isolates and assessment of nanopore sequencing to detect and genotype potato viruses. Viruses 12, 478.
Ferdousi, A., Shahhossein, M., Bayat, M., Hashemi, S., Ghahri, M., 2014. Comparison of polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) for diagnosis of Fusarium solani in human immunodeficiency virus (HIV) positive patients. African Journal of Biotechnology 13.
Ghosh, D., Roy, K., Williamson, K.E., White, D.C., Wommack, K.E., Sublette, K.L., Radosevich, M., 2008. Prevalence of Lysogeny among Soil Bacteria and Presence of 16S rRNA and trzN Genes in Viral-Community DNA. Appl. Environ. Microbiol. 74, 495-502.
Hanssen, I.M., Thomma, B.P.H.J., 2010. Pepino mosaic virus: a successful pathogen that rapidly evolved from emerging to endemic in tomato crops. Mol. Plant Pathol. 11, 179-189.
ISHI-Veg, 2019. Tomato brown rugose fruit virus. ISHI-veg develops new, specific detection method.
Kawanobe, M., Toyota, K., Ritz, K., 2021. Development and application of a DNA metabarcoding method for comprehensive analysis of soil nematode communities. Appl. Soil Ecol. 166, 103974.
Kenmotsu, H., Uchida, K., Hirose, Y., Eki, T., 2020. Taxonomic profiling of individual nematodes isolated from copse soils using deep amplicon sequencing of four distinct regions of the 18S ribosomal RNA gene. PLOS ONE 15, e0240336.
Kim, D.-R., Gang, G.-H., Jeon, C.-W., Kang, N.J., Lee, S.-W., Kwak, Y.-S., 2016. Epidemiology and Control of Strawberry Bacterial Angular Leaf Spot Disease Caused by Xanthomonas fragariae. Plant Pathol. J. 32, 290-299.
Liefting, L.W., Waite, D.W., Thompson, J.R., 2021. Application of Oxford Nanopore Technology to Plant Virus Detection. Viruses 13.
Oya, H., Nakagawa, H., Saito, N., Uematsu, H., Ohara, T., 2008. Detection of Acidovorax avenae subsp. citrulli from seed using LAMP method. Japanese Journal of Phytopathology (Japan).
Qiao, N., Dai, H., Liu, J., Zhu, X., Li, J., Zhang, D., Liu, Y., 2020. Detection of melon necrotic spot virus by one-step reverse transcription loop-mediated isothermal amplification assay. PLOS ONE 15, e0230023.
Tian, Q., Zeng, D., Li, B., Zhang, H., Wang, Y., Zheng, X., 2016. Rapid detection of Verticillium albo-atrum using a loop-mediated isothermal amplification assay. Journal of Nanjing Agricultural University 39, 582-588.
Treder, K., Chołuj, J., Zacharzewska, B., Mielczarek, M., 2017. Detection of potato virus Y (PVY) by reverse-transcription loop-mediated nucleic acid amplification (RT-LAMP). Plant Breeding and Seed Science 75, 77-85.
Wang, Y., Wang, C., Wang, L., Zhang, X., Yan, J., Wang, J., Wang, M., 2020. Development of loop-mediated isothermal amplification (LAMP) assay for rapid detection of Fusarium proliferatum causing ear and kernel rot on maize. Crop Prot. 132, 105142.
Yasuhara-Bell, J., Kubota, R., Jenkins, D.M., Alvarez, A.M., 2013. Loop-Mediated Amplification of the Clavibacter michiganensis subsp. michiganensis micA Gene Is Highly Specific. Phytopathology® 103, 1220-1226.