Plant Disease • 2022 • 106:2927-2939 • https://doi.org/10.1094/PDIS-10-21-2312-RE

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Genome-Informed Design of a LAMP Assay for the Specific Detection of the Strain of '*Candidatus* Phytoplasma asteris' Phytoplasma Occurring in Grapevines in South Africa

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Abstract

Grapevine yellows is one of the most damaging phytoplasma-associated diseases worldwide. It is linked to several phytoplasma species, which can vary regionally due to phytoplasma and insect-vector diversity. Specific, rapid, and reliable detection of the grapevine yellows pathogen has an important role in phytoplasma control. The purpose of this study was to develop and validate a specific loop-mediated isothermal amplification (LAMP) assay for detection of a distinct strain of grapevine '*Candidatus* Phytoplasma asteris' that is present in South Africa, through implementation of a genome-informed test design approach. Several freely available, user-friendly, web-based tools were coupled to design the specific LAMP assays. The criteria for selection of the assays were set for each step of the process, which resulted in four experimentally operative LAMP assays that targeted the *ftsH/hflB* gene region, specific to the aster yellows phytoplasma strain from South Africa. A real-time PCR was developed, targeting the same genetic region, to provide extensive validation of the LAMP assay. The validated molecular assays are highly specific to the targeted aster yellows phytoplasma strain from South Africa, with good sensitivity and reproducibility. We show a genome-informed molecular test design and an efficient validation approach for molecular tests if reference and sample materials are sparse and hard to obtain.

Keywords: aster yellows, fruit, LAMP, molecular detection, pathogen detection, phytoplasma, Prokaryotes

Grapevine yellows (GY) is a disease of *Vitis vinifera* associated with several phytoplasmas that is present in grapevine-growing regions throughout the world, and that can lead to severe yield losses (Dermastia et al. 2017). The epidemiological cycles of GY show regional variations due to diversity of phytoplasma species and insect vectors (Bertaccini et al. 2014).

In South Africa, symptoms of GY were observed for the first time in 2006 (Botti and Bertaccini 2006). The main pathogen that causes GY in South Africa is an aster yellows phytoplasma strain (aster yellows from South Africa [AY-SA]) classified within the 16SrI-B subgroup, which correspond to '*Candidatus* Phytoplasma asteris' (Engelbrecht et al. 2010; Carstens et al. 2011). This pathogen was first confirmed in vineyards in South Africa in the Vredendal region of the Olifantsriver Valley (Engelbrecht et al. 2010), and has since been reported in the wine-producing regions of Waboomsrivier, and Robertson (Carstens, 2014). As well as their main host (*V. vinifera*), AY-SA have been detected in various species of wild and crop plants that grow in the vicinity of vineyards (Kruger et al. 2015). These phytoplasmas are transmitted by a leafhopper indigenous to South Africa, *Mgenia fuscovaria* (Kruger et al. 2018). The common symptoms of GY often lead to abortion of immature berries (Engelbrecht et al. 2010) and, consequently, result in high yield losses, which can reach 30% for certain grapevine cultivars (Carstens 2014; Constable and Bertaccini 2017).

Knowledge gaps in the disease cycles of GY remain, with the need for specific, rapid, and reliable detection of the pathogen, preferably in an on-site setting. Loop-mediated isothermal amplification (LAMP) is among the promising methods for detection of phytoplasmas. This molecular method generates high quantities of amplified DNA from a specific DNA fragment using a set of four to six primers (Notomi et al. 2000). The advantages of LAMP include isothermal amplification that does not require expensive apparatus, speed, and high resilience to various inhibitors present in samples (Hara-Kudo et al. 2007; Kaneko et al. 2007; Wang et al. 2009; Zhang et al. 2013). These characteristics allow detection of pathogenic bacteria in the field, including phytoplasmas, with minimum sample preparation (Kogovšek et al. 2015, 2017). LAMP tests have already been developed for universal detection of different phytoplasmas of the 16Sr groups (Bekele et al. 2011; Dickinson 2015; Kogovšek et al. 2015; Tomlinson et al. 2010). A LAMP test that was designed for the detection of grapevine "flavescence dorée" phytoplasmas was paired with a sample homogenization procedure for grapevine samples (leaf vein, flower, or berry) that allows direct testing of crude homogenates in LAMP assays, with sensitivity on par with quantitative PCR (Kogovšek et al. 2015, 2017).

The purpose of the present study was to develop and validate a LAMP assay for specific detection of the GY phytoplasma in South Africa (i.e., AY-SA). No adequate LAMP assays could be designed based on commonly used phytoplasma target sequences (data not shown); therefore, we exploited the available genomic data to identify novel diagnostic markers and to develop LAMP and real-time PCR assays for specific detection of AY-SA. The LAMP test was then validated in-house and in an interlaboratory test performance study.

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Funding: This study was funded by the European Union Horizon 2020 Research and Innovation Programme under grant agreement 727459, project "Insect-borne prokaryote-associated diseases in tropical and subtropical perennial crops" TROPICSAFE, and Javna Agencija za Raziskovalno Dejavnost RS (the Slovenian Research Agency) (contracts P4–0165 and 1000-15-0105).

*The *e*-Xtra logo stands for "electronic extra" and indicates that supplementary files, supplementary figures, and supplementary tables are published online.

The author(s) declare no conflict of interest.

Accepted for publication 1 April 2022.

Materials and Methods

In silico identification of novel diagnostic markers. Automated analysis was carried out using a freely available program for rapid identification of PCR primers for unique core sequences: RUCS (Thomsen et al. 2017). RUCS was used to identify genome sequences specific to the AY-SA; namely, two draft assemblies of the AY-SA genome that consisted of 16 and 550 contigs. Assemblies were refined and combined in a complete genome sequence (GenBank accession number CP035949), and later published by Coetzee et al. (2019). The negative dataset (Supplementary Table S1) provides complete or draft genome sequences of other phytoplasmas (18 genomes) and mycoplasmas (55 genomes) from publicly available databases (Supplementary Table S1 for NCBI/GenBank accession numbers).

Design of LAMP and real-time PCR assays. The novel diagnostic markers identified by RUCS (Thomsen et al. 2017) were filtered based on their suitability for the test design, in terms of length of at least 200 bp, and sequence uniqueness confirmed by blastn (Altschul et al. 1990). Altogether, three suitable unique sequences were selected, and are indicated as Seq1, Seq3, and Seq11. LAMP primer sets were designed using the PrimerExplorer V5 software (Eiken Genome; http://primerexplorer.jp/lampv5e/index.html), and the primers and hydrolysis probe for real-time PCR were designed using Primer Express version 2.0 (Applied Biosystems). The LAMP primer design was run with the default parameters adapted to AT-rich template sequences, because all target sequences had very low GC content (18 to 35%).

The following modifications were implemented: the lengths of the F2/B2 and F3/B3 primers were adapted to 15 to 25 bp and the GC rate was expanded to 25 to 65%. The proposed LAMP assays were checked manually and filtered using mainly the following predefined minimum quality parameter criteria: (i) dG of the 3′ end at region F2, the 5′ end at region F1c, the 3′end at region B2, and the 5′ end at region B1c lower than −4.0 kcal/mol; (ii) melting temperature (Tm) differences among the primer pairs was <3°C; and (iii) the high specificity of each primer (i.e., F3, B3, F2, B2, F1c, and B1c) was confirmed by the BLAST program.

The quality of the designed oligonucleotides was checked in silico using Oligo Analyzer (Integrated DNA Technologies), and the specificities of the primers and probes were evaluated in silico using blastn (Altschul et al. 1990) (Fig. 1).

Material for test evaluations. *Synthetic reference material.* The synthetic double-stranded (ds)DNAs were based on the unique genomic sequences of Seq1, Seq3, and Seq11 (Supplementary Table S2). These were synthesized commercially (gBlock Gene Fragments; Integrated DNA Technologies) and used to prepare defined concentrations of the target DNAs in Tris-EDTA buffer (Sigma-Aldrich) with salmon sperm DNA (final concentration = 25 mg/ml). Absolute concentrations of the target copy numbers were determined using digital PCR with the set of primers and probe of the AY-SA_ftsH real-time PCR assay. Synthetic reference material was used to optimize the LAMP tests, and preparation of defined spiked grapevine leaf vein extracts were used for evaluation of different on-site sample preparation techniques.

Phytoplasma collection. The specificities of the designed PCR and LAMP tests were determined on DNA from an international phytoplasma collection (Paltrinieri et al. 2015). Altogether, 21 different phytoplasmas were tested that belonged to the 16Sr group: 16SrI-A, 16SrI-B (n = 7), 16SrI-C, 16SrI-F, 16SrII-C, 16SrIII-A, 16SrIII-B (n = 2), 16SrVII-A, 16SrIX-C, 16SrX-A, and 16SrXII-A (n = 4) (Table 1). All phytoplasma strains were propagated in periwinkle, except CH1, which was propagated in the original host (Table 1).

Plant material. Plant material was collected from naturally infected and healthy grapevines over the 2016 to 2018 growing seasons in South Africa. Grapevines from a commercial vineyard (cultivar Colombar) in the Vredendal region of the Western Cape (South Africa) were selected based on their symptomatology. The selection and preparation of the grapevine samples was described by van der Vyver et al. (2019). The grapevines included in the analysis were selected based on their visual symptomatology in February 2016. Of 49 grapevines chosen for this study, 37 were symptomatic and 12 did not show any phytoplasma symptoms (van der Vyver et al. 2019). Altogether, 114 grapevine samples were collected at four different times from 2016 to 2018 (Tables 2 and 3). DNA from the grapevine samples was extracted from phloem scrapings using the cetyltrimethylammonium bromide method, as described by van der Vyver et al. (2019). The grapevine samples were divided into two groups, symptomatic and asymptomatic, based on their symptom expression in 2016. The presence of AY was confirmed using AY nested PCR (van der Vyver et al. 2019).

Six carrot samples (*Daucus carota* subsp. *sativus*) with symptoms of an AY infection were obtained in Slovenia in 2017 (Table 1) (Mehle et al. 2018). The DNA was extracted and purified using magnetic-bead-based plant DNA kits (QuickPick SML; Bio-Nobile, Turku, Finland) on an automated DNA purification system (KingFisher mL; Thermo Labsystem), as described previously (Pirc et al. 2009), with a minor modification (440 µl of lysate used in the purification). The presence of AY was confirmed using diagnostic real-time PCR (Angelini et al. 2007; Christensen et al. 2004; Nikolić et al. 2009).

On-site homogenization was tested using grapevine leaf veins spiked with target DNA. Two types of on-site sample preparation were tested: a dipstick method and a direct homogenate method. The dipstick method was performed accordingly to Zou et al. (2017), with minor modifications. Here, the leaf veins were homogenized manually with vigorous shaking for 2 min in cell lysis buffer (20 mM Tris [pH 8.0], 25 mM NaCl, 2.5 mM Na_2EDTA, and 0.05% sodium dodecyl sulfate) in 4.5-ml tall prep A lysing matrix tubes (Matrix A; MP Biomedical). For the direct homogenate technique, leaf veins were homogenized manually with vigorous shaking with enzyme-linked immunosorbent assay (ELISA) buffer (264 mM Tris, 236 mM Tris-HCl, 137 mM NaCl, 2% polyvinylpyrrolidone K-25, 2 mM polyethylene glycol 6000, and 0.05% Tween 20, pH 8.2) for 2 min in 4.5-ml tall prep A lysing matrix tubes (Matrix A; MP Biomedical). The homogenate was diluted 10-fold in molecular-grade water (Top-Bio) and tested directly in the LAMP and real-time PCR assays.

LAMP. The LAMP reactions were performed on a sequence detection system (QuantStudio 3 and QuantStudio 7 Flex real-time PCR systems; Applied Biosystems, Thermo Fisher Scientific), or using portable 8-well strip LAMP detection systems (Genie II; OptiGene).

Optimization of the assays was conducted for four assays that gave promising results in the preliminary experimental evaluation. In the optimization step, the following LAMP conditions were varied: reaction temperature, concentration of inner primers, concentration of Mg^{2+} ions, and form of synthetic target DNA (denatured or double stranded). To determine the best primer concentration, three different forward inner primer (FIP) and backward inner primer (BIP) concentrations were tested (0.8, 1.2, and 1.6 µM), without and with the addition of 2 mM Mg^{2+} ions (Supplementary File S1).

The optimized LAMP AY-SA_ftsH assay (Table 4) used a reaction volume of 25 µl that contained (final concentrations) 0.2 µM F3 and B3 primers (Sigma Aldrich, Integrated DNA Technologies), 1.6 µM high-performance liquid chromatography purified FIP and BIP primers (Sigma Aldrich, Integrated DNA Technologies), 1× isothermal master mix ISO-001 (OptiGene), and 5 µl of sample. When the LAMP reaction was performed on the instruments that required a reference dye (i.e., QuantStudio 3 and QuantStudio 7 Flex real-time PCR systems), ROX dye was added to the reaction (final concentration 0.1×; Premix Ex Taq, Takara and Kapa Probe Fast qPCR Master Mix; KapaBiosystems). The LAMP assay was conducted under isothermal conditions at 63°C for 30 min (except where stated otherwise). The isothermal reaction followed the measurement of the Tm by heating the reaction to 98°C and cooling to 60°C (ramp = 0.05°C/s). Data were analyzed using the QuantStudio real-time PCR software v1.3 or the QuantStudio Design and Analysis desktop software v1.5.1 (Applied Biosystems, Thermo Fisher Scientific), with automatic baseline and a manual threshold of 0.3. Data from the portable 8-well strips (Genie II; OptiGene) were analyzed automatically by the instrument software. A positive reaction was defined by a curve of sigmoidal shape above the background, a time of positivity <30 min, and a Tm from 80 to 83°C.

The optimized test was validated and evaluated for the following matrices: (i) serial dilution of heat-denatured synthetic target dsDNA (concentration range = 10^8 to 10^2 target molecules/ml), (ii) phytoplasma DNA from the phytoplasma collection (Paltrinieri et al. 2015), (iii) carrot samples from Slovenia, and (iv) grapevine samples from South Africa. Some natural samples and phytoplasma DNA from the phytoplasma collection were tested as 10-fold dilutions, due to the limited quantities of the samples.

The stability of the reaction mixture was tested for the optimized LAMP AY-SA_ftsH assay. The reaction mixture was incubated at 30°C for 0, 3, and 6 h in closed tubes. After the incubation, the synthetic target dsDNA (dilutions = 10^7 to 10^2 target molecules/ml) was added to the reaction mixture, and the LAMP reaction was performed.

Real-time PCR. The real-time PCR assay with AY-SA_ftsH (Table 4) targets the same *ftsH* gene as the LAMP AY-SA_ftsH assay. All of the real-time PCR assays were performed in triplicate (QuantStudio 3 and QuantStudio 7 Flex real-time PCR systems; Applied Biosystems, Thermo Fisher Scientific) using the following universal cycling conditions: 2 min at 50°C, 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C, using standard temperature ramping mode. The reaction volume of 10 µl contained (final concentrations) 900 nM primers and 200 nM 6-carboxyfluorescein (FAM) and black hole quencher (BHQ)-1 labeled probes (all Integrated DNA Technologies), 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific), and 1 or 2 µl of sample DNA. The real-time PCR data were analyzed using the QuantStudio real-time PCR software v1.3 or the QuantStudio Design and Analysis desktop software v1.5.1 (Applied Biosystems, Thermo Fisher Scientific), with automatic baseline and a manual threshold of 0.04. The real-time PCR data are given as quantification cycle (Cq) values (i.e., real-time PCR quantification cycles).

The presence of phytoplasma DNA in the samples was confirmed by universal real-time PCR assays for phytoplasmas, as described by Christensen et al. (2004). The test was performed as described for the real-time PCR AY-SA_ftsH assay, with the following modifications (final concentrations): 300 nM forward primer, 900 nM reverse

1.	**RUCS**	
	Entry point	Find unique core sequences
	Positive genomes	AY_Mgenia[a] AY_Periwinkle[a]
	Reference	AY_Periwinkle[a]
	Negative genomes	73 phytoplasma and mycoplasma genomes (Annex A)
	K-mer size	20 bp
	Read length	250 bp
	Unique core sequences	802
	Unique core sequences (>200 bp)	11
2.	**Blastn**	
	Parameters	Default
	Database	nr/nt
	Input sequences	11 unique core sequences (>200 bp)
3.	**Primer Explorer v5**	
	Parameter set	Automatic judgment
	Detail settings:	
	Parameter condition	AT rich
	Length F2/B2	15-25 bp
	Length F3/B3	15-25 bp
	GC rate	25-65
	Results:	
	Seq1	258 assays
	Seq2	0 assays
	Seq3	54 assays
	Seq4	0 assays
	Seq5	0 assays
	Seq6	0 assays
	Seq7	0 assays
	Seq8	0 assays
	Seq9	0 assays
	Seq10	0 assays
	Seq11	774 assays
4.	**Parameter-based selection[b]**	
	Seq1	8 assays
	Seq3	5 assays
	Seq11	7 assays
5.	**Amplicon genetic position**	
	Seq1	hypothetical protein
	Seq3	hypothetical protein
	Seq11	*ftsH* gene

Fig. 1. Overview of the loop-mediated isothermal amplification (LAMP) assay development process and the parameters defined for each step. The process is divided into five steps: 1. rapid identification of PCR primers for unique core sequences (i.e., RUCS); 2. specificity confirmation using blastn; 3. design of LAMP primers using Primer Explorer v5; 4. selection of appropriate LAMP assays based on the minimum selection criteria; 5. identification of the LAMP amplicon genetic position. Superscript "a" indicates genome of grapevine yellows phytoplasmas from South Africa and "b" indicates complying with defined minimum criteria for choosing LAMP assays.

primer, and 100 nM FAM and BHQ-1 labeled probe. The real-time PCR data analysis parameters included an automated baseline and a manual threshold of 0.02.

Digital PCR. For digital PCR (QX100 Droplet Digital PCR system; Bio-Rad), the real-time PCR AY-SA_ftsH assay (Table 4) was transferred to digital PCR format and used to determine the concentration of the target copy numbers in the test items and controls. The reactions contained 12 µl of dPCR Supermix for probes (Bio-Rad) and 8 µl of sample DNA, with the primer and probe concentrations of 900 nmol (each primer) and 200 nmol per reaction, respectively. After droplet generation, 40 µl of the generated droplet emulsion was transferred to a new 96-well PCR plate (Eppendorf) and amplified in a thermal cycler (Bio-Rad). The amplification conditions consisted of 10 min of DNA polymerase activation at 95°C, followed by 45 cycles of a two-step thermal profile of 15 s at 95°C for denaturation and 60 s at 60°C for annealing and extension, followed by a final hold of 10 min at 98°C for droplet stabilization, and cooling to 4°C. The temperature ramp rate was set to 3°C/s, and the lid was heated to 105°C, according to the manufacturer's recommendations. After the thermal cycling, the plates were transferred to a droplet reader (QX100; Bio-Rad). The software package provided with the digital PCR system was used for data acquisition (QuantaSoft, version 17.4.0917; Bio-Rad). A minimum of 10,000 accepted droplets per reaction was required for the reaction to be considered valid, and no nonvalid reactions were observed. A fixed manual global threshold that discriminated between negative and positive droplets was selected. A reaction was interpreted as positive if the number of positive droplets was ≥3. Positive and no-template controls were used in each run. Each sample was analyzed as three technical repeats. The expended measurement uncertainty was calculated for each sample.

Validation of new LAMP and real-time PCR. Validations were conducted following the European and Mediterranean Plant Protection Organization guidelines on validation (EPPO 2018), which include analytical specificity, analytical sensitivity, diagnostic specificity and sensitivity, repeatability, reproducibility, and robustness (Fig. 2).

The analytical specificity was evaluated on synthetic DNA, phytoplasma DNA from the phytoplasma collection (Paltrinieri et al. 2015), and the carrot samples from Slovenia (Table 1). The presence of phytoplasma DNA in the samples was confirmed, and the concentrations were determined by the universal real-time PCR assays for phytoplasmas (Christensen et al. 2004).

The analytical sensitivity of the designed LAMP AY-SA_ftsH and the real-time PCR AY-SA_ftsH assays were determined on 10-fold serial dilutions of synthetic target DNA sequences (Supplementary Table S2) as 10^8 to 10^1 target molecules/ml. Each dilution was tested in two replicates and on two instruments (QuantStudio 3 and QuantStudio 7 Flex real-time PCR systems [Applied Biosystems, Thermo Fisher Scientific] and Genie II [OptiGene]), and in three replicates and two instruments (QuantStudio 3 and QuantStudio 7 Flex real-time PCR systems) for the LAMP AY-SA_ftsH and real-time PCR AY-SA_ftsH assays, respectively.

Diagnostic sensitivity and specificity were assessed on a collection of infected and healthy grapevine samples collected from 2016 to 2018 (Tables 2 and 3). The samples were collected within the study described by van der Vyver et al. (2019) and the presence of phytoplasma DNA in the grapevine samples was tested with nested PCR. Some DNA samples isolated from symptomatic grapevines were diluted 10-fold in molecular-grade water due to limited sample quantities. The DNA samples from asymptomatic grapevines were tested in an undiluted form. The samples were tested by LAMP AY-SA_ftsH and real-time PCR AY-SA_ftsH assays, along with the universal real-time PCR assay for phytoplasmas (Christensen et al. 2004). Both real-time PCR assays were performed in duplicate, and the LAMP assay in a single reaction. Molecular-grade water was used as a no-template control and synthetic target DNA as a positive

Table 1. Testing of assay specificity against characterized phytoplasma isolates obtained from the International Phytoplasmologist Working Group and from carrot samples[a]

				Assay for phytoplasma[b]		AY-SA-specific results	
Sample	Host plant	Origin	Group	Result	Cq	PCR[c]	LAMP[d]
D310/17 A	*Daucus carota* subsp. *sativus*	Slovenia	16SrI	Pos	18.3	Neg	Neg
D310/17 B	*D. carota* subsp. *sativus*	Slovenia	16SrI	Pos	22.1	Neg	Neg
D322/17 A	*D. carota* subsp. *sativus*	Slovenia	16SrI	Pos	16.5	Neg	Neg
D322/17 B	*D. carota* subsp. *sativus*	Slovenia	16SrI	Pos	20.7	Neg	Neg
D357/17 A	*D. carota* subsp. *sativus*	Slovenia	16SrI	Pos	15.5	Neg	Neg
D357/17 B	*D. carota* subsp. *sativus*	Slovenia	16SrI	Pos	21.3	Neg	Neg
NJ-AY[e]	Aster	United States	16SrI-A	Pos	19.9	Neg	Neg
AY2192[e]	NA	NA	16SrI-B	Pos	19.0	Neg	Neg
NA[e]	Periwinkle	Italy	16SrI-B	Pos	19.6	Neg	Neg
PRIVA[e]	Primula	Germany	16SrI-B	Pos	19.4	Neg	Neg
RV[e]	Brassicaceae	France	16SrI-B	Pos	15.7	Neg	Neg
SIL[e]	*Silene vulgaris*	Italy	16SrI-B	Pos	17.9	Neg	Neg
CVT[e]	Periwinkle	Thailand	16SrI-B	Pos	15.6	Neg	Neg
DIV[e]	*Diplotaxis erucoides*	Spain	16SrI-B	Pos	14.8	Neg	Neg
C-CP[e]	Trifolium	Canada	16SrI-C	Pos	15.7	Neg	Neg
ACLR[e]	NA	NA	16SrI-F	Pos	14.2	Neg	Neg
FBPSA[e]	*Crotalaria saltiana*	Sudan	16SrII-C	Pos	16.3	Neg	Neg
CX[e]	*Prunus*	Canada	16SrIII-A	Pos	15.7	Neg	Neg
KVI[e]	Trifolium	Italy	16SrIII-B	Pos	13.4	Neg	Neg
GYU[e]	*Vitis vinifera*	Italy	16SrIII-B	Pos	18.5	Neg	Neg
ASHY[e]	NA	NA	16SrVII-A	Pos	14.1	Neg	Neg
PEY[e]	*Pichris echioides*	Italy	16SrIX-C	Pos	19.0	Neg	Neg
AP-15[e]	Malus	Italy	16SrX-A	Pos	16.1	Neg	Neg
ASLO[e]	Aster	Slovenia	16SrXII-A	Pos	14.7	Neg	Neg
CH1[e]	*V. vinifera*	Italy	16SrXII-A	Pos	16.4	Neg	Neg
STOL[e]	Capsicum	Serbia	16SrXII-A	Pos	15.2	Neg	Neg
MOL[e]	*Prunus*	France	16SrXII-A	Pos	13.4	Neg	Neg

[a] Group = ribosomal group, AY-SA = aster yellows from South Africa, NA = not available, Pos = positive, and Neg = negative.
[b] Universal real-time PCR assay for phytoplasma (Christensen et al. 2004). Cq = quantification cycle.
[c] Real-time PCR AY-SA_ftsH assay. Negative real-time PCR after 45 cycles.
[d] Loop-mediated isothermal amplification (LAMP) AY-SA_ftsH assay. Result for 10× dilution of the original sample. Negative LAMP reaction after 30 min.
[e] Samples from the International Phytoplasmologist Working Group collection.

amplification control in every experiment. The cross-reactivity of the designed LAMP and real-time PCR assays with the plant tissue, its microflora, and other phytoplasmas was monitored by testing a total of 114 grapevine DNA extracts. Diagnostic sensitivity and specificity were evaluated in terms of the grapevine symptomatology, the AY nested PCR (results from the study described by van der Vyver et al. [2019]), and the universal real-time PCR assay for phytoplasmas (Christensen et al. 2004).

Repeatability was analyzed across triplicates of an assay for the real-time PCR and as duplicates for the LAMP assay (i.e., within run). Reproducibility was determined across two repeated-day assays (i.e., between runs), between two different laboratories, between two detection systems for real-time PCR (QuantStudio 3 and QuantStudio 7 Flex real-time PCR systems), and between three detection systems for LAMP (QuantStudio 3 and QuantStudio 7 Flex real-time PCR systems and Genie II). For the assessment of repeatability and

Table 2. Results of the molecular tests of the grapevine samples collected in February and November 2016[a]

	February 2016				November 2016			
Grapevines	**PCR[b]**	**LAMP[c]**	**Universal[d]**	**AY[e]**	**PCR[b]**	**LAMP[c]**	**Universal[d]**	**AY[e]**
Asymptomatic								
A01	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
A12	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg
A13	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
A19	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
A02	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
A25	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
A26	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
A29	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
A03	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
A05	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
A07	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
A08	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Symptomatic								
S14	Neg	Neg[f]	Neg	Pos	/	/	/	Neg
S15	Pos	Pos	Pos	Pos	/	/	/	Neg
S17	Pos	Pos[f]	Pos	Pos	/	/	/	Neg
S18	Pos	Pos[f]	Pos	Pos	Neg	Neg[f]	Neg	Neg
S19	Pos	Pos[f]	Pos	Pos	/	/	/	Neg
S23	Pos	Pos	Pos	Pos	/	/	/	Neg
S24	Pos	Pos	Pos	Pos	/	/	/	Neg
S26	Neg	Neg[f]	Pos	Pos	/	/	/	Neg
S29	Disc	Pos	Pos	Pos	/	/	/	Neg
S31	Pos	Neg	Pos	Pos	/	/	/	Neg
S32	Pos	Pos	Pos	Pos	/	/	/	Neg
S33	Pos	Pos	Pos	Pos	/	/	/	Neg
S34	Pos	Pos[f]	Pos	Pos	/	/	/	NA
S35	Pos	Pos[f]	Pos	Pos	/	/	/	Neg
S39	Pos	Neg[f]	Pos	Pos	Pos	Pos	Pos	Pos
S04	Pos	Neg	Pos	Pos	/	/	/	Neg
S40	Pos	Pos[f]	Pos	Pos	Pos	Pos[f]	Pos	Pos
S43	Pos	Pos	Pos	Pos	/	/	/	Neg
S44	Pos	Neg[f]	Pos	Pos	Neg	Neg[f]	Neg	Neg
S46	Pos	Pos[f]	Pos	Pos	Pos	Pos	Pos	Pos
S47	Pos	Neg[f]	Pos	Pos	/	/	/	Neg
S48	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Pos
S49	Pos	Pos[f]	Pos	Pos	/	/	/	Neg
S05	Pos	Neg[f]	Pos	Pos	/	/	/	Neg
S51	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
S52	Pos	Pos[f]	Pos	Pos	Neg	Neg[f]	Neg	Neg
S54	Pos	Pos[f]	Pos	Pos	Pos	Pos[f]	Pos	Pos
S55	Pos	Pos	Pos	Pos	Neg	Neg[f]	Neg	Neg
S56	Pos	Neg[f]	Pos	Pos	/	/	/	Neg
S57	Pos	Neg[f]	Pos	Pos	/	/	/	Neg
S58	Pos	Pos	Pos	Pos	Neg	Neg[f]	Neg	Neg
S59	Pos	Pos	Pos	Pos	/	/	/	Neg
S06	Pos	Pos	Pos	Pos	/	/	/	Neg
S60	Pos	Pos	Pos	Pos	/	/	/	Neg
S07	Pos	Pos	Pos	Pos	/	/	/	Neg
S08	Pos	Pos	Pos	Pos	/	/	/	Neg
S09	Pos	Pos[f]	Pos	Pos	/	/	/	Neg

[a] Symptomatic and asymptomatic grapevines were defined in February and November 2016 based on their apparent symptomatology. Asymptomatic grapevine samples were analyzed as undiluted DNA extracts. Symptomatic grapevine samples were analyzed as undiluted DNA extracts in nested PCR and as 10-fold dilutions in real-time PCR assays. For the loop-mediated isothermal amplification (LAMP) assays, symptomatic grapevine samples were predominantly analyzed as undiluted DNA, although some samples needed to be 10-fold diluted due to shortage of the DNA extract (as indicated). Neg = negative, Pos = positive, NA = not available (lack of sample material), and / indicates samples not analyzed with all of the methods due to lack of sample material.
[b] Real-time PCR AY-SA_ftsH assay.
[c] LAMP AY-SA_ftsH assay.
[d] Universal phytoplasma real-time PCR.
[e] Aster yellows (AY) nested PCR.
[f] Samples 10-fold diluted.

reproducibility, a standard curve of the synthetic target DNA was used (analytical sensitivity). Reproducibility of the LAMP assay was additionally assessed in a test performance study (TPS).

TPS. The performance of the LAMP_AY-SA_ftsH assay was evaluated in the TPS. The TPS sample panel was composed of 12 test items: positive samples containing synthetic dsDNA of AY-SA; positive samples containing samples naturally contaminated with AY-SA; and negative samples containing other AY or host-plant *V. vinifera* DNA, with the corresponding control samples. Qualitative reference values were assigned to the test items based on the results of the LAMP AY-SA_ftsH assay. Quantitative reference values (target concentrations) were determined using digital PCR analysis of the test items and controls. Stability of the test items and controls was tested with the LAMP AY-SA_ftsH assay. The stability testing was carried out under conditions that mimicked transport and storage conditions (< −15°C).

Table 3. Results of the molecular tests of the grapevine samples collected in February 2017 and November 2018[a]

	February 2017				November 2018			
Grapevines	**PCR[b]**	**LAMP[c]**	**Universal[d]**	**AY[e]**	**PCR[b]**	**LAMP[c]**	**Universal[d]**	**AY[e]**
Asymptomatic								
A01	Neg	Neg	Neg	Neg	Neg	/	/	Neg
A12	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg
A13	Neg	Neg	Neg	Neg	/	/	/	Neg
A19	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
A02	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg
A25	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg
A26	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
A29	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
A03	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg
A05	Neg	Neg	Neg	Neg	/	/	/	Neg
A07	Neg	Neg	Neg	Neg	/	/	/	Neg
A08	Neg	Neg	Neg	Neg	Disc.	Neg	Neg	Neg
Symptomatic								
S14	/	/	/	Neg	/	/	/	Neg
S15	/	/	/	Neg	/	/	/	Neg
S17	/	/	/	Neg	/	/	/	Pos
S18	Pos	Pos[f]	Pos	Pos	Neg	Neg	Neg	Neg
S19	/	/	/	Neg	/	/	/	Neg
S23	/	/	/	Neg	/	/	/	Neg
S24	/	/	/	Neg	/	/	/	Neg
S26	/	/	/	Neg	/	/	/	Neg
S29	/	/	/	Neg	/	/	/	Neg
S31	/	/	/	Neg	/	/	/	Neg
S32	/	/	/	Neg	/	/	/	Neg
S33	/	/	/	Neg	/	/	/	NA
S34	/	/	/	NA	/	/	/	NA
S35	/	/	/	Neg	/	/	/	Neg
S39	Neg	Neg[f]	Neg	Neg	Neg	Neg	Neg	Neg
S04	/	/	/	Neg	/	/	/	Pos
S40	Neg	Neg	Neg	Neg	Neg	Neg[f]	Neg	Pos
S43	/	/	/	Neg	/	/	/	Neg
S44	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg
S46	Neg	Neg[f]	Neg	Neg	Neg	Neg	Pos	Pos
S47	/	/	/	Neg	/	/	/	Neg
S48	Neg	Neg[f]	Neg	Neg	Neg	Neg	Neg	Pos
S49	/	/	/	Neg	/	/	/	NA
S05	Neg	Neg[f]	Neg	Neg	Neg	Neg	Neg	Neg
S51	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Neg
S52	Neg	Neg[f]	Neg	Neg	Neg	Neg	Neg	Neg
S54	Neg	Neg[f]	Neg	Neg	Neg	Neg	Pos	Neg
S55	/	/	/	Neg	/	/	/	Neg
S56	/	/	/	Neg	/	/	/	Neg
S57	/	/	/	Neg	/	/	/	Neg
S58	Neg	Neg[f]	Neg	Neg	Neg	Neg	Neg	Neg
S59	/	/	/	Neg	/	/	/	Neg
S06	/	/	/	Neg	/	/	/	Pos
S60	/	/	/	Neg	/	/	/	Neg
S07	/	/	/	Neg	/	/	/	Neg
S08	/	/	/	Neg	/	/	/	Neg
S09	/	/	/	Neg	/	/	/	Neg

[a] Symptomatic and asymptomatic grapevines were defined in February 2017 based on their apparent symptomatology. Asymptomatic grapevine samples were analyzed as undiluted DNA extracts. Symptomatic grapevine samples were analyzed as undiluted DNA extracts in nested PCR and as 10-fold dilutions in real-time PCR assays. For the loop-mediated isothermal amplification (LAMP) assays, symptomatic grapevine samples were predominantly analyzed as undiluted DNA, although some samples needed to be 10-fold diluted due to shortage of the DNA extract (as indicated). Neg = negative, Pos = positive, Disc. = discordant or inconclusive, NA = not available (lack of sample material), and / indicates samples not analyzed with all of the methods due to lack of sample material.

[b] Real-time PCR AY-SA_ftsH assay.

[c] LAMP AY-SA_ftsH assay.

[d] Universal phytoplasma real-time PCR.

[e] Aster yellows (AY) nested PCR.

[f] Samples 10-fold diluted.

The data for each participating laboratory were analyzed based on the numbers of positive agreements (as positive detected from positive expected), negative agreements (as negative detected from negative expected), positive deviations (as positive detected from negative expected), and negative deviations (as negative detected from positive expected). All of the samples were analyzed in two separate reactions by each participant. A sample was considered positive if it produced at least one positive reaction, according to the exponential amplification profile, the time of positivity <30 min, and the Tm from 82 to 83°C.

Analysis of the collected TPS results was based on the concordance with the assigned reference values (Supplementary File S2).

Adaptation of LAMP for on-site use. Two different on-site sample preparation methods were tested: a dipstick method and direct use of a crude homogenate. The nucleic acid purification using the dipstick method was performed according to Zou et al. (2017), with the following modifications: (i) the size of the nucleic acid binding active zone was 3 by 3 mm, (ii) the grapevine leaf veins (approximately 200 mg) were added to a TallPrep Lysing Matrix A 4.5-ml tube (MP Biomedicals) that contained 1 ml of cell lysis buffer, (iii) the grapevine tissue was macerated by shaking the tube for approximately 2 min, and (iv) the prepared homogenate was spiked with synthetic target DNA or isolated AY-SA DNA. The nucleic acids from the dipsticks were eluted directly into the LAMP reaction or into the real-time PCR mix using preadded molecular-grade water for the sample volume. Direct use of crude homogenates was tested as described by Kogovšek et al. (2015), using ELISA buffer and grapevine leaf veins. The prepared homogenate was spiked with synthetic target DNA or isolated AY-SA DNA. The dipstick cell lysis buffer and the ELISA buffer were spiked with the synthetic target DNA sequence to final concentrations of 10^7 to 10^4 DNA molecules/ml, or with the DNA isolate of sample S40N16 to a final concentration of approximately 8×10^4 genomes/ml. Both of these sample preparation methods were evaluated for use with the LAMP and real-time PCR assays.

The stability of the DNA and the possibility for short-term storage using the dipstick approach was tested by adding the following steps to the method described: the dipsticks were placed in new 2-ml centrifuge tubes after the wash step, the dipsticks were dried at room temperature (22 ± 1°C) in the open centrifuge tubes for 30 min, and the dried dipsticks were stored in the closed centrifuge tubes in the dark at room temperature. The stability of the target DNA on these stored dipsticks was tested after 3 weeks with the LAMP assay. The dipsticks were first briefly dipped into 100 µl of wash buffer (to rehydrate the dipstick nucleic acid binding zone), then eluted directly into the LAMP reaction mixture.

Statistical analysis. Nonlinear modeling of the probability of detection of the target was calculated in the R statistical environment (R Core Team 2020) using the *drc* package (Ritz et al. 2015), along with determination of the target concentration that was detected with 95% probability (LOD_{95}). Dichotomous positive and negative results from the LAMP and real-time PCR assays were analyzed using a 2-by-2 contingency table.

Results

Design of the LAMP and real-time PCR assays. The sequence analysis tool RUCS (Thomsen et al. 2017) was applied to the 75

Table 4. Primers and probes for the molecular tests designed and used in this study

Molecular assay, target gene, primer, or probe	Sequence (5′–3′)
Real-time PCR, *ftsH*	
AY-SA_ftsH_R	CCCAAAAGGTGCAAAAAAATAACTA
AY-SA_ftsH_P[a]	FAM–CGTGTGGGAATTCGGGCGGTTATAA–BHQ[b]
AY-SA_ftsH_R	AAAGAAAGTTTCTGTTTCTGGTGTCA
LAMP, *ftsH*[c]	
AY-SA_ftsH_F3	TGAAGCAGGACACGCTAT
AY-SA_ftsH_B3	CAAAAATTAATTCTTCAGCCACA
AY-SA_ftsH_FIP[a]	CCGAATTCCCACACGGAATAATTAAGTTGGAACATGCCCA
AY-SA_ftsH_BIP[a]	AATGACACCAGAAACAGAAACTTTCCGTCCCCCTAAATAAGATGT

[a] High-performance liquid chromatography purified.
[b] FAM = 6-carboxyfluorescein and BHQ = black hole quencher.
[c] LAMP = loop-mediated isothermal amplification.

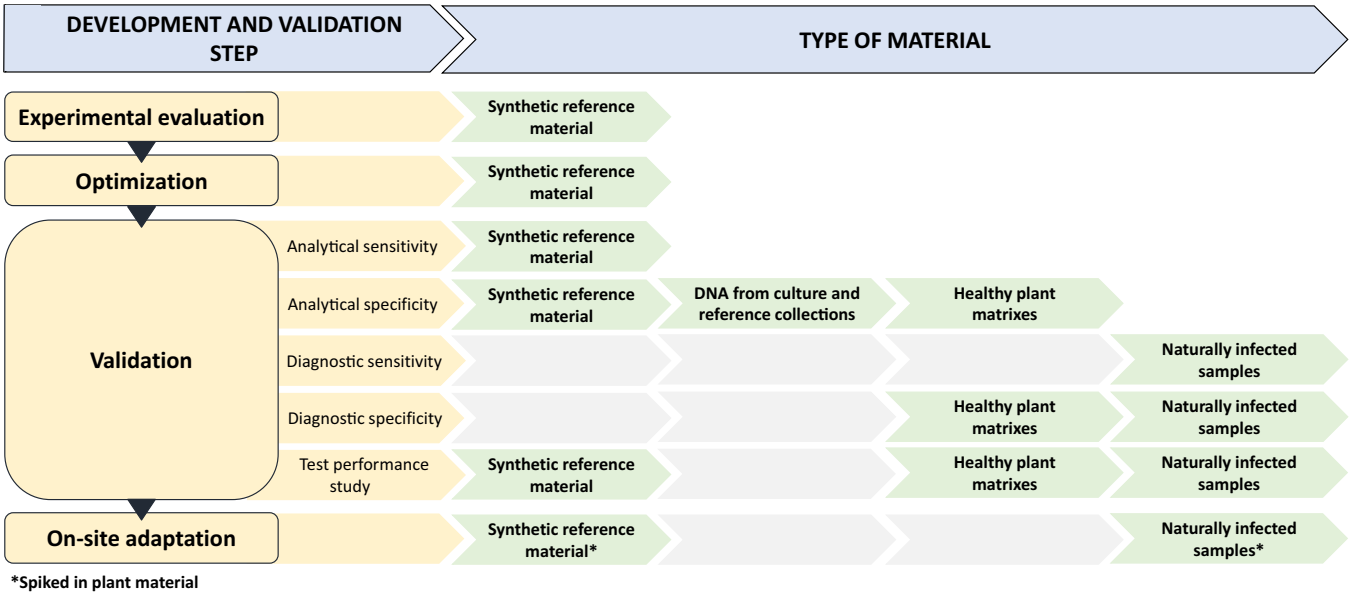

Fig. 2. Development and validation steps and types of reference material used for the loop-mediated isothermal amplification and real-time PCR assays.

selected genomes, which resulted in the identification of 802 sequences unique to the AY-SA genome. The great majority (99%) of these specific sequences identified were shorter than 200 bp. In the range suitable for LAMP design (i.e., 200 to 700 nucleotides), 11 sequences were identified, and these were used as target sequences (Fig. 3). The unique sequences of all lengths were distributed throughout the AY genome.

All target sequences had very low GC content (18 to 35%); therefore, the LAMP primer design was run with the default parameters adapted to AT-rich template sequences, with the implementation of the following modifications: the lengths of the F2/B2 and F3/B3 primers were adapted to 15 to 25 bp and the GC rate was expanded to 25 to 65%. The proposed LAMP assays were checked manually and filtered using mainly the following predefined minimum quality parameter criteria: (i) dG of the 3′ end at region F2, the 5′ end at region F1c, the 3′end at region B2, and the 5′ end at region B1c lower than −4.0 kcal/mol; (ii) Tm differences among the primer pairs was <3°C; and (iii) the high specificity of each primer (i.e., F3, B3, F2, B2, F1c, and B1c) was confirmed by the BLAST program. The LAMP primers of sufficient quality were successfully designed on 3 of the 11 selected target sequences as Seq1, Seq3, and Seq11 (Supplementary Table S3). Altogether, 20 assays were selected for experimental evaluation (Supplementary Table S4), of which 8 primer sets were developed on Seq1, 5 on Seq3, and 7 on Seq11 (Supplementary Table S3). The selected sequences were associated with their corresponding genes based on the annotated genome. Seq1 and Seq3 represent partial regions of the same hypothetical protein, while Seq11 represents a sequence linked to the cell-division protein FtsH.

The real-time PCR assays were developed on the target sequence that successfully produced the functional LAMP primer sets. The assays were successfully developed using default design parameters to provide the specific TaqMan real-time PCR assay (Table 4).

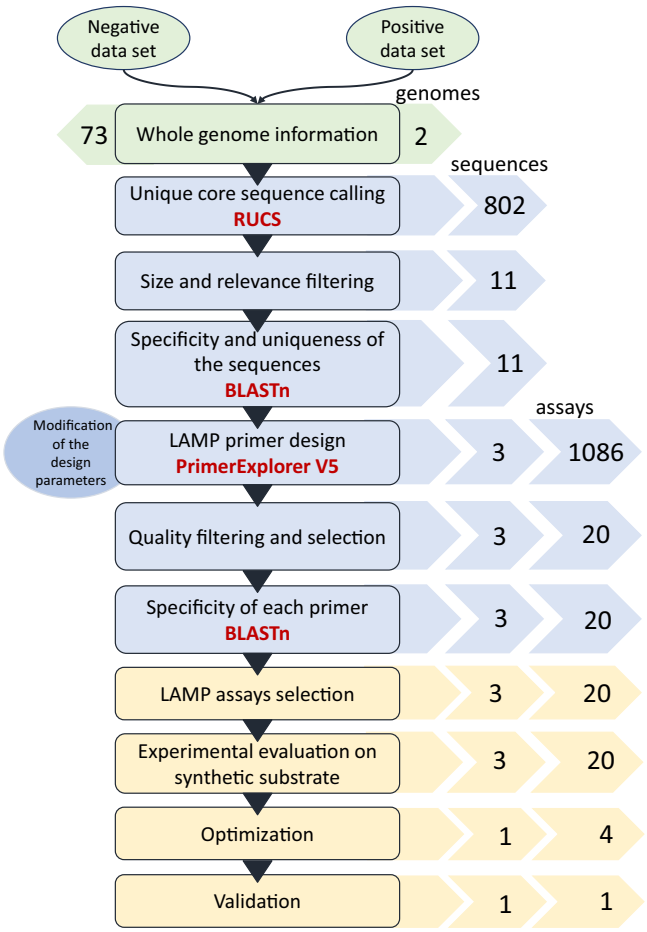

Fig. 3. Schematic overview of the complete loop-mediated isothermal amplification (LAMP) design process.

Performances of the LAMP and real-time PCR assays. *Optimization of the LAMP assays.* Twenty LAMP primer sets were tested empirically on synthetic target and nontarget dsDNA at the three different reaction temperatures of 60, 62, and 65°C. Four LAMP assays that successfully detected target DNA with no cross-reactivity were selected for optimization (Supplementary File S1). The other assays tested did not produce any positive signals or show evident cross-reactivity with nontarget DNA or the LAMP reagents (Supplementary File S1).

The shortest detection time was at the reaction temperature of 62°C for all of the specific assays; therefore, the optimization step was performed at this reaction temperature. In further testing, it was noted that the LAMP assay performance and reaction characteristics did not change if the reaction temperature was 62 or 63°C. Hence, validation of the LAMP assay developed was performed at 63°C, to allow combination of the runs with the assays for general phytoplasma detection and for identification of different 16Sr groups described by Dickinson (2015).

The optimal LAMP reaction mix conditions identified in the optimization step used 1.6 µM FIP and BIP primers (final concentration) without addition of Mg^{2+} ions. Throughout the optimization of the LAMP assay, the LAMP AY-SA_ftsH assay produced the best performance characteristics and, therefore, was chosen for validation.

Analytical specificity. The specificity of the LAMP assay developed was tested on synthetic nontarget dsDNA, carrot samples with symptoms of AY infection, and phytoplasma DNA from the phytoplasma collection (Paltrinieri et al. 2015) (Table 1). AY-SA-specific LAMP did not give positive results with any of these sample materials. Also, 21 different phytoplasma isolates from the phytoplasma collection were tested, which corresponded to the 16Sr groups 16SrI-A, 16SrI-B (7 isolates), 16SrI-C, 16SrI-F, 16SrII-C, 16SrIII-A, 16SrIII-B (2 isolates), 16SrVII-A, 16SrIX-C, 16SrX-A, and 16SrXII-A (4 isolates). None of the isolates represented AY-SA. All of the tested phytoplasma isolates were negative using both the LAMP and the real-time PCR AY-SA-specific assays, which confirmed high specificity of the tests for the variety of the 16SrI-B subgroup from South Africa. The amounts of DNA in the phytoplasma samples were confirmed using the universal real-time PCR assay (Christensen et al. 2004). The DNA concentration of the isolates was high (Cq < 20) and, therefore, the negative results recorded with the specific LAMP assay did not arise due to low target concentrations. No cross reactivity with any of the host plant DNAs was detected for either of the assays tested (Table 1).

Analytical sensitivity. Analytical sensitivity was assessed on dilution of synthetic target dsDNA (Supplementary Fig. S1). The LAMP assay showed very high sensitivity. With LOD_{100} defined as the lowest target amount that gave positive results in all the parallel reactions, this was 10^4 target dsDNA molecules/ml; the LOD_{95} was 8.2×10^3 target dsDNA molecules/ml (Supplementary Fig. S2). These calculations were based on pure solutions of synthetic target DNA without any plant matrix background.

Diagnostic specificity and sensitivity. The performance parameters of the tests (Table 5) were calculated based on the sanitary status of the grapevines, the results of the universal phytoplasma real-time PCR (Christensen et al. 2004), and the results of the AY nested PCR (Tables 2 and 3) (van der Vyver et al. 2019). The performance parameters in terms of the sanitary status of the grapevines were determined on 49 grapevine samples collected in 2016, because the sanitation status of the grapevines was not recorded for the later samples. The LAMP assay showed up to 27% lower sensitivity in comparison with the other tests; however, it was very specific (100% specificity). The overall accuracy was adequate for the purpose of the designed test.

Correlations between the LAMP time of positivity and the corresponding Tm of the amplification products showed that samples with lower time of positivity produced amplification products with higher Tm, and vice versa (Fig. 4). The amplification products produced showed Tm from 82.2 to 83.2°C. The samples and controls with

time of positivity <23 min generally had Tm > 82.6°C, and the samples with higher time of positivity melted at <82.6°C.

Repeatability and reproducibility. Repeatability and reproducibility of both assays was assessed on synthetic target material (i.e., the analytical sensitivity) in two different laboratories and using different laboratory equipment (e.g., pipettes and detection systems). The analysis of precision within the duplicates and across two runs showed 100% agreement at 10^4 target dsDNA molecules/ml. Below 10^4 target dsDNA molecules/ml, agreement varied across the different concentrations and different instruments. Considering the reproducibility between runs using different detection instruments and laboratory equipment, there were no effects on the reproducibility of the results. Here, 100% reproducibility was maintained above LOD_{100}. Additionally, the reproducibility of the LAMP assay was assessed in the TPS. The TPS results demonstrated reproducibility of the LAMP assay on all of the types of test material.

Robustness. The temperature stability of the LAMP assay reaction mixture was tested to evaluate the ease of use for on-site testing. The LAMP reaction mixture gradually lost its activity over time. Incubation at 30°C resulted in gradual loss of the reagent mixture activity. After 6 h of incubation, the reagent mixture gave no positive reaction signal in the positive control (data not shown). However, no false-positive results were observed for any of the times tested.

The robustness of the LAMP assay was assessed in terms of small temperature variations of the isothermal reaction. The reaction temperatures tested (62 and 63°C) did not influence the results of the LAMP reaction. Neither time of positivity nor Tm of the samples tested changed.

Test performance study. Two laboratories took part in the TPS to evaluate the specific LAMP AY-SA_ftsH assay. Both laboratories used identical reagents and instruments, as the isothermal master mix ISO-001 (OptiGene) and Genie II (OptiGene) instruments. The TPS sample panel was composed of synthetic target dsDNA, grapevine samples infected with AY-SA, and negative samples containing other GY phytoplasmas or host-plant *V. vinifera* DNA. The controls provided with the test panel were used for the quality check of the dataset. If the results of the controls were concordant with the expected results, the test panel data were considered as valid. All TPS results were valid, and in 100% concordance with the expected results. The TPS confirmed that the LAMP AY-SA_ftsH assay does not cross-react with other GY phytoplasmas or host-plant DNA, and that it is very sensitive and reproducible. The detailed results of the TPS are presented in Supplementary File S2.

Real-time PCR. The AY-SA-specific real-time PCR was developed as a control assay for the LAMP assay, to enable more comprehensive evaluation of the LAMP assay and sample material used in the validation process. Real-time PCR was validated on the same material and in parallel with the LAMP assay. Like the LAMP assay, the real-time PCR showed 100% analytical specificity for all of the sample material tested. The analytical sensitivity of the real-time PCR was very close to the sensitivity of the LAMP assay. The LOD_{100} was the same for both assays, at 10^4 target dsDNA molecules/ml, although the LOD_{95} was slightly different for the real-time PCR assay, at 6.8×10^3 target dsDNA molecules/ml (Supplementary Fig. S2). The diagnostic specificity of the real-time PCR was in line with the LAMP assay. However, real-time PCR showed greater diagnostic sensitivity, at >90%. Consequently, the overall accuracy of the real-time PCR was approximately 21% higher compared with the LAMP assay. The repeatability of the real-time PCR was assessed within triplicates and between two runs, and showed 100% agreement at 10^4 target dsDNA molecules/ml, as had the LAMP assay. Similarly, the reproducibility of the real-time PCR assay was the same as that of the LAMP assay.

The concordance of the results between the two tests was 92% (104 concordant of 113); however, the discrepancies were mainly observed for the samples analyzed as 10-fold dilutions in the LAMP assay (Tables 2 and 3), which provided 1/10th the sensitivity of the LAMP assay on natural samples, in comparison with the real-time PCR. Considering only the results of the undiluted samples, the sensitivities of the AY-SA-specific LAMP and the real-time PCR were similar (2 discrepancies of 81), as determined for the analytical sensitivity. However, no correlation was seen between the real-time PCR Cq values and the LAMP time-of-positivity values (Fig. 5). Samples from the asymptomatic grapevines (as assessed in 2016) consistently tested negative with both the AY-SA-specific LAMP and the real-time PCR assays.

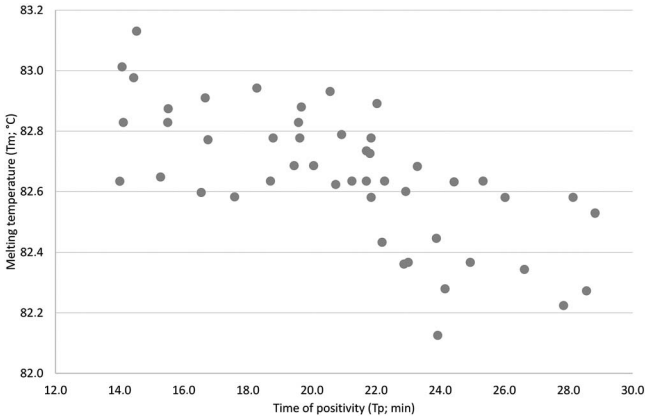

Fig. 4. Correlation between time of positivity (Tp) and corresponding melting temperature (Tm) of the loop-mediated isothermal amplification AY-SA_ftsH assay amplification product. Samples with lower Tp produced amplification products with higher Tm, and vice versa. Tm of the amplification products ranged from 82.1 to 83.1°C.

Table 5. Comparison of performance parameters determined for individual tests according to sanitary status of the grapevines in February 2016, and according to the universal phytoplasma real-time PCR assay (Christensen et al. 2004) and aster yellows (AY) nested PCR (van der Vyver et al. 2019)[a]

	According to sanitary status				According to universal phytoplasma real-time PCR assay		
Performance parameter	**PCR**	**LAMP**	**Universal**	**Nested**	**PCR**	**LAMP**	**Nested**
Total analyzed samples	48	49	49	49	113	114	114
Prevalence	0.75	0.76	0.76	0.76	0.45	0.46	0.46
Sensitivity	0.94	0.73	0.97	1.00	0.84	0.69	0.87
Specificity	1.00	1.00	0.92	1.00	0.98	1.00	0.94
False-positive rate	0.00	0.00	0.08	0.00	0.02	0.00	0.06
False-negative rate	0.06	0.27	0.03	0.00	0.16	0.31	0.13
Positive predictive value	1.00	1.00	0.92	1.00	0.98	1.00	0.92
Negative predictive value	0.86	0.55	0.03	1.00	0.88	0.79	0.89
Positive likelihood ratio	NA	NA	11.68	NA	52.28	NA	13.41
Negative likelihood ratio	0.06	0.27	0.03	0.00	0.16	0.31	0.14
Accuracy	0.96	0.80	0.96	1.00	0.92	0.86	0.90
Diagnostic odds ratio	NA	NA	396.00	NA	327.88	NA	93.21

[a] PCR = aster yellows from South Africa (AY-SA) real-time PCR, LAMP = AY-SA loop-mediated isothermal amplification, Universal = universal phytoplasma real-time PCR, Nested = AY nested PCR, and NA = not applicable.

The specificities of the LAMP and real-time PCR assays developed were narrow and limited only to AY-SA. In contrast, universal real-time PCR (Christensen et al. 2004) and nested PCR (van der Vyver et al. 2019) have broader specificities and, therefore, discrepancies between the tests can be expected. However, variations in the results between the universal real-time PCR and the nested PCR can also be explained by differences in the specificities and concentrations of the samples analyzed in the assays.

Biological case study. The presence of phytoplasma DNA in the collected grapevine field samples was initially analyzed by AY-specific nested PCR (van der Vyver et al. 2019) and, within this study, also with the universal phytoplasma real-time PCR assay (Christensen et al. 2004) and the LAMP AY-SA_ftsH assay and specific real-time PCR AY-SA_ftsH assay developed here (Tables 2 and 3).

The symptomatology of the grapevines was not in complete agreement with the results of the tests. Discordance was mainly observed in the pool of symptomatic grapevines, because 2 of 37 (5.4%) tested negative with the AY-SA-specific real-time PCR. No phytoplasmas were detected in any of the samples from the asymptomatic grapevines with either of these specific molecular tests. However, five samples from the asymptomatic grapevines gave positive results only with the universal phytoplasma real-time PCR assay. The majority of these samples (4 of 5; 80.0%) were collected in November 2018. However, no other molecular tests detected target sequences in these samples; therefore, these samples most likely contained other types of phytoplasmas, and not those from the AY group, or they potentially contained a cross-reactive DNA sequence. Furthermore, this indicates the possibility of mixed phytoplasma infections in these samples.

In the initial sampling in February 2016, all of the samples from the symptomatic grapevines were positive in at least one of the assays tested. However, 35 of 37 grapevines (94.6%) were positive in the AY-SA-specific molecular tests. The number of AY-SA-positive grapevines was inconsistent, and decreased through the sampling period. Eleven samples from the symptomatic grapevines were analyzed with all four phytoplasma assays at all four sampling times. The number of positive samples here decreased from 11 to 5 from February to November 2016 and, by November 2018, only 1 sample was still positive. Furthermore, the concentrations of the target phytoplasma DNA in the samples were quantified relatively by AY-SA real-time PCR, through comparisons with the synthetic target dsDNA standard curves. Positive grapevine samples contained from 4.3×10^4 up to 6.5×10^6 target molecules/ml; however, because the samples needed to be diluted prior to the real-time PCR analysis, the lower target values fell below the LOD of the assays.

On-site sample adaptation of the LAMP assay. A dipstick method and the direct use of a crude homogenate were evaluated for on-site sample preparation, based on speed, simplicity, and performance. Because grapevine material with detectable concentrations of AY-SA was not available, the evaluation was performed on spiked extracts of healthy grapevine leaf veins. The same manual plant tissue maceration step was used for both methods, with different buffers used. Both of these methods were simple and relatively fast to execute; however, each of the methods had its advantages and disadvantages.

The dipstick method was adaptable, given that no pipetting was needed on-site, because the DNA extraction step does not require pipetting (Supplementary Fig. S1). A wash step eliminated any molecular test inhibitors and, hence, there was no need to prepare sample dilutions. However, although the dipsticks are cheap to prepare, they are not commercially available and have to be prepared manually. The process is hard to standardize, because it requires hand cutting of the sticks. Furthermore, the preparation of the sticks must be performed in a DNA-clean environment, to avoid their contamination.

On the other hand, the direct use of the crude homogenate requires dilutions of the samples and, therefore, there is the need for pipetting. The process can be adapted to be more on-site friendly by replacing the pipetting of the samples with a transfer with a single-use inoculation loop. However, imprecise preparation of the dilutions can result in inaccurate results and the introduction of contamination.

The performance of each method was evaluated with the LAMP and real-time PCR assays. The methods gave very similar results with the LAMP assay. The LOD was 10^5 target dsDNA molecules/ml of spiked grapevine extract for both methods, which indicated successful elution of the DNA from the dipsticks into the LAMP reaction mix. Furthermore, the dipstick method also was successfully used on a sample spiked with phytoplasma DNA. The crude homogenate method also gave good results on the real-time PCR detection system; however, here, the dipstick did not provide satisfactory results. The elution of the samples from dipsticks appeared to be very inefficient, because only two samples with 10^7 target dsDNA molecules/ml gave positive results. No cross-reactivity or influence of the grapevine material was observed with any of the sample preparation and detection system combinations.

The dipstick method was also suitable for short-term storage of the DNA for use in molecular tests such as LAMP. Synthetic DNA stored on washed and dried dipsticks was detected after 3 weeks of storage if the initial sample concentration was 10^6 target dsDNA molecules/ml or higher. However, because a LAMP detection system was used, the DNA loss on the dipstick could not be assessed. The integrity of the DNA after storage is not known, although it was sufficient for successful detection with the LAMP assay.

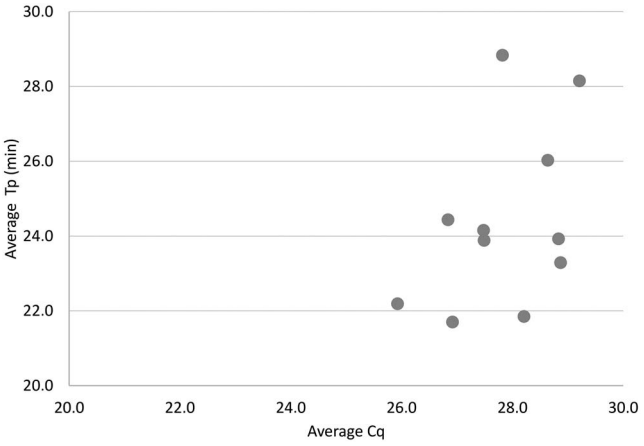

Fig. 5. Correlation between average real-time PCR AY-SA_ftsH assay's quantification cycle and loop-mediated isothermal amplification AY-SA_ftsH assay's time of positivity (Tp) for positive grapevine samples (10-fold dilutions tested in both assays).

Discussion

Molecular detection of phytoplasmas is mostly focused on a limited number of target genes, because targeting these regions is useful for phylogenetic and taxonomic studies (Bertaccini et al. 2019). However, this can present a limitation for the design of tests for specific detection of a phytoplasma group or with very specific requirements for the target sequence, such as with LAMP. On the other hand, genomic data are becoming more available and can be tapped as a source of potential novel target sequences for test design.

Here, we report on the identification of novel target sequences in the genomes of AY-SA through analysis of the genomic sequences, followed by design of a specific LAMP assay and the testing of its performance on a range of reference DNA samples and naturally contaminated samples. We used the molecular tests developed for a case study of phytoplasma occurrence in an experimental vineyard in the Vredendal region in South Africa, and adapted the LAMP assay for on-site diagnostic use.

Although relatively few genomes are available for the phytoplasmas associated with diseases in plants, the amount of information is nevertheless too substantial to be analyzed manually. Therefore, we adapted and applied an automated analysis approach based on comparisons of genomic data (Thomsen et al. 2017). A custom-assembled composite pipeline was used for the design of the LAMP and real-time PCR

assays, starting from whole-genome information. The programs and pipelines designed to implement genomic data for the development of molecular assays are, at present, mainly adapted to the development of PCR assays (Pritchard et al. 2012; Thomsen et al. 2017), and these cannot be easily transferred to the design of LAMP assays. The present shortage of tools that would enable LAMP development from whole-genome data puts the method at a disadvantage in comparison with PCR and real-time PCR. Furthermore, some software packages that were designed to identify signature candidates that are appropriate for LAMP assays are no longer available, such as LAVA. At the time of the present study, there was no tool available, to the best of our knowledge, for LAMP design from whole-genome data that would have suited our needs. On this note, a new Linux-based LAMP primer design tool was described relatively recently (Jia et al. 2019), although this was not available at the initiation of our study.

The ability to rapidly and accurately identify plant-pathogenic organisms represents a critical aspect for the management of plant diseases. As well as the need to be rapid, an ideal disease identification method should be simple and able to be performed on site. Among the different molecular diagnostic approaches, LAMP technology can provide rapid and reliable detection in resource-limited settings and, unlike PCR, LAMP can be transferred easily to an on-site environment (Kogovšek et al. 2015; Mori and Notomi 2020).

Our pipeline identified DNA regions that enabled the development of specific LAMP and real-time PCR assays. However, the wise selection of a negative dataset is crucial to the production of target sequences of sufficient specificity (Thomsen et al. 2017). To ensure specificity of the regions identified, a BLAST step was included, which compared the target regions against negative samples that were not included in the training data. The modularity of the pipeline also allows the user to adapt it to their needs. All of the tools included in the pipeline are also available as web services with user-friendly graphic interfaces and, therefore, are convenient for less-proficient users of bioinformatics tools. Quality control assessed with bioinformatics tools will not guarantee a good and successful LAMP assay design. From 20 assays with similar quality parameters, only 4 worked adequately under the required experimental conditions.

The LAMP assays with acceptable performance characteristics that were developed had the same target region: the *ftsH* (syn. *hflB*) gene coding for ATP-dependent zinc-binding membrane proteases FtsH or HflB. The *ftsH* genes are conserved and are usually present in multiple copies in phytoplasma genomes. It was suggested that the abundance of *ftsH* genes in phytoplasma genomes corresponded with horizontal gene transfer, because they are often clustered within potential mobile units (PMU) (Bai et al. 2006; Seemüller et al. 2011). Four *ftsH* genes were identified in the *Catharanthus roseus* AY strain De Villa chromosome (GenBank accession number CP035949) (Coetzee et al. 2019); however, none directly corresponded to a PMU region because the genomes lacked any identifiable PMU region (Huang et al. 2022). Some indications have linked the aggressiveness of phytoplasmas and the structure of their FtsH proteins, making the *ftsH* gene an interesting target for their detection (Seemüller et al. 2011, 2013).

The amplicon sequence of the developed LAMP AY-SA_ftsH assay corresponds to the genomic sequence from 50691 bp to 50891 bp of the *C. roseus* AY strain De Villa chromosome (GenBank accession number CP035949) (Coetzee et al. 2019). Only one target sequence was identified in the draft AY-SA genome.

The sensitivity of the AY-SA-specific LAMP assay was comparable with that of real-time PCR, with the LOD_{95} for both assays in the range of 7 to 8×10^3 target dsDNA molecules/ml. However, the sample volume in the LAMP assay was 2.5-fold that in the real-time PCR, per reaction. This means that, for successful detection at 95% confidence, the LAMP reaction should contain at least 40 target copies and the real-time PCR at least 13 target copies. The sensitivity did not significantly deviate from the theoretical LOD of real-time PCR. The LOD_{95} for real-time PCR under error-free conditions is based on the calculation of three target copies per reaction. However, the LOD of real samples is affected by noise caused by the sampling, extraction, and amplification reaction; therefore, the experimentally determined LOD can be substantially higher (Forootan et al. 2017). The sensitivity of the LAMP reaction can also be affected by the form of the target DNA. LAMP assays can be performed using nondenatured template DNA (Nagamine et al. 2001); however, it was reported that an additional denaturation step can increase the sensitivity of the LAMP reaction (Aryan et al. 2010). Indeed, heat denaturation increased the sensitivity of the AY-SA LAMP assay for synthetic dsDNA (data not shown).

Rapid development and deployment of molecular tests is essential to support new findings, and for monitoring of novel pathogens and efficient diagnostics. Therefore, the appropriate reference material is needed for test development and validation. Here, we have described the combination of different reference materials and material sources that enable molecular test development when reference and sample materials are scarce or difficult to obtain (Fig. 2).

Both the AY-SA-specific LAMP and real-time PCR showed good performance criteria. Some discordance between molecular tests and the symptomatology of the tested grapevines was expected. The disease symptoms caused by phytoplasmas are similar to those of plant stress (Bertaccini et al. 2014). Moreover, phytoplasmas are not distributed evenly within a plant, and can appear to be present at low concentrations. Therefore, it is impossible to be certain that a negative result means that the plant is actually phytoplasma free, or whether the result is effectively a false negative (Christensen et al. 2004; Constable et al. 2003). For some host plants, it has been reported that the degree of phytoplasma infection is correlated with the level of the visual symptoms (Christensen et al. 2004). A similar correlation can be seen for AY-SA in grapevines, because the sanitation status of the grapevines and the results of the AY-SA-specific real-time PCR here corresponded at 94% (46 of 49 grapevines tested).

Furthermore, the comparison between the different universal and AY-SA-specific tests can confirm the possibility of mixed infections with non-AY phytoplasmas (Botti and Bertaccini 2006). Infections with non-AY phytoplasmas have increased over the years, while infections with AY-SA have decreased. This drop in phytoplasma infections is most likely connected to the extreme drought that severely affected the Western Cape region in 2016 (Mahlalela et al. 2020). Moreover, fewer grapevines were included in the further analysis in this study and, therefore, some positive samples might have been missed.

Concentration of target molecules in positive grapevine samples varied. Variability was observed on the level of the plant and between plants. Variability in target concentrations between plants was expected, because phytoplasmas are known to be inconsistently distributed through the plant (Christensen et al. 2004). Differences in seasonal distribution were clearly observed in 2016. Concentration of the target in the test plants was typically higher in autumn, similar to that described by Constable et al. (2003). In the following years, post extreme drought, (years 2017 and 2018), the number of positive samples was too low to perform comparisons between sampling seasons.

This newly developed LAMP assay is specific; therefore, it has the potential to address key gaps in our knowledge of phytoplasmas through the extension of the study to vectors and other host plants. The assays can be adopted in diagnostic laboratories and for epidemiological studies. It is shown here to be very repeatable and reproducible, because consistent results were obtained regardless of the instruments used, the experimental location, and the people who performed the tests. The primary advantage of the LAMP method is its applicability to on-site settings. However, it needs to be paired with a user-friendly on-site sample preparation method. Both of the methods tested here (the dipstick method and the direct use of crude homogenates) performed well in combination with the LAMP assay. Moreover, the dipsticks provided an easy and adequate way to store samples with high DNA concentrations at room temperature. Storing DNA on filter paper, on chromatography paper, or on Flinders Technology Associates cards are known long-term storage options (Owens and Szalanski 2005; Thompson and Hrabak 2018). However, none of these systems provides fast and easy elution of the sample nucleic acids directly into the reaction mixture of the detection assay. They require either direct addition to the reaction mixture of the paper with the bound sample,

or a separate nucleic acid elution step. Direct addition of the paper is not an acceptable option for fluorometer detection systems such as LAMP, because this disrupts the optical detection. Therefore, the dipstick method would be especially convenient for sample transportation if immediate freezing is not possible.

In this study, we have shown the easy and accessible design of molecular detection assays based on LAMP and real-time PCR using whole-genome data. Although the LAMP assays require experimental testing, the approach appears to be a viable way of increasing the pool of potential target sequences of phytoplasmas. We have shown an efficient validation approach if reference and sample materials are sparse. The molecular assays developed here are highly specific to AY-SA, and show good sensitivity and reproducibility. The main advantage of this LAMP assay is its applicability to the on-site diagnosis setting. This study is also the first to show the applicability of the dipstick DNA isolation technique to a LAMP detection system, and its potential for DNA storage.

Acknowledgments

We thank A. Bertacccini and N. Contaldo (UNIBO) for the phytoplasma DNA from the international phytoplasma collection; N. Mehle, Ž. Ramšak, and M. Pirc (NIB) for helpful discussions, help with nonlinear modeling, and for technical assistance, respectively, and C. Berrie for critical reading and English language editing.

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