Supplementary File S1

Experimental evaluation an optimisation of AY-SA LAMP assays

Twenty LAMP primer sets were tested empirically using a 30 min amplification at 60, 62 and 65 °C. LAMP assays performance was tested using synthetic target and non-target DNA. For all the LAMP assays. any amplification curve in 30 min was considered a positive reaction.

To determine optimal reaction temperature. LAMP reactions were run at three different temperatures 60, 62 and 65°C. Reaction temperature range was selected based on the optimal temperature activity of GspSSD LF DNA Polymerase used in mastermix (Optigene). The concentration of synthetic DNA template was 5×10^4 molecules/reaction. Specific positive signal was observed for the assays ID55, ID71, ID90 and AY-SA_ftsH (ID 58) at all tested temperatures (Table 1). Other tested assays did not produce any positive signal or exhibited evident cross-reactivity with non-target DNA and/or LAMP reagents. However, on average, the shortest detection time was observed at reaction temperature of 62°C for all the specific assays.

Table 1 Results of experimental evaluation of 20 selected LAMP assays on synthetic target DNA at three different amplification temperatures.

Sequence	Assay	Results of LAMP reaction at different reaction temperature[a]			Characteristic Tm for positive samples
		60 °C	62 °C	65 °C	
Seq.1	ID34	cross reactivity	cross reactivity	cross reactivity	no
	ID77	negative	cross reactivity	cross reactivity	no
	ID120	negative	cross reactivity	cross reactivity	no
	ID163	cross reactivity	cross reactivity	cross reactivity	no
	ID206	cross reactivity	cross reactivity	cross reactivity	no
	ID209	cross reactivity	cross reactivity	cross reactivity	no
	ID254	negative	cross reactivity	cross reactivity	no

	ID30	cross reactivity	cross reactivity	cross reactivity	no
Seq.3	ID4	negative	negative	cross reactivity	no
	ID15	negative	negative	cross reactivity	no
	ID17	negative	negative	cross reactivity	no
	ID27	negative	negative	cross reactivity	no
	ID31	negative	negative	cross reactivity	no
Seq.11	ID24	cross reactivity	cross reactivity	cross reactivity	no
	ID8	cross reactivity	cross reactivity	cross reactivity	no
	ID10	cross reactivity	cross reactivity	cross reactivity	no
	ID55	positive	positive	positive	yes
	AY-SA_ftsH	positive	positive	positive	yes
	ID71	positive	positive	negative	yes
	ID90	positive	positive	positive	yes

Primer concentration and ratio of the inner and outer primers can affect the speed of the LAMP reaction. The concentration of the outer F3 and B3 primers was maintained at 0.2 µM. Increasing the concentration of the inner primers FIP and BIP resulted in a decrease in time of positivity (Table 2). Optimal primer mixture was considered to contain 1.6 µM FIP and BIP primer, and 0.8 µM F3 and B3 primers. Free Mg^{2+} availability affects primer annealing and DNA polymerase activity, therefore addition of $MgCl_2$ to reaction mixture was evaluated. Addition of 2 mM $MgCl_2$ in the LAMP reaction decreased the speed of the LAMP reaction, regardless of the primer concentration. The results were consistent for all 4 tested assays.

Table 2 Optimization of FIP and BIP primer concentration. Optimal primer concentration was determined based on the shortest time of positivity.

	Time of positivity (min) at FIP/BIP primer concentration*		
	0.8 µM	1.2 µM	1.6 µM
ID55	20.4	20.2	13.9
AY-SA_ftsH	24.3	17.1	16.2
ID71	21.9	20.5	17.7
ID90	21.2	17.0	15.6

*Target DNA concentration was $5x10^4$ molecules/reaction.

LAMP reaction does not reach temperatures high enough to facilitate DNA denaturation. Accessibility of double stranded DNA to DNA-polymerase is therefore lower, effecting the speed of the reaction. Time of positivity for heat denatured target DNA was 3 to 4 minutes (3 to 4 cycles) shorter in comparison to double stranded target DNA for all 4 tested assays.

The sensitivity of the developed assay was assessed by testing tenfold serial dilutions of denatured synthetic target sequence qBlock_Seq11 ranging from 10^6 molecules/mL to 10^2 molecules/mL. The results are shown in

Table 3. LOD of the LAMP assays was in the range of 10^4 to 10^5 target DNA molecules/mL.

Based on the results of optimisation and sensitivity testing, assay AY-SA_ftsH (ID58) was chosen for final optimisation step and validation. In the final optimisation step. the selected LAMP assay was transferred to reaction temperature of 63°C to enable combining LAMP runs with the assays for general phytoplasma detection and detection of different 16Sr groups described by

Dickinson (2015). The LAMP assay's performance and reaction characteristics did not change, if reaction temperature was 62°C or 63 °C. Therefore, validation was performed at reaction temperature of 63°C.

Table 3 Sensitivity of the LAMP assays assessed on synthetic target DNA.

	Tp			
Target sequence conc.	ID55	AY-SA_ftsH	ID71	ID90
1.00E+06	15.1	14.7	19.1	15.0
1.00E+05	16.6	16.7	20.9	17.9
1.00E+04	neg	21.9	neg	neg
1.00E+03	neg	neg	neg	neg
1.00E+02	neg	neg	neg	neg

Reference:

Dickinson, M. 2015. Loop-mediated isothermal amplifi cation (lamp) for detection of phytoplasmas in the field. Methods Mol. Biol. 1302:98-111.