Final Report

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Project Title (Acronym)
Grapevine flavescence dorée (FD) follow up Vitisens, GRAFDEPI and Qdetect (GRAFDEPI2)

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2. Executive Summary

Project Summary

Grapevine flavescence dorée (FD) follow up Vitisens, GRAFDEPI and Qdetect (GRAFDEPI2)

INTRODUCTION

Europe is the world's main producer and exporter of grapevine planting material and wine. This important economic sector is facing epidemic threats of at least 10 grapevine yellows diseases (GY) caused by phytoplasmas. In Europe the main phytoplasmas associated with GY are '*Candidatus*' phytoplasma solani' (BNp), which is a causal agent of bois noir and FDp, which causes flavescence dorée. Phytoplasmas are notoriously difficult to detect and identify and their specific detection relies exclusively on the molecular methods. Recently new methods, which are reliable, sensitive, fast, less expensive and suitable for using on-sites, have been introduced. Among them is a loop-mediated isothermal amplification (LAMP) method, with several advantages (e.g. low sensitivity to plant extracts inhibitors, speed, robustness, simplicity of use) over the other methods (e.g. the real-time PCR). In a recently finished FP7 project VITISENS, a new LAMP protocols have been developed for specific detection of FDp, however, they have not been tested in the interlaboratories trials. In addition, there is no validated LAMP protocol available for the specific detection of BNp at the moment.

The main **objective**s of this project were:

(1) Development of new loop-mediated isothermal amplification (LAMP) based protocols for accurate, reliable, fast and affordable diagnostics of '*Candidatus* Phytoplasma solani' (BNp), which will be applicable in-field.

(2) To study new possible hosts plants and insect vectors of phytoplasma FDp.

(3) To organize an interlaboratory test performance study (TPS) to obtain validation parameters for the selected LAMP protocols for BNp, as well as for the LAMP assay for FDp detection developed in the course of the FP7 project VITISENS.

METHODS

Using bioinformatics approaches the available gene sequences were assessed for their suitability for the development of LAMP assays for the specific amplification of DNA of 16SrXII phytoplasmas including BNp. Multiple LAMP primer sets were designed to the selected sequences and tested. With further analysis of performance of individual assay, best performing assay was selected. .

For establishing the most suitable sample processing method in-field, different tissues were tested in different homogenization buffers and several approaches for mechanical homogenization of plant material were applied and compared to standard homogenization procedure.

To find new potential host plants and insect vectors of FDp in the Slovene and Austrian field monitoring started in 2008, was continued in 2013 (GRAFDEPI1) and 2014 (GRAFDEPI2). The collected insects and plant samples were tested for the presence of FDp.

Several transmission trial set-ups were prepared to evaluate a potential role of *Orientus ishidae*, *Oncopsis alni* and *Scaphoideus titanus* as vectors in transmission of FDp to known or alternative hosts.

TPS was performed to validate LAMP protocols for the detection of 16SrV phytoplasmas including FDp and 16SrXII phytoplasmas including BNp. Ten laboratories from the research and plant protection area from Europe and Australia participated in this TPS. Additionally, LAMP FDp assay was compared with a Qualiplante/Hyris isothermal amplification assay for FD (code: ISOA FD) by three laboratories (FR-ANSES, IT-CRA-PAV and SI-NIB).

RESULTS AND CONCLUSIONS

Gene *secA* was chosen as the most suitable gene for developing of the LAMP assay for the specific 16SrXII phytoplasmas including BNp detection. The assay was validated in accordance with EPPO recommendations. The LAMP assay was shown to be only up to 3-times less sensitive than qPCR.

For the on-site sample preparation protocol a homogenization of the leaf veins with an Ultra Turrax Tube Drive (IKA) device and ELISA buffer 1 was selected.. No preceding extraction of DNA is needed for LAMP testing.

In Austria individuals of *Phlogotettix cyclops* (Auchenorryncha: Cicadellidae) were tested positively with qPCR for FDp. From all tested wild plants only *Clematis vitalba* could be shown to harbor FDp. In Slovenia, *C. vitalba*, *Alnus glutinosa*, *A. incana* and *Alianthus altissima* are infected with different types of FDp. In addition, alder trees also harbor alder yellows phytoplasma, which is from the same ribosomal group 16SrV-C as phytoplasma FDp that infects *C. vitalba* and grapevine. Other 285 samples of native plants were tested negative to FDp. Among the insects, 7% of *Scaphoideus titanus* individuals were infected with FD-D, while 50 to 62 % of *Orientus ishidae* and *Oncopsis alni*, respectively were mix-infected with different types of phytoplasma from the group 16SrV.

The results from the transmission trials show that very few *S. titanus* nymphs are able to acquire the FDp from infected *A. glutinosa* twigs and to transmit it to grapevine. These preliminary results might support the hypothesis that alder tree populations are a possible resource for a primary infection. In addition, the transmission experiments of FDp from *O. ishidae* to the artificial media showed positive results in 15% of cases after 2 days of feeding of insects on the medium, and in 43% after 3 to 4 days of feeding. However, their role in the transmission of FDp to grapevine remained unclear because there is no evidence of the successful transmission to the plant.

During TPS eight different devices were used for amplification with LAMP. Ten laboratories have analyzed 18 unknown samples using LAMP FD and LAMP BN assays. In addition, those samples were also analyzed by ISOA FD Qualiplante assay in three laboratories. The accuracy of all assays is higher than 98%. In one single case, one BNp positive sample was concluded as negative after using LAMP BN assay. FDp negative samples were concluded as FDp suspected samples with LAMP FD assay three times (no. of all results with this assay 179), and once when using ISOA FD Qualiplante assay (no. of all results with this assay 54). All other results were correct, indicating that all tested assays are suitable for reliable and accurate detection of BNp and FDp.

3. Report

3.1 INTRODUCTION

Europe is the world's main producer and exporter of grapevine planting material and wine. This important economic sector is facing epidemic threats of at least 10 grapevine yellows diseases (GY). GY are caused by phytoplasmas – taxonomically unrelated wall-less phytopathogenic bacteria from the Mollicutes class.

Phytoplasmas are transmitted from plant to plant by sap-feeding insect vectors from the order Hemiptera, and they propagate within the cytoplasm of both insects and plants. In plants they exclusively inhabit phloem that is responsible for transport of carbohydrates and nutrients to plant sink tissues. In Europe the main phytoplasmas associated with GY are '*Candidatus* phytoplasma solani' (BNp), which is a causal agent of bois noir, and FDp, which causes flavescence dorée, the most devastating phytoplasmal disease of grapevine. While BNp is widespread in Europe, FDp is a quarantine pest in Europe, listed in the EU2000/29 Council Directive on harmful organisms, as well as in the EPPO A2 quarantine list of pests. Its only known natural insect vector is *Scaphoideus titanus*, which is continuously spreading from western Europe in the NE direction. The FD spread follows the spreading of *S. titanus* and it was reported for the first time in France around 1950, in Italy in 70's, in Slovenia in 2005 and few isolated FD outbreaks have been recorded in Austria since 2009. Because of the FD spread, there are more and more reports that both phytoplasmas appear in the same vineyard or even in the same plant.

The collected data support the hypothesis that FDp has the European origin having first been accidentally introduced to grapevine from alter plant reservoirs, and then diffused in vineyards by *S. titanus* that turned out to be a competent vector. Other reservoirs than already known alder and clematis may exist that we have not yet identified, and new potential exotic vectors species to grapevine besides *Orientus ischidae* may be introduced in the future.

Phytoplasmas are notoriously difficult to detect and identify, being present at low concentrations with spatial and temporal fluctuating distribution in the phloem of the host plant and cannot be routinely culture-grown. Consequently, our knowledge and understanding of their hosts, resistance, insect transmission and mechanisms of phytopathogenicity is severely limited, and their specific detection relies exclusively on the molecular methods. In past, different molecular approaches have been applied in BNp and FDp diagnostics. More recently, new methods which are more reliable, sensitive, fast, less expensive and suitable for using on-sites have been introduced. Among them is a loop-mediated isothermal amplification (LAMP) method, with several advantages (e.g. low sensitivity to plant extracts inhibitors, speed, robustness, simplicity of use) over the other methods (e.g. the real-time PCR). In a recently finished FP7 project VITISENS, a new LAMP protocol has been developed for specific detection of FDp, however, it has not been tested in the interlaboratory trials. In addition, there is no LAMP protocol available for the specific detection of

BNp at the moment. Because the symptoms caused by both phytoplasmas on the grapevine are undistinguishable by the visual inspection, a new specific protocol for very fast detection of BNp in grapevine is urgently needed.

3.2 OBJECTIVES

The main goals of the project were:

(1) Development of a new loop-mediated isothermal amplification (LAMP) based protocols for accurate, reliable, fast and affordable diagnostics of '*Candidatus* Phytoplasma solani' (BNp), which will be applicable in-field.

(2) To study new possible hosts plants and insect vectors of phytoplasma FDp.

(3) To organize an interlaboratory trial to obtain validation parameters (EPPO PM7/98) (a ring-test) for the selected LAMP protocols for BNp, as well as for the LAMP assay for FDp detection developed in the course of the FP7 project VITISENS.

Work on the project was divided in 5 work packages. The project was led by NIB, whose role was clarified in WP 5 "Consortium management and dissemination of results". NIB reviewed and assessed compliance by the partners with their contractual obligations under the project. It ensured that all aspects of communication and reporting were met and that important results of the project will be disseminated beyond the consortium to a wide and relevant audience in order to maximize the project's impact.

There were no deviations from the project objectives; all tasks were concluded with the proposed deliverables.

3.3 METHODS AND RESULTS

3.3.1 WP1: Loop-mediated isothermal amplification (LAMP) protocol for BNp

Deliverable: Standard protocol for BNp DNA amplification by LAMP
Current status: Confidential before the publication

3.3.1.1 Lamp primer design and reactions

Sequences for rRNA genes (*16S*, *23S* and *ITS* region), *secA*, *secY*, *stamp* and *tuf* were retrieved from NCBI and aligned in the VectorNTI software (InforMax). Regions of homology, specific for stolbur phytoplasma strain (16SrXII-A), were determined. LAMP assays were designed to all seven regions using LAMP Designer software (Premier Biosoft) and were synthesized by Integrated DNA Technologies. All LAMP reactions were performed in a 25 µL reaction volume, containing 5 µL of sample DNA or homogenate, 2x Isothermal Master Mix (OptiGene), 0.2 µM F3 and B3 primers, 2 µM FIP and BIP primers and 1 µM F-loop and B-loop primers. LAMP reactions were performed in 8-well strips, 96-well or 384-well plates in a GenieII (Optigene) or in a Roche LC480 instrument, respectively. For LAMP product annealing temperature determination (Tm), the fluorescence was detected (on the FAM channel for the Roche LC480) during the cooling of the samples from 98°C to 80°C (GenieII) or during the heating of the samples from 62°C to 98°C (Roche LC480).

3.3.1.2 Lamp assay selection and optimization

The performance of all seven LAMP assays was initially tested on a small set of samples (BNp positive and negative grapevine samples, phytoplasma isolates from other 16Sr groups, bacteria). LAMP assays were run at 62°C or at 65°C. The LAMP assays that showed specific amplification and gave shortest time of positivity (Tpos) at selected temperature of amplification were selected for further testing.
Sets of primers were designed to the 16S rRNA, 23SrRNA, ITS, *secA*, *secY*, *stamp* and *tuf* genes, where regions of sequence specific for 16SrXII-A phytoplasmas were identified. The performance of each LAMP assay, in terms of time to positive reaction (Tpos), specificity and sensitivity, was evaluated by testing samples with different amount of BNp and BNp negative plant samples. Almost all assays performed better at 62°C then at 65°C. Out of four LAMP assays (23SrRNA, *secA*, *stamp* and *tuf* assay), the secA and tuf assays showed high specificity. SecA LAMP assay however, showed higher sensitivity than tuf LAMP assay and was selected for further validation.

3.3.1.3 Validation of the LAMP secA assay

SecA LAMP assay was validated in accordance with EPPO recommendations (EPPO 2010). The analytical sensitivity of the secA LAMP assay was estimated to be up to 9 - 27 BNp DNA copies in a reaction, which is only 3-times lower sensitivity than that of the BNp specific qPCR assay.

SecA LAMP assay was further evaluated by testing grapevine leaf vein samples with different amounts of the BNp DNA (i.e. diagnostic sensitivity), which was estimated with qPCR (lower Cq values represent higher BNp DNA quantity). With the secA LAMP assay it was possible to detect BNp in altogether 57 tested samples. In 5 samples with low BNp titre (Cq above 34.5) BNp DNA was confirmed only when the undiluted sample DNA was retested with LAMP assay. All positive reactions were observed before 30 minutes of amplification.

Analytical specificity of the secA LAMP assay was firstly evaluated by in silico analysis, which showed a high predicted specificity to 16SrXII-A phytoplasmas, including BNp. No difference in the specificity was observed when different types of BNp (according to tuf-type) were tested. Phytoplasma DNA from other 16Sr groups, bacterial and fungal isolates and healthy hosts were tested and in no case secA LAMP assay give positive reactions.

Anneal temperature (Tm) analysis of the LAMP product showed that all signals obtained in the case of BNp infected samples were specific. The Tm for the specific amplicon ranged from 84°C to 85°C, when samples were analyzed on the GenieII machine, and from 85°C to 86°C when samples were analyzed on the Roche LC480 machine. In five samples with low BNp titre (Cq above 34.5), up to 2°C higher Tm was determined when analyzed 10-times diluted, while undiluted sample DNA gave Tm in the expected range.

Various grapevine cultivars and different plant tissues were analyzed with secA LAMP assay to evaluate the selectivity of the assay. All the results obtained with the secA LAMP were in accordance with the qPCR results.

Repeatability and reproducibility of the assay were evaluated by analysing several replicates of DNA sample with various BNp DNA concentrations. When testing replicates of the same sample with high and medium concentrations of the BNp DNA, e.g. qPCR Cq value lower than 33, the assay was shown to be 100 % repeatable, where all replicates gave positive result. At lower concentrations the detection of the BNp DNA by the secA LAMP assay varied, which can be attributed to stochastic effects in target copy distribution in replicates (Hren et al. 2007). Results were 100 % reproducible when tested with different devices, by different operators, on different days and with different reaction mixes. The reproducibility of the assay was additionally tested in a TPS.

3.3.2 WP2: Homogenization and extraction protocol

Deliverable: Sample collection and homogenization/DNA extraction protocol

3.3.2.1 Sample collection and homogenization/DNA extraction protocol

For the on-site detection of BNp in grapevine samples, a sample preparation procedure developed for the on-site FDp testing (Kogovšek et al., 2015) was applied and additional homogenization approaches were tested as well. Altogether 101

grapevine samples infected with BNp were submerged to the direct homogenate testing.

FastPrep-24 and ELISA buffer were used for preparation of 67 samples, from which in 61 homogenates BNp was confirmed (i.e. 91 %). Bertin Minilys device and portable hand-held FastPrep-1 device showed high performance as well, however both were tested only on a limited number of samples.

4.3.2.1 Sensitivity and selectivity of the on-site BNp testing approach

For the on-site applicable homogenization approach, the Ultra Turrax Tube Drive (UTTD, IKA) device and ELISA buffer were used (Kogovšek et al., 2015). Analytical sensitivity of the on-site procedure, which includes homogenization of the leaf veins with UTTD and direct homogenate testing with LAMP assay, was compared to the standard in-lab BNp detection procedure with FastPrep homogenization, KingFisher assisted DNA extraction and qPCR analysis. The whole procedure was repeated 5-times and in average the on-site procedure was shown to detect BNp DNA in samples where as low as 9-27 copies of BNp DNA were present.

3.3.3 WP3: Searching for new hosts and vectors of FDp

Deliverables: Discovery of new potential host plants and insect vectors, if present

Evaluated possible role of *Orientus ishidae* and *Oncopsis alni* in the transmission of FDp

Evaluated possible role of other potential vectors in the transmission of FDp

3.3.3.1 Austrian field monitoring for alternative insect vectors and reservoir plants of FDp

To find new potential host plants and insect vectors of FDp several wild plants and insects commonly present inside or outside the vineyards will be collected and tested for the presence of FDp in order to evaluate their potential role as alternative hosts/vectors. The field monitoring started in 2012 was continued in 2013 (GRAFDEPI 1) and 2014 (GRAFDEPI 2) at the same monitoring sites as in 2012 in the South of Styria (Glanz) and in the southeast of Styria (Bayrisch Kölldorf). All monitoring sites are located close to vineyards where FDp has been detected in single grapevines in the previous years and within FD-focus zones therefore. Additionally, monitoring sites in non-infested vine growing areas in Burgenland (Eisenberg, Siegendorf, Deutschkreuz, Wulkaprodersdorf) were sampled.

Insects were collected by beating net, vacuum sampling and yellow sticky traps. Beat sampling and vacuum suction sampling were carried out in August and yellow sticky traps were installed in August and September. Collected insect specimens were

immediately cooled in cool boxes and stored at -18°C for later species and phytoplasma identification.

The plant samples were taken after the first symptoms of grapevine yellowing appeared (late August and September). The collected insects and plant samples were tested for the presence of FDp using the molecular techniques described in Angelini et al. (2007) and in EPPO (2007).

In the course of the field monitoring in total 43 individuals of *Phlogotettix cyclops* (Auchenorryncha: Cicadellidae) were caught at the sites in Burgenland in 2014. At Siegendorf, Deutschkreuz, Wulkaprodersdorf. *P. cyclops* was frequently found on *Clematis vitalba* and on *Vitis vinifera*. At each site several individuals were found to habor FDp. Also in the focus zone of South Styria (Glanz) *P. cyclops* (caught from *C. vitalba*) was tested positively with real-time PCR for FDp. All isolates sustained from *P. cyclops* had the same RFLP patterns and this pattern could be allocated to the 16SrV-C reference isolate from Austria. In contrast to the results of GRAFDEPI 1, FDp could not be detected in the tested individuals of *O. alni* and *Psylla alni*, caught in Deutschkreutz. In 2014 *O. ishiadae* could not been found at any monitoring site. From all tested wild plants only *C. vitalba* could be shown to harbor FDp.

3.3.3.2 Slovenian field monitoring for alternative insect vectors and reservoir plants of FDp

All data on alternative host plants and vectors collected during the official survey of the Phytosanitary Administration of Slovenia, during GRAFDEPI1 and GRAFDEPI2 were reanalyzed (Table 1). Their potential role as alternative hosts/vectors was estimated according to detected type of FDp in the sample (Table 2).

Table 1: FDp infected alternative host plants and vectors from 2008 to 2014.

	No. of samples	No. of FDp positive samples	% of FDp positive samples	FDp type
Clematis vitalba	142	86	61	FD-C
Alnus glutinosa & A. incana	31	28	90	FD-C
Alianthus alitissima	131	6	5	5x FD-C; 1x FD-D
Other plant species in the vicinity of the infected vineyard	285	0	0	
Scaphoideus titanus	59	4	7	FD-D
Orientus ishidae	21	13	62	Fd70, FD-D, mix (FD-C, FD-D, FD70, ALY)
Oncopsis alni	4	2	50	mix (FD-C, FD-D, FD70, ALY)
Dictyophara europaea	5	0	0	

Table 2: FDp and BNp infected plants of grapevine from 2005 to 2014.

Year	No. of samples	BNp positive	FDp positive	FDp+BNp positive	FD-C	FD-D	Other
2005	148	102 (69%)	9 (6%)	4			
2006	164	99 (60%)	13 (8%)	0	2	6	0
2007	148	93 (63%)	4 (3%)	0	4	0	0
2008	217	145 (67%)	14 (6%)	3	1	6	0
2009	375	289 (77%)	46 (12%)	8	7	26	0
2010	331	231 (70%)	37 (11%)	4	2	31	FD70+FD-D, ALY
2011	350	233 (67%)	34 (10%)	1	1	31	0
2012	344	272 (79%)	42 (12%)	11	4	35	0
2013	333	243 (73%)	30 (9%)	7	0	29	FD70
2014	349	253 (72%)	15 (4%)	7	5	16	FD70

3.3.3.3 Transmission trial with FDp infected *Alder glutinosa* and *S.titanus* to grapevine

Transmission trials were conducted with FDp-positive *A. glutinosa* twigs as source of infection because results from studies in previous years have shown that *S. titanus* was not able to survive on *C. vitalba*. *A. glutinosa* twigs were tested for FDp infection before starting the transmission trials. The grape variety “Scheurebe” (=Sämling) was chosen as test plant because this variety is very susceptible to FDp, displaying symptoms very early. A laboratory rearing for *S. titanus* was established in an environmental chamber as described in Privet et al. (2007).

FDp infected alder twigs were put together with a young potted grapevine (cv. Scheurebe) in a plexiglas cylinder (height: 102. 5 cm; diameter: 25 cm). At the start of the transmission trial the alder leaf mass was higher than that of the potted grapevines. Five replicates (cylinders) were set up. The cylinders had a fine net at the upper end for ventilating as well as several openings for manipulation and watering of plants (Fig.1). The transmission trial was carried out in an environmental chamber at 20°C and 75% RH.

Forty *S. titanus* nymphs taken from the laboratory rearing were placed in each of the five plexiglas cylinders. Mainly the second and the third instar were used for the transmission trial.

Every 5 to 8 days the following parameters were recorded:

- the position of *S. titanus* nymphs: sit on the alder leaves, sit on grapevine
- the number of *S. titanus* alive
- the number of *S. titanus* dead

Dead individuals were removed for later PCR analysis. The duration of the transmission trials was 57 days. After that all *S. titanus* alive were collected and cool stored for PCR analysis.

Figure 1: Transmission trial set-up, plexiglas cylinder with *Alnus glutinosa* twigs in water bottles and potted grapevines (cv. Scheurebe).

In general very few *S. titanus* could be relocated in the plexiglas cylinder (54 out of 240 individuals). The mortality rate was very high indicating that *A. glutinosa* is not a thriving host plant for *S. titanus*. After 57 days four *S. titanus* were alive. The total number of *S. titanus* tested for FDp infection was 54 (51 adults and 3 larvae in the developmental stage fife). By visual control several larvae and exuviae of *S. titanus* was observed on alder leaves (Fig. 2).

Figure 2: Detail of plexiglas cylinder with *Alnus glutinosa* twigs and grapevine (cv. Scheurebe). Red marked a larva of *S. titanus* on an alder leaf.

PCR analysis revealed that three adult and one nymph of *S. titanus* and 2 potted grapevines were tested positive for FDp (Table 3).

Table 3: Results from transmission trial with *S. titanus*, *A. glutinosa* as FD infection source and grapevine (cv. Scheurebe).

Replicate	Tested *S. titanus*	Number of FDp positive *S. titanus*	FDp status of potted grapevines (cv. Scheurebe)
cylinder 1	3	1 adult	negative
cylinder 2	16	0	negative
cylinder 3	5	0	negative
cylinder 4	5	1 larva	negative
cylinder 5	13	2 adults	positive
cylinder 6	12	0	negative
In total:	54	3 adults, 1 larva	2 positive grapevines

3.3.3.4 Transmission trial of FDp infected *O. ishidae* and *O. alni* to grapevine

A number of phytoplasma transmission tests have been successfully performed on artificial media using leafhoppers (references listed in Bosco and Tedeschi, 2013), therefore similar experiments was chosen also to test vectoring ability of leafhopper *O. ishidae*.

157 adults of *O. ishidae* were collected by catcher from *A. glutinosa* trees that are infected with different FD genetic clusters. Five *O. ishidae* were killed and plunged into feeding medium (10 % sucrose, 0.2 % fructose, 0.375 % K2HPO4, 0.028 % MgCl2, pH 7.5) for one day (negative control of possible contamination of media by insect surface), and the rest of them were used in transmission trial. 152 feeding chambers was prepared by filling the lid of a microcentrifuge tube with 200 µl of feeding medium and closing the lid with parafilm. The bottom of the tube was cut off and one field-collected adult of *O. ishidae* was isolated inside one microcentrifuge tube, then the tube was closed with a small cotton wool ball. Tubes were maintained with the cap facing a light source to attract the insects to the feeding medium. 60% of 152 *O. ishidae* specimens died in the first day of transmission trial, and those were excluded from further testing. The rest of specimens were survived in feeding chambers up to 4 days. At the end of the inoculation period (2-4 days after starting), the feeding medium were collected with a pipette. From each of feeding medium DNA was extracted using King Fisher procedure according to Mehle et al. (2013) with minor modification: 200 µl of feeding medium was mixed with 400 µl of lysis buffer and 25 µl of proteinase K solution, after the lysis step 300 µl of lysate was washed and finally DNA was eluted in 100 µl of elution buffer. DNA was extracted also from 34 *O. ishidae* (crushed in liquid nitrogen) using the same procedure. Each DNA sample was then analyzed by a real time PCR (Hren et al., 2007). All samples of O. ishidae were positive for FDp, and further characterization of FDp in some samples revealed the mix of FDp types. FDp was detected also in 15% out of 46 feeding media where *O. ishidae* has been feeding 2 days; and in 6 out of 7 (43%) feeding media where *O. ishidae* has been feeding longer (3-4 days), however it was not detected in any of 5 feeding media where *O. ishiade* has been plunged into them for one day (Table 4). Only successful transmission to the plant is the final evidence of vectoring ability, however successful transmission to artificial feeding media confirmed the transmission capability of *O. ishidae*.

Table 4: Transmission of FDp to the artificial feeding medium by field-collected adults of *Orientus ishidae*

Type of sample tested*	No. of samples tested	No. of positive samples	Range of Cq values	Average Cq values
O. ishidae	34	34 (100%)	19-35	24
Days in feeding media: 2	46	7 (15%)	33-37	36
Days in feeding media: 3 to 4	14	6 (43%)	33-38	35
Days in feeding media: 1	5	0 (0%)	neg	neg

Less than 10 adults of *O. alni* were collected by catcher from *A. glutinosa* trees that are infected with different FDp types. Additionally high mortality of insects after catching was observed; therefore transmission of FDp to artificial feeding medium by field-collected adults of *O. alni* was not performed. Additionally, two *Catharantus roseus* and 12 grapevines were planted close to the infected *A. glutinosa* trees, but the transmission of FDp from *A. glutinosa* to the *C. roseus* and grapevines by any species of insects were not confirmed in limited period of the experiment.

3.3.4 WP4: Test performance study (TPS) (ring test) of the LAMP assays for detection of BNp and FDp

Deliverable: The validated parameters of LAMP assays for Fdp and BNp

3.3.4.1 Context and goal of the test performance study

A test performance study (TPS) was performed to validate LAMP protocols for the detection of 16SrV phytoplasmas including FD phytoplasma (FDp) and 16SrXII phytoplasmas including '*Candidatus* Phytoplasma solani' (BNp) with LAMP assays. LAMP assay for BNp detection was developed in the frame of WP 1 of this Euphresco GRAFDEPI2 project and is not published yet, while the LAMP for FDp assay was described in Kogovšek et al. (2015). The validation data for testing FDp by LAMP obtained before the TPS was organized are deposited with the EPPO database on Diagnostic Expertise: http://dc.eppo.int/validationlist.php.

Ten laboratories from the research and plant protection area from Europe and Australia participated in this TPS (Table 5).

Additionally, LAMP FDp assay was compared with a Qualiplante/Hyris isothermal amplification assay for FD (code: IsoA.FD/80; hereinafter ISOA FD Qualiplante) by three laboratories (FR-ANSES, IT-CRA-PAV and SI-NIB).

Table 5: List of participants in alphabetical order

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[1] The protocol and samples for this TPS were sent to two additional laboratories, but we did not get the results of their analysis till the end of September 2015 (the reason of one lab was the lack of appropriate equipment, and the other one did not have time to perform analysis).

[2] SI-NIB was the organizer of the TPS and was also a participating laboratory. The staff involved in the analysis as a participating laboratory was not the same as those involved in the TPS preparation (listed in this table).

3.3.4.2 Material and methods

3.3.4.2.1 Methods included in TPS

LAMP assay for BNp detection developed in the frame of WP 1 of this EUPHRESCO GRAFDEPI2 project (hereinafter LAMP BN): the protocol was prepared by SI-NIB and distributed to participants. The protocol is in Appendix 1.

LAMP for FDp assay described by Kogovšek et al. (2015) (hereinafter LAMP FD): the protocol was prepared by SI-NIB and distributed to participants. The protocol is in Appendix 1.

Qualiplante/Hyris isothermal amplification assay for FD (code: IsoA.FD/80): a scan of the protocol is in Appendix 2.

3.3.4.2.2 Chemicals

BNp primer mix, FDp primer mix and Isothermal Master Mix (Optigene Ltd; Cat. No. ISO-001) used for LAMP BN and for LAMP FD: supplied by SI-NIB. For testing of the homogeneity and stability of those chemicals see point 3.3.4.2.4.

Qualiplante/Hyris isothermal amplification kit for FD (code: IsoA.FD/80; batch number: 1505005).

3.3.4.2.3 Samples

Eighteen samples (labeled as 1-18), and FDp and BNp positive controls (labeled as PC FD and PC BN) were supplied by SI-NIB (Table 6). DNA samples from FD-infected clematis, FD-infected, BN-infected and healthy grapevine plants were provided by SI-NIB. Grapevine leaf samples were collected in the field from several grapevine plants, including different cultivars and different winegrowing regions of Slovenia. Additionally, one sample of fungi DNA and one sample of bacterial DNA were included in this TPS.

DNA extraction procedure used for FD-infected, BN-infected and healthy plant samples: 1g of leaf mid-vein tissue was homogenized in 2 mL of ELISA (264 mM Tris, 236 mM Tris-HCl, 137 mM NaCl, 2% PVP K-25, 2 mM PEG 6000, 0.05 % Tween 20, pH 8.2) or lysis buffer (from QuickPick^{TM} SML Plant DNA kit, Bio-Nobile) using tissue homogenizer (FastPrep^{R}-24 with TN 12x15-TeenPrep^{TM} Adapter (MP Biochemicals)). Total DNA was extracted using QuickPick^{TM} SML Plant DNA kit (Bio-Nobile) and a magnetic particle processor (KingFisher^{R} mL, Thermo Scientific) (Mehle et al., 2013a). Total DNA extract was eluted in 200 µl of elution buffer (QuickPick^{TM} SML Plant DNA kit + KingFisher). For grapevine leaf mid-vein tissue tenfold diluted DNA and for clematis sample 100-fold diluted DNA were prepared. Twenty DNA of fungal strains of the genus *Ascomycota* (two *Cladosporium* cf.*,* three *Aureobasidium pullulans,* two *Thysanophora penicillioides,* two *Aspergillus jensenii,*

two *Penicillium brevicompactum,* two *Cordycipitaceae,* two *Fusarium cf. tricinctum, Phaeomoniella, Botryotinia fuckeliana, Didymella exitialis, Meyerozyma guilliermondii, Taphrina like*) were obtained from the collection of the Agricultural Institute of Slovenia, Slovenia. Some of these strains are present in the grapevine microflora. A mix of tenfold diluted DNA from all of these strains in the same proportion was prepared and used for this TPS.

Additionally, 20 bacterial isolates from plant extracts of various grapevine cultivars grown on nutrient agar (NA) (Bacto Nutrient Agar, Difco) or YPGA (yeast extract 7·0 g, proteose peptone 5·0 g, glucose 10·0 g, agar 15·0 g, distilled water 1 L, pH 7·0) were prepared by SI-NIB. A mix of tenfold diluted DNA from all of these isolates in the same proportion was used for this TPS.

All samples were analyzed for the presence of FDp and BNp with a real time PCR procedure (Hren et al., 2007; Mehle et al., 2013b). Additionally, samples were analyzed also with phytoplasma universal real time PCR (Christensen et al., 2004). In order to control the DNA extraction procedure, 18S rRNA endogenous control was included in the analysis. Detailed results of real time PCR are presented in Appendix 3. Further molecular characterization was then performed on FDp positive samples by PCR with FD9R1/FD9F1 primers (Daire et al., 1997) followed by nested PCR with FD9F3b/ FD9R2 primers (Clair et al., 2003) and RFLP with *AluI* and *TaqI* enzyme (Angelini et al., 2001; Filipini et al., 2009) (Table 6). Molecular characterization of some BNp positive samples was performed by PCR with Tuf1f/Tuf1r primers followed by nested PCR with TufAYf/TufAYr primers (Schneider et al., 1997) and RFLP with *HpaII* enzyme (Langer and Maixner, 2004) (Table 6).

Samples were coded. Several aliquots of each sample were prepared and were kept at – 20 °C. For testing of the homogeneity and stability of those aliquots see point 3.3.4.2.4.

Table 6: Samples included in TPS (for details see Appendix 3)

Sample code	FD status	BN status	Details – FD/ BN isolates	Details - samples
1	pos	neg	FD-D	Mix of four FD infected grapevine samples: D546/12, D921/12, D236/14, D278/14
2	neg	pos	tuf- b	Mix of four BN infected grapevine samples: D282/13, D666/13, D375/13, D736/13
3	pos	neg	FD-C	FD infected clematis sample: D738/13
4	neg	neg	/	Mix of four healthy grapevine (cv. Chardonnay) samples: D778/14, D692/14, D481/14, D404/14
5	neg	neg	/	Mix of four healthy grapevine (cv. Rumeni muškat) samples: D555/14, D342/14, D667/13, D399/13
6	pos	pos	FD-D, tuf- b	Mix of two FD infected grapevine samples: D536/13, D693/13; and mix of two BN infected grapevine samples: D436/13, D665/13
7	neg	neg	/	A mix of DNA of 20 fungal strains of the genus Ascomycota
8	neg	pos	tuf-type not determined	Mix of four BN infected grapevine samples: D491/14, D499/14, D578/14, D605/14
9	pos	neg	FD-D	Mix of four FD infected grapevine samples: D594/12, D727/12, D917/12, D454/14

10	neg	neg	/	Mix of four healthy grapevine (cv. Malvazija) samples: D726/14, D581/14, D577/14, D576/14
11	neg	neg	/	Mix of DNA of 20 bacterial isolates from plant extracts of various grapevine cultivars
12	pos	neg	FD-C	Mix of four FD infected grapevine samples: D346/14, D397/10, D404/10, D1780/09
13	neg	pos	tuf-type a	Mix of four BN infected grapevine samples: D275/13, D564/13, D767/13, D776/13
14	neg	neg	/	Mix of four healthy grapevine (cv. Refošk) samples: D648/14, D580/14, D554/14, D541/14
15	neg	neg	/	Mix of four healthy grapevine (cv. Merlot) samples: D582/14, D308/14, D252/14, D230/14
16	neg	neg	/	Mix of four healthy grapevine (cv. Žametna črnina) samples: D462/14, D379/14, D322/14, D437/13
17	neg	pos	tuf-type not determined	Mix of four BN infected grapevine samples: D291/14, D495/14, D501/14, D732/14
18	neg	neg	/	Sterile nuclease free water
PC FD	pos	neg	FD-D	Mix of four FD infected grapevine samples: D861/12, D265/13, D710/13, D892/12
PC BN	neg	pos	tuf-type a	Mix of four BN infected grapevine samples: D657/13, D406/13, D447/13, D471/13

3.3.4.2.4 Homogeneity and stability testing

The homogeneity and stability testing were performed by SI-NIB according to methodology proposed in EPPO standard PM7/122. The aliquots of samples and chemicals were randomly chosen and tested by LAMP with two different operators and by two different devices for amplification. Stability testing was conducted in conditions that mimic transport (at room temperature) and storage conditions (at -20°C). The summary of this test of homogeneity and stability appear in Table 7 and 8.

All samples and chemicals were shown to be stable at room temperature for two days. Longer storage at room temperature caused a damage of sample 3 (FDp infected clematis sample), therefore this was taken into account when the results were analyzed.

Table 7: Homogeneity and stability of aliquots tested with LAMP FD

		Operator	1	2	1	1	1
		Device for amplification	Roche LC480	Roche LC480	Genie II	Genie II	Genie II
		Date	2.4.2015	10.4.2015	10.4.2015	14.4.2015	6.5.2015
		Aliquot of samples and chemicals	1	2	3	3	4
		Aliquot stored	**-20 °C**	**-20 °C**	**3 days at room T**	**7 days at room T**	**2 days at room T**
		details	Appendix 4.A)	Appendix 4.B)	Appendix 4.C)	Appendix 4.D)	Appendix 4.E)
Sample code	FD status	BN status					
1	**pos**	**neg**	pos	pos	pos	nt	pos
2	**neg**	**pos**	neg	neg	neg	nt	neg
3	**pos**	**neg**	pos	pos	sus	neg	pos
4	**neg**	**neg**	neg	neg	neg	nt	neg
5	**neg**	**neg**	neg	neg	nt	neg	neg
6	**pos**	**pos**	pos	pos	nt	pos	pos
7	**neg**	**neg**	neg	neg	nt	neg	neg
8	**neg**	**pos**	neg	neg	nt	neg	neg
9	**pos**	**neg**	pos	pos	nt	pos	pos
10	**neg**	**neg**	neg	neg	nt	neg	neg
11	**neg**	**neg**	neg	neg	nt	neg	neg
12	**pos**	**neg**	pos	pos	nt	pos	pos
13	**neg**	**pos**	neg	neg	nt	neg	neg
14	**neg**	**neg**	neg	neg	nt	neg	neg
15	**neg**	**neg**	neg	neg	nt	neg	neg
16	**neg**	**neg**	neg	neg	nt	neg	neg
17	**neg**	**pos**	neg	neg	neg	neg	neg
18	**neg**	**neg**	neg	neg	neg	neg	neg
PC FD	**pos**	**neg**	pos	pos	pos	pos	pos
PC BN	**neg**	**pos**	neg	nt	nt	nt	nt

nt – not tested

Table 8: Homogeneity and stability of aliquots tested with LAMP BN

		Operator	1	2	1	1	1
		Device for amplification	Roche LC480	Roche LC480	Genie II	Genie II	Genie II
		Date	2.4.2015	10.4.2015	10.4.2015	14.4.2015	6.5.2015
		Aliquot of samples and chemicals	1	2	3	3	4
		Aliquot stored	**-20 °C**	**-20 °C**	**3 days at room T**	**7 days at room T**	**2 days at room T**
		Details	Appendix 5.A)	Appendix 5.B)	Appendix 5.C)	Appendix 5.D)	Appendix 5.E)
Sample code	FD status	BN status					
1	**pos**	**neg**	neg	neg	neg	nt	neg
2	**neg**	**pos**	pos	pos	pos	pos	pos
3	**pos**	**neg**	neg	neg	neg	nt	neg
4	**neg**	**neg**	neg	neg	neg	nt	neg
5	**neg**	**neg**	neg	neg	neg	nt	neg
6	**pos**	**pos**	pos	pos	pos	nt	pos
7	**neg**	**neg**	neg	neg	neg	nt	neg
8	**neg**	**pos**	pos	pos	pos	nt	pos
9	**pos**	**neg**	neg	neg	neg	nt	neg
10	**neg**	**neg**	neg	neg	neg	nt	neg
11	**neg**	**neg**	neg	neg	neg	nt	neg
12	**pos**	**neg**	neg	neg	neg	nt	neg
13	**neg**	**pos**	pos	pos	pos	nt	pos
14	**neg**	**neg**	neg	neg	neg	nt	neg
15	**neg**	**neg**	neg	neg	neg	nt	neg
16	**neg**	**neg**	neg	neg	neg	nt	neg
17	**neg**	**pos**	pos	pos	pos	nt	pos
18	**neg**	**neg**	neg	neg	neg	neg	neg
PC FD	**pos**	**neg**	neg	nt	nt	nt	nt
PC BN	**neg**	**pos**	pos	pos	pos	pos	pos

nt – not tested

3.3.4.2.5 Data analysis and evaluation of the results

All results are presented in confidential manner (partners were coded randomly; each partner received its code in a separate e-mail).

The results for each participant were analyzed based on the numbers of positive agreements (PA), negative agreements (NA), positive deviations (PD) and negative deviations (ND) as presented in Table 9. The obtained values where then used for evaluation based on the following calculations as described in Table 10. (EPPO PM 7/122 (1))

Table 9: Definition of positive agreement (PA), negative agreement (NA), positive deviation (PD) and negative deviation (ND) (EPPO PM 7/122 (1))

Reference Participant	Assigned value= positive	Assigned value= negative
Result obtained is positive	PA = positive agreement	PD = positive deviation
Result obtained is negative	ND = negative deviation	NA = negative agreement
Result obtained is undetermined	ND = negative deviation	PD = positive deviation

Table 10: Evaluation of results (EPPO PM 7/122 (1))

Performance values	Calculation
Accuracy	$(\sum PA + \sum NA) /N \times 100\%$
Rate of true positives	$\sum PA /N+ \times 100\%$
Rate of true negatives	$\sum NA/N- \times 100\%$

N^+ = number of samples with a positive assigned value = $\sum PA+\sum ND$
N^- = number of samples with a negative assigned value = $\sum NA+ \sum PD$
N= total number of samples = $(N^+ + N^-)$

3.3.4.3 Results

Eight different devices were used for amplification with LAMP (Table 11). It was shown (see point 3.3.4.2.4) that storage of samples at room temperature was causing a damage of FDp in sample 3, therefore a duration of the transport of samples to the participant has been checked. Nine partners have received samples one day after dispatch, while a partner with a code 9 has received samples three days after dispatch (Table 11). Therefore a result of LAMP FD for sample 3 of this partner was not included in the analysis.

Table 11: Devices for amplification used by different partners and duration of transport of samples and chemicals to partners.

Partner code	Duration of transport of samples and chemicals (days)	Device for amplification used
1	1	Roche LC480
2	1	Stratagene MxPro 3005
3	1	ABI 7900 HT Fast, Applied Biosystems
4	1	Genie II, Optigene
5	1	ABI Prism 7500 Fast, Applied Biosystems
6	1*	CFX96 Real time PCR detection system, Biorad
7	1	CFX Connect, BioRad
8	1	Rotor-Gene Q, Qiagen
9	**3**	Rotor-Gene Q, Qiagen
10	1	Genie II, Optigene

*data provided by DHL company

A summary of performances of all three evaluated assays is presented in Table 12. The detailed results obtained for each sample with each assay in each laboratory are shown in Tables 13 to 15. Ten laboratories have analyzed 18 unknown samples using LAMP FD and LAMP BN assays. In addition, those samples were also analyzed by ISOA FD Qualiplante assay in three laboratories. The accuracy of all assays is higher than 98%. In one single case, one BNp positive sample was concluded as negative after using LAMP BN assay. FD negative samples were concluded as FD suspected samples with LAMP FD assay three times (no. of all results with this assay 179), and once when using ISOA FD Qualiplante assay (no. of all results with this assay 54). All other results are correct.

Table 12: A summary of performances for assays that were evaluated

	Assay		
	LAMP BN	LAMP FD	ISOA FD Qualiplante
No. of labs taking into account for the evaluation	10	10	3
No. of results	180	179	54
N^+	50	49	15
PA	49	49	15
ND	1	0	0
Undetermined (sus) of N^+	0	0	0
N^-	130	130	39
NA	130	127	38
PD	0	0	0
Undetermined (sus) of N^-	0	3	1
Accuracy	99.4%	98.3%	98.1%
Rate of true positives	98.0%	100%	100%
Rate of true negatives	100%	97.7%	97.4%

PA = positive agreements
NA = negative agreements
PD = positive deviations
ND = negative deviations
N^+ = number of samples with a positive assigned value ($\sum$ PA+$\sum$ ND)
N^- = number of samples with a negative assigned value ($\sum$ NA+ $\sum$ PD)

Table 13: Results obtained by different laboratories using LAMP BN (details are in Appendix 6)

			Partner code										
Sample code	FD status	BN status	1	2	3	4	5	6	7	8	9	10	Summary
1	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
2	neg	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	
3	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
4	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
5	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
6	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	
7	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
8	neg	pos	pos	pos	neg	pos	pos	pos	pos	pos	pos	pos	
9	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
10	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
11	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
12	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
13	neg	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	
14	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
15	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
16	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
17	neg	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	
18	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
PC BN	neg	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	
		N^+	5	5	5	5	5	5	5	5	5	5	50
		PA	5	5	4	5	5	5	5	5	5	5	49
		ND	0	0	1	0	0	0	0	0	0	0	1
		Undetermined of N^+	0	0	0	0	0	0	0	0	0	0	0
		N^-	13	13	13	13	13	13	13	13	13	13	130
		NA	13	13	13	13	13	13	13	13	13	13	130
		PD	0	0	0	0	0	0	0	0	0	0	0
		Undetermined of N^-	0	0	0	0	0	0	0	0	0	0	0
		Accuracy	100%	100%	94.4%	100%	100%	100%	100%	100%	100%	100%	99.4%
		Rate of true positives	100%	100%	80.0%	100%	100%	100%	100%	100%	100%	100%	98.0%
		Rate of true negatives	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

For abbreviations see Table 12.

Table 14: Results obtained by different laboratories using LAMP FD (details are in Appendix 7)

			Partner code										
Sample code	FD status	BN status	1	2	3	4	5	6	7	8	9	10	Summary
1	pos	neg	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	
2	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
3	pos	neg	pos	pos	pos	pos	pos	pos	pos	pos	sus*	pos	
4	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
5	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
6	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	
7	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
8	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
9	pos	neg	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	
10	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
11	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
12	pos	neg	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	
13	neg	pos	neg	neg	neg	neg	neg	neg	sus	neg	neg	neg	
14	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
15	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
16	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
17	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	
18	neg	neg	neg	neg	neg	neg	sus	neg	sus	neg	neg	neg	
PC FD	pos	neg	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	
		N^+	5	5	5	5	5	5	5	5	4*	5	49
		PA	5	5	5	5	5	5	5	5	4	5	49
		ND	0	0	0	0	0	0	0	0	0	0	0
		undetermined of N^+	0	0	0	0	0	0	0	0	0	0	0
		N^-	13	13	13	13	13	13	13	13	13	13	130
		NA	13	13	13	13	12	13	11	13	13	13	127
		PD	0	0	0	0	0	0	0	0	0	0	0
		Undetermined of N^-	0	0	0	0	1	0	2	0	0	0	3
		Accuracy	100%	100%	100%	100%	94.4%	100%	88.9%	100%	100%	100%	98.3%
		Rate of true positives	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
		Rate of true negatives	100%	100%	100%	100%	92.3%	100%	84.6%	100%	100%	100%	97.7%

* Sample 3 was excluded from the analysis, because it was shown (see point 3.3.4.2.4) that longer storage at room temperature cause a damage of FD in this sample (duration of transport of samples and chemicals to this lab took 3 days – see Table 15), for abbreviations see Table 12.

Table 15: Results obtained by different laboratories using ISOA FD Qualiplante (details are in Appendix 8)

Sample code	FD status	BN status	Partner code 1	2	5	Summary
1	pos	neg	pos	pos	pos	
2	neg	pos	neg	neg	neg	
3	pos	neg	pos	pos	pos	
4	neg	neg	neg	neg	neg	
5	neg	neg	neg	neg	neg	
6	pos	pos	pos	pos	pos	
7	neg	neg	neg	neg	sus	
8	neg	pos	neg	neg	neg	
9	pos	neg	pos	pos	pos	
10	neg	neg	neg	neg	neg	
11	neg	neg	neg	neg	neg	
12	pos	neg	pos	pos	pos	
13	neg	pos	neg	neg	neg	
14	neg	neg	neg	neg	neg	
15	neg	neg	neg	neg	neg	
16	neg	neg	neg	neg	neg	
17	neg	pos	neg	neg	neg	
18	neg	neg	neg	neg	neg	
PC FD	pos	neg	pos	pos	pos	
		N^+	5	5	5	15
		PA	5	5	5	15
		ND	0	0	0	0
		Undetermined of N^+	0	0	0	0
		N^-	13	13	13	39
		NA	13	13	12	38
		PD	0	0	0	0
		Undetermined of N^-	0	0	1	1
		Accuracy	100%	100%	94.4%	98.1%
		Rate of true positives	100%	100%	100%	100%
		Rate of true negatives	100%	100%	92.3%	97.4%

For abbreviations see Table 12.

3.4 DISCUSSION OF RESULTS AND THEIR RELIABILITY

3.4.1 WP1: Loop-mediated isothermal amplification (LAMP) protocol for BNp

A new LAMP assay for BN detection was developed and optimized for on-site detection and validated in accordance with EPPO recommendations.

3.4.2 WP2: Homogenization and extraction protocol

Analytical sensitivity of the on-site procedure using UTTD homogenization and direct homogenate testing with LAMP assay in average show that BNp DNA may be detected in leaf vein samples where as low as 9-27 copies of BNp DNA are present.

No selectivity of the direct homogenate testing over different grapevine tissue homogenates (leaf veins, berries, berry pedicels and tendrils) or various cultivars was observed.

3.4.3 WP3: Searching for new hosts and vectors of FDp

3.4.3.1 Field monitoring in Austria

In the course of the Euphresco GRAFDEPI project two new Auchenorryncha species, *Phlogotettix cyclops* and *Cixius nervosus* were found to habour FDp. The 16SrV-C strain was detected in several individuals of the non-native leafhopper species. In 2014 it could be confirmed, that *P. cyclops* is harboring the phytoplasma not only in specimens found in the focus zones but also non infested vine growing areas. It could also be demonstrated that these invasive leafhoppers can be found very frequently in vineyards. *P. cyclops* belongs to the *Deltocephalinae*, which include many phytoplasma vectors, and is closey related to the genus *Scaphoideus*. It is a polyphagous leafhopper that feeds on elm (*Ulmus* sp.), willow (*Salix* sp.), grapevine (*Vitis vinifera*), rose (*Rosa* sp.), pear (*Pyrus*), peach (*Prunus* sp.), raspberry (*Rubus* sp.), *Rhododendron* sp. and *Castanea* sp.. Chen et al. (2011) identified a phytoplasma (closest to the 16SrI-B subgroup or new 16SrI subgroup) in *P. cyclops* on periwinkle (*Catharanthus roseus*) in Taiwan but the species was not able to transmit it. Therefor the importance of this invasive plant hopper species for the spread of FDp within the wild plant flora in the surroundings of the vineyards or in the vineyards remains unclear, although the frequency of infected specimens in vineyards points to an increased risk.

In 2014 *O. ishiadae* could not been found at any monitoring site. In the years before FDp infected individuals of O*. ishidae* were mainly recorded in southeast Styria. Many of the infected individuals were found on willow and alder but in three vineyards infected individual were trapped, one in each vineyard. In the course of the official monitoring for FD and *S. titanus* that is conducted every year in Styria the occurrence of *O. ishidae* is also recorded. It became clear that this non indigenous leafhopper can be frequently found in the monitoring area from August until September, mainly on *Salix* sp. and *Alnus* sp.. While it is not yet proofed as a vector,

O. ishidae is present during months when phytoplasma titer in alder trees is high, consequently leading to a higher infection risk of *O. ishidae*.

In the Euphresco GRFDEPI project several individuals of *Psylla alni* collected on *Alnus glutinosa* southeast Styria were found to habour phytoplasma isolates. In contrast FDp could not be detected in the tested individuals of *Oncopsis alni* and *Psylla alni* collected at sites in Burgenland in 2014. Several psyllid species are known to transmit different phytoplasmas e.g. '*C.*P. mali', '*Ca*. P. pyri' and '*Ca*. P. prunorum'. *P. alni* is a monophagous species, developing on grey alder (*A. incana*) and black alder (*A. glutinosa*). The high degree of phytoplasma infestation within black alder populations may be traced back to the spread by *Psylla alni*. It can be stated that black alder and clematis plants are the main reservoir hosts for FDp in the wild flora.

3.4.3.2 Field monitoring in Slovenia

In Slovenia FD-D type of FDp prevailed in the grapevine. This type was also found in *S. titanus*, in one sample of *O. ishidae*, in one sample of *Alianthus altisima* and in mixed infected *Alnus spp*, *O. Ishidae* and *O. alni*. FDp typeFD-C was occasionally found in grapevine, but in all infected plants of *C. vitalba* and five samples of *A. altisima.*

3.4.3.3 Transmission trials

The visual observation of *S. titanus* on alder leaves together with the FD-positive testing indicates that the nymphs had sucked plant sap from FDp infected alder. The results from the transmission trial show that very few *S. titanus* nymphs are able to aquire the FDp from infected *A. glutinosa* twigs and to transmit it to grapevine (cv. Scheurebe). These preliminary results might support the hypothesis that alder tree populations are a possible resource for a primary infection. Nevertheless, it should be considered that the experimental set-up of the performed transmission trial is an artificial, self-contained system. The high mortality rate of *S. titanus* could be also due to the artificial conditions of the system. Further trials are needed to proof this transmission pathway.

Transmission experiments of FDp on the grapevine in a net-chamber with the alternative possible vectors for FDp *O. ishidae* and *O. alni* have been performed in three successive years. In addition, the transmission experiments of FDp from *O. ishidae* to the artificial media showed positive results in 15% of cases after 2 days of feeding of insects on the medium, and in 43% after 3 to 4 days of feeding. However, their role in the transmission of FDp to grapevine remained unclear because there is no evidence of the successful transmission to the plant.

3.5 MAIN CONCLUSIONS

During the project GRAFDEPI 2 a LAMP assay for the specific detection of BNp has been developed and validated in accordance with EPPO recommendations. The LAMP assay was shown to be only up to 3-times less sensitive than qPCR specific for BNp.

The assay was also tested for the on-site application. Therefore, the pre-assay experiments that included cost-efficient preparation of samples were also included in the project. The most reliable procedure has been chosen and proposed for the on-site diagnostic. It includes the application of the UTTD homogenization and direct homogenate testing with LAMP assay without a prestep of DNA extraction.

A new protocol for BNp testing is simple, reliable, cost-efficient and accurate and can be therefore used for in-lab or on-site detection of BNp in grapevine samples.

Test performance study was organized in the frame of GRAFDEPI2 in which 10 laboratories from Europe and Australia participated. It includes testing of the LAMP assay developed in this project for the specific detection of BNp, testing of the LAMP assay for the specific detection of FDp developed in VITISENS project and assay ISOA FD of Qualiplante. TPS proved that all three assays are suitable for reliable use in diagnostics.

Although a lot of efforts supporting with the results from this project, have been put in searching for new host plants and vectors of FDp and in transmission trials, the results still remain inconclusive and require new research in the future.

3.6 DISSEMINATION ACTIVITIES

Title of the event/Date	Description of event (Oral presentation, poster, dissemination of material, etc.)	Event website
Meeting of the Plant Protection Society of Slovenia / 5/12/2014	Oral presentaion	
Presentation of the project	Dissemination material	www.euphresco.net/media/project_slides/GRFDEPI2_1.pdf
Success story - presentation of a work packages 1 and 4	Dissemination material	http://www.euphresco.net/media/success_stories/euphresco_success_story_GRFDEPI2.pdf
Validation data at the EPPO	Dissemination material	http://dc.eppo.int/validationlist.php/ Grapevine flavescence dorée phytoplasma/ Detection of flavescence doree phytoplasma by LAMP in grapevine
Mehle, N., Ravnikar, M., Kogovšek, P., Jakomin, T., Pugelj, A., Dermastia, M. New diagnostic tools for improved diagnostics of grapevine phytoplasmas. In: Testa - EPPO Conference on diagnostics for plant pests (and associated workshops) : programme, summaries of presentations and posters, group lists for Workshops, participant lists, 2015-11-30 to 2015-12-04, Angers (FR). [S. l.: s. n., 2015], p. 22	Dissemination material	

3.7 ACKNOWLEDGEMENTS

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4. Appendices

APPENDIX 1
LAMP FD and LAMP BN – protocol

This booklet provides a detailed description of method that can be used for the detection of 16SrV phytoplasmas including FD phytoplasma (FDp) and 16SrXII phytoplasmas including '*Candidatus* Phytoplasma solani' (BNp) with LAMP assays. LAMP assay for BNp detection was developed in the frame of Euphresco GRFDEPI2 project and is not published yet, while the LAMP for FDp assay was described in *LAMP assay and rapid sample preparation method for on-site detection of flavescence dorée phytoplasma in grapevine; Kogovšek et al., Plant Pathology, 2015, 64: 286–296*. The validation data for testing FDp by LAMP are deposited with the EPPO database on Diagnostic Expertise: http://dc.eppo.int/validationlist.php.

The procedure of testing for BNp and FDp by LAMP assays is at the moment adapted to the laboratory conditions (working on freezer block or ice) and is in present form not easily applicable on-site yet. Further development of a kit with increased stability of the chemicals is expected (Optigene Ltd.).

This booklet was prepared by Polona Kogovšek and Nataša Mehle, National Institute of Biology, Večna pot 111, 1000 Ljubljana, Slovenia

NATIONAL INSTITUTE OF BIOLOGY

In case you have specific questions about the LAMP assay, please contact: Polona Kogovšek [polona.kogovsek@nib.si]

As the result of this part of ring test is filled excel file (results_LAMP_FD_BN.xls; sheets: results and details). All results should be sending by e-mail to **polona.kogovsek@nib.si** and in cc to **natasa.mehle@nib.si** till 20 June 2015.

Samples and materials to be supplied by NIB

One box with freezer block will contain:

- 18 unknown samples (40 µl of DNA extracts labelled as 1-18; for this LAMP analysis you will need at least 30 µl of each)*
- FDp and BNp positive DNA controls (40 µl of DNA extracts labelled as: FD+, BN+)
- BNp primer mix
- FDp primer mix
- Isothermal Master Mix (Optigene Ltd; Cat. no. ISO-001)

Note: Samples and all chemicals should be kept at -20 ºC. The freezer block should be stored at -20 ºC (you will need it later).

*laboratories that already expressed a wish to do some extra analyses will get two sets of samples.

Chemicals that you need to buy:

- Molecular biology grade water (nucleic acid- and nuclease-free) or sterile-filtered double deionized water

Controls that you need:

- neg: sterile deionized water (the same as used for LAMP master mix preparation)

Protocol Summary
Includes conditions to be used, source of reagents and options for deviation from protocol

Step	Default condition*	Supplied by	Alternative
Reaction volume	25 µl	-	No
Replicates	Samples run in triplicate	-	No
Controls	As per list above	NIB (pos) and participant (neg)	No
Plate type	8-well strip	-	96-well
Reaction plate	Optigene Ltd.	Participant	Yes (record which plate/strips were used)
LAMP reagents	Isothermal Master Mix (Optigene)	NIB	No
Reaction composition	As per list below	-	No
DNA added	5 µl	NIB	No
Device for amplification	GenieII, Optigene	Participant	Yes (e.g. device for real time PCR: Roche LC480) (record which device was used)
Thermal cycling conditions	As per list below	-	No
Analyzing data and interpretation of results	As per details given below	-	No

*LAMP for the detection of FDp and BNp was originally developed on Roche LC480 device using Isothermal Master Mix (Optigene Ltd.).

Important: Fill the excel file (results_LAMP_FD_BN.xls; sheets: details)!

Protocol: LAMP set-up

Note: At all stages while setting up LAMP reactions, precautions should be taken to avoid unspecific amplification and contamination of samples and reagents. These are as follows:

- Use the freezer block (pre-cool it at **-20 °C**) during the whole reaction mixture preparation and adding sample DNA procedure! Alternatively ice can be used, note that the exterior of the reaction strips/plate should be kept dry (protect it with foil,...)
- The device used for amplification should be cooled at room temperature before putting the reaction strips/plate in (to avoid incubation at undefined temperatures).
- Use only dedicated pipettors (NOT the ones used for DNA extraction etc).
- For general pipetting advice, see e.g. www.gilson.com/Literature/pipetting.asp.
- Use only filtered tips and use a fresh tip for each pipetting step.
- Close reagent / sample tubes once desired aliquot has been removed.
- Wear gloves and change them if they become contaminated (i.e. when you leave the room, pick something up off the floor etc).
- Use only clean, sterile plastic ware.
- Following steps of analysis must be performed in separated places with separated laboratory equipment (pipettes, tips, tubes, racks for tubes, freezer block*, lab coat, gloves):
  - reaction mix preparation (without DNA)
  - adding DNA (UV chamber)
  - amplification of the target sequence.

*One freezer block is provided by NIB, but it is advised to have two: one for reaction mix preparation and the other for adding sample DNA. If the same freezer block is used for both steps, it should be put at UV light for 15 min before used for the next reaction mix preparation.

1. Remove all reagents from freezer and allow to thaw. Once thawed, mix all reagents well (invert tubes a number of times/ flick the tube/vortex) and spin briefly (~5 s) in a centrifuge.

2. Take a microtube and make up a test assay master mix (separate for FD and BN), including sufficient reagents for the number of samples and controls to be tested IN TRIPLICATE. Include 10% extra volume to allow for pipetting errors. For each reaction the reagents are added in the order and quantity shown below.

3. Take a 8-well strips or 96 -well qPCR plate. Fill the appropriate number of wells with 20 µl of master mix. The samples of the scheme are shown below. When pipetting, keep the strips/plate/ on the cooled freezer block.

4. Add 5 µl of each DNA extract or control in to each test well, as required. When using 96-well plate, always start by adding water for first NTC (NTC 1), continue with sample DNA and positive control. Finish with water for the last NTC (NTC 2).

This enables to follow the source of contamination more easily. When pipetting, keep the strips/plate/ on the cooled freezer block.

5. After adding DNA, the strips/plate should be covered with caps/an optical adhesive cover.

6. Tap the strips / centrifuge the plate 1 – 2 min at 1000g to ensure the reaction mix and the DNA are collected at the bottom of the wells.

   Note: prepared reactions should be processed immediately.

7. Transfer strips/plate onto the GenieII/real-time PCR device and run the program as defined below.

Controls

1. positive (extracted DNA supplied by NIB):
   FD+: positive control for LAMP FD assay (PC FD)
   BN+: positive control for LAMP BN assay (PC BN)

2. NTC (no DNA, add water; 'no template control') (For every reaction mix one or two NTCs are prepared per series of wells with same reaction mix tested in GenieII or qPCR cycler, respectively. When two NTCs are used, first should be added at the start of pipetting (NTC1) and second at the end (NTC2).)

Samples:

1. S1 – S18 (unknown sample 1 – unknown sample 18) (extracted DNA supplied by NIB)

Important: Before using all DNA samples should be removed from freezer and allow to thaw. Once thawed, mix all samples well and spin briefly (~5 s) in a centrifuge.

Reaction Composition:
Detection assays:

Master mix for BN:

Components	Working concentration	Volume per reaction (µl)	Volume for 65 wells (µl)*	Volume for 71 wells (µl)**
Molecular grade water	n.a.	5	325	355
Isothermal Master Mix (Optigene)	2x	12.5	812.5	887.5
Primer mix - BN	10x	2.5	162.5	177.5
Total volume		*20.0*	*1300*	*1420*

*If 96-well PCR plate is used
**If two 8-well strips are used in parallel. Only two 8-well strips can be analysed per one run in GenieII (Optigene) device. Master mix can be prepared in advance for all samples and stored at 4ºC for max 5h. If it is not feasible to perform all reactions in such short term, master mix for fewer samples should be prepared at once.

Master mix for FD:

Components	Working concentration	Volume per reaction (µl)	Volume for 65 wells (µl)*	Volume for 71 wells (µl)**
Sterile nuclease free water	n.a.	5	325	355
Isothermal Master Mix (Optigene)	2x	12.5	812.5	887.5
Primer mix - FD	10x	2.5	162.5	177.5
Total volume		*20.0*	*1300*	*1420*

*If 96-well PCR plate is used
**If two 8-well strips are used in parallel. Only two 8-well strips can be analysed per one run in GenieII (Optigene) device. Master mix can be prepared in advance for all samples and stored at 4ºC for max 5h. If it is not feasible to perform all reactions in such short term, master mix for fewer samples should be prepared at once.

Add 5 µl of each DNA sample or control to each test well, as required.

The sample of the scheme (examples for assay BN are given below)**:**

- If two 8-well strips are used in parallel (for each assay (BN and FD) separate batch of strips):

	1	2	3	4	5	6	7	8
A	Sample 1			Sample 2				
B	Sample 3			Sample 4			NTC	PC BN

	1	2	3	4	5	6	7	8
A	Sample 5			Sample 6				
B	Sample 7			Sample 8			NTC	PC BN

	1	2	3	4	5	6	7	8
A	Sample 9			Sample 10				
B	Sample 11			Sample 12			NTC	PC BN

	1	2	3	4	5	6	7	8
A	Sample 13			Sample 14				
B	Sample 15			Sample 16			NTC	PC BN

	1	2	3	4	5	6	7	8
A	Sample 17			Sample 18			NTC	PC BN

- If 96-well PCR plate is used (for each assay (BN and FD) separate plate):

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1			Sample 9			Sample 17					
B	Sample 2			Sample 10			Sample 18					
C	Sample 3			Sample 11			PC BN					
D	Sample 4			Sample 12			NTC1	NTC2				
E	Sample 5			Sample 13								
F	Sample 6			Sample 14								
G	Sample 7			Sample 15								
H	Sample 8			Sample 16								

Amplification conditions:
Switch on the thermal cycler in advance as per producer's instructions to warm it up and stabilize conditions. If GenieII device is used, there is no need for warming up.
Set amplification parameters:
GenieII:

40 min at 62 °C	amplification
98 °C – 80 °C; 0.05 °C per second	Melting T analysis

If using thermal cycler (e.g. Roche LC480) for isothermal amplification, care must be taken when the amplification program is set. The thermal cyclers need to be programmed for cycling to measure fluorescence at each minute. Therefore 40 cycles with two steps are set, each as 1s at 62 °C and 59 s at 62 °C with single acquisition of fluorescence (see table below). Same temperature in both cycling steps ensures isothermal amplification. Melting temperature analysis is set as standard melting curve analysis given in the software with continuous acquisition of fluorescence (from 62 °C to 98 °C, with 5 acquisition of fluorescence per °C).

1 s at 62 °C	UNG activation step
1 s at 62 °C	polymerase activation
40 cycles of 1 s at 62 °C 59 s at 62 °C	DNA denaturation annealing and extension
98 °C	Melting curve

Adjust reaction volume if needed and select the combination of filters for FAM (483-533).
Assign samples to locations on a plate.
Save file.

Analyzing data:
Analysis of LAMP results on GenieII:

- Check the amplification and melting temperature analysis results on the Results tab
- Record the Tpos and Tm values on data-collection sheet

For analysis of data obtained on qPCR cycler there are usually different options available with regard to setting signal and noise limits: automatic and manual.
The following are instructions for analysis of LAMP results for Roche LC480 analysis software, please adapt them as suitable to your instrument.

- use Abs Quant/2nd Derivative Max analysis for time to positive (minutes, Tpos) (when using Roche LC480 this is Cp value)
- Record Tpos values on data-collection sheet

- Use Tm Calling analysis for melting temperature (Tm)
- Record Tm values on data-collection sheet

Interpretation of results:

Verification of controls:

- NTC should produce no fluorescence, otherwise contamination of reaction mix should be considered.
- The PC amplification curve should be exponential and the Tpos should be less than 30 min, otherwise some errors in LAMP reaction should be considered.
- The Tm of the PC for FD LAMP assay should be between 84.0 °C and 85.1 °C when samples are analysed on GenieII and between 84.9 °C and 85.9 °C when samples are analysed on the Roche LC480 device. Similar Tm range is expected when analysed on any other device, but needs to be verified.*
- The Tm of the PC for BN LAMP assay should be between 84.9 °C and 86.0 °C when samples are analysed on the Roche LC480 device. When samples were analysed on GenieII Tm was in range between 84.0 °C and 85.0 °C. Similar Tm range is expected when analysed on any other device, but needs to be verified.*

* The Tm of the positive samples should be in the frame of Tm of the positive control ± 0.5 °C (e.g. if the average Tm of PC is 85.4 than Tm of the positive samples should be between 84.9 and 85.9).

When these conditions are met:

- A test will be considered positive if it produces a positive reaction as defined for PC (curve should be exponential, Tpos should be less than 30 min and Tm should be in the range of PC ± 0.5°C)
- A test will be considered negative, if it produces no fluorescence.

Estimation of the amplification curve (amplification curve exponential yes/no), Tpos and Tm values of the samples for each amplicon and for each replicate should be given in the excel file (results_LAMP_FD_BN.xls; sheets: results).

Important: All raw data should be saved!

APPENDIX 2
ISOA FD Qualiplante – protocol

ISOA[illegible]AL AMPLIFICATION ASSAY
[illegible]tection of *Flavescence dorée*

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Flavescence dorée

Flavescence dorée (FD) is one of the greatest threats for grapevine in Europe and included in European legislation as a quarantine pest (directive 2000/29 EC). It is caused by a phytoplasma belonging to the 16SrV group, efficiently transmitted by the vector *Scaphoidus titanus*.

Principle

Isothermal Amplification assay for the detection of *Flavescence dorée*. The product should be used only for research purposes.

bKIT *Flavescence dorée* component

Reference: IsoA.FD/80

▪ FD IsoA Master Mix (2 tubes) *(do not contain ROX)*	2x40 tests
▪ Positive Control (1 tube)	15 µl
▪ Negative Control (1 tube)	15 µl

Specificity

The FD bKIT offers a sensitive diagnostic method to detect *Flavescence dorée*. Six primers were designed on the FD gene *rpl14*[(*)] by International Plant Analysis and Diagnostics (www.ipadlab.eu). The FD assay was developed in collaboration with Hyris Ltd (www.hyris.net).

Storage

-20°C.
Avoid prolonged exposure to light and repeated freeze and thaw cycles.

Shelf life

If the bKIT is correctly stored, at constant-temperature freezer, its performance is guaranteed until the shelf life indicated on the tubes.

Additional material/reagents required

- DNA extraction tools and reagents
- Nuclease-free water
- Gloves
- Pipettes
- Real-time PCR instrument[(**)] with filters calibrated for SYBR®-Green or isothermal amplification instrument
- Real-time PCR plate or strips according to thermal cycler / isothermal amplification instrument specifications

[(**)] This assay was especially developed for being used in association with the bCUBE instrument, available from Hyris Ltd, but can be used with any other thermal cycler or isothermal amplification instrument.

DNA extraction

Extract DNA from samples according to your usual protocol. If necessary, Qualiplante can recommend you an extraction method.

Reaction Set-Up

a) Thaw and store FD IsoA Master Mix by placing it on ice.
b) Gently mix the FD IsoA Master Mix by swirling the tube and vortex it.
c) For each sample, included the negative control and the positive control of the bKIT, combine in a PCR strip or PCR plate well, the components as shown in the table below:

Components	Volume/well or strip
DNA sample or Positive control or Negative control	5 µl
FD IsoA Master mix	20 µl
Total Volume / well or strip	25 µl

Run Method Set Up

Set up the run method using the following conditions, depending on the instrument you use:

1. For the use with a conventional thermal cycler:

Steps	Temp	Time	Stage
Amplification 1 [(1)]	65°C	1 sec	30
Amplification 2 [(1)]	65°C	55 sec	
Dissociation [(2)]	Follow the standard condition of your thermal cycler		

[(*)] International patents issued and pending

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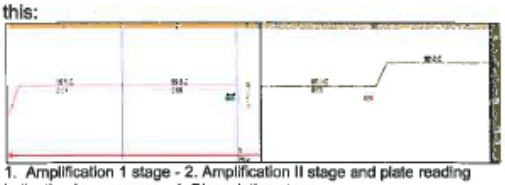
ISOTHERMAL AMPLIFICATION ASSAY
Detection of *Flavescence dorée*

[1] The assay is based on an isothermal nucleic acid amplification technique that is carried out at a constant temperature, and does not require a thermal cycler. However, Qualiplante/Hyris bKIT[*] was developed for being used with thermal cycler instrument, especially with Hyris Ltd. bCUBE instrument[*]. For this reason, it is necessary to set up 2 steps of amplification at 65°C in order to read, in real-time, the amplification at every stage. **Be careful to fix the plate reading after the Amplification 2 stage, as indicated by the green camera in the picture below.**

[2] For the parameters of dissociation stage, please follow the indication of your real-time PCR instrument.

For example, in the case of Biorad CFX 96 instrument, the Run Method screen should look like this:



1. Amplification 1 stage - 2. Amplification II stage and plate reading indicating by a camera - 4. Dissociation stage

2. For the use with an isothermal amplification instrument (e.g. Genie Reader):

Steps	T (°C)	Duration
Amplification step	65°C	30 min
Dissociation step	98°C to 80°C	

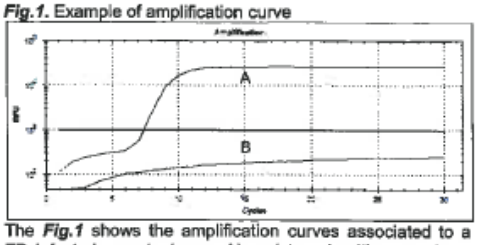
Plate run

a) **Seal carefully the Real-Time PCR plate / strips with adhesive film in order to avoid any contamination**
b) Load the Real-time PCR plate into the Real-time PCR instrument or the strips into the isothermal amplification instrument, then start the run.

Results analysis

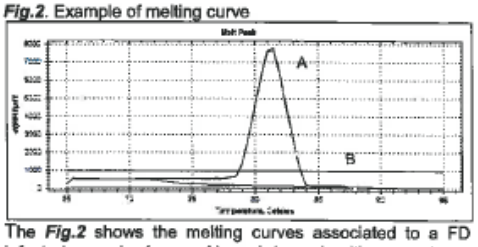
The specific product for FD will generate an amplification curve ***(Fig.1)*** and a specific melting curve ***(Fig.2)*** with a peak between 81°C and 82°C. An amplification curve could appear also for negative sample; in this case, it is necessary to examine the melting curve to interpret the results ***(Fig.1)***.

Fig.1. Example of amplification curve



The ***Fig.1*** shows the amplification curves associated to a FD infected sample (curve A) and to a healthy sample or negative control (curve B).
A= FD-infected grapevine sample; **B=** healthy grapevine sample (negative control)

Fig.2. Example of melting curve



The ***Fig.2*** shows the melting curves associated to a FD infected sample (curve A) and to a healthy sample or negative control (curve B).
A= FD-infected grapevine sample; **B=** healthy grapevine sample (negative sample)

ANALYSIS VALIDATION

The PCR plate is validated only when:
- ✓ the positive control of the kit generates an amplification curve and a melting curve with a peak between 81°C and 82°C.
- ✓ the negative control of the kit and the no template control does not generate any peak between 81°C and 82°C.

RESULTS INTERPRETATION

A sample is positive when:
- ✓ the Ct of the amplification curve is below or equal to 25.
- ✓ the peak of the melting curve is included between 81°C and 82°C.

A sample is negative when:
- ✓ there is no amplification curve or the Ct is higher than 25.
- ✓ there is no melting curve or the peak is not included between 81°C and 82°C.

[*] International patents issued and pending

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The table below can help you for the interpretation:

Melting peak	Ct value	
	Ct ≤ 25	Ct > 25
Included between 81°C to 82°C	Pos.	Ind.
No peak	Neg.	Neg.
Not included between 81°C and 82°C	Neg.	Neg.

Pos: positive – Neg: Negative – Ind: Indeterminated

If the Ct is between 25 to 30, with the presence of a melting peak, a contamination could be possible; we recommend you to test again the sample

TROUBLESHOOTING

Post-PCR data analysis shows no amplification, or amplification plots look grossly abnormal:

Possible causes	Corrective actions
Evaporation of the sample due to inadequate sealing of the plate/strips	Repeat the test using the appropriate tools to seal correctly the plate/strips
Consumables are not appropriate for the method	Repeat the test using consumables recommended by the supplier of the thermal cycler/isothermal amplification instrument
The quality of nucleic acid extracted is low	Repeat the extraction step. Ensure that the method of extraction has been performed correctly. In any doubt, contact us
Abnormal amplification	Centrifuge the plate briefly to spin down the contents and eliminate any air bubbles

No amplification reaction is observed in the positive control well, while other samples are positive:

Possible causes	Corrective actions
The positive control provided with the assay was not added into the reaction well	Repeat the test. If the problem persists, contact us

An amplification plot is observed in the negative control well with a specific melting peak:

Possible causes	Corrective actions
Contamination of the negative control or the Master Mix with target-positive DNA	Repeat the test by applying appropriate quality procedures to prevent contamination Seal correctly the plate/strips

Warning and Precautions

Do not mix the components of kits with different batch number. This kit is designed to be used by laboratory personnel trained to follow the usual molecular biology precautions.

Warranty and Responsibilities

Qualiplante SAS guarantees the buyer exclusively concerning the quality of reagents and of the components used to produce the Kits. Qualiplante SAS is not responsible and cannot anyway be considered responsible or jointly responsible for possible direct and indirect damages resulting from the utilization of the Kits by the user. The user consciously and under his/her own responsibilities decides for the utilization purposes of the Kits and uses it the way he/she considers most suitable in order to reach his/her goals and/or objectives.

Qualiplante is not responsible for the data resulting from the use of the Kits, for the utilization that the user independently decides to make of them or for the direct or indirect damages possibly resulting from the disclosure or transmission of the data themselves to third parties under any form or circumstance. This clause is automatically accepted by the user when purchasing the Kits.

The patent for performing PCR and Real-Time PCR is held by Hoffmann/La Roche. Authorization to use PCR and Real-Time PCR can be obtained on licence from Hoffmann-La Roche. The product, equipment and information included in the Kits consist of assembled reagents. The licence and authorisation for PCR and Real-Time PCR use are not included in the Kits. The user is responsible for setting prefixed goals, choosing whether or not to perform the PCR or Real-Time PCR reaction and to apply for register his/her own licence.

The Kits are designed for the services supply, quality control or any other application that is not exclusively an internal company's research and requires a specific licence for PCR and Real-Time PCR use. The Kits require the use of Taq Polymerase enzyme and/or fluorochromes, often registered as trademark by companies. TaqMan® is a trademark of Roche Molecular Systems, Inc. FAM, VIC and ROX are a trademark of Applera Corporation or its affiliate. Cy5 is a trademark of Amersham Biosciences Ltd.
The assay should be used only for research purposes.

The Kits have been internally tested by our quality control. Any responsibility is waived if the warranty of quality control does not refer to the specific Kits. The user is personally responsible for data that he/she will obtain and/or he/she will supply to third parties using these kits. Once the sealed package is opened the user accepts all the conditions without fail; if the package is still sealed the kit can be returned and the user can be refunded.

(*) International patents issued and pending

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APPENDIX 3
Detailed results of real time PCR:

Code	18s		Universal		FDgen		BNgen	
	Cq	Cq average	Cq	Cq average	Cq	Cq average	Cq	Cq average
1	19,65	19,54	30,81	30,80	32,27	32,28	undet	
	19,37		30,67		32,39		undet	
	19,61		30,93		32,16		undet	
2	19,18	19,25	25,69	25,70	undet		27,17	27,19
	19,33		25,65		undet		27,14	
	19,24		25,76		undet		27,27	
3	25,44	25,57	28,02	28,32	29,88	29,97	undet	
	25,60		28,48		30,04		undet	
	25,67		28,45		30,00		undet	
4	18,63	18,71	undet		undet		undet	
	18,90		undet		undet		undet	
	18,59		undet		undet		undet	
5	17,63	17,83	undet		undet		undet	
	17,91		undet		undet		undet	
	17,93		undet		undet		undet	
6	17,40	17,56	23,08	23,08	25,56	25,61	25,56	25,68
	17,55		23,00		25,61		25,61	
	17,72		23,15		25,66		25,86	
7	18,30	18,35	undet		undet		undet	
	18,22		undet		undet		undet	
	18,53		undet		undet		undet	
8	18,69	18,57	30,14	30,47	undet		32,30	32,23
	18,45		30,85		undet		32,48	
	18,57		30,40		undet		31,89	
9	19,59	19,77	28,64	28,86	30,19	30,49	undet	
	19,84		28,99		30,90		undet	
	19,89		28,95		30,39		undet	
10	19,10	19,24	undet		undet		undet	
	19,40		undet		undet		undet	
	19,21		undet		undet		undet	
11	34,87	34,88	undet		undet		undet	
	34,99		undet		undet		undet	
	34,79		undet		undet		undet	
12	18,80	19,02	24,93	25,02	26,67	26,79	undet	
	19,12		25,01		26,87		undet	
	19,14		25,13		26,83		undet	
13	18,27	18,33	27,11	27,21	undet		28,83	28,91
	18,41		27,18		undet		28,97	
	18,31		27,33		undet		28,94	
14	18,85	18,85	undet		undet		undet	
	18,93		undet		undet		undet	
	18,76		undet		undet		undet	
15	18,20	18,29	undet		undet		undet	
	18,34		undet		undet		undet	
	18,35		undet		undet		undet	
16	20,86	19,82	undet		undet		undet	
	19,35		undet		undet		undet	
	19,24		undet		undet		undet	
17	18,76	18,86	28,31	28,38	undet		29,95	30,16
	18,85		28,40		undet		30,24	
	18,96		28,43		undet		30,30	
18	39,62	39,05	undet		undet		undet	
	40,39		undet		undet		undet	
	37,14		undet		undet		undet	
PC FD	18,27	18,06	26,45	26,35	28,00	28,04	undet	
	17,96		26,26		28,03		undet	
	17,96		26,35		28,07		undet	
PC BN	18,06	18,16	24,63	24,65	undet		26,15	26,23
	18,22		24,67		undet		26,25	
	18,20		24,65		undet		26,30	

APPENDIX 4
Homogeneity and stability of aliquots tested with LAMP FD

4.A)
Operator: 1; Device for amplification: Roche LC480; Date: 2.4.2015; Aliquot 1 stored at -20°C

	LAMP FD assay									
	replicate 1			replicate 2			replicate 3			result FD
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	pos/neg**
sample										
1	yes	20,03	85,64	yes	16,43	85,64	yes	17,11	85,61	pos
2	no	/	/	no	/	/	no	/	/	neg
3	yes	13,26	85,78	yes	11,81	85,7	yes	13,25	85,64	pos
4	no	/	71,34	no	/	71,36	no	/	71,37	neg
5	no	/	71,28	no	/	/	no	/	71,28	neg
6	yes	10,74	85,69	yes	9,86	85,6	yes	9,73	85,56	pos
7	no	/	/	no	/	/	no	/	/	neg
8	no	/	71,54	no	/	71,87	no	/	72,98	neg
9	yes	15,67	85,66	yes	13,06	85,61	yes	16,84	85,59	pos
10	no	/	/	no	/	/	no	/	/	neg
11	no	/	70,57	no	/	71,37	no	/	/	neg
12	yes	10,31	85,71	yes	10,46	85,58	yes	10,88	85,57	pos
13	no	/	/	no	/	73,35	no	/	71,87	neg
14	no	/	70,53	no	/	/	no	/	71,34	neg
15	no	/	/	no	/	/	no	/	71,37	neg
16	no	/	71,37	no	/	71,37	no	/	71,37	neg
17	no	/	/	no	/	/	no	/	/	neg
18	no	/	/	no	/	/	no	/	/	neg
PC FD	yes	12,12	85,65	yes	10,9	85,57	yes	10,97	85,52	pos
PC BN	no	/	/	no	/	66,63	no	/	73,02	neg
NTC	no	/	/	no	/	/	nt	nt	nt	neg

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

**pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C ; neg - none of replicates produce fluorescence; sus - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

nt - not tested

4.B)

Operator: 2; Device for amplification: Roche LC480; Date: 10.4.2015; Aliquot 2 stored at -20°C

	LAMP FD assay									
	replicate 1			replicate 2			replicate 3			
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	result FD pos/neg**
sample										
1	yes	15,08	85,72	yes	17,54	85,65	yes	17,21	85,59	pos
2	no	/	/	no	/	/	no	/	/	neg
3	yes	15,67	85,58	yes	14,49	85,49	yes	13,14	85,45	pos
4	no	/	/	no	/	/	no	/	/	neg
5	no	/	/	no	/	/	no	/	/	neg
6	yes	8,57	85,60	yes	8,42	85,49	yes	8,01	85,44	pos
7	no	/	/	no	/	/	no	/	/	neg
8	no	/	/	no	/	/	no	/	/	neg
9	yes	11,16	85,52	yes	12,71	85,41	yes	12,36	85,39	pos
10	no	/	/	no	/	/	no	/	/	neg
11	no	/	/	no	/	/	no	/	/	neg
12	yes	10,02	85,41	yes	10,67	85,34	yes	9,17	85,27	pos
13	no	/	/	no	/	/	no	/	/	neg
14	no	/	/	no	/	/	no	/	/	neg
15	no	/	/	no	/	/	no	/	/	neg
16	no	/	/	no	/	/	no	/	/	neg
17	no	/	/	no	/	/	no	/	/	neg
18	no	/	/	no	/	/	no	/	/	neg
PC FD	yes	10,15	85,22	yes	9,76	85,19	yes	10,47	85,17	ok
NTC	no	/	/	no	/	/	nt	nt	nt	ok

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

**pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C ; neg - none of replicates produce fluorescence; sus - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

nt-not tested

4.C)

Operator: 1; Device for amplification: GenieII; Date: 10.4.2015; Aliquot 3 stored at room T three days

	LAMP FD assay									
	replicate 1			replicate 2			replicate 3			
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	result FD pos/neg**
sample										
1	yes	17,30	85,03	yes	17,15	85,04	yes	15,45	85,08	pos
2	no	/	/	no	/	/	no	/	/	neg
3	no	/	/	yes	23,45	84,79	yes	36,16	84,38	sus
4	no	/	/	no	/	/	no	/	/	neg
17	no	/	/	no	/	/	no	/	/	neg
18	no	/	/	no	/	/	no	/	/	neg
PC FD	yes	14,00	84,88	yes	13,30	84,88	nt	nt	nt	OK
NTC	no	/	/	no	/	/	nt	nt	nt	OK

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

**pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C ; neg - none of replicates produce fluorescence; sus - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

nt - not tested

4.D)

Operator: 1; Device for amplification: GenieII; Date: 14.4.2015; Aliquot 3 stored at room T seven days

	LAMP FD assay									
	replicate 1			replicate 2			replicate 3			
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	result FD pos/neg**
sample										
1	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
2	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
3	no	/	/	no	/	/	no	/	/	neg
4	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
5	no	/	/	no	/	/	no	/	/	neg
6	yes	12,50	84,83	yes	12,30	84,82	yes	12,30	84,77	pos
7	no	/	/	no	/	/	no	/	/	neg
8	no	/	/	no	/	/	no	/	/	neg
9	yes	13,45	84,03	yes	15,00	84,99	yes	15,00	85,03	pos
10	no	/	/	no	/	/	no	/	/	neg
11	no	/	/	no	/	/	no	/	/	neg
12	yes	13,45	84,77	yes	13,15	84,91	yes	13,15	84,91	pos
13	no	/	/	no	/	/	no	/	/	neg
14	no	/	/	no	/	/	no	/	/	neg
15	no	/	/	no	/	/	no	/	/	neg
16	no	/	/	no	/	/	no	/	/	neg
17	no	/	/	no	/	/	no	/	/	neg
18	no	/	/	no	/	/	no	/	/	neg
PC FD	yes	14,00	84,77	yes	13,45	84,78	yes	12,30	84,78	OK
PC FD	yes	13,45	84,77	nt	nt	nt	nt	nt	nt	OK
NTC	no	/	/	no	/	/	no	/	/	OK
NTC	no	/	/	nt	nt	nt	nt	nt	nt	OK

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

**pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C ; neg - none of replicates produce fluorescence; sus - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

nt-not tested

4.E)

Operator: 1; Device for amplification: GenieII; Date: 6.5.2015; Aliquot 4 stored at room T two days

	LAMP FD assay									
	replicate 1			replicate 2			replicate 3			result FD
	curve yes/	Tpos (mir	Tm (oC)	curve yes/n	Tpos (min)	Tm (oC)	curve yes/n	Tpos (mir	Tm (oC)	pos/neg**
sample										
1	yes	15,45	85,08	yes	15,45	85,04	yes	14,15	85,08	pos
2	no	/	/	no	/	/	no	/	/	neg
3	yes	22,30	84,86	yes	16,15	84,79	yes	20,45	84,82	pos
4	no	/	/	no	/	/	no	/	/	neg
5	no	/	/	no	/	/	no	/	/	neg
6	yes	12,30	84,83	yes	12,15	84,92	yes	12,30	84,77	pos
7	no	/	/	no	/	/	no	/	/	neg
8	no	/	/	no	/	/	no	/	/	neg
9	yes	14,45	85,02	yes	14,15	84,98	yes	13,00	84,92	pos
10	no	/	/	no	/	/	no	/	/	neg
11	no	/	/	no	/	/	no	/	/	neg
12	yes	13,15	84,77	yes	13,30	84,90	yes	14,00	84,85	pos
13	no	/	/	no	/	/	no	/	/	neg
14	no	/	/	no	/	/	no	/	/	neg
15	no	/	/	no	/	/	no	/	/	neg
16	no	/	/	no	/	/	no	/	/	neg
17	no	/	/	no	/	/	yes	13,00	84,92	contamination in one well! - the analysis was repeated
18	no	/	/	no	/	/	no	/	/	neg
PC FD	yes	12,45	84,78	yes	13,30	84,77	yes	13,15	84,82	ok
	yes	13,45	84,83	yes	13,45	84,76	n.t	n.t	n.t	
NTC	no	/	/	no	/	/	no	/	/	ok
	no	/	/	no	/	/	n.t	n.t	n.t	
PC FD was most probably added by mistake also in one well for sample 17 , therefore the analysis for sample 17 has been repeated with using the same aliquot of sample and chemicals:										
17	no	/	/	no	/	/	n.t	n.t	n.t	neg
PC FD	yes	13,30	84,87	n.t	n.t	n.t	n.t	n.t	n.t	ok
NTC	no	/	/	n.t	n.t	n.t	n.t	n.t	n.t	ok

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

**pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C ; neg - none of replicates produce fluorescence; sus - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

nt - not tested

APPENDIX 5
Homogeneity and stability of samples tested with LAMP BN

5.A)
Operator: 1; Device for amplification: Roche LC480; Date: 2.4.2015; Aliquot 1 stored at -20°C

	LAMP BN assay									
	replicate 1			replicate 2			replicate 3			result BN
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	pos/neg**
sample										
1	no	7,05	79,86	no	/	79,78	no	35	79,82	neg
2	yes	6	85,37	yes	7,2	85,2	yes	6	85,13	pos
3	no		79,77	no	/	79,95	no	25,87	79,79	neg
4	no	/	80,15	no	10,18	80,15	no	10,18	80,15	neg
5	no	29,96	80,15	no	7,61	80,15	no	7,61	80,15	neg
6	yes	5	85,38	yes	5	85,38	yes	5,53	85,37	pos
7	no	7,83	79,44	no	29,27	79,39	no	35	79,31	neg
8	yes	13,5	85,38	yes	8,19	85,23	yes	13,83	85,19	pos
9	no	/	79,82	no	/	79,8	no	8,41	79,74	neg
10	no	/	80,15	no	/	80,15	no	21,02	80,15	neg
11	no	8,65	/	no	16,57	/	no	30,12	/	neg
12	no	/	79,95	no	29,73	79,87	no	/	79,78	neg
13	yes	7,37	85,37	no	22,51	80,45	yes	11,13	84,67	pos
14	no	/	80,09	no	35	80,17	no	/	80,15	neg
15	no	9	80,15	no	/	80,15	no	/	80,15	neg
16	no	/	80,15	no	17,28	79,52	no	7,83	79,44	neg
17	yes	7,71	85,37	yes	8,66	85,2	yes	8,29	85,15	pos
18	no	6,65	79,4	no	10,04	79,39	no	6,66	79,34	neg
PC FD	no	9,33	79,82	no	14,54	79,77	no	35	79,76	neg
PC BN	yes	5	85,37	yes	6	85,37	yes	6	85,21	pos
NTC	no	35	79,47	no	27,78	79,41	nt	nt	nt	neg

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

**pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C ; neg - none of replicates produce fluorescence; sus - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

nt - not tested

5.B)

Operator: 2; Device for amplification: Roche LC480; Date: 10.4.2015; Aliquot 2 stored at -20°C

	LAMP BN assay									
	replicate 1			replicate 2			replicate 3			
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	result BN pos/neg**
sample										
1	no	22,84	/	no	14,84	79,89	no	/	79,87	neg
2	yes	6,00	85,40	yes	6,00	85,40	yes	6,00	85,27	pos
3	no	/	79,83	no	29,00	79,70	no	29,00	79,63	neg
4	no	/	/	no	16,54	76,59	no	/	79,82	neg
5	no	15,37	79,95	no	/	79,29	no	7,29	79,30	neg
6	yes	5,00	85,40	yes	5,00	85,40	yes	5,00	85,40	pos
7	no	/	79,75	no	7,82	79,53	no	17,54	79,51	neg
8	yes	9,95	85,52	yes	8,72	85,40	yes	7,85	85,40	pos
9	no	8,15	79,76	no	/	79,62	no	16,19	79,54	neg
10	no	13,38	79,63	no	7,00	/	no	17,82	79,52	neg
11	no	6,77	/	no	/	/	no	8,21	/	neg
12	no	15,19	79,62	no	/	79,58	no	35,00	79,74	neg
13	yes	6,00	85,23	yes	6,00	85,12	yes	7,02	85,12	pos
14	no	/	79,76	no	31,44	/	no	23,28	79,48	neg
15	no	27,28	79,81	no	12,41	79,67	no	/	79,56	neg
16	no	24,13	79,87	no	22,88	/	no	/	79,35	neg
17	yes	6,00	85,12	yes	8,02	85,12	yes	7,13	85,12	pos
18	no	27,53	79,74	no	/	79,29	no	8,97	79,74	neg
PC BN	yes	5,00	85,04	yes	5,00	84,93	yes	5,00	85,04	ok
NTC	no	32,16	79,74	no	15,24	79,74	nt	nt	nt	ok

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C; **neg - none of replicates produce fluorescence; **sus** - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

nt-not tested

5.C)

Operator: 1; Device for amplification: GenieII; Date: 10.4.2015; Aliquot 3 stored at room T three days

	LAMP BN assay									
	replicate 1			replicate 2			replicate 3			
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	result BN pos/neg**
sample										
1	no	/	/	no	/	/	no	/	/	neg
2	yes	8,15	84,44	yes	8,15	84,33	yes	8,00	84,28	pos
3	no	/	/	no	/	/	no	/	/	neg
4	no	/	/	no	/	/	no	/	/	neg
5	no	/	/	no	/	/	no	/	/	neg
6	yes	7,30	84,39	yes	7,15	84,34	yes	7,30	84,29	pos
7	no	/	/	no	/	/	no	/	/	neg
8	yes	11,15	84,28	yes	9,15	84,41	yes	12,45	84,36	pos
9	no	/	/	no	/	/	no	/	/	neg
10	no	/	/	no	/	/	no	/	/	neg
11	no	/	/	no	/	/	no	/	/	neg
12	no	/	/	no	/	/	no	/	/	neg
13	yes	8,45	84,53	yes	8,45	84,54	yes	8,45	84,47	pos
14	no	/	/	no	/	/	no	/	/	neg
15	no	/	/	no	/	/	no	/	/	neg
16	no	/	/	no	/	/	no	/	/	neg
17	yes	9,15	84,58	yes	9,30	84,54	yes	10,00	84,48	pos
18	no	/	/	no	/	/	no	/	/	neg
PC BN	yes	7,45	84,33	yes	7,45	84,34	yes	8,15	84,34	ok
PC BN	yes	8,00	84,27	yes	8,15	84,27	nt	nt	nt	ok
NTC	no	/	/	no	/	/	no	/	/	ok
NTC	no	/	/	no	/	/	nt	nt	nt	ok

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C; **neg** - none of replicates produce fluorescence; **sus** - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

nt-not tested

5.D)

Operator: 1; Device for amplification: GenieII; Date: 14.4.2015; Aliquot 3 stored at room T seven days

	LAMP BN assay									
	replicate 1			replicate 2			replicate 3			
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	result BN pos/neg**
sample										
2	yes	8,15	84,51	yes	8,15	84,47	yes	8,15	84,46	pos
18	no	/	/	no	/	/	no	/	/	neg
PC BN	yes	8,3	84,11	nt	nt	nt	nt	nt	nt	pos
NTC	no	/	/	nt	nt	nt	nt	nt	nt	neg

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C; **neg** - none of replicates produce fluorescence; **sus** - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

nt-not tested

5.E)

Operator: 1; Device for amplification: GenieII; Date: 6.5.2015; Aliquot 4 stored at room T two days

	LAMP BN assay									
	replicate 1			replicate 2			replicate 3			result BN
	curve yes/n*	Tpos (min)	Tm (oC)	curve yes/n	Tpos (min)	Tm (oC)	curve yes/n	Tpos (min)	Tm (oC)	pos/neg**
sample										
1	no	/	/	no	/	/	no	/	/	neg
2	yes	8,45	84,34	yes	9,00	84,34	yes	9,00	84,29	pos
3	no	/	/	no	/	/	no	/	/	neg
4	no	/	/	no	/	/	no	/	/	neg
5	no	/	/	no	/	/	no	/	/	neg
6	yes	8,00	84,35	yes	8,00	84,34	yes	7,45	84,34	pos
7	no	/	/	no	/	/	no	/	/	neg
8	yes	10,15	84,24	yes	10,45	84,42	yes	10,30	84,42	pos
9	no	/	/	no	/	/	no	/	/	neg
10	no	/	/	no	/	/	no	/	/	neg
11	no	/	/	no	/	/	no	/	/	neg
12	no	/	/	no	/	/	no	/	/	neg
13	yes	9	84,54	yes	9,00	84,50	yes	9,00	84,53	pos
14	no	/	/	no	/	/	no	/	/	neg
15	no	/	/	no	/	/	no	/	/	neg
16	no	/	/	no	/	/	no	/	/	neg
17	yes	9,30	84,40	yes	11,15	84,38	yes	10,30	84,38	pos
18	no	/	/	no	/	/	no	/	/	neg
PC BN	yes	8,00	84,28	yes	8,15	84,33	yes	8,15	84,29	
	yes	8,15	84,33	yes	8,00	84,34	n.t	n.t	n.t	ok
NTC	no	/	/	no	/	/	no	/	/	
	no	/	/	no	/	/	n.t	n.t	n.t	ok

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C; **neg - none of replicates produce fluorescence; **sus** - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

*** n.t= not tested

APPENDIX 6
Detailed results obtained by different laboratories using LAMP BN

Partner code: 1

	LAMP BN assay									
	replicate 1			replicate 2			replicate 3			
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	result BN pos/neg**
sample										
1	no	13,39	79,85	no	15,17	79,86	no	23,56	79,80	neg
2	yes	6,66	85,48	yes	7,10	85,41	yes	6,00	85,32	pos
3	no	11,83	79,59	no	11,48	79,54	no	30,78	79,52	neg
4	no	25,61	79,85	no	7,47	79,77	no	9,38	79,69	neg
5	no	/	79,74	no	/	79,74	no	23,00	79,67	neg
6	yes	6	85,47	yes	5,00	85,37	yes	5,00	85,34	pos
7	no	19,08	79,59	no	/	79,53	no	28,56	79,49	neg
8	yes	13,04	85,48	yes	8,84	85,48	yes	12,48	85,48	pos
9	no	11,59	79,70	no	14,31	79,61	no	12,46	79,60	neg
10	no	25,26	79,65	no	7,37	79,56	no	14,02	79,47	neg
11	no	16,3	79,78	no	13,90	79,75	no	28,00	79,57	neg
12	no	/	79,61	no	7,23	79,51	no	13,78	79,45	neg
13	yes	8,62	85,27	yes	8,76	85,27	yes	8,05	85,14	pos
14	no	/	79,56	no	/	79,54	no	8,03	79,50	neg
15	no	29,88	79,61	no	/	79,53	no	/	79,47	neg
16	no	10,71	79,59	no	/	79,47	no	/	79,47	neg
17	yes	8,63	85,17	yes	14,41	85,12	yes	8,87	85,00	pos
18	no	7,43	79,41	no	14,19	79,49	no	7,42	79,49	neg
PC BN	yes	8,06	85,00	yes	6,00	85,00	yes	6,00	85,00	pos
NTC	no	9,12	79,33	no	24,14	79,31				neg

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C; **neg** - none of replicates produce fluorescence; **sus** - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

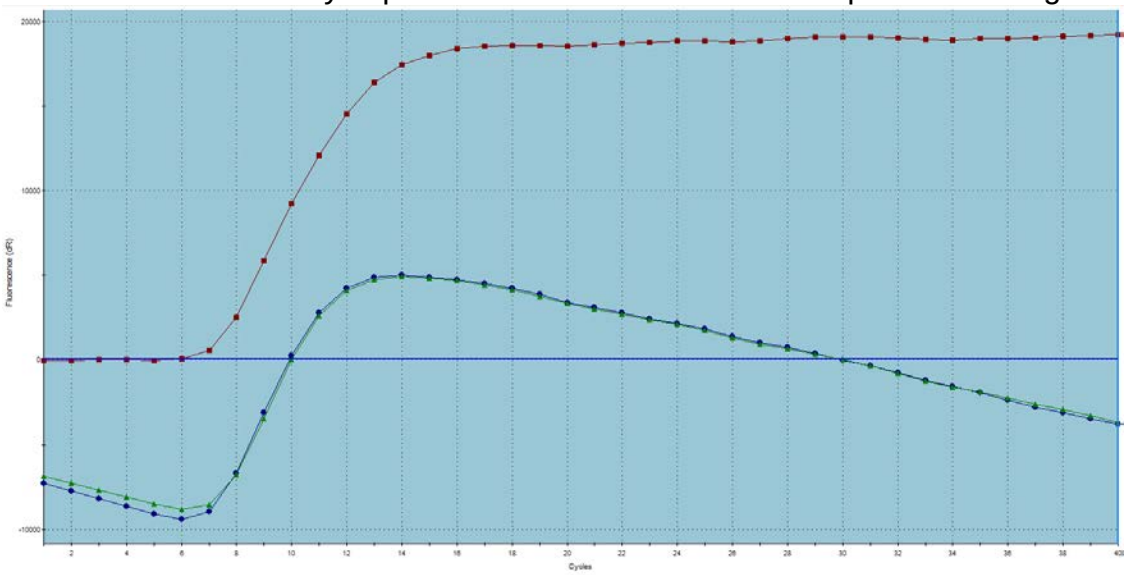
Partner code: 2

	LAMP BN assay									
	replicate 1			replicate 2			replicate 3			result BN
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	pos/neg**
sample										
1	no	No Ct	64.75	no	No Ct	64.75	no	No Ct	64.75	neg
2	yes	5.60	86.28	yes	6.33	86.28	yes	5.97	86.28	pos
3	no	7.10	63.71	no	No Ct	64.80	no	No Ct	64.80	neg
4	no	29.50	64.28	no	32.79	64.85	no	28.91	64.28	neg
5	no	No Ct	64.86	no	39.52	64.86	no	27.40	64.86	neg
6	yes(!)	No Ct	85.85	yes(!)	No Ct	85.85	yes(!)	No Ct	85.85	pos
7	no	38.42	64.31	no	26.19	64.31	no	32.86	64.86	neg
8	yes	7.35	86.33	yes	6.84	85.83	yes	6.42	85.83	pos
9	no	No Ct	63.71	no	29.41	64.75	no	28.07	64.75	neg
10	no	38.93	64.22	no	27.71	64.22	no	39.74	64.22	neg
11	no	30.89	63.71	no	28.62	87.28	no	31.55	64.83	neg
12	no	No Ct	65.39	no	No Ct	64.28	no	39.18	64.83	neg
13	yes	6.52	85.78	yes	6.89	85.78	yes	6.34	85.78	pos
14	no	35.87	63.77	no	27.55	64.84	no	No Ct	64.84	neg
15	no	29.92	64.86	no	No Ct	64.86	no	No Ct	64.86	neg
16	no	No Ct	63.77	no	13.00	64.86	no	No Ct	64.86	neg
17	yes	6.47	86.25	yes	6.90	86.25	yes	6.57	86.25	pos
18	no	37.92	64.22	no	27.64	65.33	no	38.69	64.22	neg
PC BN	yes(!)	No Ct	85.78	yes	5.97	85.78	yes(!)	10.29	85.78	pos
NTC	no	No Ct	65.35	no	39.20	65.35				neg

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

****pos** - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C; **neg** - none of replicates produce fluorescence; **sus** - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

Note for curves marked with "(!)" given by partner 2:
The curves were clearly exponential but there was a sort of problem during reading.



Explanation (SI-NIB):
Here is a problem of the normalisation of fluorescence by the software. It is suggested to use manual settings for analysis of the curves.

Partner code: 3

	LAMP BN assay									
	replicate 1			replicate 2			replicate 3			result BN
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	pos/neg**
sample										
1	no	Undet	61,80	no	Undet	61,80	no	Undet	61,80	neg
2	yes	6,6278	85,20	no	26,8670	85,20	yes	Undet	85,10	pos
3	no	17,4974	62,10	no	Undet	62,90	no	23,3958	61,80	neg
4	no	Undet	61,80	no	Undet	62,90	no	25,2889	61,80	neg
5	no	Undet	78,80	no	Undet	71,30	no	Undet	61,80	neg
6	no	Undet	84,90	no	Undet	86,00	yes	Undet	85,50	sus
6 retested	yes	4,0727	85,60	yes	6,0234	85,60	yes	6,0337	85,30	pos
7	no	Undet	85,50	no	Undet	91,90	no	Undet	86,3	neg
8	no	Undet	81,10	no	Undet	62,90	no	Undet	85,50	neg
9	no	12,8929	61,80	no	36,8024	61,80	no	6,6015	61,80	neg
10	no	37,526	62,00	no	32,1965	61,80	no	29,5753	61,80	neg
11	no	Undet	61,80	no	Undet	62,00	no	4,3582	61,80	neg
12	no	33,3554	61,80	no	6,4302	61,80	no	20,4786	61,80	neg
13	yes	6,9559	85,70	yes	6,2287	85,70	yes	Undet	85,70	pos
14	no	36,9007	61,80	no	Undet	61,80	no	Undet	61,80	neg
15	no	Undet	78,20	no	Undet	81,60	no	Undet	62,60	neg
16	no	Undet	91,30	no	Undet	84,80	no	Undet	80,40	neg
17	yes	Undet	85,00	yes	7,8486	85,00	yes	Undet	85,00	pos
18	no	11,1394	61,80	no	16,0205	61,70	no	16,1597	61,70	neg
PC BN	yes	4,0584	85,40	yes	Undet	85,40	yes	Undet	85,30	pos
NTC	no	22,8404	61,80	no	Undet	61,70				neg

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C; **neg - none of replicates produce fluorescence; **sus** - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

Note:
The partner 3 decided to do re-testing for sample 6 because of ambiguous results (it was assumed for a loading error, spill over, etc.). Therefore, the result of a repetition is taken into account for further analysis.

Note (SI-NIB):
It seems that partner 3 has similar problem as partner 2 (no Tpos for some positive samples – the problem of the normalisation of fluorescence by the software).

Partner code: 4

	LAMP BN assay									
	replicate 1			replicate 2			replicate 3			result BN
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	pos/neg**
sample										
1	NO			NO			NO			NEG
2	YES	8:45	86,03	YES	8:30	86.08	YES	8:30	86.53	POS
3	NO			NO			NO			NEG
4	NO			NO			NO			NEG
5	NO			NO			NO			NEG
6	YES	8:00	85,83	YES	8:00	86.08	YES	8:00	86.14	POS
7	NO			NO			NO			NEG
8	YES	10:00	84,36	YES	12:15	84.44	YES	11:30	84.51	POS
9	NO			NO			NO			NEG
10	NO			NO			NO			NEG
11	NO			NO			NO			NEG
12	NO			NO			NO			NEG
13	YES	9:45	85.88	YES	9:00	86.18	YES	9:45	86.10	POS
14	NO			NO			NO			NEG
15	NO			NO			NO			NEG
16	NO			NO			NO			NEG
17	YES	10:15	84,46	YES	10:30	86.06	YES	10:15	86.28	POS
18	NO			NO			NO			NEG
PC BN	YES	8:15	84,28	YES	9:00	84.23	YES	8:30	84.34	POS
NTC	NO			NO			NO			NEG

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C; **neg - none of replicates produce fluorescence; **sus** - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

Note (SI-NIB):

Tm of positive samples was not always in the range of PC ± 0.5°C. Since positive samples gave exponential amplification curves and the Tpos were lower than 30 min, the interpretation of results done by partner 4 is accepted. From this results it is obvious that Tm range need to be verified for each device (as it was already mentioned in the protocol – see Appendix 1)

Partner code: 5

	LAMP BN assay									
	replicate 1			replicate 2			replicate 3			result BN
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	pos/neg**
sample										
1	NO			NO			NO			NEG
2	SI	9,40	84,61	SI	9,64	84,75	SI	8,56	84,84	POS
3	NO			NO			NO			NEG
4	NO			NO			NO			NEG
5	NO			NO			NO			NEG
6	SI	7,03	84,84	SI	6,89	85,01	SI	8,57	85,26	POS
7	NO			NO			NO			NEG
8	SI	10,98	84,75	SI	14,04	84,84	SI	10,97	84,89	POS
9	NO			NO			NO			NEG
10	NO			NO			NO			NEG
11	NO			NO			NO			NEG
12	NO			NO			NO			NEG
13	SI	9,01	85,44	SI	10,97	85,46	SI	9,49	85,40	POS
14	NO			NO			NO			NEG
15	NO			NO			NO			NEG
16	NO			NO			NO			NEG
17	SI	10,88	84,91	SI	10,37	84,87	SI	10,68	84,84	POS
18	NO			NO			NO			NEG
PC BN	SI	8,51	85,30	SI	7,84	85,30	SI	7,41	85,26	POS
NTC	NO			NO			/			NEG

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C; **neg - none of replicates produce fluorescence; **sus** - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

Note:
Tm of the three replicates is slightly out from the range PC ± 0.5 °C – see comment SI-NIB on page 43.

Partner code: 6

	LAMP BN assay									
	replicate 1			replicate 2			replicate 3			result BN
	curve yes/no*	Tpos * (min)	Tm (oC)	curve yes/no*	Tpos * (min)	Tm (oC)	curve yes/no*	Tpos * (min)	Tm (oC)	pos/neg**
sample										
1	no			no			no			neg
2	yes	7,05	84,60	yes	7,26	84,60	yes	6,81	84,60	pos
3	no			no			no			neg
4	no			no			no			neg
5	no			no			no			neg
6	yes	6,09	84,60	yes	6,02	84,60	yes	6,04	84,60	pos
7	no			no			no			neg
8	yes	8,27	84,60	yes	8,23	84,60	yes	8,04	84,60	pos
9	no			no			no			neg
10	no			no			no			neg
11	no			no			no			neg
12	no			no			no			neg
13	yes	7,47	84,60	yes	7,01	84,60	yes	7,00	84,60	pos
14	no			no			no			neg
15	no			no			no			neg
16	no			no			no			neg
17	yes	7,28	84,60	yes	8,50	84,60	yes	7,49	84,60	pos
18	yes			yes			yes			neg
PC BN	yes	6,47	84,60	yes	6,22	84,60	yes	6,23	84,60	pos
NTC	no			no			no			neg

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C; **neg - none of replicates produce fluorescence; **sus** - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

*: the Cq values were converted in min as 1 cycle corresponded to 1 minute

Partner code: 7

	LAMP BN assay									
	replicate 1			replicate 2			replicate 3			result BN
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	pos/neg**
sample										
1	no			no			no			neg
2	yes	8,27	84,50	yes	8,41	85,00	yes	8,14	85,00	pos
3	no			no			no			neg
4	no			no			no			neg
5	no			no			no			neg
6	yes	7,48	85,00	yes	7,64	84,50	yes	7,62	84,50	pos
7	no			no			no			neg
8	yes	12,27	85,00	yes	9,69	85,00	yes	9,02	84,50	pos
9	no			no			no			neg
10	no			no			no			neg
11	no			no			no			neg
12	no			no			no			neg
13	yes	10,21	84,50	yes	10,81	84,50	yes	9,20	84,50	pos
14	no			no			no			neg
15	no			no			no			neg
16	no			no			no			neg
17	yes	11,73	84,50	yes	11,15	84,50	yes	9,86	84,50	pos
18	no			no			no			neg
PC BN	yes	8,34	84,50	yes	7,78	84,50	yes	7,20	84,50	pos
NTC	no			no			no			neg

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C; **neg** - none of replicates produce fluorescence; **sus** - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

Partner code: 8

	LAMP BN assay									
	replicate 1			replicate 2			replicate 3			result BN
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	pos/neg**
sample										
1	No			No			No			
2	Yes	5,09	85,10	Yes	5,21	85,10	Yes	5,01	85,14	pos
3	No			No			No			
4	No			No			No			
5	No			No			No			
6	Yes	5,00	85,14	Yes	5,00	85,14	Yes	5,04	85,14	pos
7	No			No			No			
8	Yes	5,73	85,10	Yes	5,90	85,10	Yes	5,33	85,14	pos
9	No			No			No			
10	No			No			No			
11	No			No			No			
12	No			No			No			
13	Yes	5,07	85,10	Yes	5,06	85,14	Yes	5,10	85,14	pos
14	No			No			No			
15	No			No			No			
16	No			No			No			
17	Yes	5,04	85,10	Yes	5,33	85,10	Yes	5,33	85,14	pos
18	No			No			No			
PC BN	Yes	4,93	85,10	Yes	4,94	85,10		1)		pos
NTC	No			No			No			

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C; **neg - none of replicates produce fluorescence; **sus** - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

1) For positive controls, only duplicates were performed

Partner code: 9

	LAMP BN assay									
	replicate 1			replicate 2			replicate 3			result BN
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	pos/neg**
sample										
1	no			no			no			neg
2	yes	< 10 min	84,66	yes	< 10 min	84,64	yes	< 10 min	84,60	pos
3	no			no			no			neg
4	no			no			no			neg
5	no			no			no			neg
6	yes	< 10 min	84,70	yes	< 10 min	84,66	yes	< 10 min	84,66	pos
7	no			no			no			neg
8	yes	< 10 min	84,66	yes	< 10 min	84,66	yes	< 10 min	84,66	pos
9	no			yes	> 20 min	86,44	no			neg
10	no			no			no			neg
11	yes	> 30 min	86,70	no			no			neg
12	no			no			no			neg
13	yes	< 10 min	84,66	yes	< 10 min	84,66	yes	< 10 min	84,66	pos
14	no			no			no			neg
15	no			yes	> 25 min	87,40	no			neg
16	no			no			no			neg
17	yes	< 10 min	84,70	yes	< 10 min	84,64	yes	< 10 min	84,70	pos
18	no			no			no			neg
PC BN	yes	< 10 min	84,66	yes	< 10 min	84,70	yes	< 10 min	84,70	pos
NTC	no			no			yes	< 30 min	85,90	neg

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C; **neg - none of replicates produce fluorescence; **sus** - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

Partner code: 10

	LAMP BN assay									
	replicate 1			replicate 2			replicate 3			result BN
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	pos/neg**
sample										
1	No	-	-	No	-	-	No	-	-	Neg
2	Yes	9,00	84,20	Yes	9,15	84,25	Yes	9,00	84,24	Pos
3	No	-	-	No	-	-	No	-	-	Neg
4	No	-	-	No	-	-	No	-	-	Neg
5	No	-	-	No	-	-	No	-	-	Neg
6	Yes	8,15	84,20	Yes	8,15	84,21	Yes	8,15	84,26	Pos
7	No	-	-	No	-	-	No	-	-	Neg
8	Yes	13,00	84,25	Yes	12,15	84,05	Yes	13,00	84,05	Pos
9	No	-	-	No	-	-	No	-	-	Neg
10	No	-	-	No	-	-	No	-	-	Neg
11	No	-	-	No	-	-	No	-	-	Neg
12	No	-	-	No	-	-	No	-	-	Neg
13	Yes	9,45	84,27	Yes	10,00	84,50	Yes	10,00	84,33	Pos
14	No	-	-	No	-	-	No	-	-	Neg
15	No	-	-	No	-	-	No	-	-	Neg
16	No	-	-	No	-	-	No	-	-	Neg
17	Yes	8,45	84,06	Yes	8,30	84,16	Yes	10,15	84,17	Pos
18	No	-	-	No	-	-	No	-	-	Neg
PC BN	Yes	8,45	84,20	Yes	8,45	84,44	Yes	8,45	84,20	Pos
NTC	No	-	-	No	-	-	No	-	-	Neg

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C; **neg - none of replicates produce fluorescence; **sus** - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

APPENDIX 7
Detailed results obtained by different laboratories using LAMP FD

Partner code: 1

	LAMP FD assay									
	replicate 1			replicate 2			replicate 3			result FD
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	pos/neg**
sample										
1	yes	17,05	85,72	yes	16,62	85,7	yes	17,63	85,62	pos
2	no			no			no			neg
3	yes	15,84	85,61	yes	17,73	85,54	yes	20,41	85,53	pos
4	no			no			no			neg
5	no			no			no			neg
6	yes	10,27	85,65	yes	10,36	85,59	yes	10,08	85,54	pos
7	no			no			no			neg
8	no			no			no			neg
9	yes	15,96	85,53	yes	14,17	85,37	yes	16,56	85,41	pos
10	no			no			no			neg
11	no			no			no			neg
12	yes	10,9	85,49	yes	11,77	85,37	yes	10,58	85,31	pos
13	no			no			no			neg
14	no			no			no			neg
15	no			no			no			neg
16	no			no			no			neg
17	no			no			no			neg
18	no			no			no			neg
PC FD	yes	14,38	85,22	yes	16,35	85,24	yes	15,46	85,16	pos
NTC	no			no						neg

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

**pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C ; neg - none of replicates produce fluorescence; sus - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

Partner code: 2

	LAMP FD assay									
	replicate 1			replicate 2			replicate 3			result FD
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	pos/neg**
sample										
1	yes	18.64	86.78	yes	15.96	86.25	yes	13.87	86.25	pos
2	no	No Ct	63.17	no	No Ct	63.17	no	No Ct	63.17	neg
3	yes	17.77	86.28	yes	28.29	86.28	yes	23.10	86.28	pos
4	no	No Ct	63.74	no	No Ct	63.23	no	No Ct	62.66	neg
5	no	No Ct	63.23	no	No Ct	63.23	no	No Ct	62.67	neg
6	yes	10.69	86.33	yes	10.47	86.33	yes	10.50	86.33	pos
7	no	No Ct	63.24	no	No Ct	62.69	no	No Ct	62.69	neg
8	no	No Ct	63.83	no	No Ct	63.25	no	No Ct	62.70	neg
9	yes	13.11	86.25	yes	13.46	86.25	yes	12.78	86.78	pos
10	no	No Ct	63.17	no	No Ct	63.17	no	No Ct	63.17	neg
11	no	No Ct	63.17	no	No Ct	62.64	no	No Ct	63.19	neg
12	yes	11.47	86.30	yes	11.30	86.30	yes	11.14	86.30	pos
13	no	No Ct	62.67	no	No Ct	63.23	no	No Ct	63.23	neg
14	no	No Ct	62.67	no	No Ct	63.23	no	No Ct	63.23	neg
15	no	No Ct	62.69	no	No Ct	62.69	no	No Ct	63.25	neg
16	no	No Ct	62.70	no	No Ct	63.24	no	No Ct	63.81	neg
17	no	No Ct	63.70	no	No Ct	63.15	no	No Ct	63.15	neg
18	no	No Ct	63.17	no	No Ct	63.17	no	No Ct	63.17	neg
PC FD	yes	11.23	86.28	yes	11.48	86.28	yes	12.44	86.28	pos
NTC	no	No Ct	62.66	no	No Ct	63.23				

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

**pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C ; neg - none of replicates produce fluorescence; sus - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

Partner code: 3

	LAMP FD assay									
	replicate 1			replicate 2			replicate 3			result FD
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	pos/neg**
sample										
1	yes	11,3544	85,20	yes	12,5744	85,20	yes	12,5930	85,20	pos
2	no	36,6713	61,60	no	36,2141	61,60	no	Undet	61,90	neg
3	yes	13,2888	85,50	yes	13,0841	85,80	yes	12,0443	85,50	pos
4	no	Undet	61,60	no	Undet	61,60	no	Undet	61,90	neg
5	no	Undet	61,60	no	Undet	61,60	no	Undet	61,90	neg
6	yes	10,9059	85,50	yes	9,9470	85,80	yes	4,2960	85,80	pos
7	no	12,5083	61,60	no	Undet	61,60	no	24,3916	61,90	neg
8	no	4,5107	61,60	no	Undet	61,60	no	Undet	61,90	neg
9	yes	10,4219	85,20	yes	10,4841	85,20	yes	9,1864	85,10	pos
10	no	Undet	61,90	no	35,7206	61,80	no	10,5111	61,80	neg
11	no	Undet	61,90	no	Undet	61,80	no	10,4076	61,80	neg
12	yes	9,9349	86,10	yes	7,5584	85,70	yes	8,0759	85,70	pos
13	no	Undet	61,90	no	Undet	61,80	no	Undet	61,80	neg
14	no	Undet	61,90	no	16,1728	61,80	no	Undet	61,80	neg
15	no	Undet	61,90	no	2,0201	61,80	no	Undet	61,80	neg
16	no	Undet	61,90	no	Undet	61,80	no	21,7890	61,80	neg
17	no	32,9366	61,80	yes	18,0286	85,10	no	11,6022	61,80	sus
17 retested	no	Undet	61,8	no	Undet	61,8	no	Undet	61,8	neg
18	no	Undet	61,80	no	Undet	61,80	no	Undet	61,80	neg
PC FD	yes	10,8051	85,70	yes	8,0402	85,70	yes	8,0774	85,70	pos
NTC	no	21,1688	61,80	no	32,0557	61,80				neg

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

**pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C ; neg - none of replicates produce fluorescence; sus - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

Note:
The partner 3 decided to do re-testing for sample 17 because of ambiguous results (it was assumed for a loading error, spill over, etc.). Therefore, the result of a repetition is taken into account for further analysis.

Partner code: 4

	LAMP FD assay									
	replicate 1			replicate 2			replicate 3			result FD
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	pos/neg**
sample										
1	YES	15:30	86.57	YES	15:00	86.47	YES	17:15	86.49	POS
2	NO			NO			NO			NEG
3	YES	16:00	84.72	YES	20:30	84.88	YES	16:30	84.76	POS
4	NO			NO			NO			NEG
5	NO			NO			NO			NEG
6	YES	12:30	86.29	YES	12:15	86.48	YES	12:00	86.39	POS
7	NO			NO			NO			NEG
8	NO			NO			NO			NEG
9	YES	15:15	86.18	YES	12:30	86.38	YES	15:00	86.44	POS
10	NO			NO			NO			NEG
11	NO			NO			NO			NEG
12	YES	13:15	84.77	YES	13:00	84.80	YES	13:15	84.92	POS
13	NO			NO			NO			NEG
14	NO			NO			NO			NEG
15	NO			NO			NO			NEG
16	NO			NO			NO			NEG
17	NO			NO			NO			NEG
18	NO			NO			NO			NEG
PC FD	YES	13:15	84.79	YES	13:15	84.84	YES	12:45	84.79	POS
NTC	NO			NO			NO			NEG

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

**pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C ; neg - none of replicates produce fluorescence; sus - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

Note (SI-NIB):
Tm of positive samples was not always in the range of PC ± 0.5°C. Since positive samples gave exponential amplification curves and the Tpos were lower than 30 min, the interpretation of results done by partner 4 is accepted. From this results it is obvious that Tm range need to be verified for each device (as it was already mentioned in the protocol – see Appendix 1)

Partner code: 5

	LAMP FD assay									
	replicate 1			replicate 2			replicate 3			result FD
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	pos/neg**
sample										
1	SI	22,99	85,07	SI	18,72	85,07	SI	13,03	85,07	POS
2	NO			NO			NO			NEG
3	SI	18,90	85,07	SI	15,64	85,43	SI	25,66	85,52	POS
4	NO			NO			NO			NEG
5	NO			NO			NO			NEG
6	SI	11,58	85,07	SI	10,83	85,52	SI	10,85	85,61	POS
7	NO			NO			NO			NEG
8	NO			NO			NO			NEG
9	SI	14,41	85,07	SI	13,50	85,16	SI	12,75	85,16	POS
10	NO			NO			NO			NEG
11	NO			NO			NO			NEG
12	SI	12,25	85,79	SI	11,53	85,79	SI	12,89	85,82	POS
13	NO			NO			NO			NEG
14	NO			NO			NO			NEG
15	NO			NO			NO			NEG
16	NO			NO			NO			NEG
17	NO			NO			NO			NEG
18	SI	13,73	85,52	NO			NO			**SUS**
PC FD	SI	12,59	85,73	SI	11,79	85,70	SI	12,97	85,61	POS
NTC	NO			NO			/			NEG

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

**pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C ; neg - none of replicates produce fluorescence; sus - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

Note:
Tm of some replicates is slightly out from the range PC ± 0.5 °C – see comment SI-NIB on page 43.

Partner code: 6

	LAMP FD assay									
	replicate 1			replicate 2			replicate 3			result FD
	curve yes/no*	Tpos * (min)	Tm (oC)	curve yes/no*	Tpos * (min)	Tm (oC)	curve yes/no*	Tpos * (min)	Tm (oC)	pos/neg**
sample										
1	yes	13,70	85,00	yes	15,44	85,00	yes	12,63	85,00	pos
2	no			no			no			neg
3	yes	16,73	84,80	yes	13,69	84,80	yes	20,19	84,80	pos
4	no			no			no			neg
5	no			no			no			neg
6	yes	10,24	84,80	yes	10,09	84,80	yes	10,24	84,80	pos
7	no			no			no			neg
8	no			no			no			neg
9	yes	10,86	85,00	yes	12,13	85,00	yes	12,07	85,00	pos
10	no			no			no			neg
11	no			no			no			neg
12	yes	11,09	84,80	yes	10,86	84,80	yes	10,89	84,80	pos
13	no			no			no			neg
14	no			no			no			neg
15	no			no			no			neg
16	no			no			no			neg
17	no			no			no			neg
18	no			no			no			neg
PC FD	yes	11,38	84,80	yes	11,49	84,80	yes	11,27	84,80	pos
NTC	no			no			no			neg

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

**pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C ; neg - none of replicates produce fluorescence; sus - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

*: the Cq values were converted in min as 1 cycle corresponded to 1 minute

Partner code: 7

	LAMP FD assay									
	replicate 1			replicate 2			replicate 3			result FD
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	pos/neg**
sample										
1	yes	18,07	85,00	yes	15,96	85,00	yes	25,06	85,00	pos
2	no			no			no			neg
3	yes	21,95	85,00	yes	15,73	85,00	yes	18,77	85,00	pos
4	no			no			no			neg
5	no			no			no			neg
6	yes	10,27	85,00	yes	10,33	85,00	yes	11,03	85,00	pos
7	no			no			no			neg
8	no			no			no			neg
9	yes	14,96	85,00	yes	14,37	85,00	yes	14,68	85,00	pos
10	no			no			no			neg
11	no			no			no			neg
12	yes	12,14	85,00	yes	12,17	85,00	yes	12,64	85,00	pos
13	no			no			yes	20,40	84,50	sus
14	no			no			no			neg
15	no			no			no			neg
16	no			no			no			neg
17	no			no			no			neg
18	no			yes	12,49	85,00	no			sus
PC FD	yes	12,69	85,00	yes	12,54	85,00	no			pos
NTC	no			no						neg

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

**pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C ; neg - none of replicates produce fluorescence; sus - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

Partner code: 8

	LAMP FD assay									
	replicate 1			replicate 2			replicate 3			result FD
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	pos/neg**
sample										
1	yes	20,21	85,36	yes	9,06	85,34	yes	12,38	85,40	pos
2	No			No			No			
3	yes	8,68	85,36	yes	8,64	85,40	yes	9,48	85,40	pos
4	No			No			No			
5	No			No			No			
6	yes	5,47	85,50	yes	5,70	85,50	yes	5,34	85,40	pos
7	No			No			No			
8	No			No			No			
9	yes	7,42	85,46	yes	6,98	85,46	yes	7,03	85,44	pos
10	No			No			No			
11	No			No			No			
12	yes	6,27	85,44	yes	6,34	85,46	yes	6,58	85,50	pos
13	No			No			No			
14	No			No			No			
15	No			No			No			
16	No			No			No			
17	No			No			No			
18	No			No			No			
PC FD	yes	6,74	85,36	yes	6,48	85,36		1)		pos
NTC	No			No			No			

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

**pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C ; neg - none of replicates produce fluorescence; sus - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

1) For positive controls, only duplicates were performed

Partner code: 9

	LAMP FD assay									
	replicate 1			replicate 2			replicate 3			result FD
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	pos/neg**
sample										
1	yes	< 15 min	84,90	yes	< 15 min	84,86	yes	< 15 min	84,84	pos
2	no			no			no			neg
3	yes	< 15 min	84,84	no			no			sus
4	no			no			no			neg
5	no			no			no			neg
6	yes	< 15 min	84,84	yes	< 15 min	84,90	yes	< 15 min	84,84	pos
7	no			no			no			neg
8	no			no			no			neg
9	yes	< 15 min	84,84	yes	< 15 min	84,84	yes	< 15 min	84,80	pos
10	no			no			no			neg
11	no			no			no			neg
12	yes	< 15 min	84,80	yes	< 15 min	84,80	yes	< 15 min	84,86	pos
13	no			no			no			neg
14	no			no			no			neg
15	no			no			no			neg
16	no			no			no			neg
17	no			no			no			neg
18	no			no			no			neg
PC FD	yes	< 15 min	84,84	yes	< 15 min	85,10	yes	< 15 min	84,86	pos
NTC	no			no			no			neg

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

**pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C ; neg - none of replicates produce fluorescence; sus - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

Note (SI-NIB):
Sample 3 was excluded from the analysis, because it was shown (see point 3.3.4.2.4) that longer storage at room temperature cause a damage of FD in this sample (duration of transport of samples and chemicals to this lab took 3 days – see Table 11).

Partner code: 10

	LAMP FD assay									
	replicate 1			replicate 2			replicate 3			result FD
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	pos/neg**
sample										
1	Yes	26,00	84,80	Yes	28,00	84,75	Yes	17,00	84,74	Pos
2	No	-	-	No	-	-	No	-	-	Neg
3	Yes	17,00	84,78	Yes	17,00	84,78	Yes	15,15	84,78	Pos
4	No	-	-	No	-	-	No	-	-	Neg
5	No	-	-	No	-	-	No	-	-	Neg
6	Yes	12,15	84,81	Yes	12,30	84,76	Yes	12,15	84,81	Pos
7	No	-	-	No	-	-	No	-	-	Neg
8	No	-	-	No	-	-	No	-	-	Neg
9	Yes	15,00	84,71	Yes	14,00	84,56	Yes	14,30	84,62	Pos
10	No	-	-	No	-	-	No	-	-	Neg
11	No	-	-	No	-	-	No	-	-	Neg
12	Yes	13,15	84,78	Yes	13,15	84,83	Yes	13,00	84,73	Pos
13	No	-	-	No	-	-	No	-	-	Neg
14	No	-	-	No	-	-	No	-	-	Neg
15	No	-	-	No	-	-	No	-	-	Neg
16	No	-	-	No	-	-	No	-	-	Neg
17	No	-	-	No	-	-	No	-	-	Neg
18	No	-	-	No	-	-	No	-	-	Neg
PC FD	Yes	13,15	84,66	Yes	13,30	84,71	Yes	13,45	84,81	Pos
NTC	No	-	-	No	-	-	No	-	-	Neg

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

**pos - at least two of three replicates: exponential curve, Tpos less than 30 min and Tm in the range of PC ± 0.5°C ; neg - none of replicates produce fluorescence; sus - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 30 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

APPENDIX 8

Detailed results obtained by different laboratories using ISOA FD Qualiplante

Partner code 1:

	ISOA FD Qualiplante									
	replicate 1			replicate 2			replicate 3			
	curve yes	Tpos (mi	Tm (oC)	curve yes	Tpos (mi	Tm (oC)	curve yes	Tpos (mi	Tm (oC)	result FD
sample										
1	yes	10,30	81	yes	10,15	81	yes	11,45	81	pos
2	no	/	/	no	/	/	no	/	/	neg
3	yes	11,00	81	yes	10,00	81	yes	13,00	81	pos
4	no	/	/	no	/	/	no	/	/	neg
5	yes	29,30	86	no	/	/	yes	26,15	81	
5 repeat	yes	26,45	83	no	/	/	no	/	/	neg
6	yes	8,45	81	yes	9,00	81	yes	9,00	81	pos
7	no	/	/	no	/	/	no	/	/	neg
8	no	/	/	no	/	/	no	/	/	neg
9	yes	10,00	81	yes	10,00	81	yes	10,00	81	pos
10	no	/	/	no	/	/	no	/	/	neg
11	no	/	/	no	/	/	no	/	/	neg
12	yes	9,30	81	yes	9,30	81	yes	9,30	81	pos
13	no	/	/	no	29,30	/	no	/	/	neg
14	no	/	/	no	/	/	no	/	/	neg
15	no	/	/	no	/	/	no	/	/	neg
16	yes	29,30	82	no	/	/	no	/	/	
16 repeat	no	/	/	no	/	/	no	/	/	neg
17	no	/	/	no	/	/	no	/	/	neg
18	no	/	/	no	/	/	no	/	/	neg
NC Q	no	/	/	no	/	/	no	/	/	neg
PC Q	yes	8,15	81	yes	8,30	81	yes	8,15	81	pos
	yes	9,45	81	yes	9,30	81	yes	9,15	81	pos
PC FD	yes	9,30	81	yes	9,45	81	yes	10,00	81	pos
	no	/	/	no	/	/	no	/	/	neg
NTC	no	29,30	82	no	/	/	no	/	/	neg

Qualiplante instruction for results interpretation:

Melting peak	Ct value	
	Ct ≤ 25	Ct > 25
Included between 81°C to 82°C	Pos.	Ind.
No peak	Neg.	Neg.
Not included between 81°C and 82°C	Neg.	Neg.

Pos: positive – Neg: Negative – Ind: Indeterminated

If the Ct is between 25 to 30, with the presence of a melting peak, a contamination could be possible; we recommend you to test again the sample

Partner code: 2

	ISOA FD Qualiplante										
	replicate 1			replicate 2			replicate 3			result FD	result FD
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	pos/neg**	pos/neg***
sample											
1	yes	9.23	83.20	yes	8.54	82.71	yes	8.81	82.71	?	pos
2	no	No Ct	66.71	no	No Ct	66.15	no	22.73	66.71	neg	neg
3	yes	9.56	82.70	yes	9.41	82.70	yes	8.78	82.22	?	pos
4	no	No Ct	66.71	no	No Ct	66.71	no	No Ct	66.20	neg	neg
5	no	No Ct	67.28	no	No Ct	67.83	no	No Ct	66.71	neg	neg
6	yes	No Ct	82.78	yes	No Ct	82.78	yes	No Ct	82.78	?	pos
7	no	No Ct	66.78	no	No Ct	65.65	no	No Ct	65.65	neg	neg
8	no	No Ct	66.80	no	No Ct	66.80	no	No Ct	66.24	neg	neg
9	yes	8.30	82.71	yes	7.63	82.71	yes	6.73	83.20	?	pos
10	no	No Ct	66.71	no	No Ct	66.15	no	No Ct	66.15	neg	neg
11	no	No Ct	87.30	no	16.37	66.17	no	17.41	66.17	neg	neg
12	yes	6.92	82.20	yes	7.03	82.20	yes	7.15	82.20	?	pos
13	no	No Ct	67.30	no	No Ct	66.20	no	No Ct	65.65	neg	neg
14	no	No Ct	66.21	no	No Ct	66.78	no	No Ct	67.33	neg	neg
15	no	No Ct	84.28	no	No Ct	67.33	no	No Ct	66.21	neg	neg
16	no	No Ct	66.80	no	No Ct	66.80	no	No Ct	66.80	neg	neg
17	no	14.71	84.70	no	No Ct	67.24	no	13.87	66.70	neg	neg
18	no	24.49	84.75	no	No Ct	66.71	no	9.63	66.15	neg	neg
PC FD	yes	7.18	82.72	yes	7.40	82.72	yes	7.36	82.74	?	pos
NTC	no	No Ct	85.28	no	9.29	65.65				neg	neg
PC Qualiplante	yes	6.77	82.75							?	pos
NC Qualiplante	no	No Ct	84.28							neg	neg

*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential

**pos - see on booklet of Qualiplante

***__pos__ - at least two of three replicates: exponential curve, Tpos less than 25 min and Tm in the range of PC ± 0.5°C; __neg__ - none of replicates produce fluorescence; __sus__ - only one of replicates meet a criteria for 'pos' or at least two of three replicates: exponential curve, but Tpos higher than 25 min and/or Tm not in the range but close (± 0.5°C) to the criteria for 'pos'

Qualiplante instruction for results interpretation:

Melting peak	Ct value	
	Ct ≤ 25	Ct > 25
Included between 81°C to 82°C	Pos.	Ind.
No peak	Neg.	Neg.
Not included between 81°C and 82°C	Neg.	Neg.

Pos: positive – Neg: Negative – Ind: Indeterminated

If the Ct is between 25 to 30, with the presence of a melting peak, a contamination could be possible; we recommend you to test again the sample

Note:
If using a recommendation of a producer (Qualiplante instruction: melting peak for positive sample should be between 81 and 82°C), there are no positive results with this method. Since positive samples gave exponential amplification curves and the range of Tm is similar to the Tm of positive controls, the interpretation of results written in the last column of the table is accepted.

Partner code: 5

	ISOA FD Qualiplante									
	replicate 1			replicate 2			replicate 3			result FD
	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	curve yes/no*	Tpos (min)	Tm (oC)	pos/neg**
sample										
1	YES	11,39	81,49	YES	10,94	81,51	YES	12,68	81,62	POS
2	YES	24,53	84,06	YES	22,78	83,54	NO			NEG
3	YES	13,55	81,40	YES	13,51	81,62	YES	12,96	81,79	POS
4	NO			NO			YES	23,85	84,74	NEG
5	NO			NO			NO			NEG
6	YES	8,66	81,76	YES	8,82	81,95	YES	8,89	82,09	POS
7	NO			NO			YES	25,60	82,11	**IND**
8	NO			NO			NO			NEG
9	YES	11,18	81,73	YES	13,31	81,79	YES	12,16	81,84	POS
10	NO			NO			NO			NEG
11	YES	20,53	84,31	NO			NO			NEG
12	YES	10,64	82,09	YES	10,13	82,15	YES	10,09	82,12	POS
13	NO			NO			NO			NEG
14	NO			NO			NO			NEG
15	NO			NO			NO			NEG
16	NO			NO			NO			NEG
17	NO			NO			NO			NEG
18	NO			NO			NO			NEG
PC FD	YES	10,60	82,22	YES	10,50	82,20	YES	10,82	82,20	POS
NTC	NO			NO						
*yes - amplification curve is exponential; no - no amplification curve or amplification curve is not exponential										

Qualiplante instruction for results interpretation:

Melting peak	Ct value	
	Ct ≤ 25	Ct > 25
Included between 81°C to 82°C	Pos.	Ind.
No peak	Neg.	Neg.
Not included between 81°C and 82°C	Neg.	Neg.

Pos: positive – Neg: Negative – Ind: Indeterminated

If the Ct is between 25 to 30, with the presence of a melting peak, a contamination could be possible; we recommend you to test again the sample

APPENDIX 8
Planned dissemination activities

Activity	Where	When	Agreement
Scientific paper on TPS	Suggested journal: Phytopathogenic Mollicutes	May 2016	All involved partners have agreed with this action
Scientific paper on LAMP BNp assay	Suggested journal: Plant Pathology	April 2016	All involved partners have agreed with this action
Master thesis	University of Ljubljana	End of 2016	