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New diagnostic tools for improved diagnostics of grapevine phytoplasmas

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Phytoplasma

- cell wall-less Gram positive bacteria
- class Mollicutes
- cell and genome size are the smallest among bacteria
- obligate intracellular parasites
- Transmitted:

- phloem-feeding leafhoppers, planthoppers and psyllids
- dodder, micropropagation, grafting and cutting

- >1000 diseases

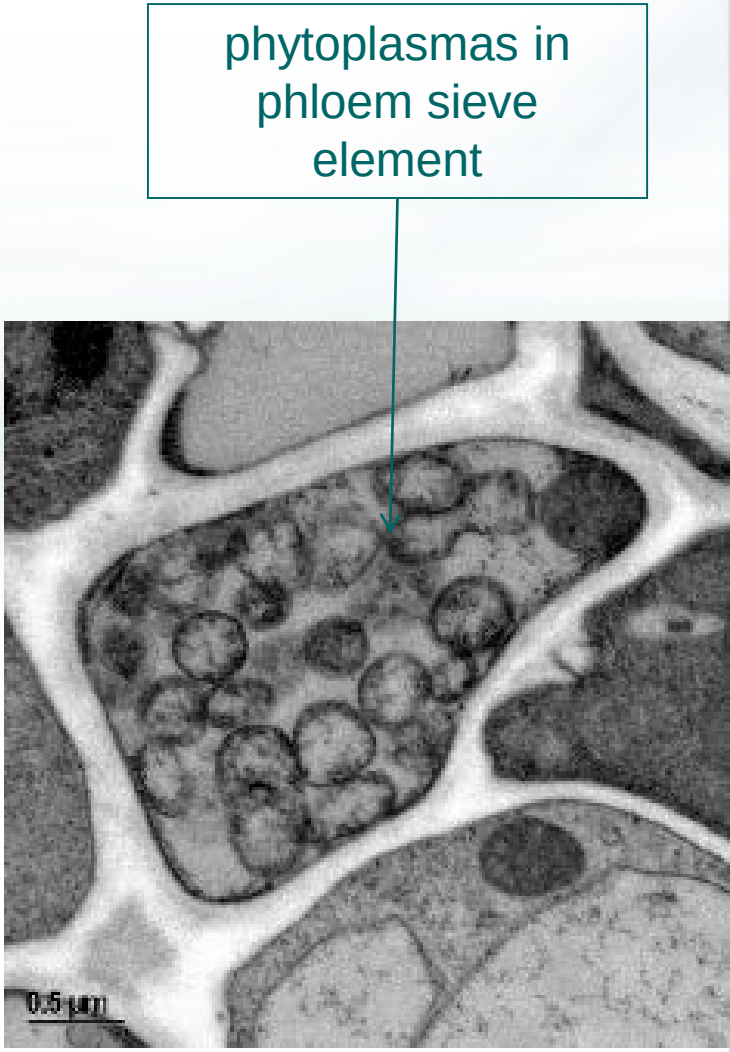


Photo: Magda Tušek Žnidarič

Grapevine yellows

- caused by different phytoplasmas (different vectors)
- indistinguishable by symptoms

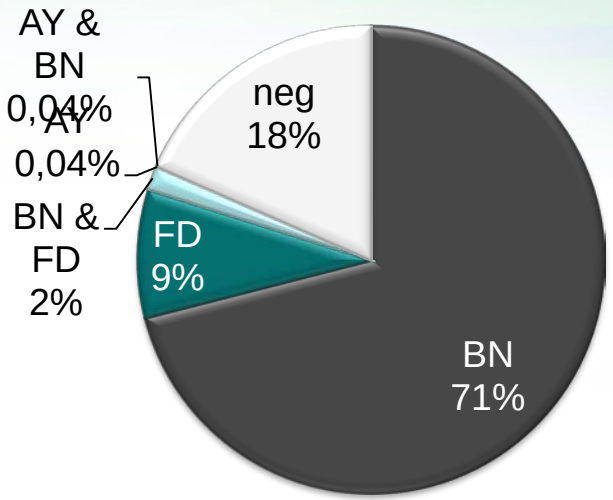


Slovenia:

-‘*Ca.* P. solani’ -> **BN**

- Flavescence dorée phytoplasma -> **FD**

-‘*Ca.* P. asteris’ -> **AY**



2005-2015 (2234 samples)

Limitations of phytoplasma detection

- the smallest by size and genome
- routinely uncultivable ~~– traditional diagnostic methods suitable for bacteria~~
- uneven distribution in the phloem (vascular tissue in stem, leaves, roots)
- low concentration
- variations in titer according to the season/plant organ

FD is listed in the EU2000/29 Council Directive on Harmful organisms and the A2 quarantine list of pests of EPPO: the destruction of diseased stocks, plants showing symptoms and surrounding plants is mandatory.

Example: FD – Izola

Reliable, sensitive and fast diagnostic procedure is needed!

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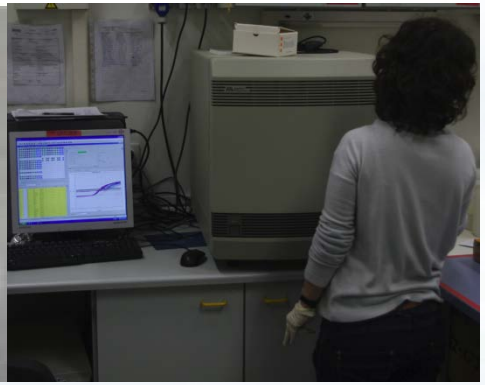
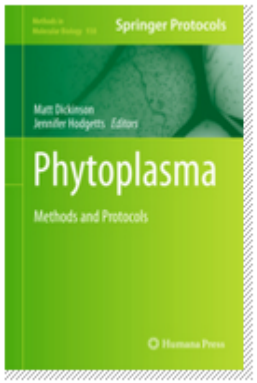
Diagnostic procedure





Phytoplasma

Methods and Protocols
Series: » Methods in Molecular Biology, Vol. 938
Dickinson, Matt; Hodgetts, Jennifer (Eds.)
2013, 2013, XIII, 421 p. 68 illus., 45 in color.
A product of Humana Press

The validation data about this method is available at EPPO website: http://dc.eppo.int/validationlist.php

SLOVENSKA AKREDITACIJA
SIST EN ISO/IEC 17025
LP-028

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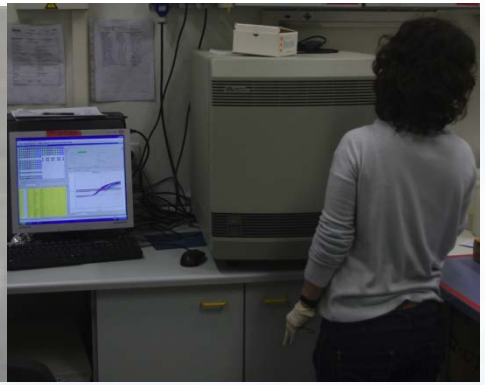
Diagnostic procedure



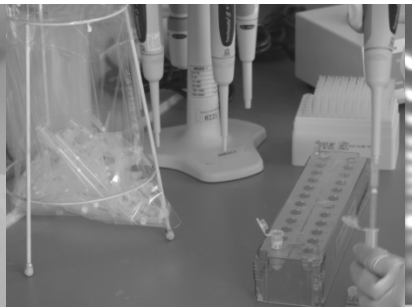
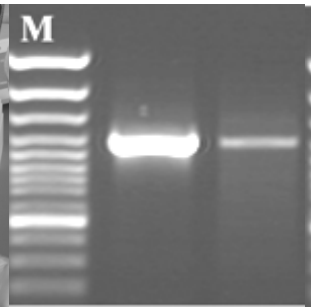
P	FP	KF	qPCR	D

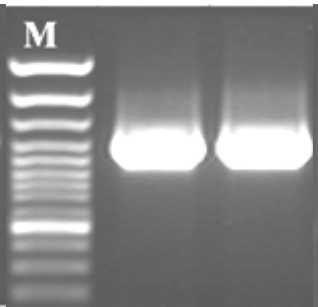
+ less contamination, higher sensitivity

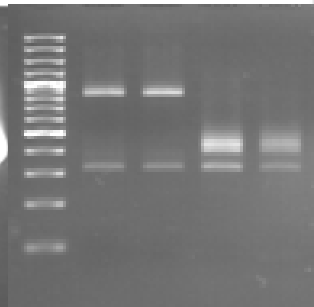
P	N2	CTAB	AGE	3x PCR	AGE	D	2x nPCR	AGE	D	nPCR	AGE	RLFP	PAGE	D

1st day | 2nd day | 3rd day | 4th day | 5th day



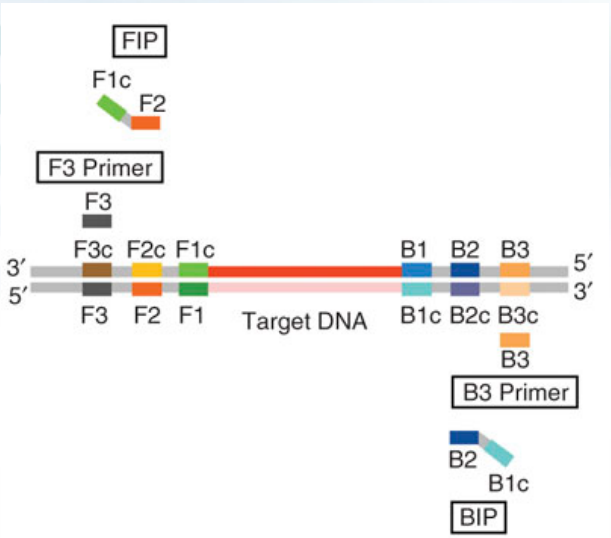


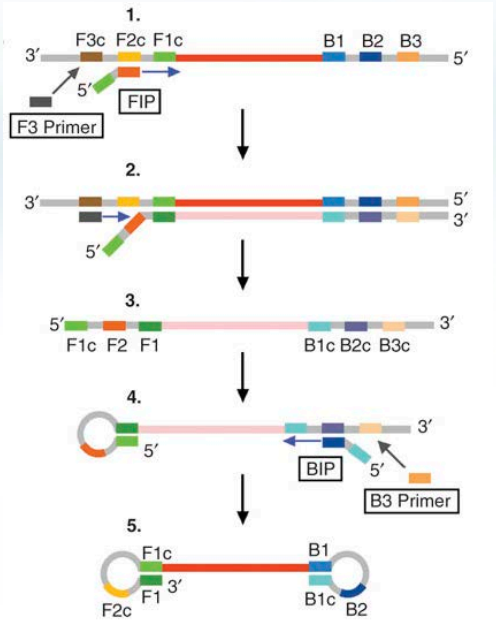


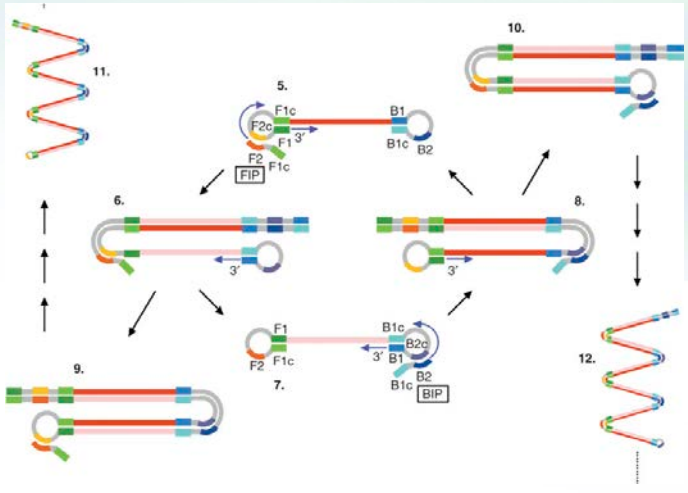
LAMP : Loop mediated isothermal AMPlification

Fast multiplication of DNA targets in one tube
at **isothermal conditions** (60-65ºC)
using a set of 4 or 6 primers

-**Relatively simple application**
-**No expensive equipment needed**







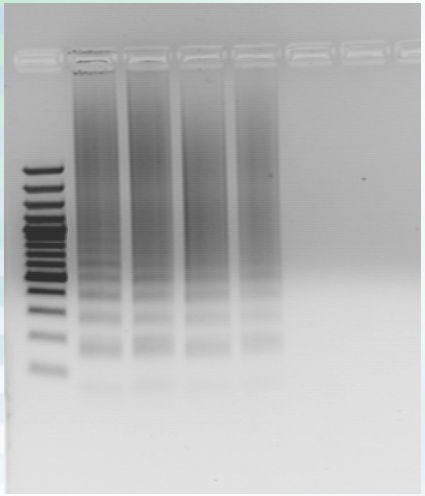
Starting structure producing step

Cycling amplification step

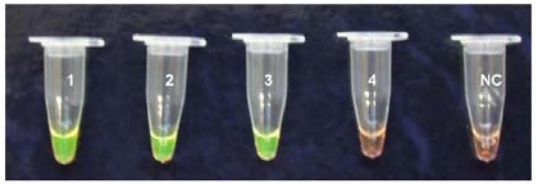
Principle of loop-mediated isothermal amplification (LAMP) method. (Tomita *et al*., 2008, Notomi *et al*., 2000).

Detection of LAMP products

- LAMP product on gel



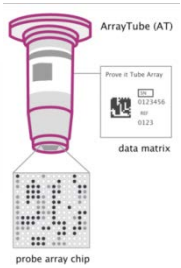
- Fluorescence



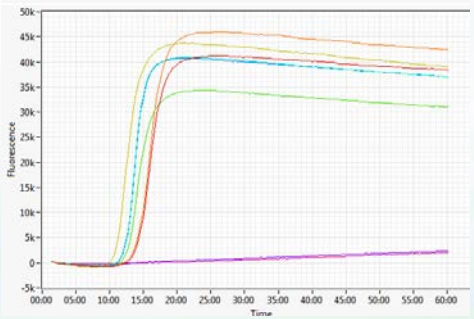
- Turbidity

- LFD

- Array tubes



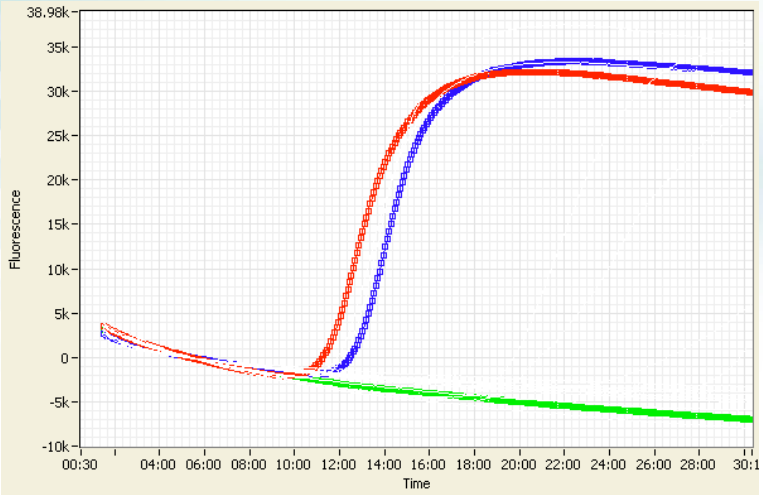
- Real time
  - Intercalating dye (!)
  - Fluorescent probes



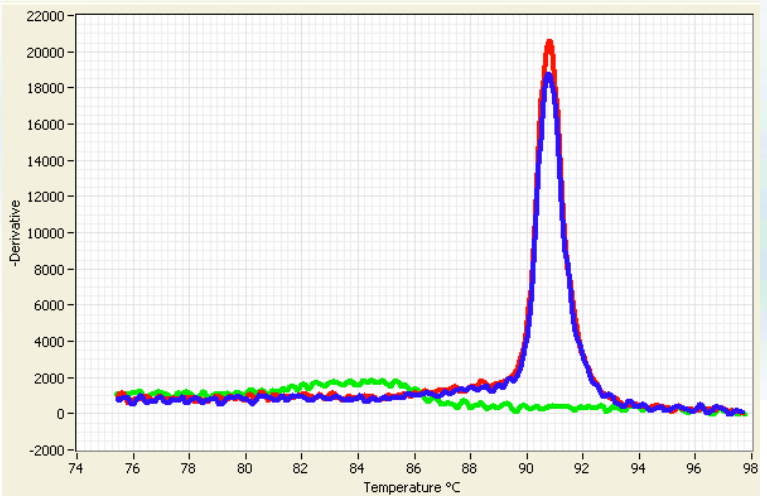
Simultaneously heater and fluorimeter (e.g., GenieII/III, SmartCycler)

Real time detection of LAMP products

1) **READING RESULTS**
pos: rise of fluorescence
neg: no rise of fluorescence



2) **CONFIRMATION OF RESULTS**
Melting temperature of the final product is pathogen specific



Legend:
- positive control (amplification-> rise of fluorescence)
- sample (comparable to positive control)
- negative control (no amplification-> no fluorescence)

LAMP detection of phytoplasmas FD and BN

- FDp:

Plant Pathology (2015) 64, 286–296 Doi: 10.1111/ppa.12266

BSPP

LAMP assay and rapid sample preparation method for on-site detection of flavescence dorée phytoplasma in grapevine

P. Kogovšek[ab]*, J. Hodgetts[c], J. Hall[c], N. Prezelj[a], P. Nikolić[a], N. Mehle[a], R. Lenarčič[a], A. Rotter[a], M. Dickinson[d], N. Boonham[c], M. Dermastia[a] and M. Ravnikar[a]

*Department of Biotechnology and Systems Biology, National Institute of Biology; [b]Department of Biology, Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1000 Ljubljana, Slovenia; [c]The Food and Environment Research Agency, Sand Hutton, York YO41 1LZ; and [d]School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK

Vitisens

-BNp:

Euphresco GRAFDEPI 2

On-site application of the FDp and BNp testing

Sampling

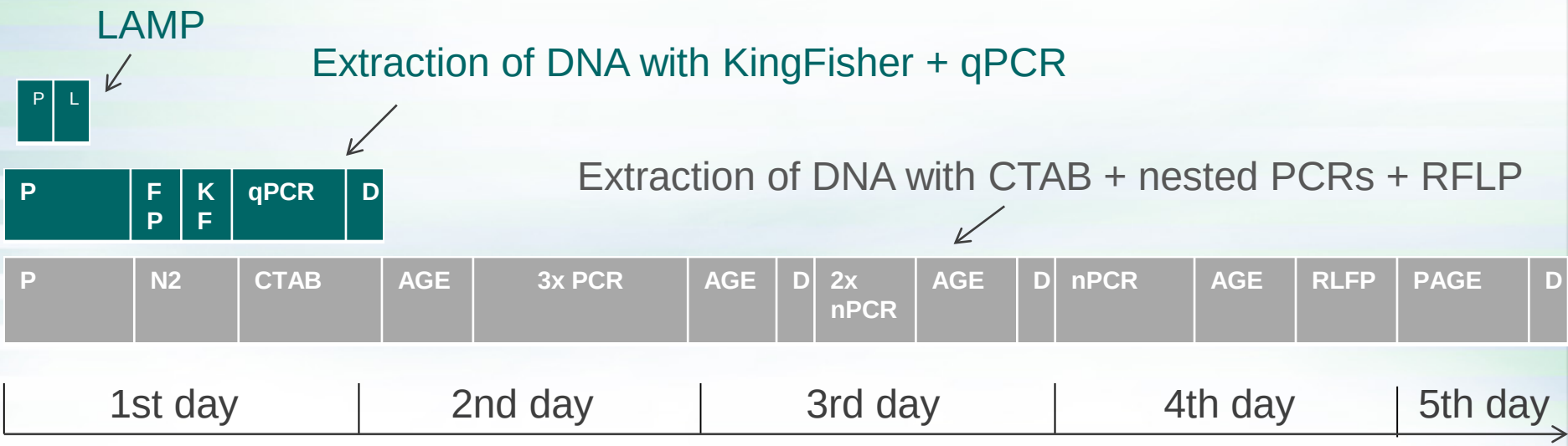
Homogenisation

No need for DNA extraction and purification (not sensitive to inhibitors)

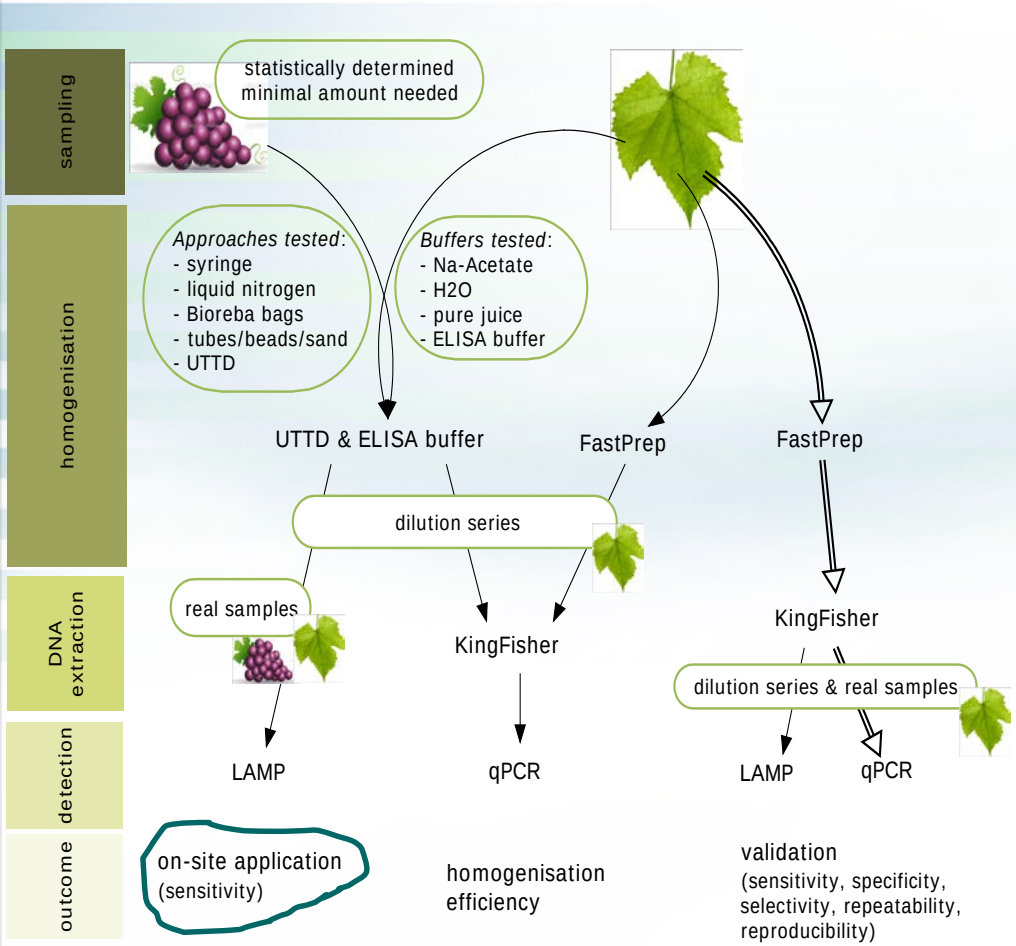
Mix the sample with LAMP reagents

Amplification & Detection

Comparison of time needed for FDp and BNp detection with different methods



LAMP – validation (FD example)

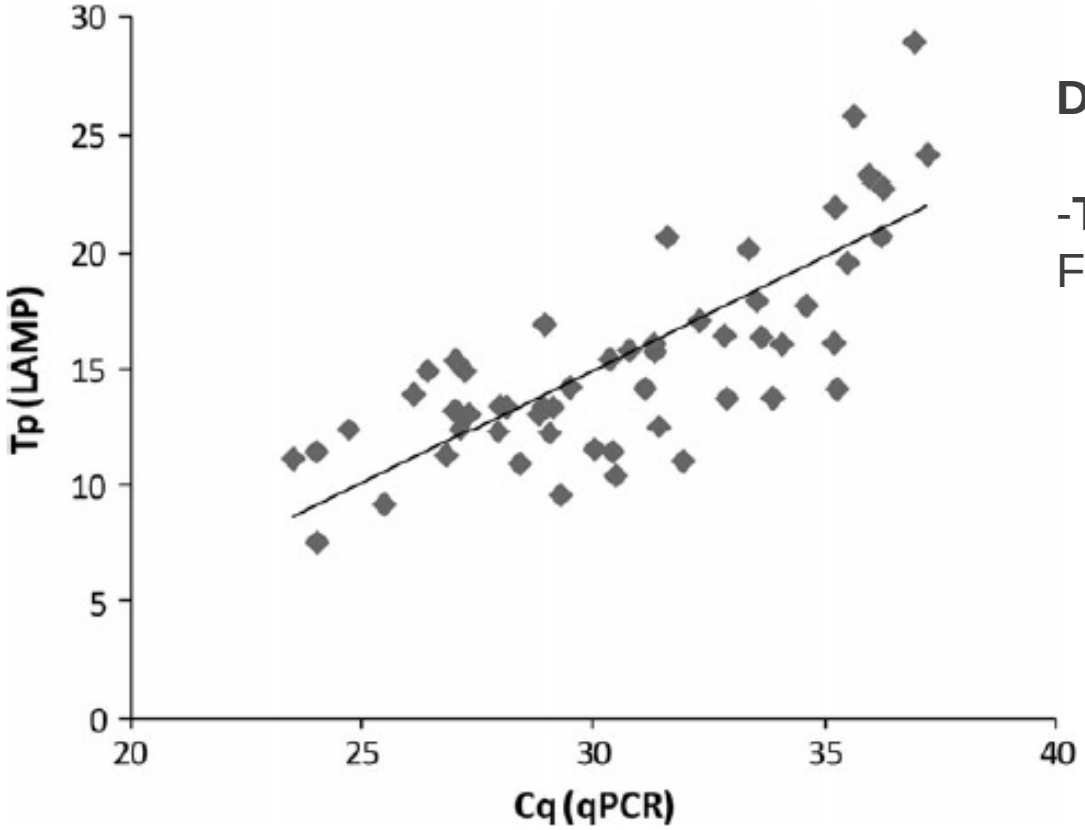


Dilution	FDp DNA copy no.	Extraction of DNA with KingFisher + **qPCR** (Cq)	**LAMP** (Tp)
3x	243-729	+ (27.9)	+ (21.1)
9x	81-243	+ (29.5)	+ (27.3)
27x	27-81	+ (31.4)	+ (25.0)
81x	9-27	+ (32.9)	+ (19.1)
243x	3-9	+ (34.4)	-
729x	1-3	+ (34.8)	-
2187x	0	-	-

LAMP is 9x less sensitive than qPCR (analytical sensitivity)

(Kogovšek et al., 2015)

LAMP – validation (FD example)



Diagnostic sensitivity:

-**Testing of extracted DNA** (52 FDp infected samples)**: 100%**

-**Testing of crude homogenates** (27 FDp infected samples)**: 100%**

(Kogovšek et al., 2015)

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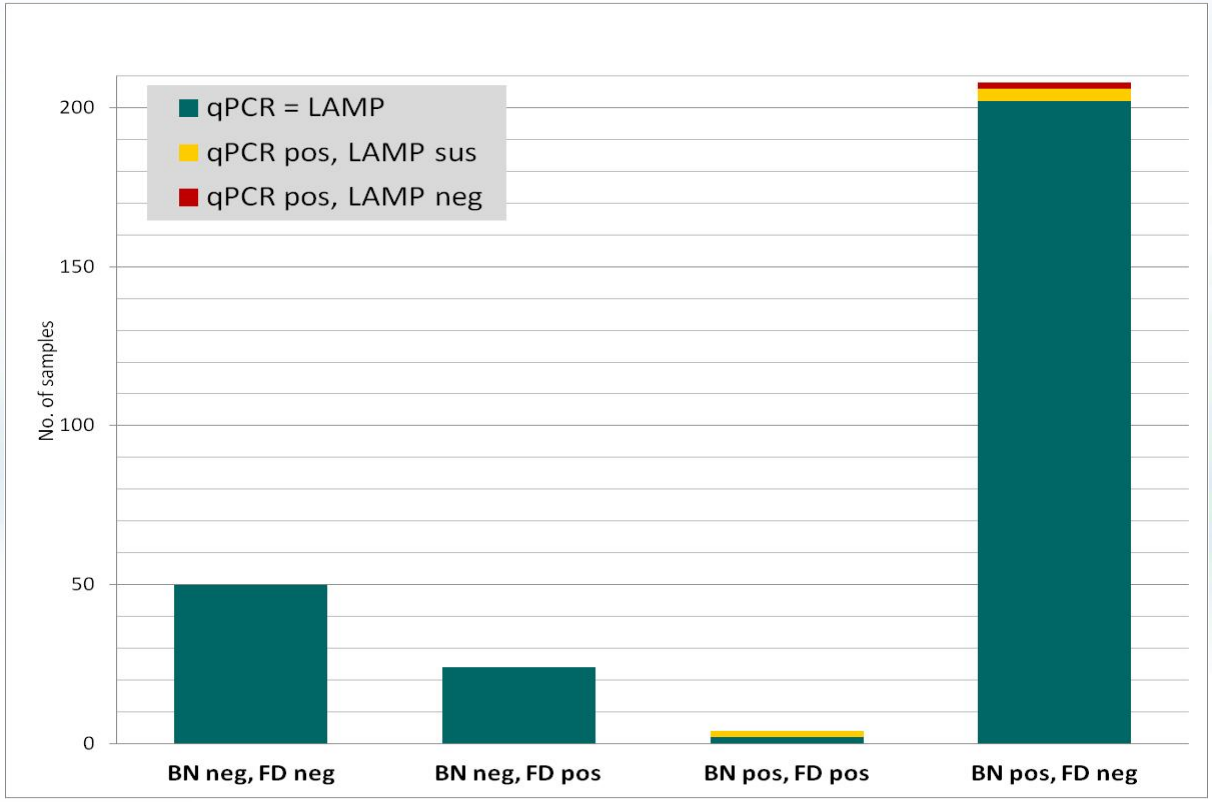
LAMP – validation

(FD & BN – testing of crude homogenates)

2015:
286 official
grapevine samples



		Cq < 32	Cq > 32			
BN pos	212	193	19	12	5	2

		Cq < 31	Cq > 31		
FD pos	28	24	4	3	1

LAMP – test performance study

(FD & BN – testing of extracted DNA)

- Euphresco project GRAFDEPI 2
- Participants: 10 laboratories (from the research and plant protection area from Europe and Australia)
- Additionally, LAMP FDp assay (Kogovšek et al., 2015) was compared with a Qualiplante/Hyris isothermal amplification assay for FD (ISOA FD Qualiplante) by 3 laboratories
- 18 DNA samples were subject of this TPS

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LAMP – test performance study

(FD & BN – testing of extracted DNA)

	Assay		
	LAMP BN	**LAMP FD** (Kogovšek et al., 2015)	**ISOA FD** Qualiplante
No. of labs taking in 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%**

The validation data for LAMP FD (Kogovšek et al., 2015) available at EPPO website: **http://dc.eppo.int/validationlist.php**

Quantification

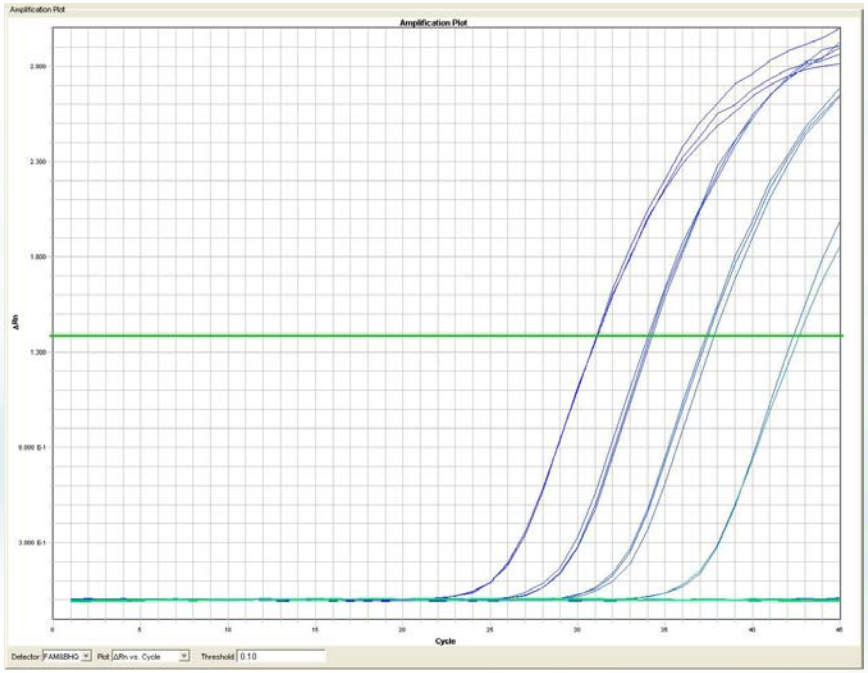
- monitor phytoplasma kinetics (progress of an infection, and variations of the phytoplasma titer through the season and in different plant tissues)

- screening plants for resistance against phytoplasma
- estimate the number of copies carried by the vectors

Quantification

- Real time PCR:

  quantification against reference material (standard curve):



No certified phytoplasma reference material (dilutions of a sample containing the target DNA sequence or a sample with known copy numbers of plasmids)

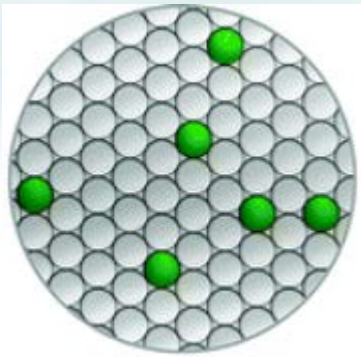
Quantification

- **Digital PCR**

  - absolute quantification of target sequences without relying on the use of standard curves

- droplet digital PCR (ddPCR):

droplet generation



amplification (PCR)

reading

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Analysis of FDp with ddPCR

- Transfer from qPCR to ddPCR

Plant Pathology (2007) **56**, 785–796 Doi: 10.1111/j.1365-3059.2007.01688.x

Real-time PCR detection systems for Flavescence dorée and Bois noir phytoplasmas in grapevine: comparison with conventional PCR detection and application in diagnostics

M. Hren[a*], J. Boben[a], A. Rotter[a], P. Kralj[b], K. Gruden[a] and M. Ravnikar[a]

- same primers and probes
- change in mastermix

Phytopathogenic Mollicutes
Vol. 4(1), June 2014, 9-15
doi : 10.5958/2249-4677.2014.00576.3
IndianJournals.com
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Research Article

Quantitative analysis of "flavescence doreé" phytoplasma with droplet digital PCR

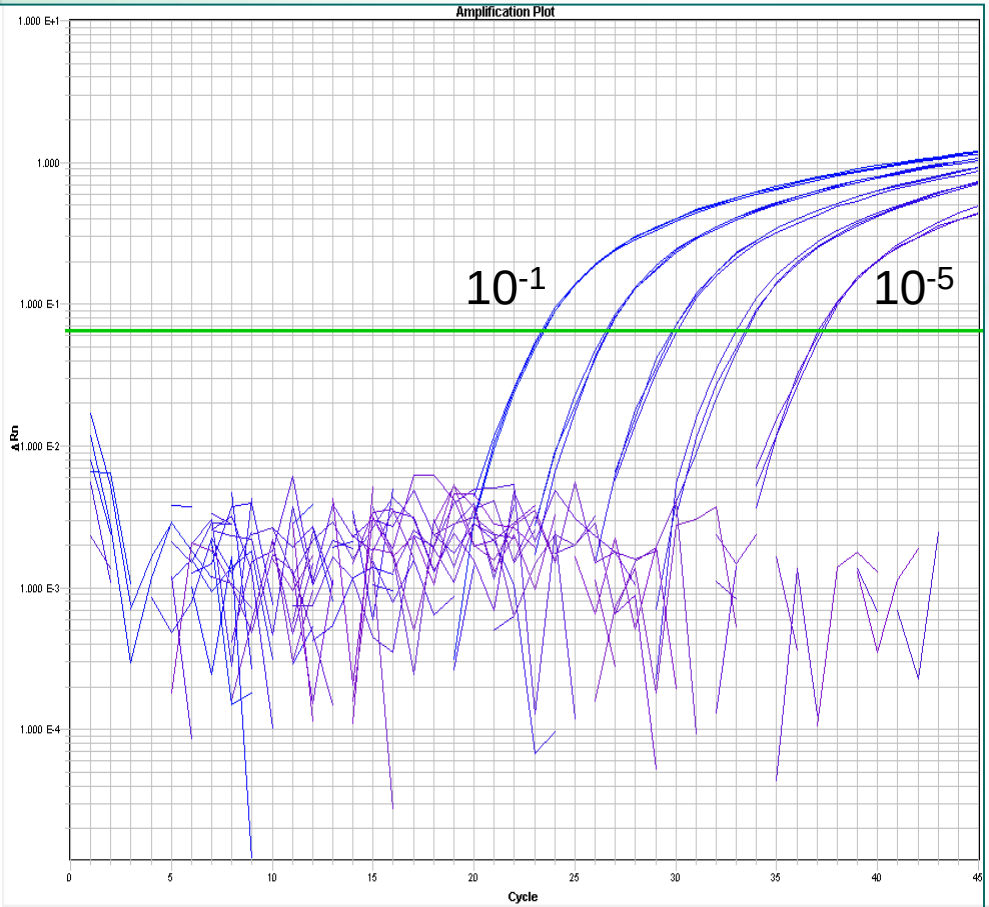
Nataša Mehle, Tanja Dreo and Maja Ravnikar

ARRS (Slovenian Research Agency) project (contract no. L4-5525)

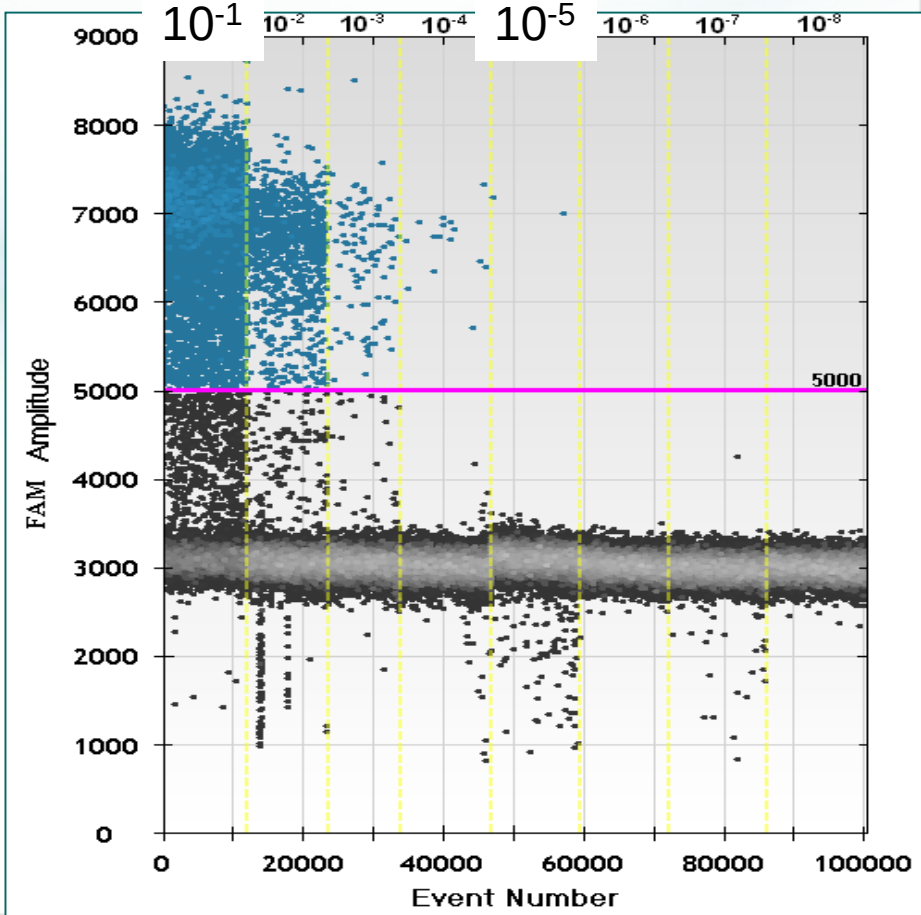
Analysis of FDp with ddPCR

Sensitivity: similar as with qPCR

qPCR

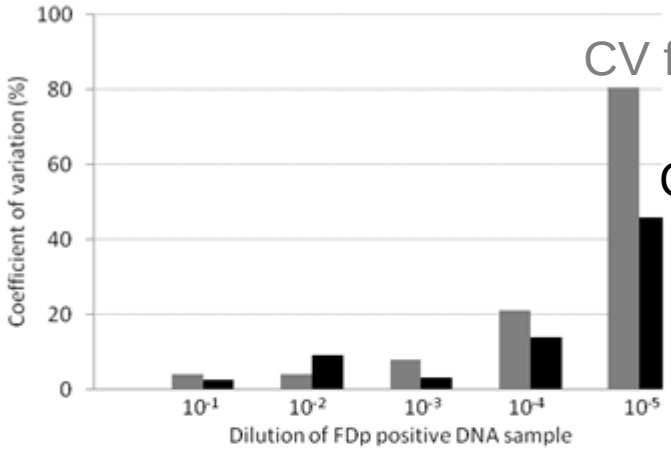


ddPCR



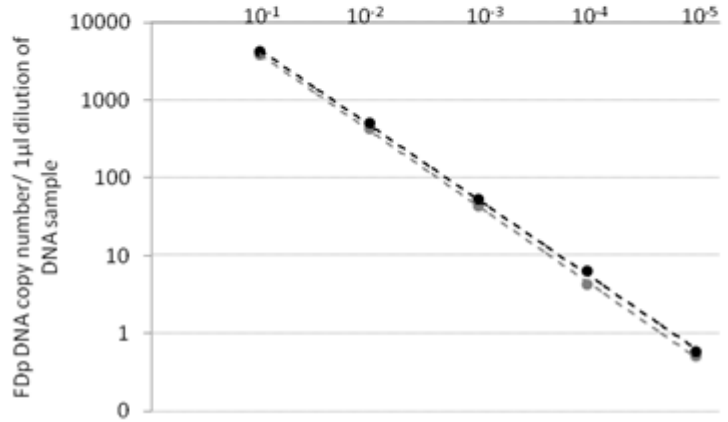
Analysis of FDp with ddPCR

Repeatability of **ddPCR** and **qPCR**:



CV for **qPCR 80%**

CV for **ddPCR 46%**



higher precision and repeatability of ddPCR for quantification of FDp at the low concentrations

Conclusions – phytoplasma detection

- **Diagnostic procedure:**

  simple&quick homogenisation step + DNA extraction based on the binding of DNA to magnetic beads + real-time PCR

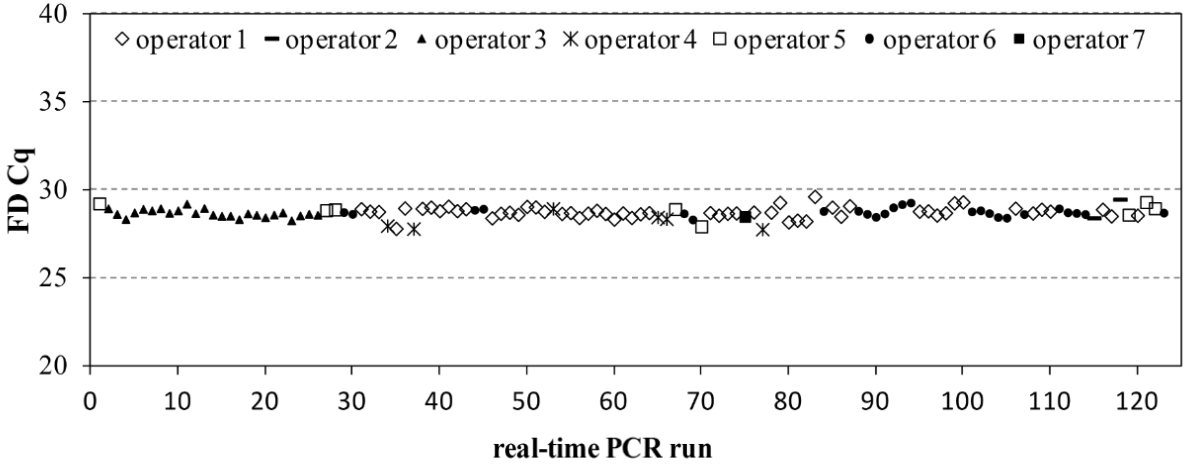
- **LAMP assay for FDp and BNp:**

- Application in laboratories (high through-put) or without expensive equipment on-site
- LAMP is less prone to inhibition therefore just homogenization of samples without NA extraction is sufficient

Conclusions – phytoplasma detection

- **ddPCR for FDp:**

- Absolutly quantify phytoplasma without the need of any calibrant (calibration curves for quantification of FDp are not needed)
- Quantification and quality control of DNA based on in-house reference materials typically used in diagnostics and metrological laboratories



Bulletin OEPP/EPPO Bulletin (2014) **44** (3), 502–509 ISSN 0250-8052. DOI: 10.1111/epp.12166

Descriptive assessment of uncertainties of qualitative real-time PCR for detection of plant pathogens and quality performance monitoring

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