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Phytoplasmas of grapevine: molecular diversity of Slovenian strains and new diagnostic challenges

Nataša Mehle, Maja Ravnikar, Polona Kogovšek, Tjaša Jakomin, Anja Pugelj, Sanda Kavčič, Marina Dermastia

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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

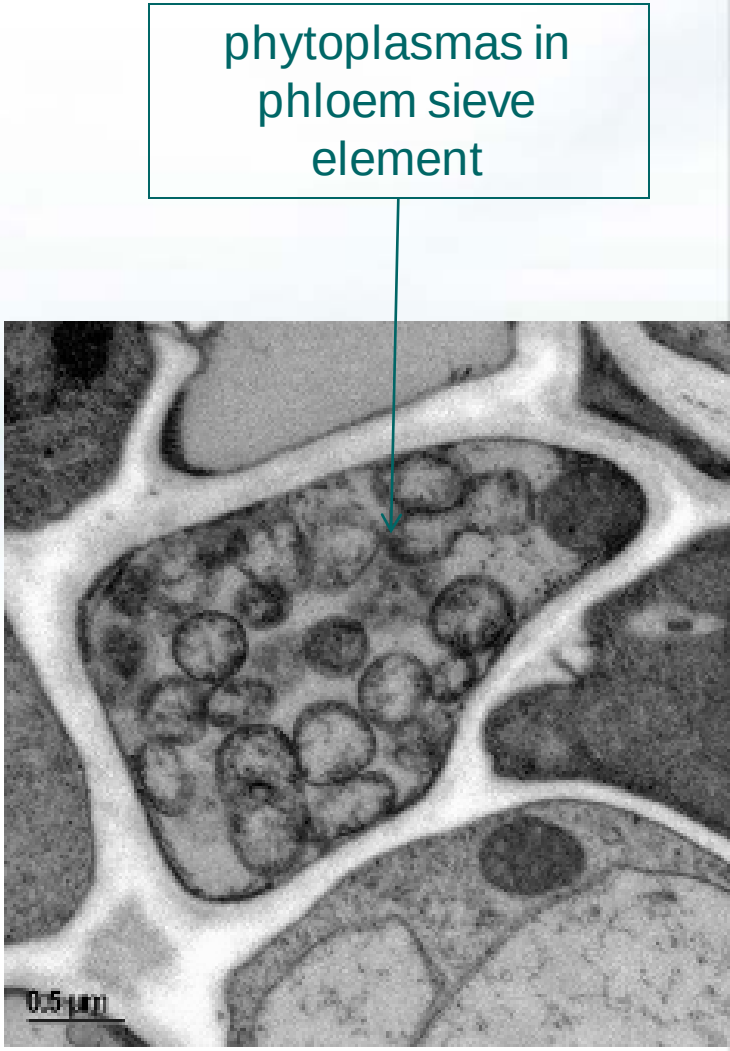


Photo: Magda Tušek Žnidarič

Grapevine yellows

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



Grapevine yellows

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

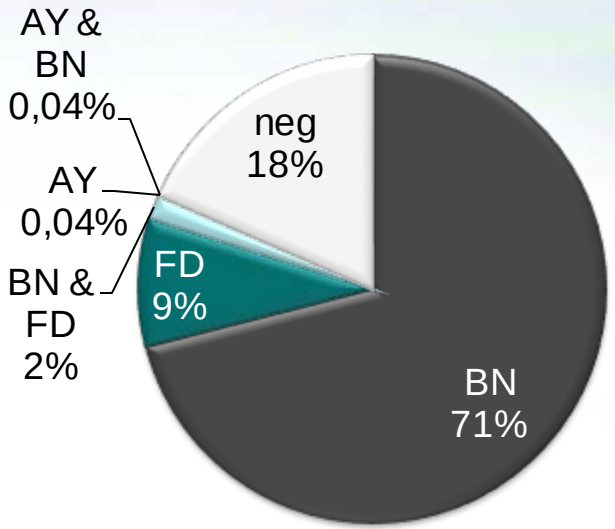


Slovenia:

-‘*Ca.* P. solani’ -> **BN**

- Flavescence dorée phytoplasma -> **FD**

-‘*Ca.* P. asteris’ -> **AY**



2005-2014 (1960 samples)

Molecular diversity of BNp

tuf (RFLP with *Hpa*II)

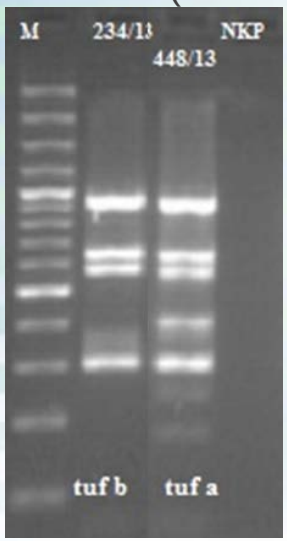


Photo: Wikipedia

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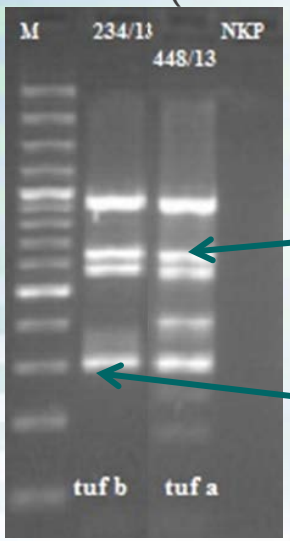


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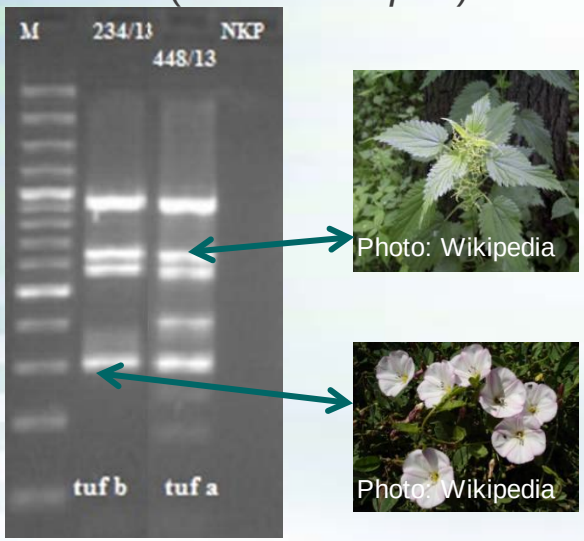
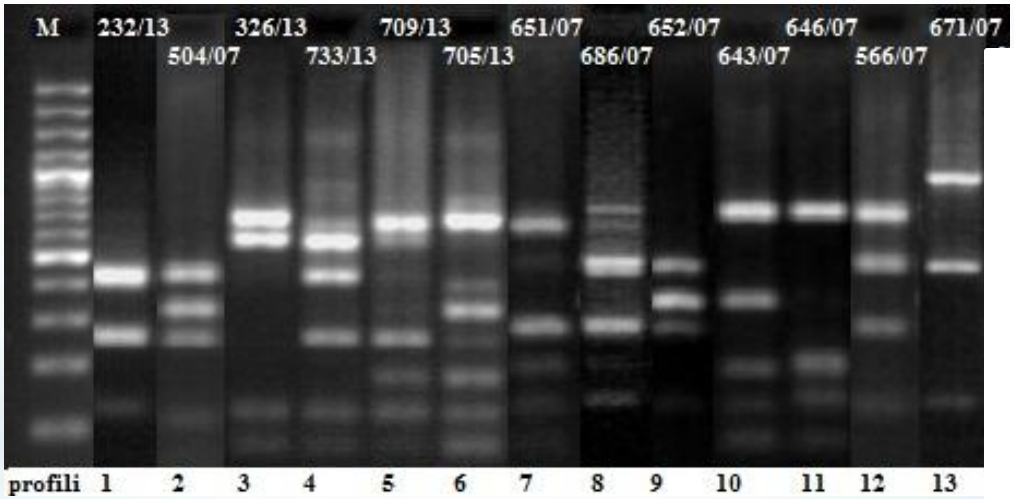


vmp 1 (RFLP with *Rsa*I)



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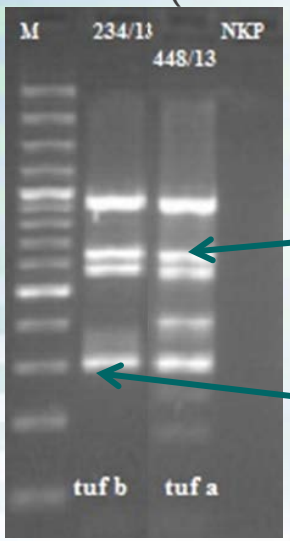
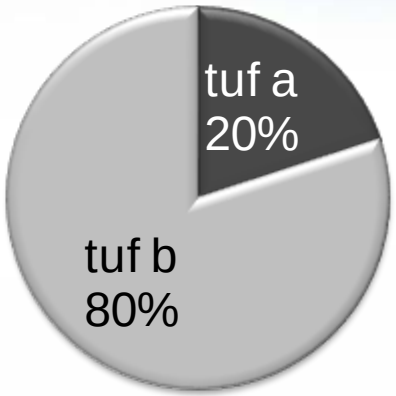
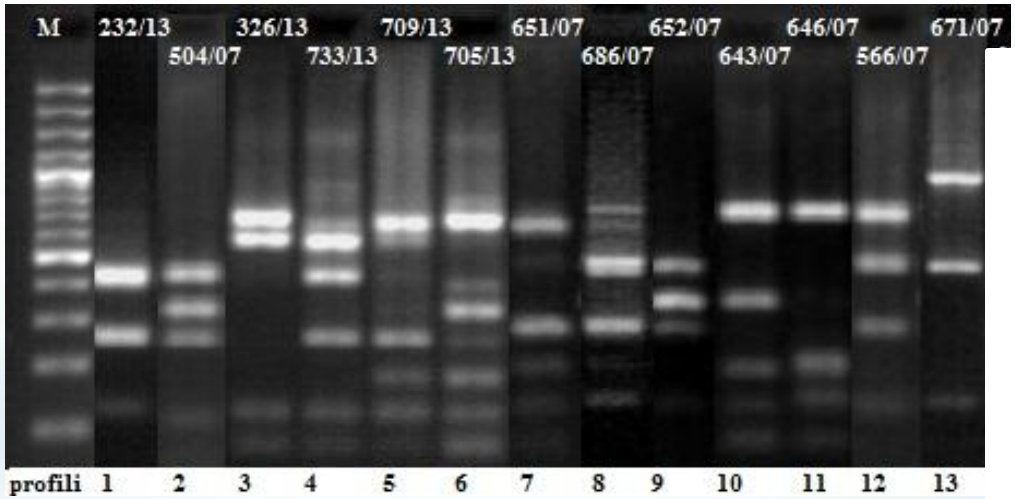
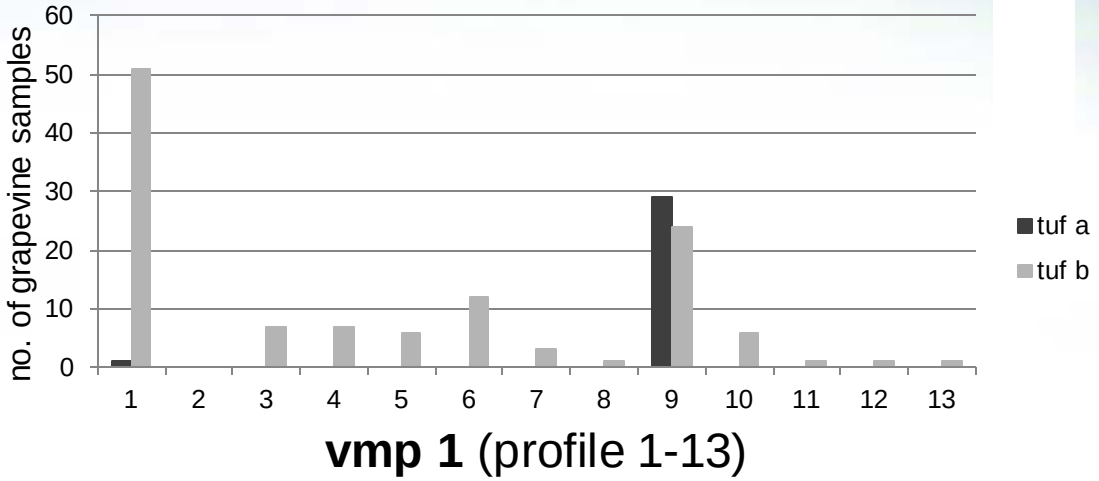




2007 and 2013 (150 samples)

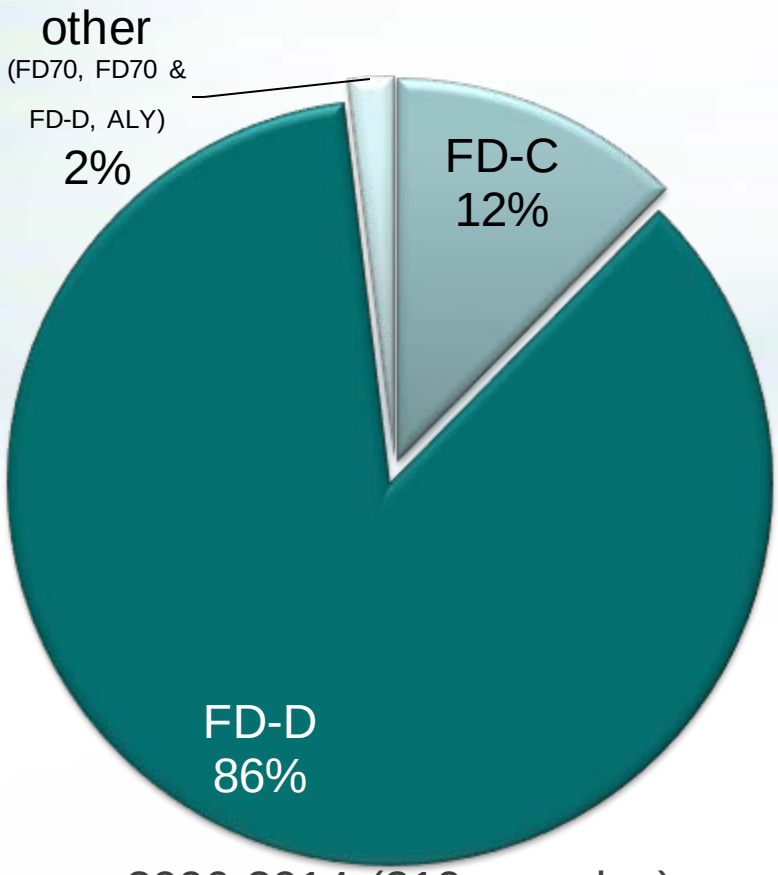
vmp 1 (RFLP with *Rsa*I)





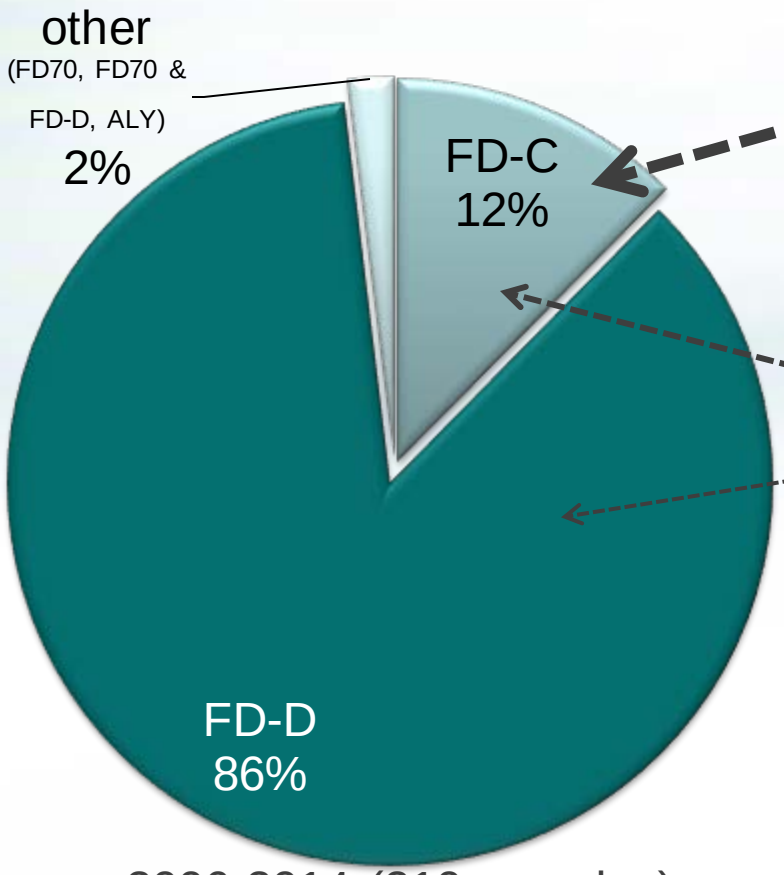
vmp 1 (profile 1-13)

Molecular diversity of FDp



2006-2014 (210 samples)

Molecular diversity of FDp



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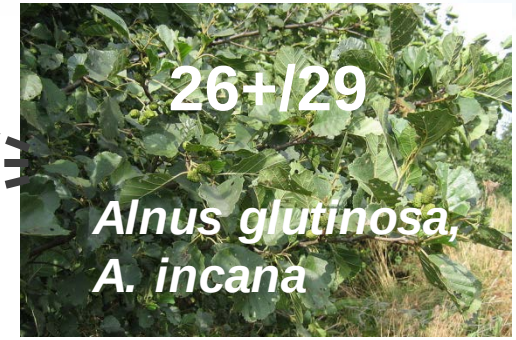
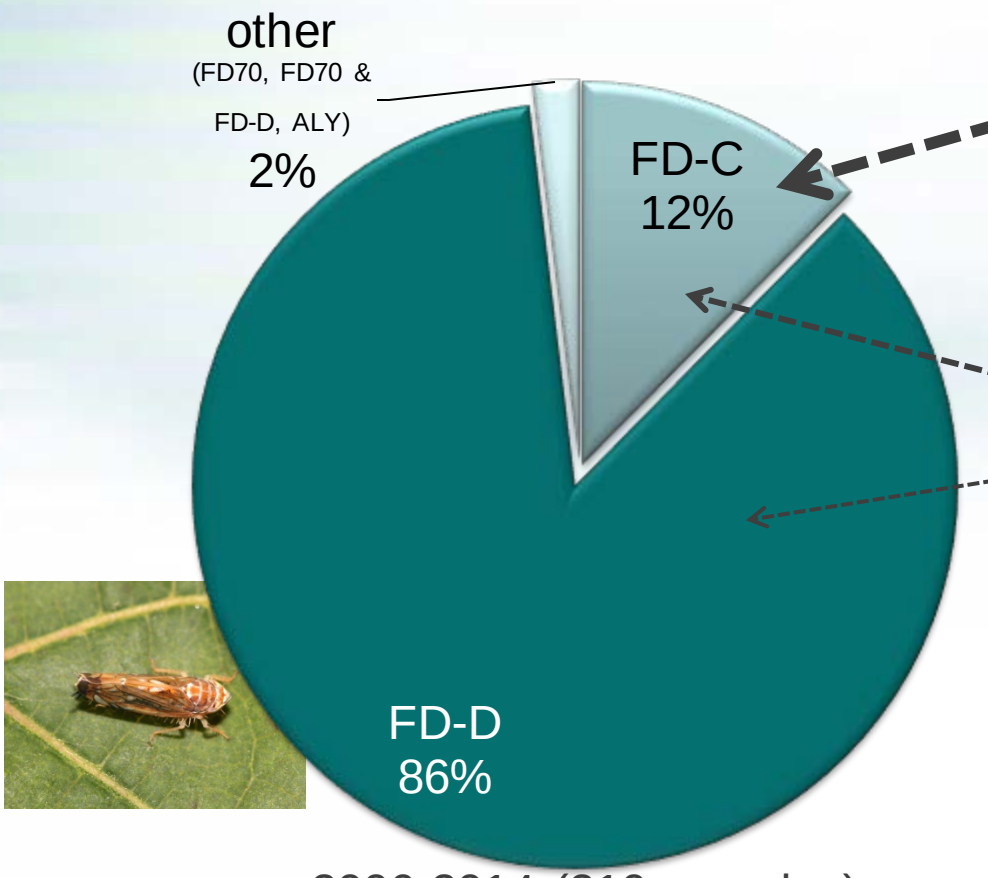


Photos: Wikipedia

Molecular diversity of FDp



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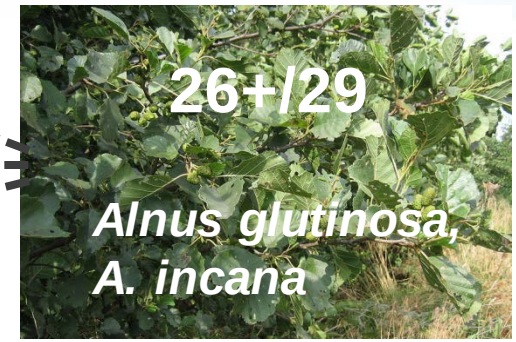

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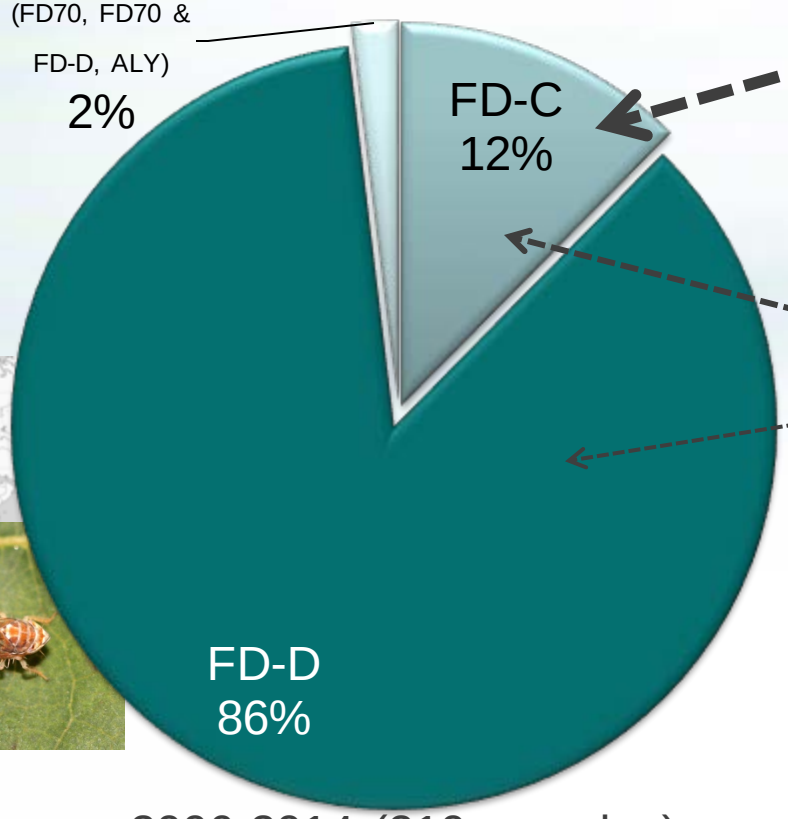

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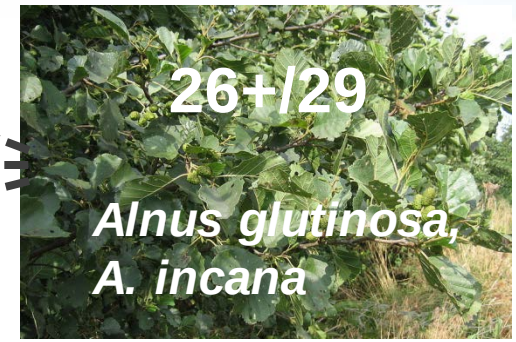


Photos: Wikipedia

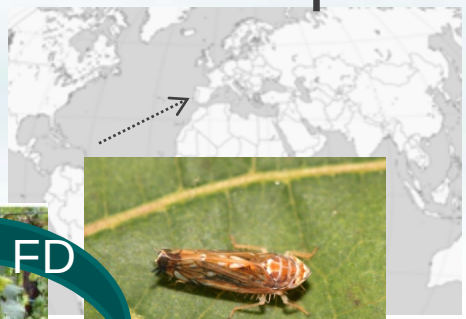
Conclusions – molecular diversity of FDp and BNp

- Several different genetic strains of FDp and BNp in Slovenian vineyards – different epidemiology and control measures

Photos: Wikipedia

BN

FD



FD

Photos: Wikipedia

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

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Example: FD – Izola

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Example: FD – Izola

Reliable, sensitive and fast diagnostic procedure is needed!

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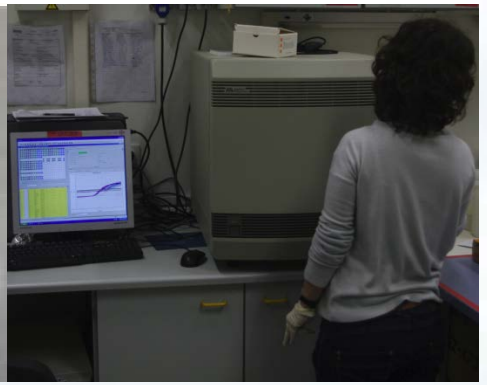
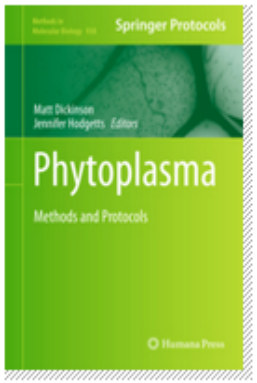
Diagnostic procedure





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

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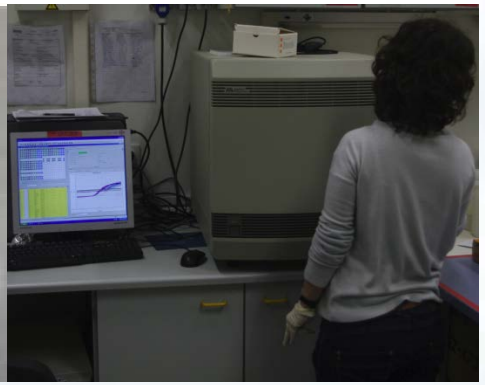
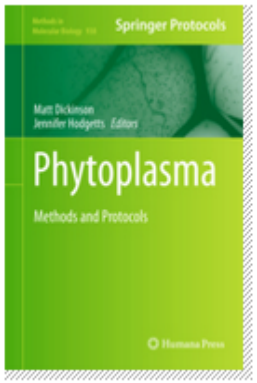
Diagnostic procedure





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SLOVENSKA AKREDITACIJA
SIST EN ISO/IEC 17025
LP-028

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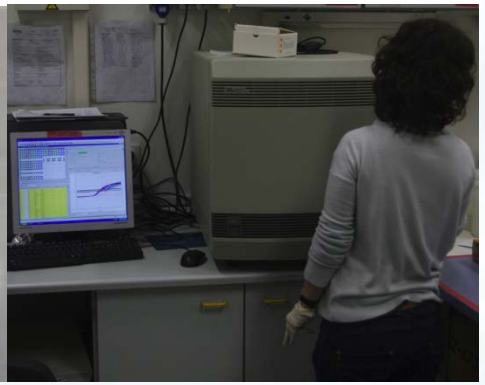
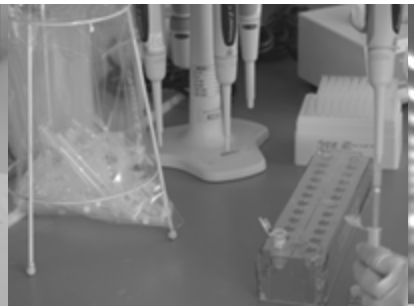
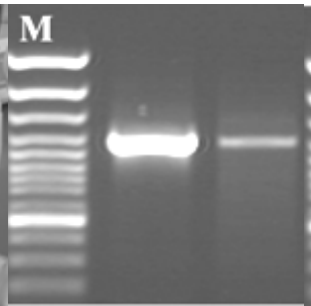


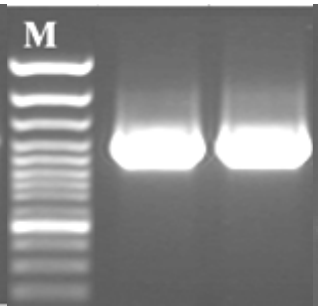
P	FP	KF	qPCR	D

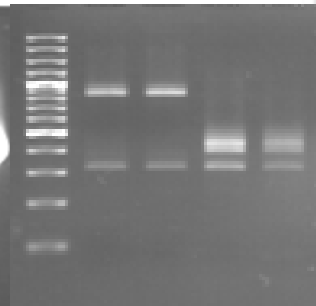
P	N2	CTAB	AGE	3x PCR	AGE	D	2x nPCR	AGE	D	nPCR	AGE	RLFP	PAGE	D

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







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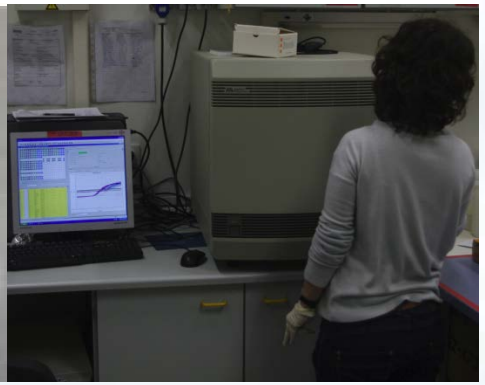
Diagnostic procedure



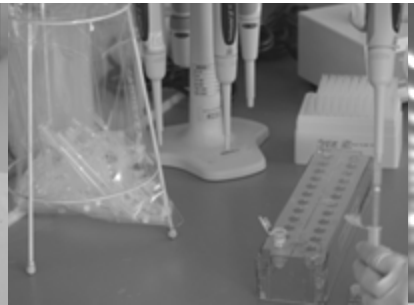
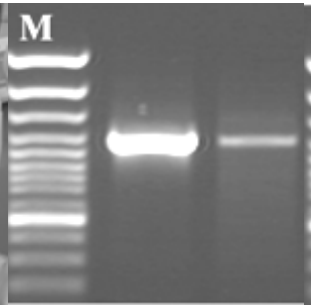
P	FP	KF	qPCR	D

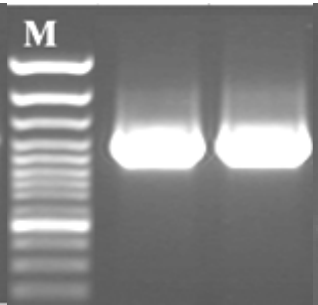
+ less contamination, higher sensitivity

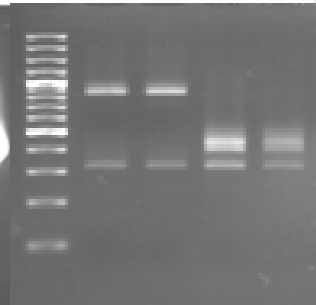
P	N2	CTAB	AGE	3x PCR	AGE	D	2x nPCR	AGE	D	nPCR	AGE	RLFP	PAGE	D

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







Quantification

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

Plant Pathology (2013) Doi: 10.1111/j.1365-3059.2012.02693.x

Spatiotemporal distribution of flavescence dorée phytoplasma in grapevine

N. Prezelj, P. Nikolić, K. Gruden, M. Ravnikar and M. Dermastia*

Quantification

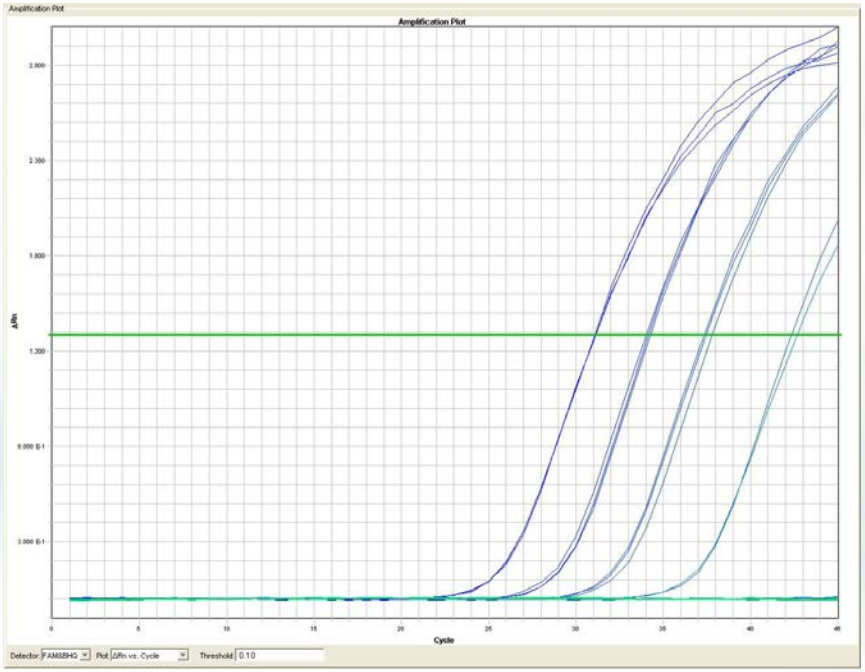
- monitor phytoplasma kinetics (progress of an infection, and variations of the phytoplasma titer through the season and in different plant tissues)

- 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):



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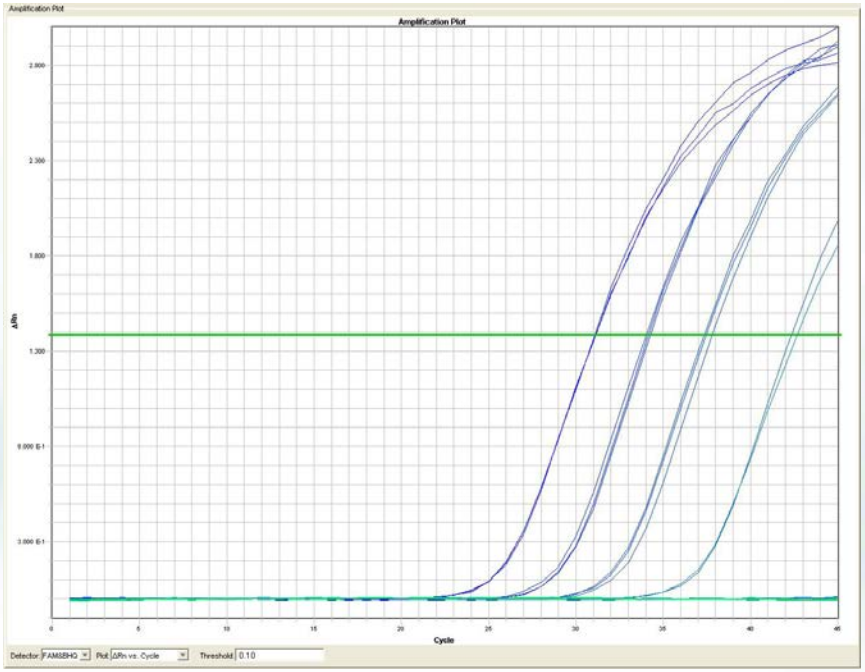


No certified phytoplasma reference material (dilutions of a sample containing the target DNA sequence or a sample with known copy numbers of plasmids)

Quantification

- **Digital PCR**

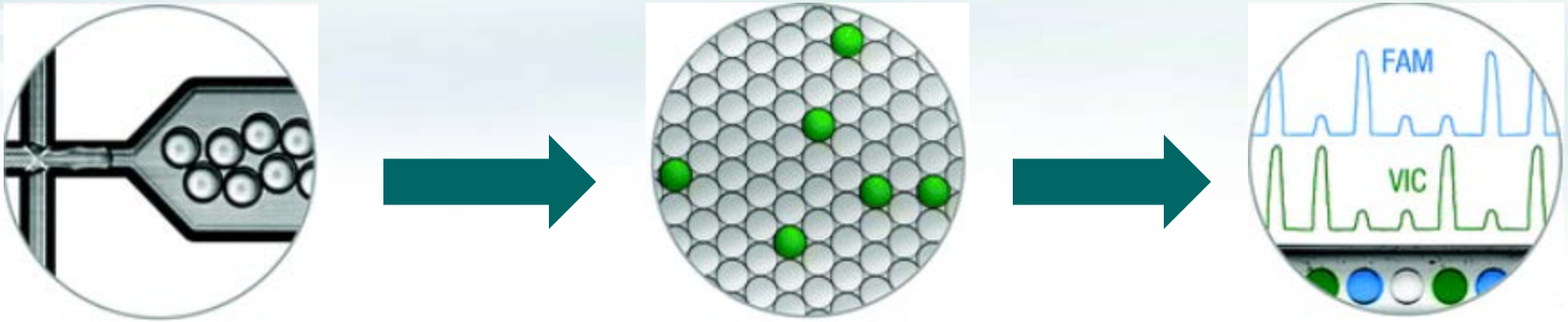
  - absolute quantification of target sequences without relying on the use of standard curves

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

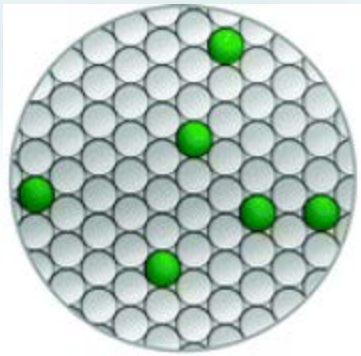
Quantification

- **Digital PCR**

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

- droplet digital PCR (ddPCR):

droplet generation



amplification (PCR)

reading

poster: Dreo et al.

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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)

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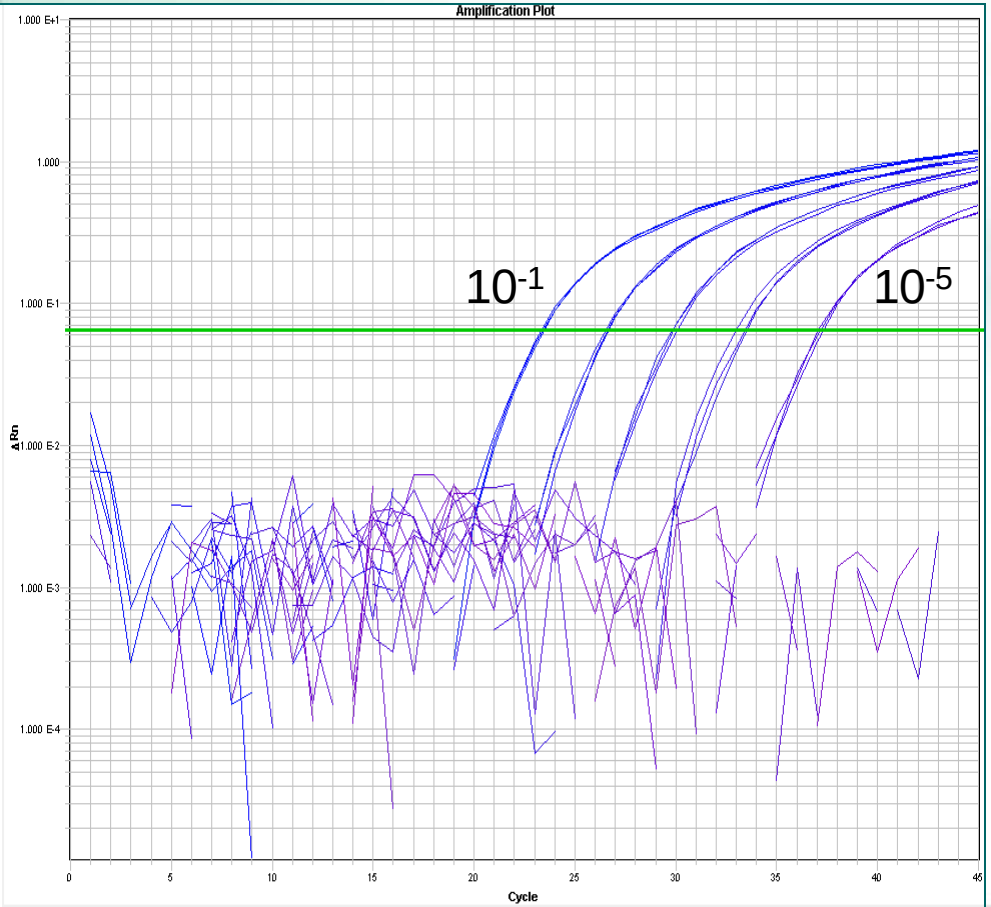
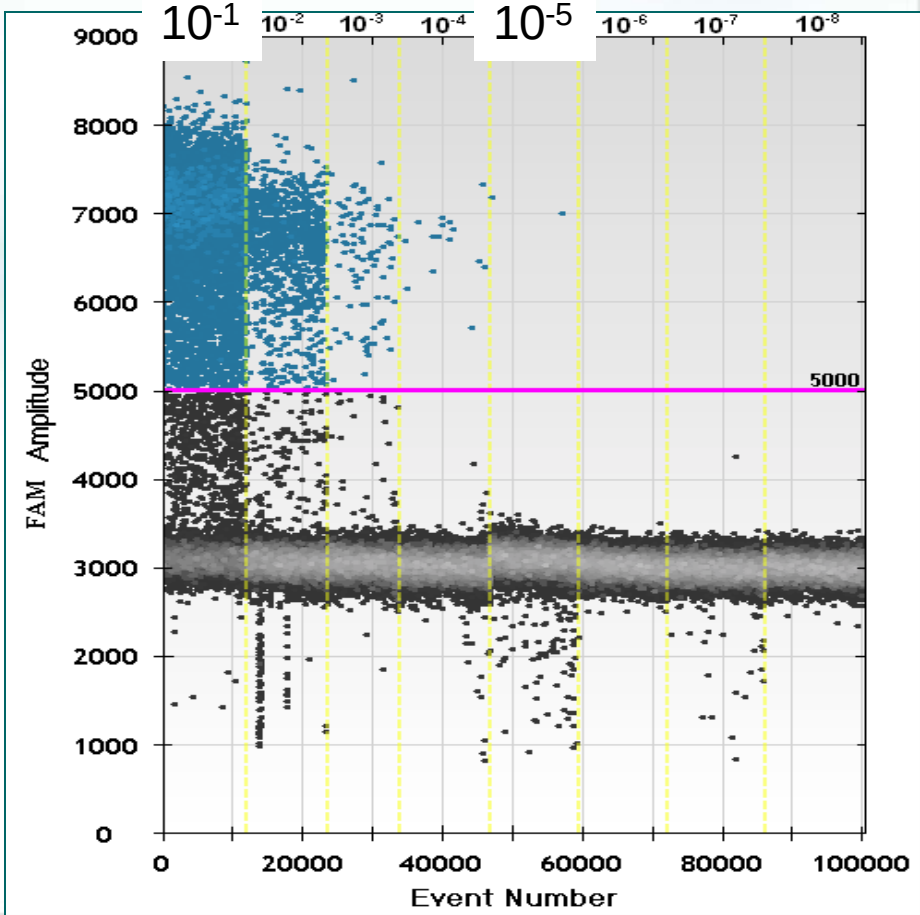
Analysis of FDp with ddPCR

Sensitivity: similar as with qPCR

qPCR

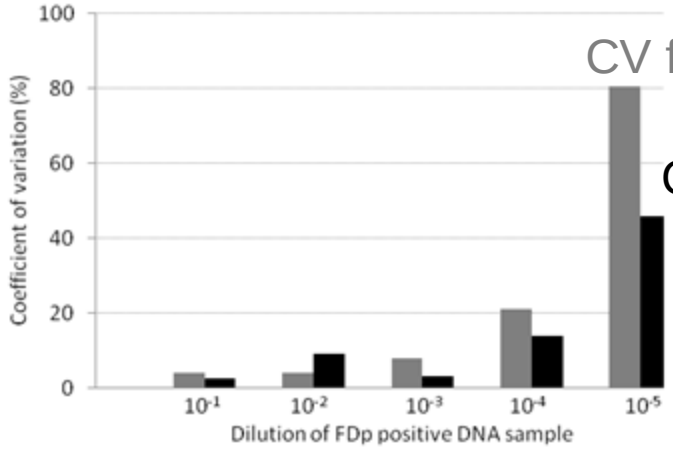


ddPCR



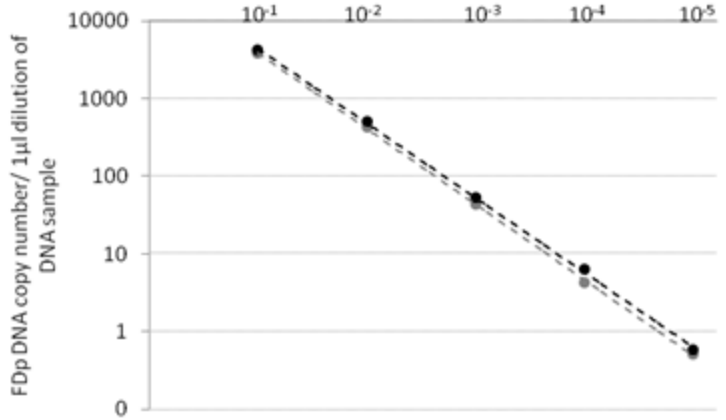
Analysis of FDp with ddPCR

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

On-site detection

LAMP : **L**oop mediated isothermal **AMP**lification

On-site detection

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Fast multiplication of DNA targets in one tube
at **isothermal conditions** (60-65ºC)

On-site detection

LAMP : Loop mediated isothermal **AMP**lification

Fast multiplication of DNA targets in one tube
at **isothermal conditions** (60-65ºC)

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

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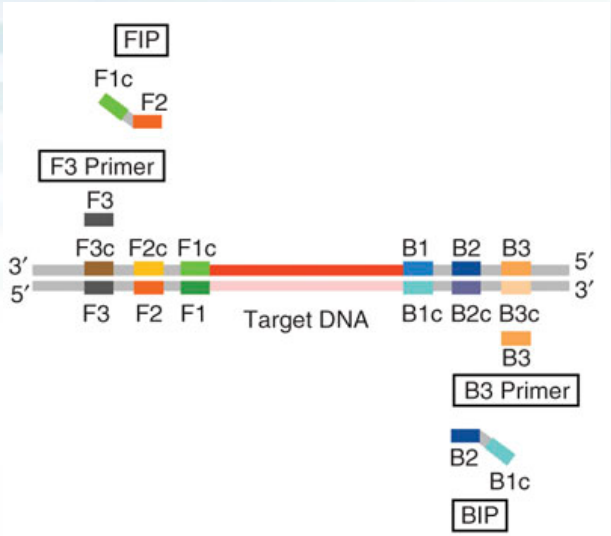
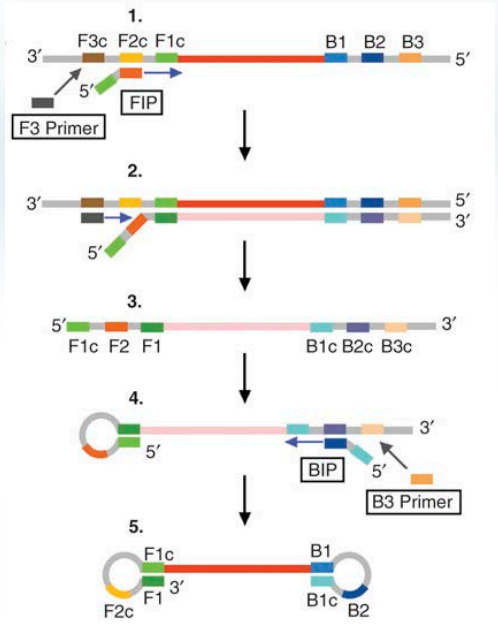
On-site detection

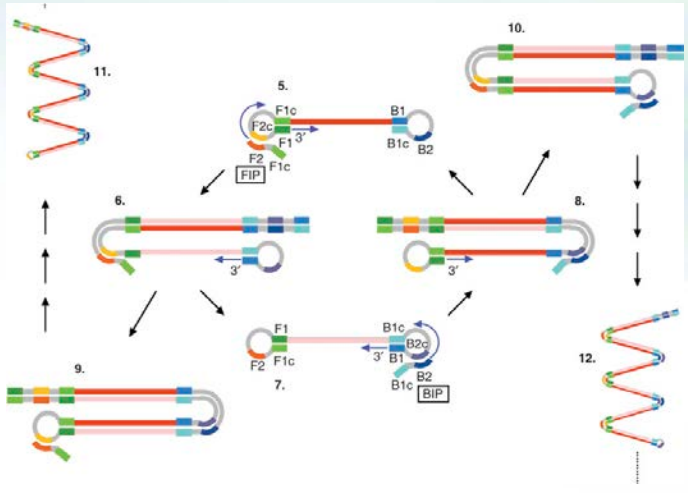
LAMP : Loop mediated isothermal AMPlification

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







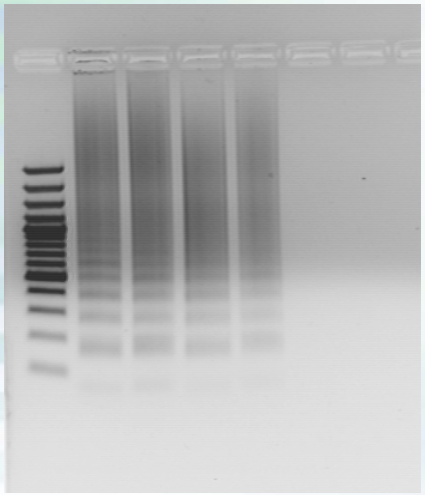
Starting structure producing step

Cycling amplification step

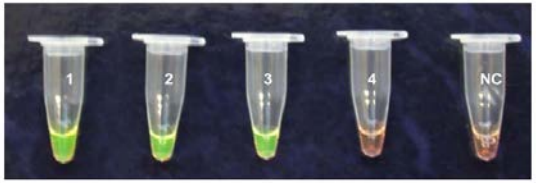
Principle of loop-mediated isothermal amplification (LAMP) method. (Tomita *et al*., 2008, Notomi *et al*., 2000).

Detection of LAMP products

- LAMP product on gel



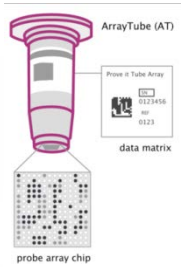
- Fluorescence



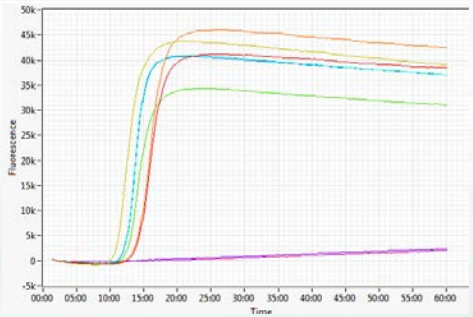
- Turbidity

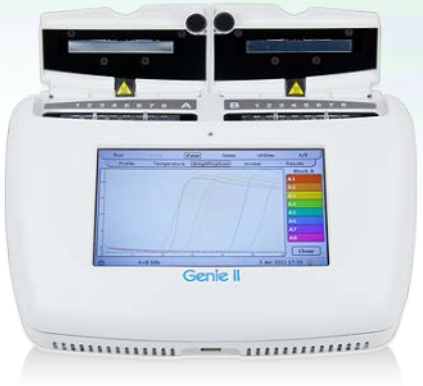
- LFD

- Array tubes



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

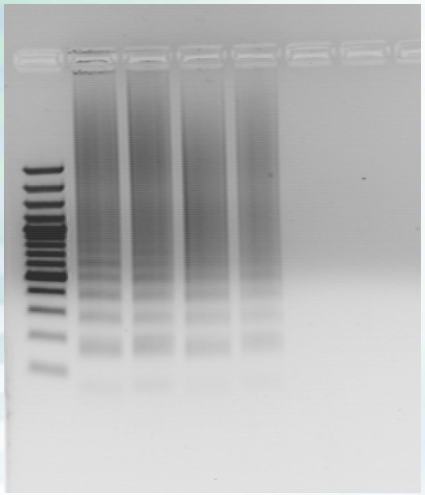




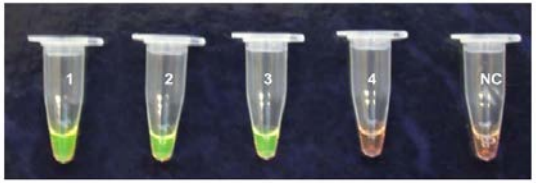
Simultaneously heater and fluorimeter (e.g., GenieII/III, SmartCycler)

Detection of LAMP products

- LAMP product on gel



- Fluorescence



- Turbidity

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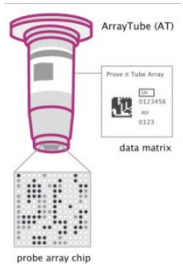

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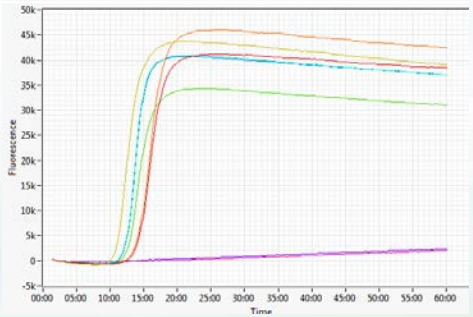
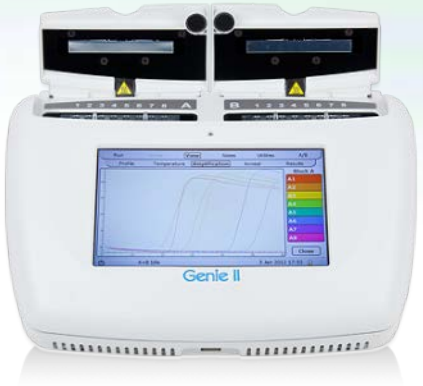
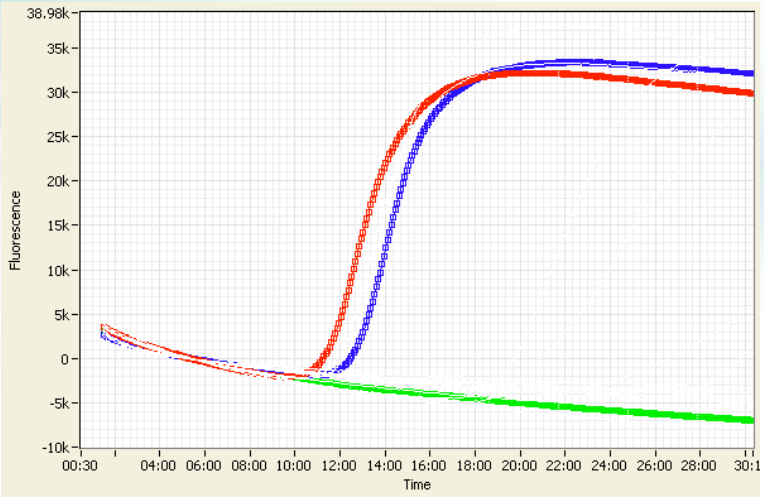




Simultaneously heater and fluorimeter (e.g., GenieII/III, SmartCycler)

Real time detection of LAMP products

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



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

Real time detection of LAMP products

1) **READING RESULTS**
pos: rise of fluorescence
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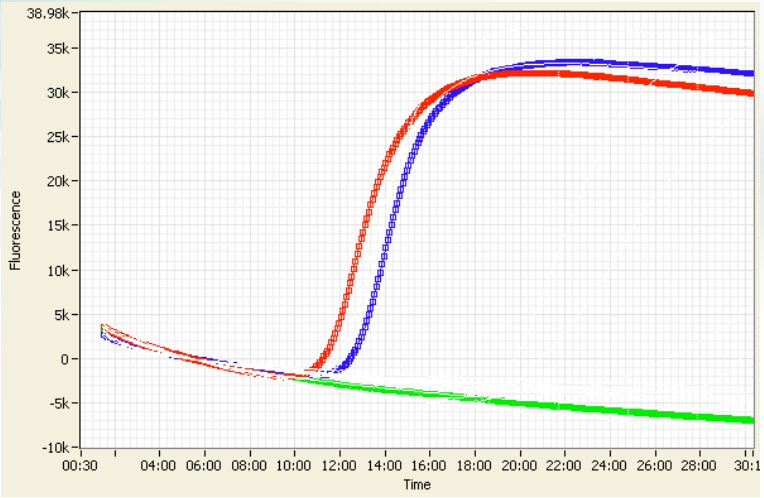
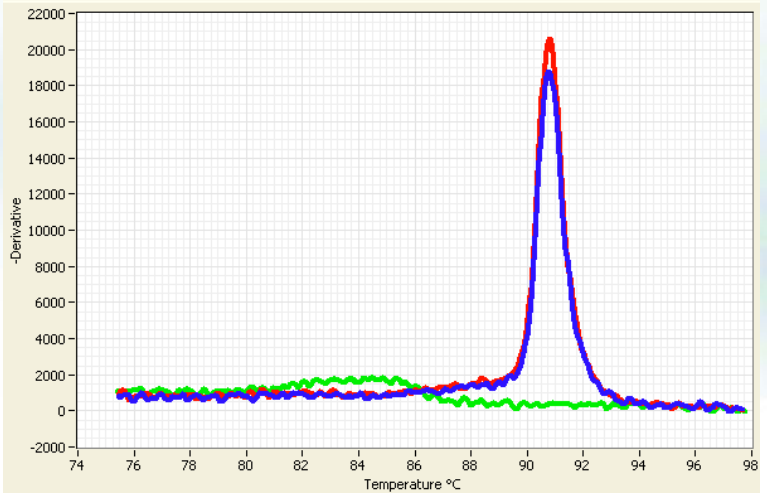


2) **CONFIRMATION OF RESULTS**
Melting temperature of the final product is pathogen specific



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- negative control (no amplification-> no fluorescence)

LAMP detection of phytoplasmas FD and BN

- FDp:

Plant Pathology (2014) Doi: 10.1111/ppa.12266

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]

[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

-BNp:

Euphresco GRAFDEPI II

On-site application of the FDp and BNp testing

Sampling

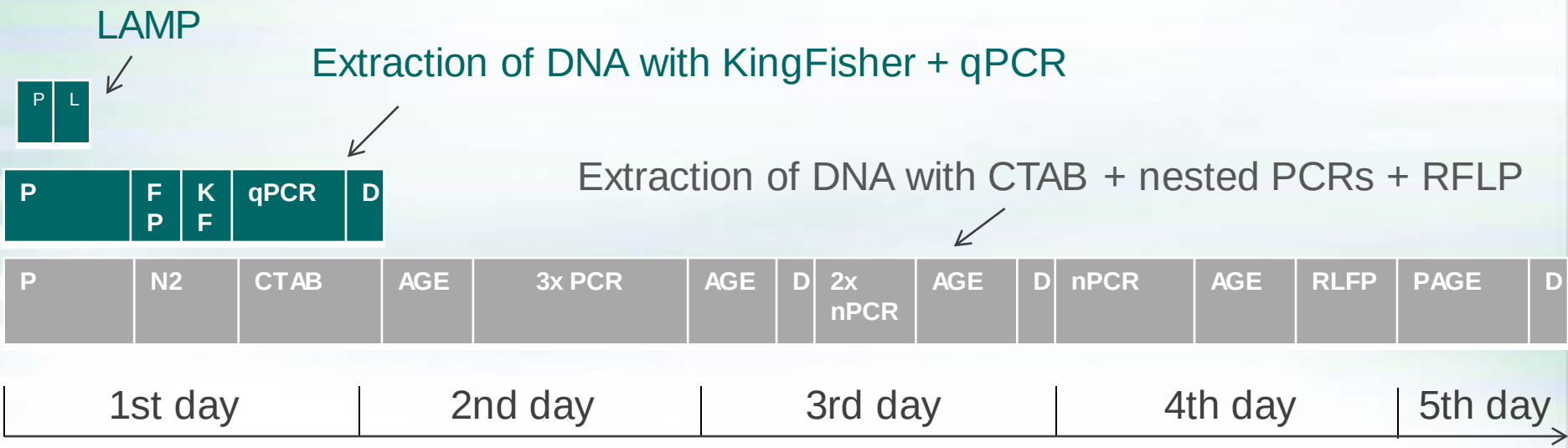
Homogenisation

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

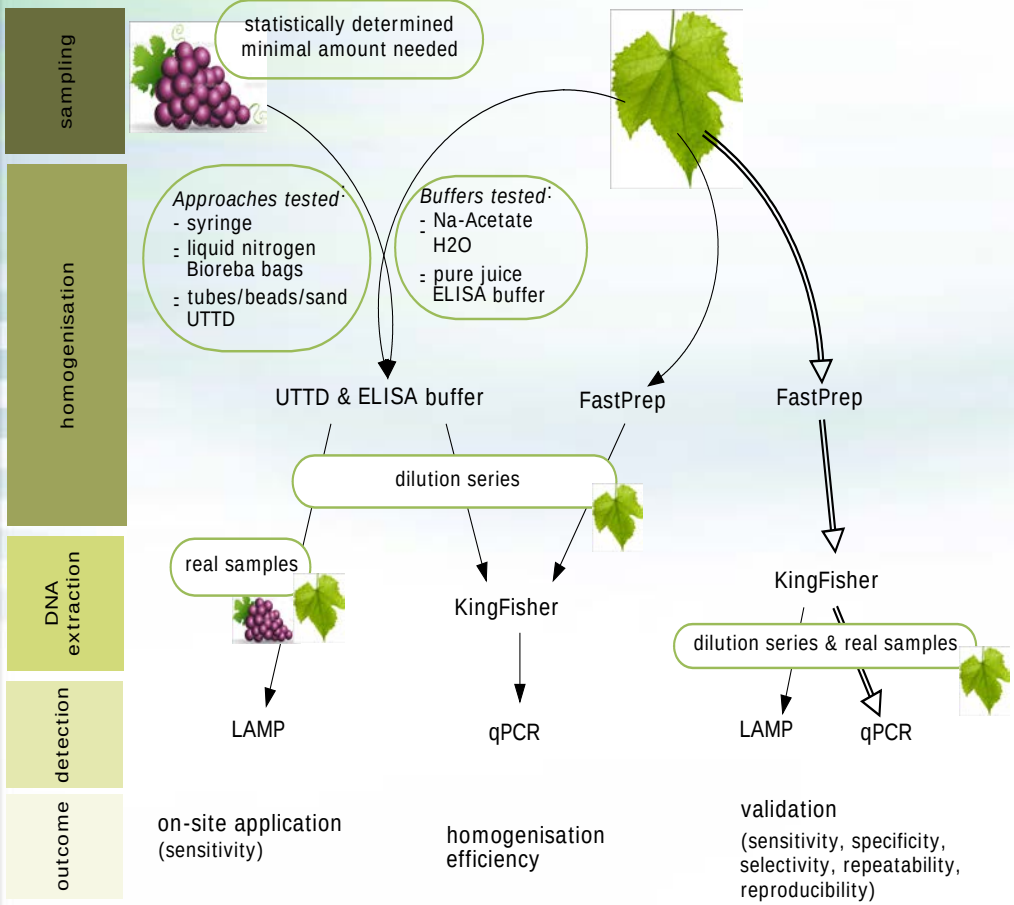
Mix the sample with LAMP reagents

Amplification & Detection

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



LAMP - validation



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

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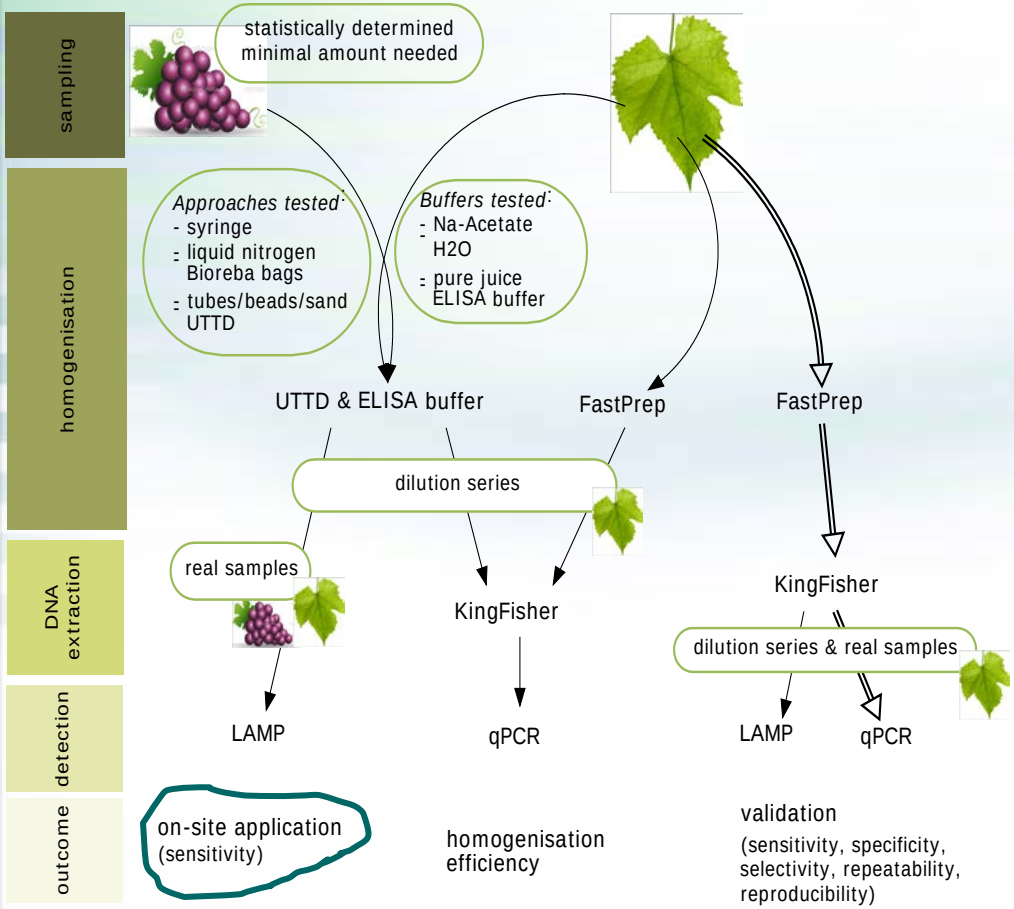
LAMP - validation



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	-	-

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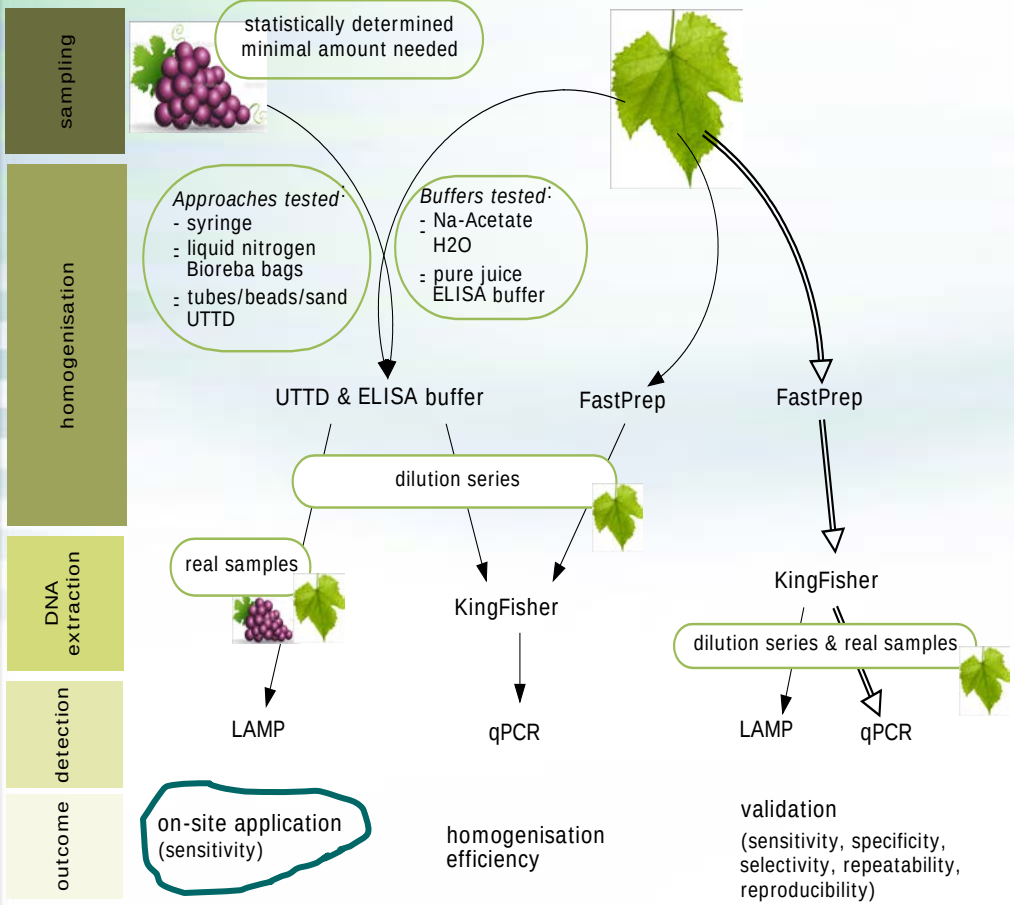
LAMP - validation



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243x	3-9	+ (34.4)	-
729x	1-3	+ (34.8)	-
2187x	0	-	-

LAMP is 9x less sensitive than qPCR

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

Conclusions – phytoplasma detection

- **Diagnostic procedure:**

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

- **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
- Potential to be used to characterise phytoplasma reference material

- **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 heating or just homogenization without NA extraction is sufficient

Omic studies

Phytoplasma – grapevine interaction (gene expression in infected grapevine):

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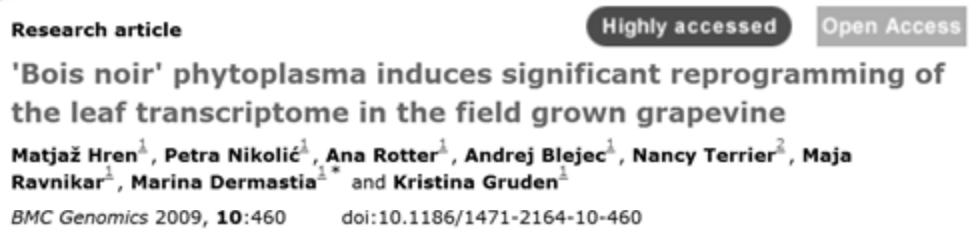
Omic studies

Phytoplasma – grapevine interaction (gene expression in infected grapevine):

- host transcriptome and metabolome levels with an emphasis on the host carbohydrate metabolism which is presumably affected by the phytoplasma infection
- bioinformatics approaches for searching of new markers for early detection of grapevine yellows to be applied in early diagnostic of disease before the formation of symptoms

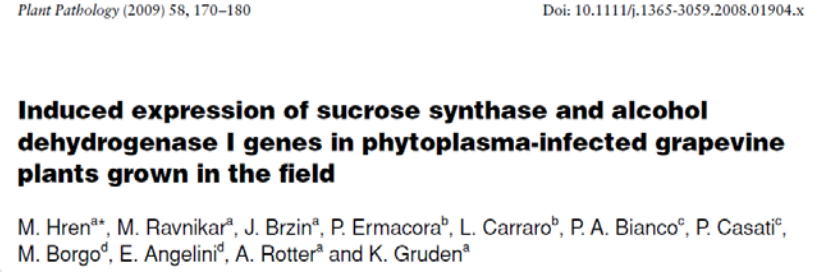


Research article Highly accessed Open Access

'Bois noir' phytoplasma induces significant reprogramming of the leaf transcriptome in the field grown grapevine

Matjaž Hren[1], Petra Nikolić[1], Ana Rotter[1], Andrej Blejec[1], Nancy Terrier[2], Maja Ravnikar[1], Marina Dermastia[1]* and Kristina Gruden[1]

BMC Genomics 2009, **10**:460 doi:10.1186/1471-2164-10-460



Plant Pathology (2009) 58, 170–180 Doi: 10.1111/j.1365-3059.2008.01904.x

Induced expression of sucrose synthase and alcohol dehydrogenase I genes in phytoplasma-infected grapevine plants grown in the field

M. Hren[a*], M. Ravnikar[a], J. Brzin[a], P. Ermacora[b], L. Carraro[b], P. A. Bianco[c], P. Casati[c], M. Borgo[d], E. Angelini[d], A. Rotter[a] and K. Gruden[a]

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- FP7 project Vitisens
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- Euphresco project GRAFDEPI (financed by the Ministry of Agriculture, Forestry and Food) and GRAFDEPI II
- Slovenian phytosanitary administration