Proficiency test results, for the testing of potato infecting phytoplasmas

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Purposes - Aims

- Proficiency testing - using the procedures established in each laboratory

Organisation: SASA; UK Potato Quarantine unit.

Participants: 5 labs PHYLIB partners took part.

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Stability evaluation

- To ensure samples would not be degraded by the test date
  - Temperature
    - 4°C
    - Room temp
    - 30°C
  - Time
    - 5
    - 10
    - 15 days

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Stability evaluation

- Two dilution series, healthy potato leaf DNA spiked with:
  - BLTVA (Beet Leafhopper Transmitted Virulent Agent) '*Ca* phytoplasma trifolli' from infected potato plants
  - '*Ca* phytoplasma asteris' plasmid (starting dilution 1:200)

- Dilutions tested by:
  - real-time PCR (Christensen *et al* 2004)
  - nested PCR (R16f2/r2 primers nested with fu5/ru3)

to determine a high, medium and low dilution to use in the stability evaluation

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Stability evaluation

- Using real time CT values the following dilutions selected:

  BLTVA – neat

  BLTVA – 10-1

  BLTVA – 10-2

  Plasmid – 10-2

  Plasmid – 10-3

  Plasmid – 10-4

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Stability results: Real-time (Christensen *et al* 2004)

	25.6.14	5 days			9 days			15 days		
	original sample	**30°C**	**room temp**	**4°C**	**30°C**	**room temp**	**4°C**	**30°C**	**room temp**	**4°C**
BLTVA Neat	28	28	28	27	28	28	28	28	28	28.
BLTVA 10-1	31	31	31	31	31	32	31	32	32	32
BLTVA 10-2	35	37	36	35	37	36	36	39	37	38
Plasmid 10-2	24	25	24	24	24	24	24	24	25	25
Plasmid 10-3	27	34	27	28	27	27	27	27	28	28
Plasmid 10-4	31	30	31	31	30	31	31	31	31	32

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Ring test - sample preparation

- dilution series in healthy potato leaf DNA:
  - '*Ca* phytoplasma asteris' plasmid, (10^{-1},10^{-2},10^{-3})
  - '*Ca* phytoplasma solani' plasmid (10^{-2},10^{-3},10^{-4})
  - '*Ca* phytoplasma trifolli' plasmid (1:20, 1:50)
  - BLTVA '*Ca* phytoplasma trifolli' from infected glasshouse grown plants (Neat,10^{-1},10^{-2})
- Bacterial suspensions (to test for cross reactions)
  - *Paenibacillus* sp.
  - *Brevundimonas* sp.
  - *Agrobacterium tumefaciens*
- Healthy potato leaf DNA

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Ring test sample preparation

- All samples were randomised
- Each Lab was given a number
- Each sample was labelled with the lab number and the sample number

Example for lab number 4:

Code	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420

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Ring test sample preparation

- DNA samples sent out to labs 4th of September
    - to be stored in the fridge on arrival
    - tested within two weeks of receipt

- A healthy and infected microplant were also sent out for extraction and testing.

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Ring test - results

Sample number

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Lab 1																						
Lab 2 M1	+	-	-	+	+	-	+	-	+	-	-	-	-	+	-	-	+	-	+	+	+	-
Lab 3 M2	+	-	-	+	+	-	+	-	+	-	+	+	-	+	-	-	+	-	+	+	-	+
M5	+ 18	-	-	+ 25	+ 23	-	+ 24	-	+ 16	-	+ 23	+ 26	-	+ 19	-	-	+ 27	-	+ 21	+ 20	-	+
Lab 4 M1	+	-	-	+	-	-	+	-	+	-	+	+	-	+	-	-	+	-	+	+	-	+
M6	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	NT	NT
Lab 5 M3	+	-	-	+	+	-	+	-	+	-	+	+	-	+	-	-	+	-	+	+	NT	NT
M4	+	-	-	+	+	-	+	-	+	-	+	+	-	+	-	-	+	-	-	+	NT	NT
P1/P7 nested with R16f2/r2 M1	+	-	-	+	+	-	+	-	+	-	+	+	-	+	-	-	+	-	+	+	NT	NT
P1/P7 nested with fu5/ru3 M2	+	-	-	+	+	-	+	-	+	-	+	+	-	+	-	-	+	-	+	+	NT	NT
P1/tint nested with fu5/ru3 M3	+	-	-	+	+	-	+	-	+	-	+	+	-	+	-	-	+	-	+	+	NT	NT
R16f2/r2 nested with fu5/ru3 M4	+	-	-	+	+	-	+	-	+	-	+	+	-	+	-	-	+	-	+	+	NT	NT
Christensen *et al.*, 2004 real-time method M5	+ 24	-	-	+ 32	+ 30	-	+ 31	-	+ 23	-	+ 30	+ 34	-	+ 26	-	-	+ 34	-	+ 28	+ 27	NT	NT

M6 Hodgett's real-time method

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Ringtest - results

- Two of the five labs obtained the correct results
- Factors which could have resulted in differences
    - operator error?
    - thermocyclers?
    - primer concentration?
    - sample added? amount of DNA added to reactions varies, some labs diluted the PCR product before nesting, some use a smaller volume of first round product.
    - master mix? majority of labs still used individual reagents ready mix has been found to work well and reduces operator error?

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Ringtest – future work

- Should we try to optimise the assays to make them standard across all labs?
- Multiplex assays with internal control primers
- Evaluate further all available taqman assays

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THANK YOU

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Ringtest preparation

number	+/-	Details
01	+	Stolbur plasmid 10-2
02	-	Healthy DNA - CV Atlantic
03	-	Bacteria PB
04	+	Potato witches broom plasmid. 1:20
05	+	Aster Yellows plasmid 10-3
06	-	Healthy DNA - CV Atlantic
07	+	Stolbur plasmid 10-4
08	-	Healthy DNA - CV Atlantic
09	+	Aster Yellows plasmid 10-1
10	-	Bacteria Br
11	+	BLTVA glasshouse plant DNA 10-1
12	+	BLTVA glasshouse plant DNA 10-2
13	-	Healthy DNA - CV Atlantic
14	+	Aster Yellows plasmid 10-2
15	-	Healthy DNA - CV Atlantic
16	-	Bacteria Agt
17	+	Potato witches broom plasmid. 1:50
18	-	Healthy DNA - CV Atlantic
19	+	Stolbur plasmid 10-3
20	+	BLTVA glasshouse plant DNA neat
21	-	Healthy microplant - CV Atlantic
22	+	BLTVA infected microplant

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Ring test – My method PCR

Master Mix PCR	µl
Jumpstart Redtaq Readymix	12.5
Forward Primer (8pmol/µl)	1
Reverse Primer (8pmol/µl)	1
Nuclease free water	9.5
DNA	1
Total	25

95°C for 5 min

35 cycles of :

94°C for 30 sec

52°C for 30 sec

72°C for 30 sec

72°C for 10min

15°C forever

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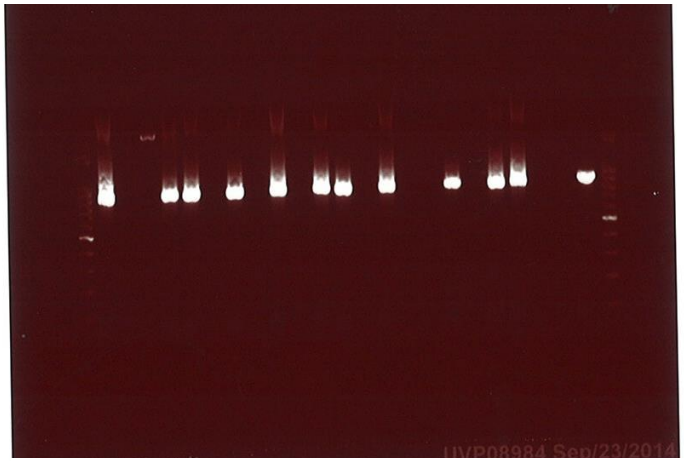
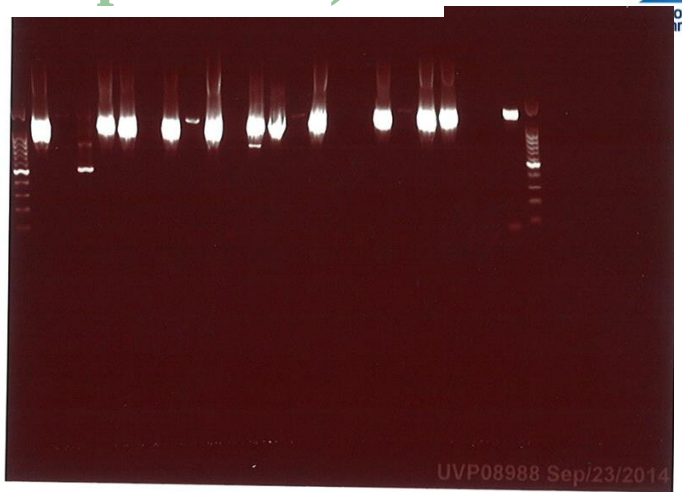
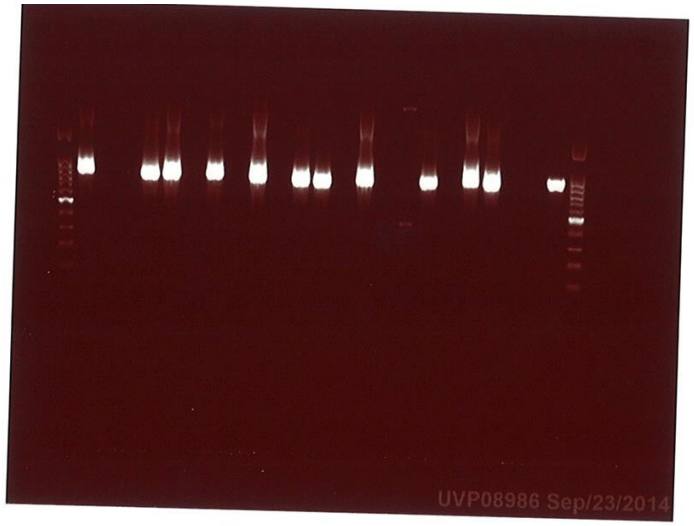
Ring test – results (18 days after samples sent)



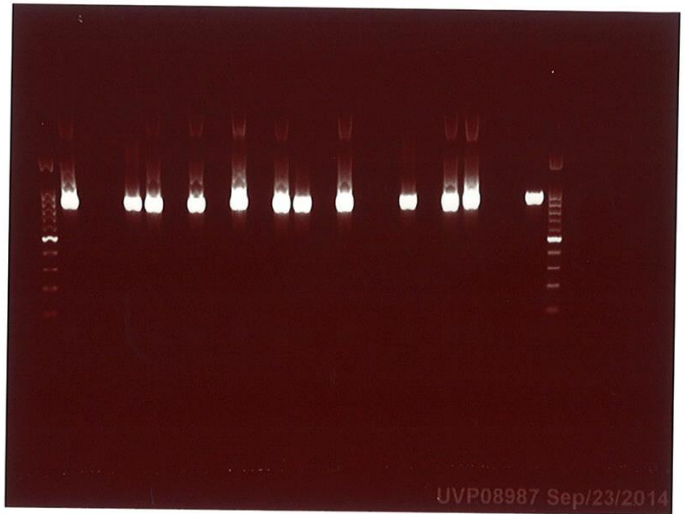
P1/tint nested with fu5/ru3



P1/P7 nested with R16f2/r2



R16f2/r2 nested with fu5/ru3



P1/P7 nested with fu5/ru3

Ring test – My method real-time

Master Mix PCR	µl
Jumpstart Readymix	12.5
Forward Primer (8pmol/µl)	0.75
Reverse Primer (8pmol/µl)	2.25
Probe	0.5
Water	8.0
DNA	1

50°C for 2 min

95°C for 10 min

40 cycles of :

95°C for 15 sec

60°C for 60 sec

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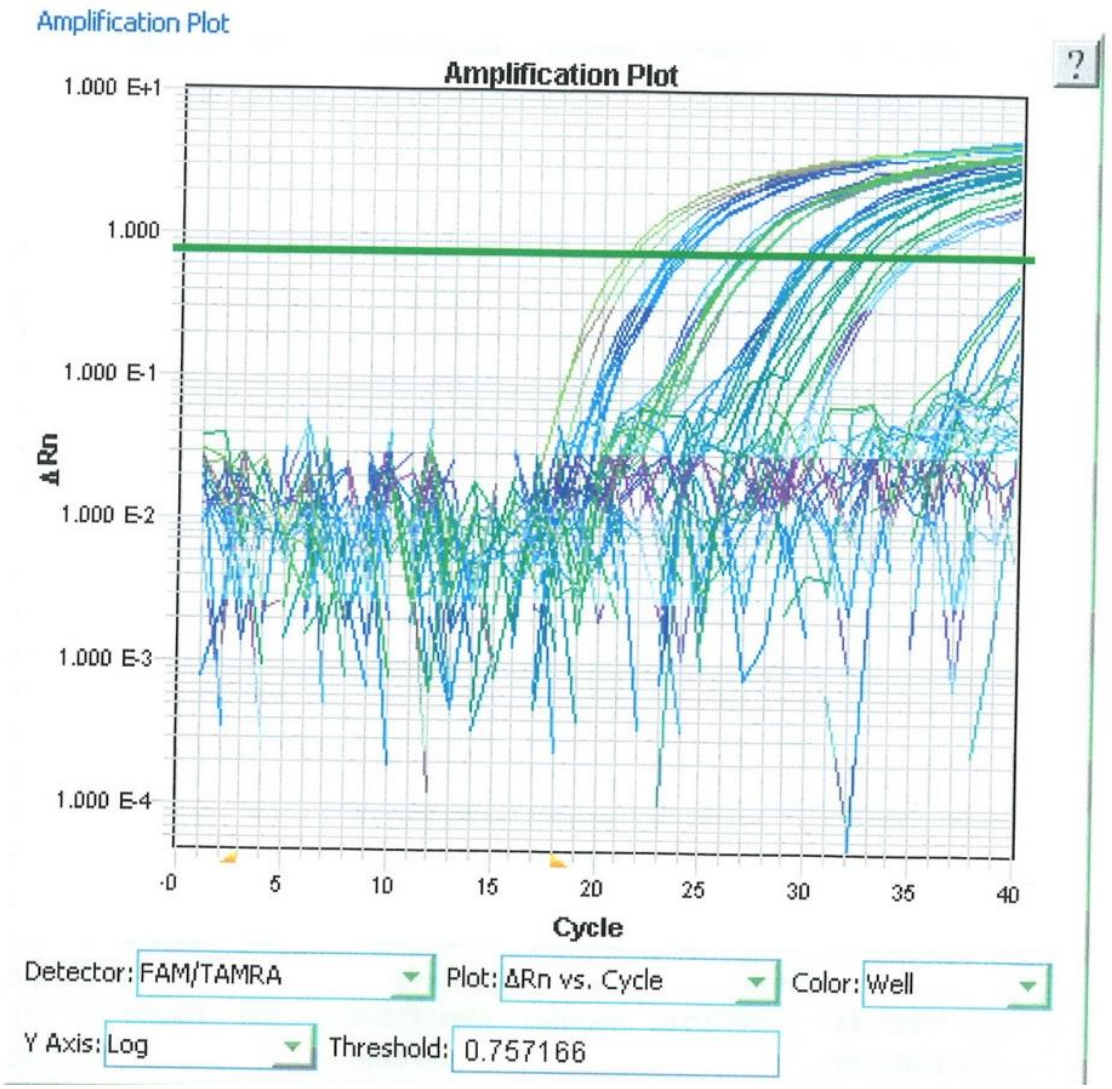
Amplification Plot



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