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Potential of spiral plating and digital real-time PCR for improved seed health testing

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Xanthomonas campestris pv. *campestris*

http://www.apsnet.org/edcenter/advanced/topics/Pages/Xanthomonadins.aspx

Disease: Black rot
The most destructive disease of crucifers

Hosts:
Members of the plant family *Brassicaceae* as cabbage, broccoli, cauliflower, kale, turnip, oilseed rape, mustard, radish,…

Symptoms:
V-shaped chlorotic to necrotic lesions extending from the leaf margins and blackening of vascular tissues, wilting, stunted growth, and stem rot symptoms

Prevention:
Using disease-free seed

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Xcc and current methods in seed testing

International Rules for Seed testing 7-019a: Detection of *Xanthomonas campestris* pv. *campestris* on *Brassica* spp.

Extraction

Soaking with prechilled sterile saline with Tween 20 on shaker for 2.5 h

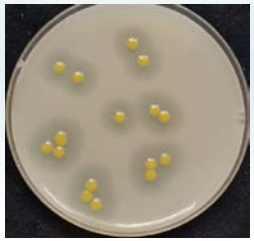
Confirmation of pathogenicity

Inoculation by stabbing major leaf veins by suspected isolates

ISTA, 2015

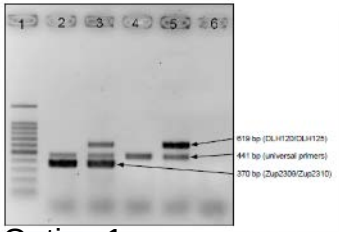
Dilution plating on semi-selective media

Fs agar medium

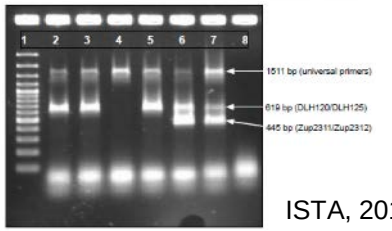


mCS20ABN agar medium

Identification of isolates with multiplex PCR (two options)



Option 1



ISTA, 2015

Option 2

Real – time PCR for detection Xc from brassicas (Berg et al, 2006)

- Target on *hrpF* gene
- Multiplex assay with internal control that applify DNA from *Brassica* spp
- Detect also other related pathovars

Seed – qPCR (Laala et al, 2015)

- Primers and probes based on Berg et al, 2006)
- Germination of seeds before qPCR

Challenges in seed testing

Dilution plating

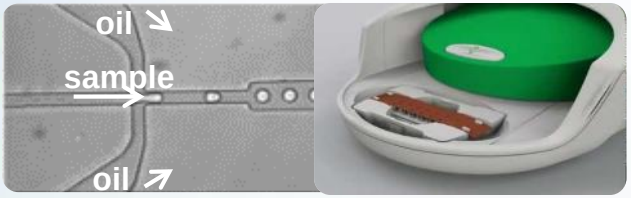
- Strain variation - Some strains don't grow –recovery rates vary
- Post-harvest seed treatments (Chemical, biological control)
- Age of seed lot
- Microflora may inhibit growth – natural antibiotic production

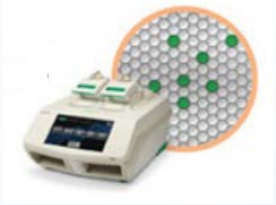
Molecular methods based on polymerase chain reaction

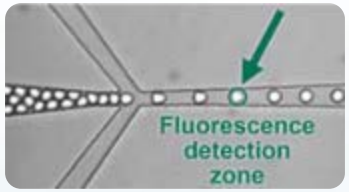
- Inhibitors present in/on seeds that inhibit reaction
- Many different DNA extraction methods but not all are appropriate for the seeds
- False positive results if primers/probes are not specific enough or false negative if are too specific
- High background

Droplet dPCR workflow

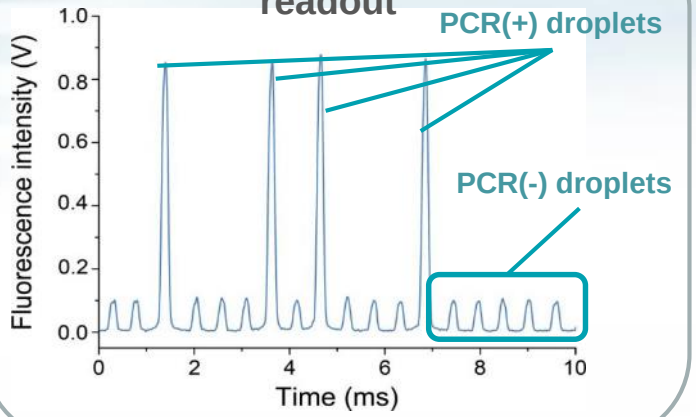
DNA + qPCR MM → Droplet formation → Thermal Cycling → Signal readout

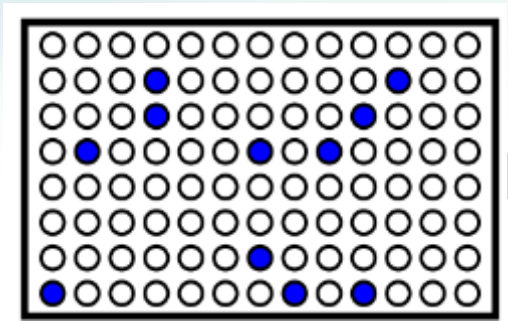


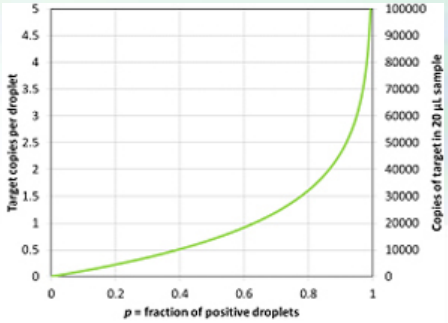




Droplet fluorescence during readout



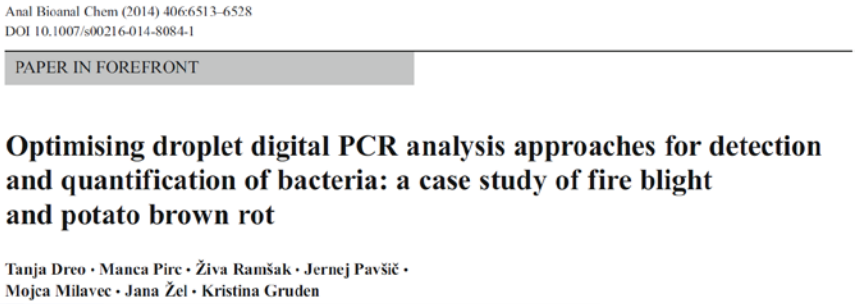




cps/rxn

Droplet dPCR benefits

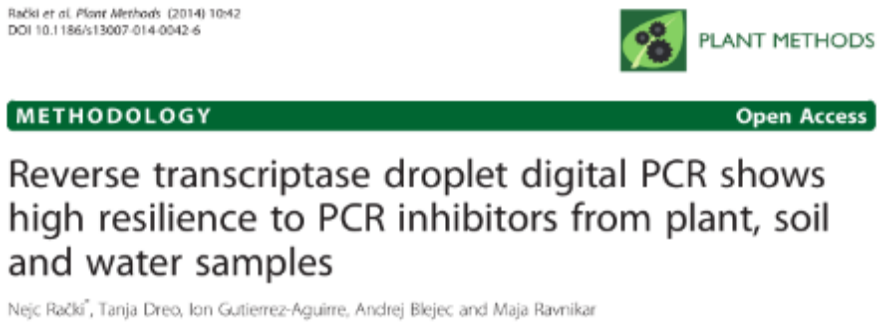
- **Absolute quantification** - no need for standard curves
- **Improved sensitivity** (rare event detection!)
- Better signal to noise ratio
- Less sensitive to **inhibition**
- Absolute quantification even at low levels
- **Validation** of in-house reference materials


Anal Bioanal Chem (2014) 406:6513–6528
DOI 10.1007/s00216-014-8084-1

PAPER IN FOREFRONT

Optimising droplet digital PCR analysis approaches for detection and quantification of bacteria: a case study of fire blight and potato brown rot

Tanja Dreo · Manca Pirc · Živa Ramšak · Jernej Pavšič · Mojca Milavec · Jana Žel · Kristina Gruden


Rački et al. Plant Methods (2014) 10:42
DOI 10.1186/s13007-014-0042-6

PLANT METHODS

METHODOLOGY Open Access

Reverse transcriptase droplet digital PCR shows high resilience to PCR inhibitors from plant, soil and water samples

Nejc Rački*, Tanja Dreo, Ion Gutierrez-Aguirre, Andrej Blejec and Maja Ravnikar

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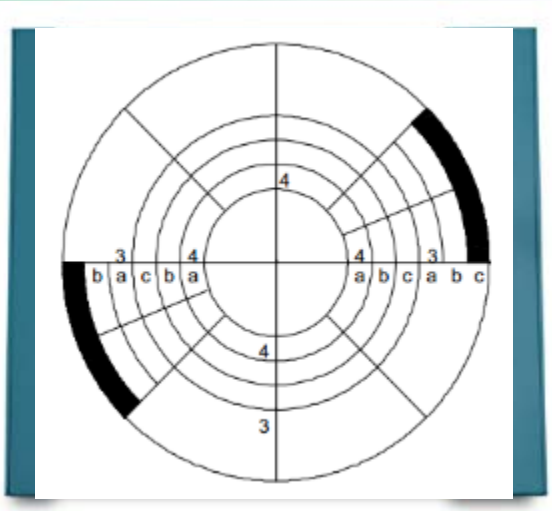
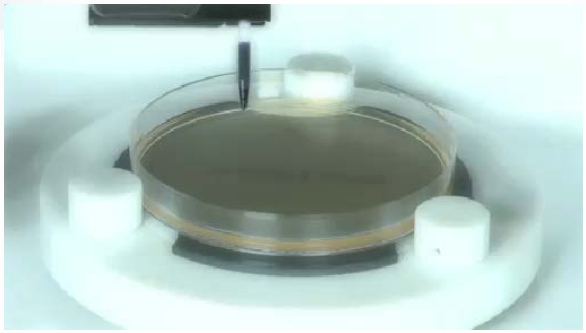
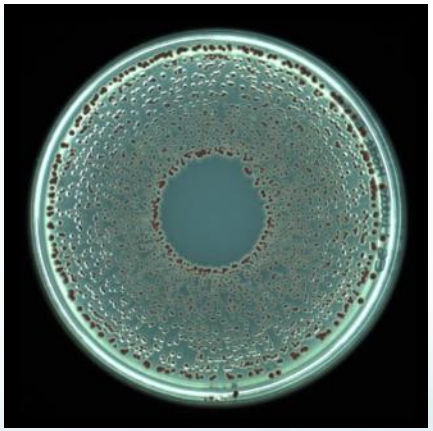
Spiral plating

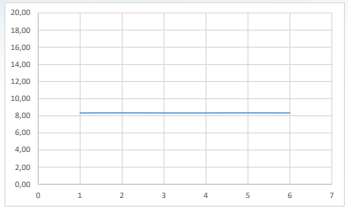
Eddy jet 2 (IUL Instruments)



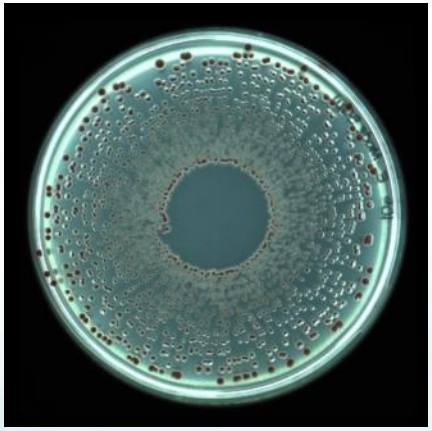




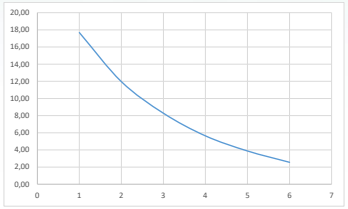
Linear mode 50µL
2,4x



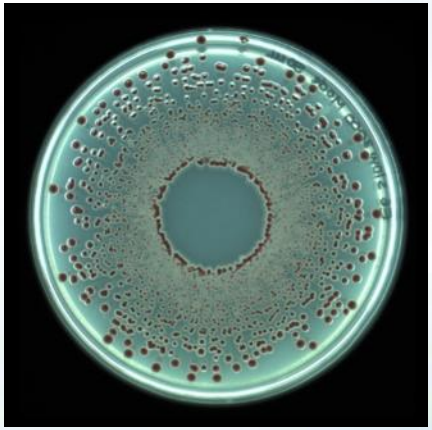
	V (µL)
4a	8,33
4b	8,34
4c	8,33
3a	8,33
3b	8,34
3c	8,33



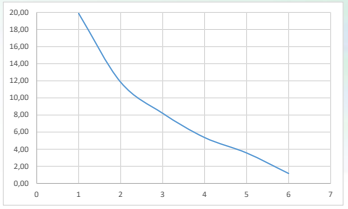
E-mode 50µL
16,7x



	V (µL)
4a	17,68
4b	11,96
4c	8,28
3a	5,64
3b	3,88
3c	2,56



Slow 3000 mode 50µL
41,5x

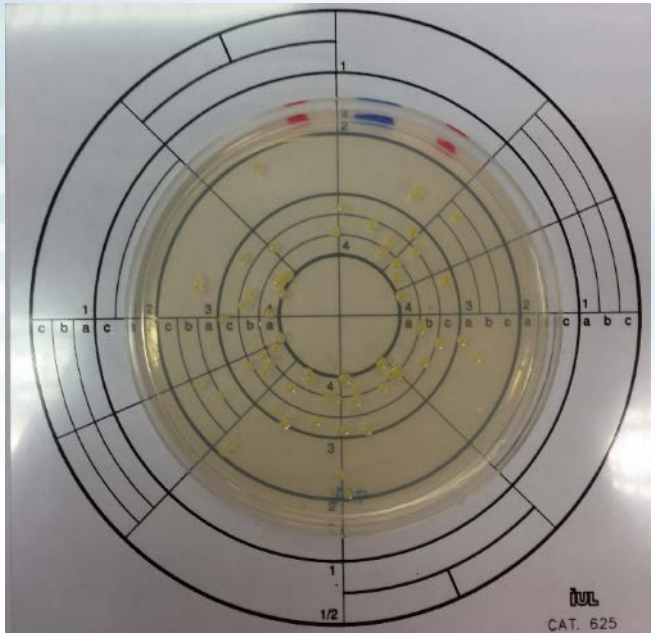


	V (µL)
4a	19,88
4b	11,84
4c	8,20
3a	5,36
3b	3,56
3c	1,16

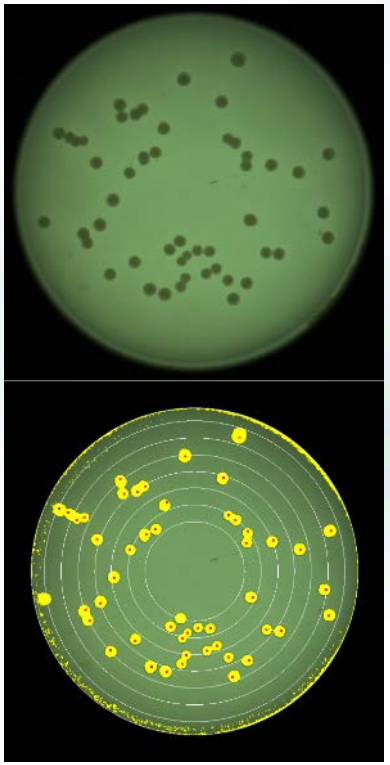
Potential of automatisation

- Colonies can be counted manually or automatic

Manually



Automatically



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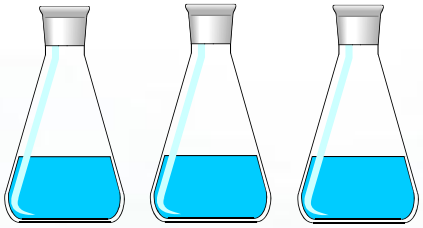
Design of the experiment I

3 different cultivars of untreated seeds of cabbage (*Brassica* oleracea L var capitata)



Extraction with soaking

Extract

Molecular methods

Two different starting volumes were tested

100 µL

1000 µL
+ 10 min 10.000 g

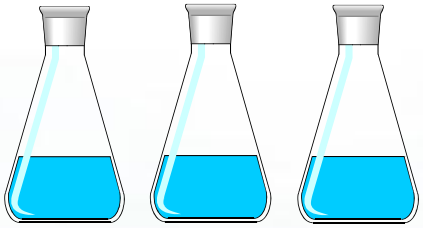
DNA extraction

Quick Pick Plant kit
Bionobile
Dilutions: 0x, 10x

1. **Modified qPCR according to Berg et al, 2006**
   - different volume (10 µL reactions; 8+2)
   - different primer and probes concentration
   - different mastermix and cycling contitions, anneling T remain 60°C
   - 3 repetitions per sample
2. **Droplet digital PCR (Biorad QX 100)**
   - reaction volume (20 µL reactions 12+8)
   - different mastermix and cycling contitions, anneling T remain 60°C
   - 1 reaction per sample

Design of the experiment II

3 different cultivars of untreated seeds of cabbage (*Brassica* oleracea L var capitata)

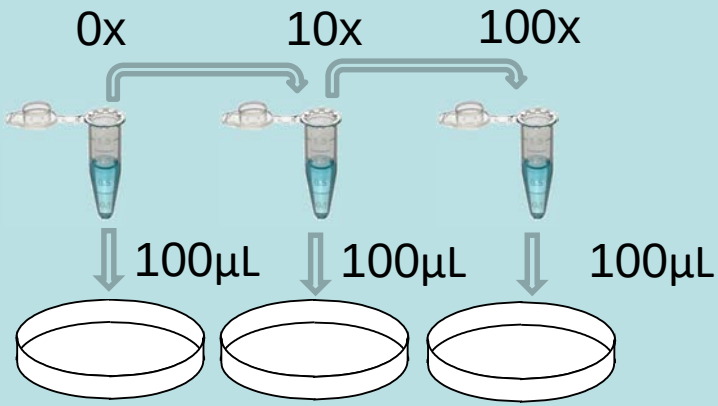


Extraction with soaking

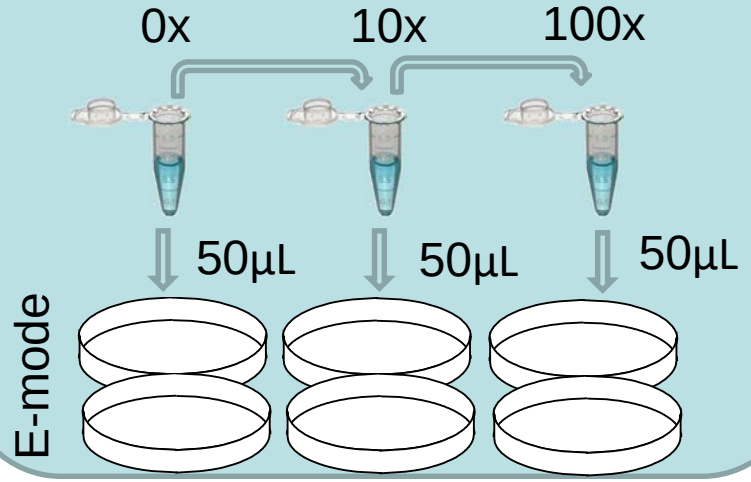
Extract

Plating on Semi – selective media

Dilution plating



Spiral plating with Eddy Jet 2



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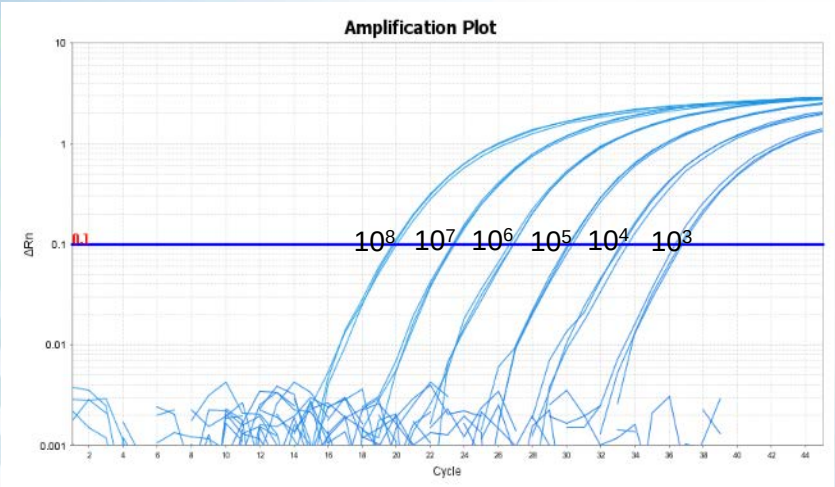
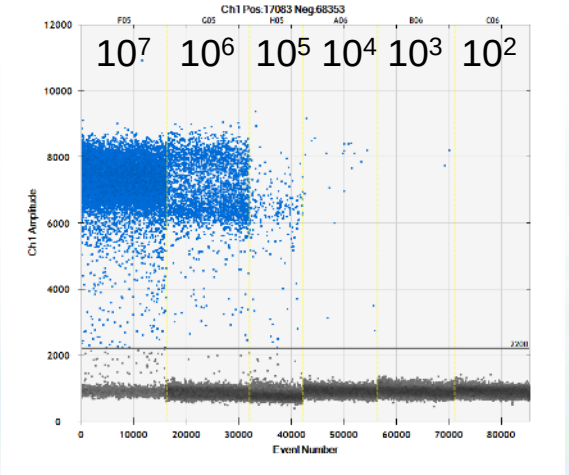
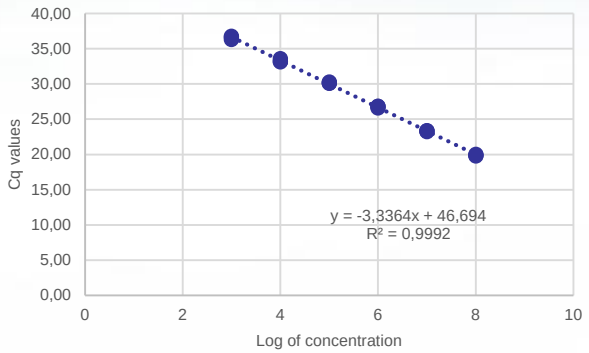
Adaptation of qPCR and transfer to droplet dPCR

DNA dilution of *Xcc* suspension (10^8- 10^1 cfu/mL)



DNA dilution of *Xcc* suspension (10^7- 10^2 cfu/mL)





$$E = 10^{-\frac{1}{s}} - 1$$

$$E = 99{,}4\%$$

Sample	Conc(copies/ µL)	Copies/20µL Well	cps/mL
10^7 cfu/mL	2332	46640	6E+06
10^6 cfu/mL	229	4580	6E+05
10^5 cfu/mL	22	440	6E+04
10^4 cfu/mL	1,7	34	4E+03
10^3 cfu/mL	0,16	3,2	4E+02
10^2 cfu/mL	0	0	0E+00

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Results of seed testing - qPCR

100µL

Sample	Average Cq Xcc	Slope Xcc	Average Cq Brassicacea	Slope Brassicacea	Result
Cultivar 1 0x	23,84	3,53	27,14	2,27	POS
10x	27,37		29,41		
Cultivar 2 0x	31,11	4,11	28,13	2,45	POS
10x	35,23		30,58		
Cultivar 3 0x	Undet	NA	26,39	3,27	NEG
10x	Undet		29,66		

1000µL

Sample	Average Cq Xcc	Slope Xcc	Average Cq Brassicacea	Slope Brassicacea	Result
Cultivar 1 0x	32,58	-8,76	35,38	-8,62	POS
10x	23,82		26,76		
Cultivar 2 0x	33,25	-1,73	30,11	-3,61	POS
10x	31,52		26,50		
Cultivar 3 0x	36,93	NA	23,04	2,19	POS
10x	Undet		25,22		

Increased sensitivity with higher volume

Increased inhibition in qPCR reaction

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Results of seed testing – droplet dPCR

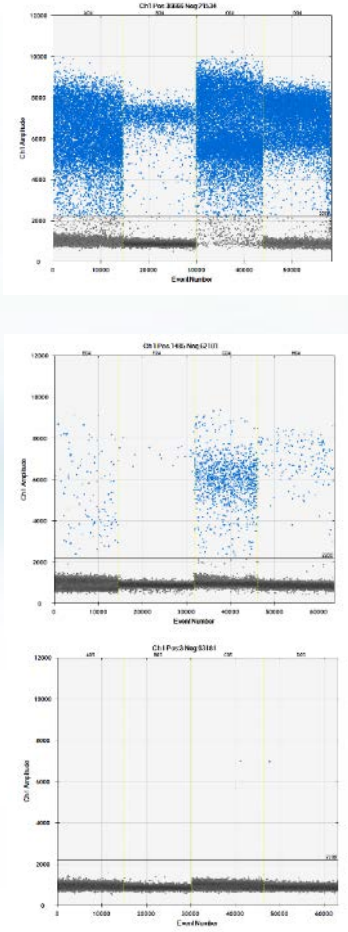
100µL

Sample	Conc(copies/µL)	Copies/20µLWell	cps/mL	Result
Cultivar 1 0x	1477	29540	4E+06	POS
10x	163	3260	4E+05	

Sample	Conc(copies/µL)	Copies/20µLWell	cps/mL	Result
Cultivar 2 0x	9,7	194	2E+04	POS
10x	1,2	24	3E+03	

Sample	Conc(copies/µL)	Copies/20µLWell	cps/mL	Result
Cultivar 3 0x	0	0	0E+00	NEG
10x	0	0	0E+00	

100µl 1000µl



1000µL

Sample	Conc(copies/µL)	Copies/20µLWell	cps/mL	Result
Cultivar 1 0x	4100	82000	1E+07	POS
10x	1585	31700	4E+06	

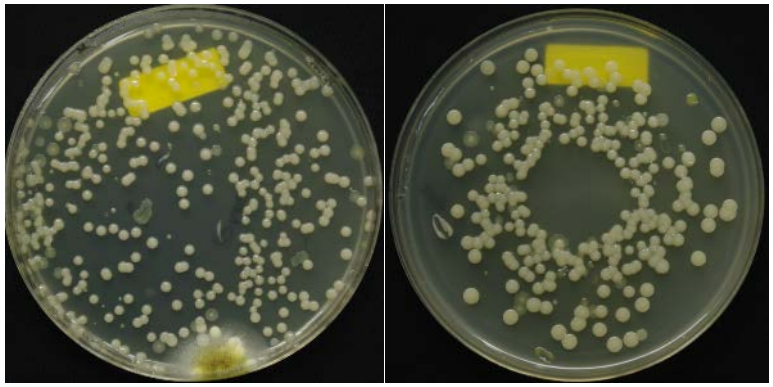
Sample	Conc(copies/µL)	Copies/20µLWell	cps/mL	Result
Cultivar 2 0x	100	2000	3E+05	POS
10x	11,3	226	3E+04	

Sample	Conc(copies/µL)	Copies/20µLWell	cps/mL	Result
Cultivar 3 0x	0,15	3	4E+02	POS
10x	0,07	1,4	2E+02	

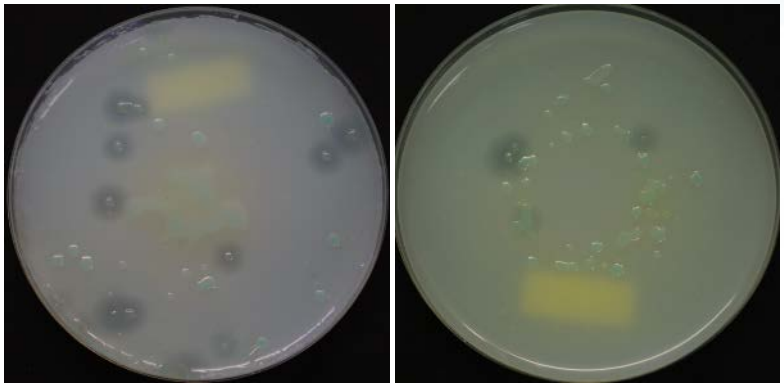
Results of seed testing - plating

	mCS20ABN agar medium			Fs agar medium		
	Dilution plating 100µL	Eddy Jet E-mode 50µL 1.	Eddy Jet E-mode 50µL 2.	Dilution plating 100µL	Eddy Jet E-mode 50µL 1.	Eddy Jet E-mode 50µL 2.
Cultivar 1 0x	1	11	5	7	3	1
10x	4	1	1	0	2	0
100x	0	0	0	0	0	1
Cultivar 2 0x	0	0	0	1	0	0
10x	0	0	0	0	0	0
100x	0	0	0	0	0	0
Cultivar 3 0x	0	0	0	0	0	0
10x	0	0	0	0	0	0
100x	0	0	0	0	0	0

mCS20ABN



FS agar medium



Conclusions I

- With qPCR and droplet dPCR Xc was detected in seed of all three cultivars
- From two out of three cultivars we isolated Xcc suspected colonies.
- One cultivar was positive only with the molecular methods and only if DNA was extracted from 1000 µL
- Level of seed contamination ranged from 10^7 – 10^2 copies/mL as determined with droplet dPCR
- Spiral plating with further optimization and validation can be promising technique without preparation dilutions (150 mm plates)

Conclusions II

- qPCR was successfully transferred to droplet dPCR
- Sensitivity of qPCR and droplet dPCR tested on diluted DNA of *Xcc* suspension was comparable (3 vs 1 reaction)
- Despite high concentration of bacteria determined with the droplet dPCR few bacteria grew on the semi-selective media (live/dead differentiation)
- As expected with higher volume of sample the sensitivity is increased but also inhibition in qPCR is greater. In droplet dPCR inhibition was not present or was much lower

Acknowledgements

- Slovenian phytosanitary administration
- NIB Bacteriological team for support and technical assistant

Thank you for your attention

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MINISTRY OF ECONOMIC DEVELOPMENT AND TECHNOLOGY
METROLOGY INSTITUTE OF THE REPUBLIC OF SLOVENIA

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European Regional Development Fund