Detection of viable cells of Xanthomonas campestris pv. campestris using a PMA-TaqMan

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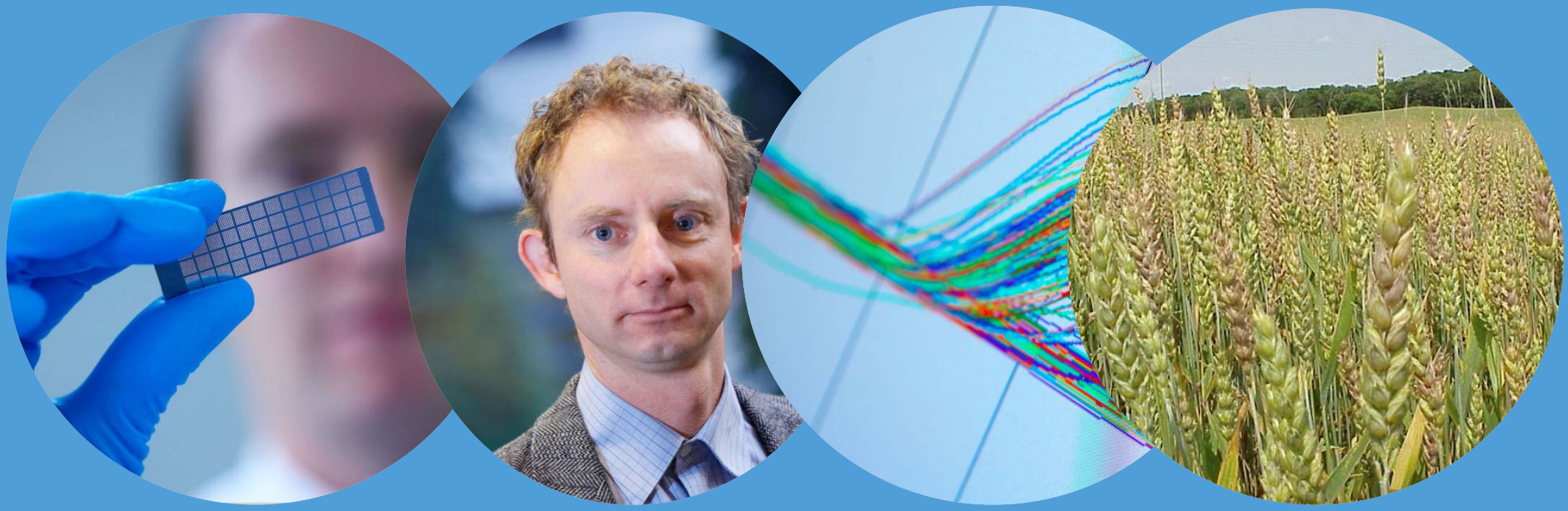
TESTA meeting 1 December 2015, Angers, France



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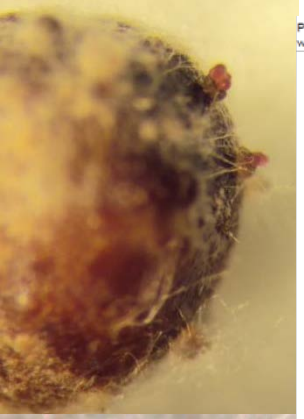
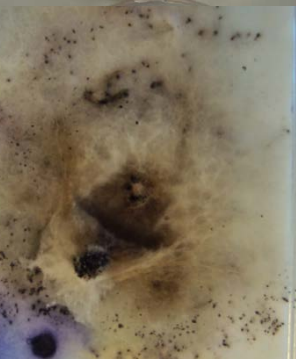
For quality of life

Detection

- Any target
  - bacteria
  - viruses
  - nematodes
  - fungi
  - insects
  - phytoplasms
- Anywhere
  - in plant (parts)
  - in water
  - in soil, compost
  - in air



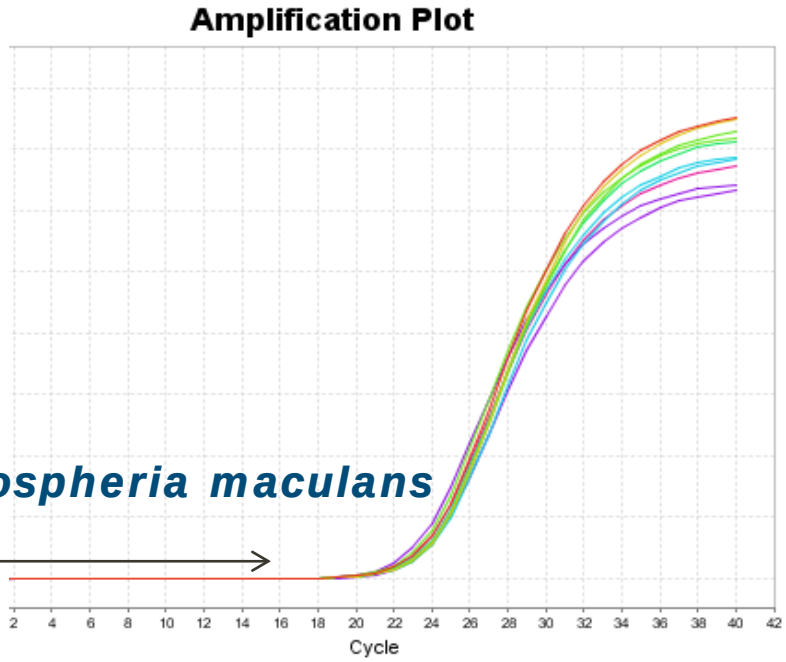


Phytopathology

collections

Taxonomy

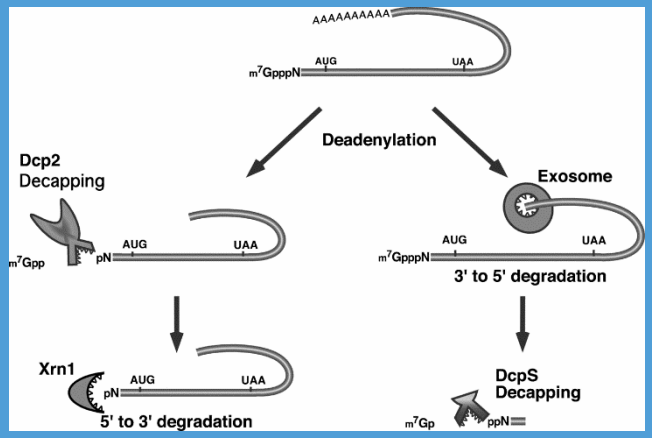
Genomics



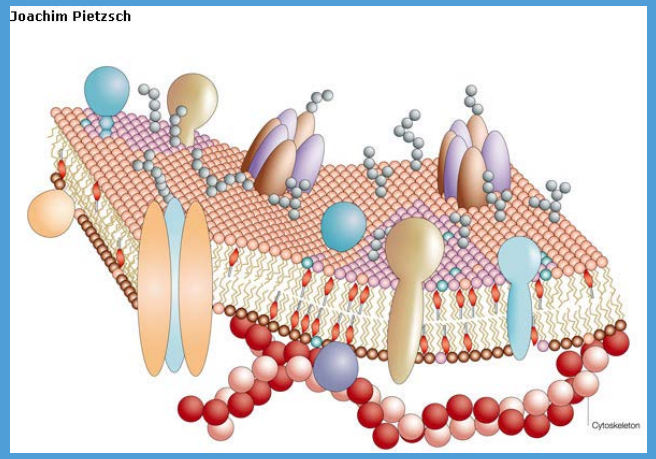
Leptospheria maculans

State of the art: detection of viable pathogens

- Biologische methods, are subjective, time consuming, show large variation, are costly, outcome may depend on the season
- Moleculaire methods Immuno or DNA based detect also non-viable organims (depending on the external conditions)



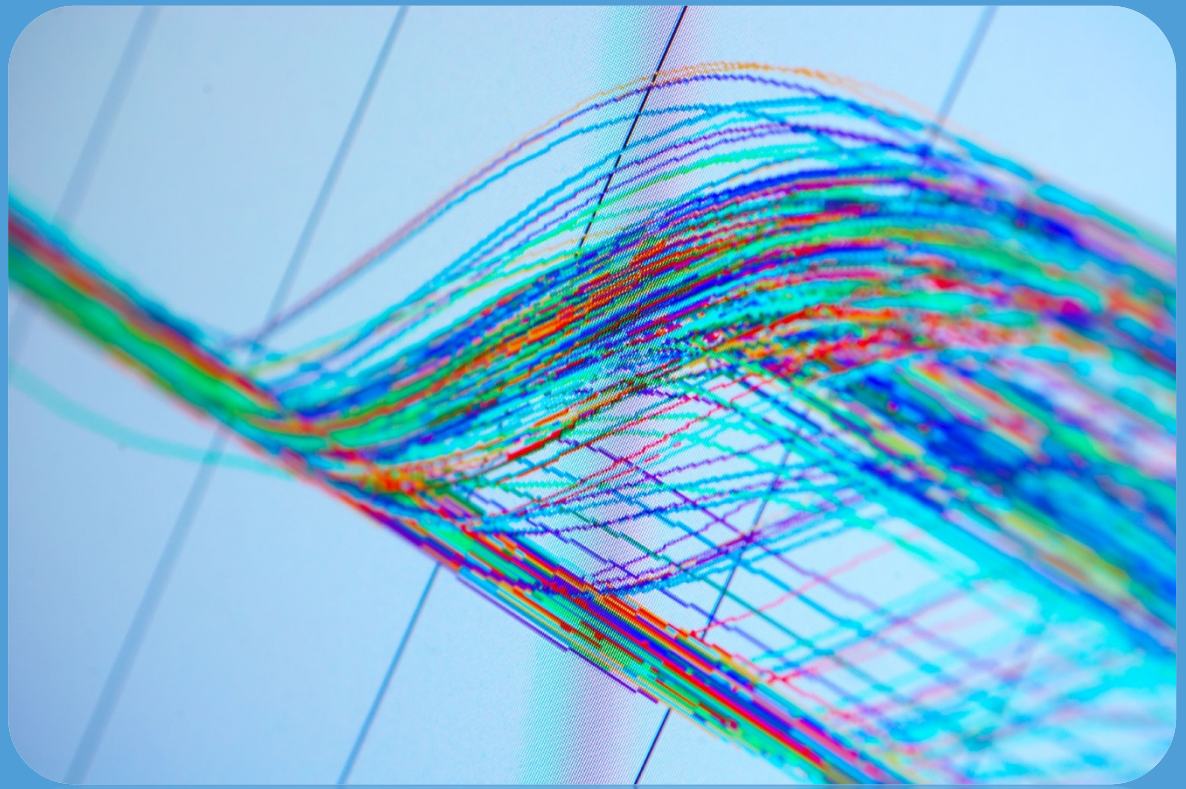
Based on RNA degradation



Membrane permeability

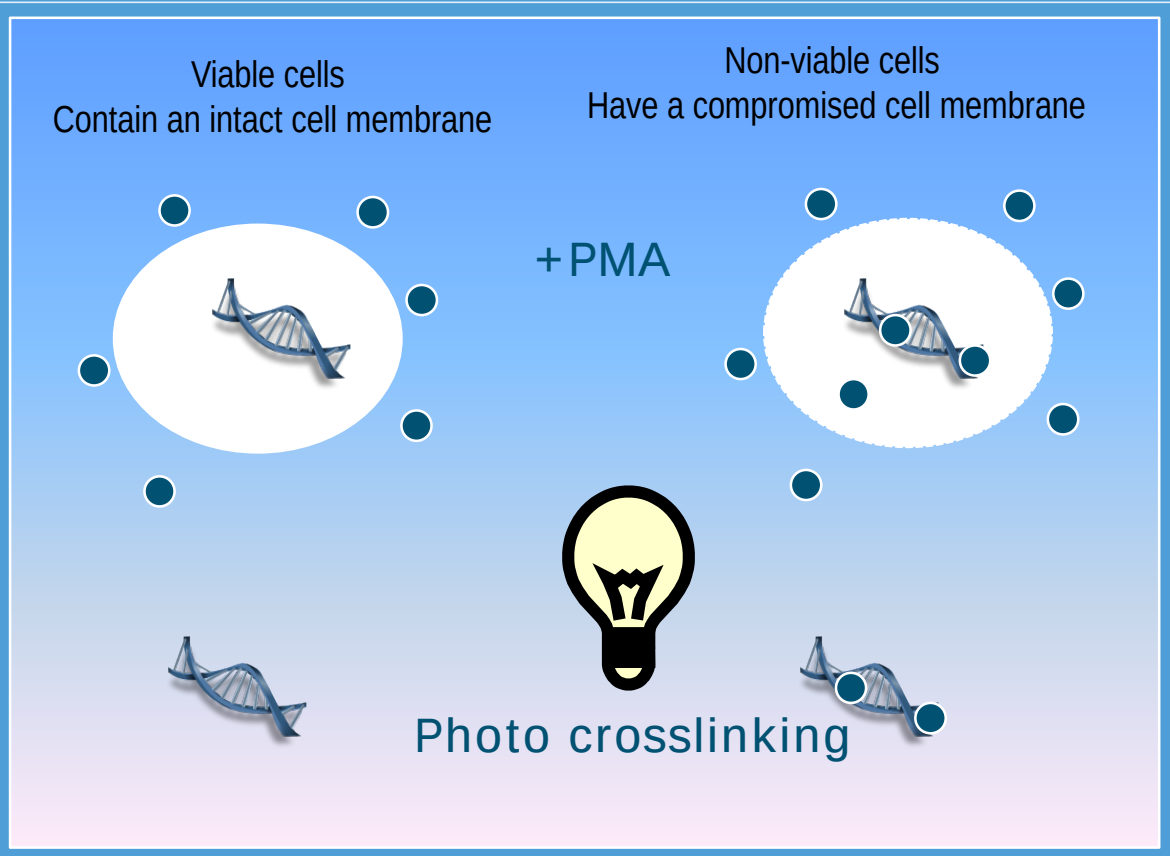
Why use TaqMan PCR?

- Sensitive
- Specific
- Quantification
- Robust
- Fast
- Automation
- Scalable
- Cost efficient
- Flexible



➢ Can we use TaqMan to specifically detect viable cells?

Detection of viable cells of *Xanthomonas campestris pv. campestris* using a PMA-TaqMan

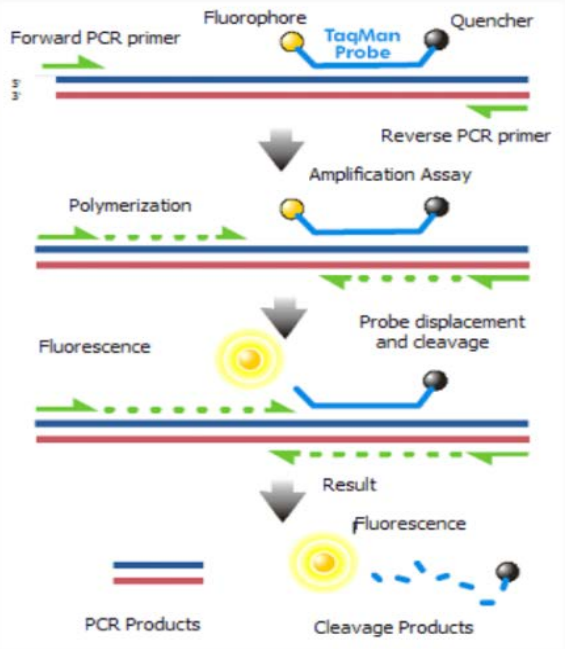


PMA= propidium monoazide:binds to DNA azide group for foto crosslinking

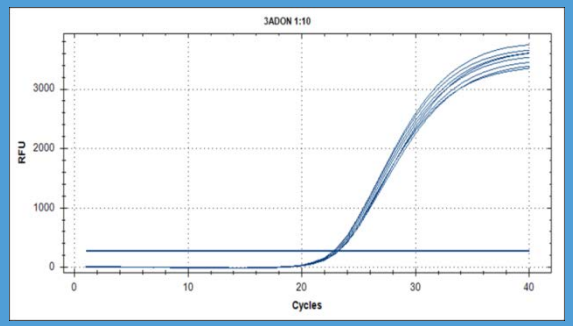
Nocker et al. 2007 Journal of microbial methods 2007

TaqMan primer and probe design

TaqMan based on a Xcc specific AFLP product (Rijlaarsdam et al., 2004)



GTC TGA GCG CAT ACC GAA GGC CTT GGC GCG AGA AGC GCT GGC TCC
TCG ACA CCT GCA AGG GAC TCC GGC CAG GGT CGA TAC AGT GCA CTC
GTG ATG CCC CGC ACC GCC CTG GGC TGC AGG CTT GCT GCT TCC AAG
AAC GCA GCC GCA CGG GTA AGG CAG CCA CGC ACC CGA CCA AGC AAT
GCC GTG GCG ATT ACC CGC CAG GGT GCA TAG GCC ACG ATG TTG GCC
CAA GCG ATG TAC TGC GGC CGT GGT GTA AGA CGC TCT GCA TCC GTA
CCG GCT AGG CCT GCG CCC GCA CCC GAC AAC GGA CCC AGA CAG CAC
TGA GGT GTC CAC GCT GTA TGG AAT CCA GAT TCT GGA CCA GTC TCT
GGA ATT AGT CGC GAC GAA AGT AGT CGG CGG CGC ACA TGA GAA GAG



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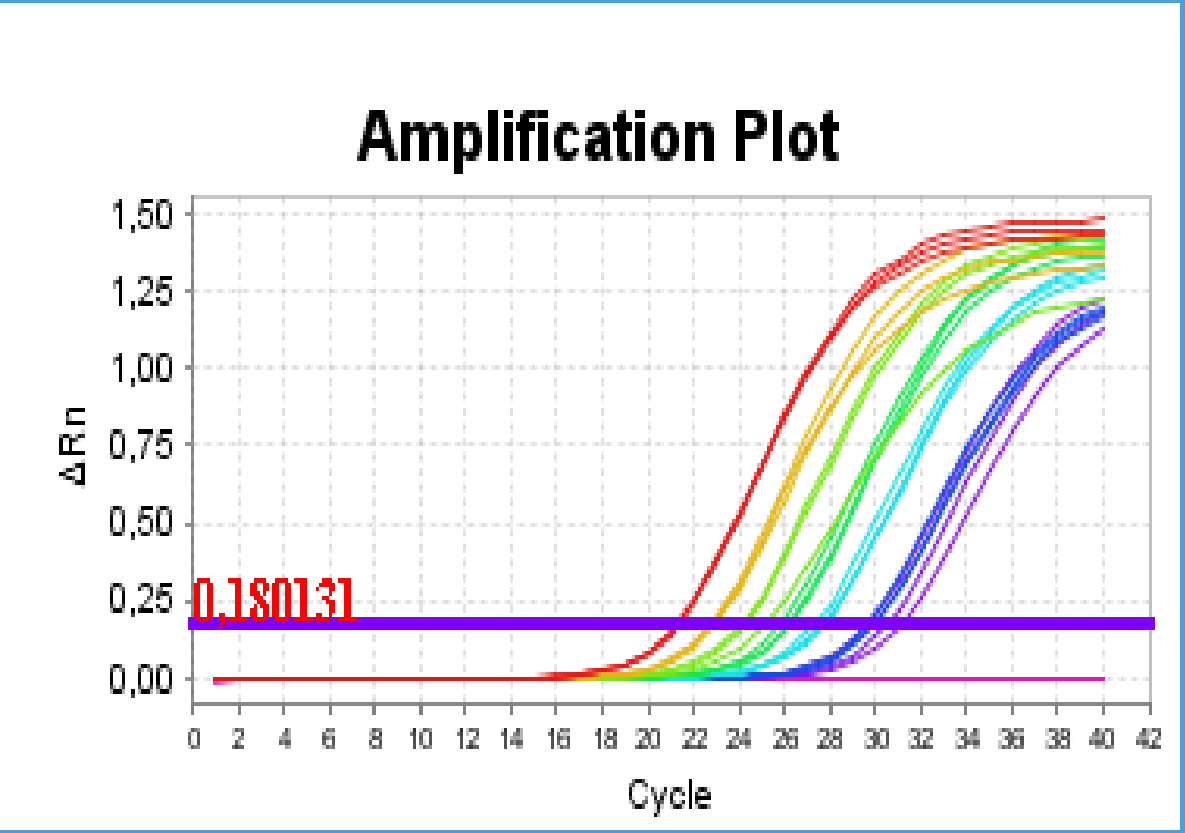
Quantification using a dilution series of Xcc 3371 DNA



Sample	Ct		
Xcc 3371 DNA(1ng/µl)	21.41	21.43	21.36
1:5 dilution	22.94	23.05	22.83
1:25 dilution	24.20	24.36	25.26
1:125 dilution	25.91	26.30	26.27
1:625 dilution	27.41	27.85	27.79
1:3125 dilution	29.67	29.96	29.99
1:15625 dilution	30.59	31.23	29.81
NC(MQ)	ND	ND	ND

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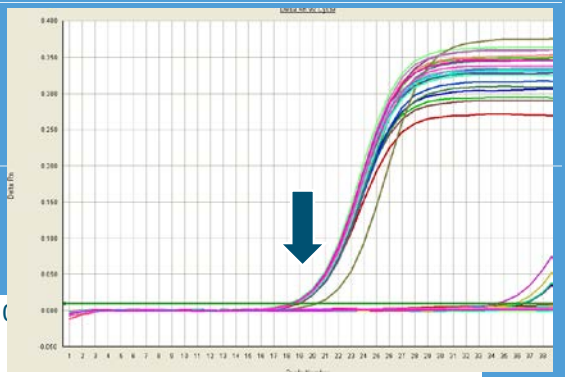
Specificity TaqMan



Validatie/specificatie TaqMan PCR Xanthomonas campestris pv. campestris 2(

Strain	PRI nr.	PD-nr.	Other collection nr.	Country of origin	Experiment 1	Experiment 2	Average	
Target strains								
X.campestris pv. campestris	3357	P5002		seed. USA California	18.34	19.02	18.68	18.68
X.campestris pv. campestris	3358	P5106		seed. Italy	18.10	18.54	18.32	18.32
X.campestris pv. campestris	3359	P5145		seed. Tasmania	18.27	18.6	18.44	18.435
X.campestris pv. campestris	3360	P5164		seed. Australia	18.15	18.44	18.30	18.295
X.campestris pv. campestris	3361	P5183		seed. New Zealand	18.23	18.82	18.53	18.525
X.campestris pv. campestris	3362	Xcc3		Collection D. Morrison phw331	17.96	18.83	18.40	18.395
X.campestris pv. campestris	3363	Xcc4		Collection D.Morrison A4 USA	18.00	18.49	18.25	18.245
X.campestris pv. campestris	3364	BR1 race 0		Broccoli. Russia	18.36	18.67	18.52	18.515
X.campestris pv. campestris	3365	M 1/3/98 race 1		Pointed Cabbage. Germany	17.98	18.38	18.18	18.18
X.campestris pv. campestris	3366	M2/198 race 4		Red Cabbage. Germany	18.32	18.59	18.46	18.455
X.campestris pv. campestris	3367	VN1 race 3		Cabbage. Russia	18.20	18.4	18.30	18.3
X.campestris pv. campestris	3368	B-172		Broccoli. Chili	18.10	18.35	18.23	18.225
X.campestris pv. campestris	3369	B-441		Broccoli. Mexico	18.43	18.78	18.61	18.605
X.campestris pv. campestris	3370		2017	Brassica sp.. Malaysia	17.98	18.37	18.18	18.175
X.campestris pv. campestris	3371		2053	Brassica oleracea. South Africa	18.08	18.44	18.26	18.26
X.campestris pv. campestris	3372		3044	Brassica sp. France	18.38	18.53	18.46	18.455
X.campestris pv. campestris	3373		3125	Cabbage. Belgium	18.05	18.36	18.21	18.205
X.campestris pv. campestris	3374		3178	Cabbage. Netherlands	18.17	18.57	18.37	18.37
X.campestris pv. campestris	3375	LMG568		Brassica oleracea. UK	18.27	18.57	18.42	18.42
X.campestris pv. campestris	3376	B-525		seed. Japan	18.07	18.19	18.13	18.13
Related strains								
X. c. pv. armoraceae	373	NCPPB 347			20.09	20.29	20.19	20.19

PMA treatment

One milliliter of the grinded cabbage seed or of the Xcc enriched medium was transferred to a 2.1 ml Eppendorf tube and PMA was added to a final concentration of 2.5 µM. Samples were mixed for 15 minutes at room temperature and cooled on ice for 15 minutes in the dark. Tubes were exposed to light by placing a light source (Tungsten Halogen Floodlight 810C) 5 cm above the Eppendorf tubes that were positioned sideways on the ice on a shaking platform. Tubes were exposed to light for 2 minutes and turned every 30 seconds. Subsequently the cells were collected by centrifugation (5 min, 14.000 rpm in an Eppendorf centrifuge). The pellet was washed with TSB by re-suspension in 500ul using a vortex and collecting the cells by centrifugation as described above and re-suspended in 500 ul TSB.

Conclusions on specifications:

- Limit of detection: (10^3 cells using a Ct <35)
- Specificity: (only *Xanthomonas campestris* are detected in 21 positive and 44 negative isolates)
- Justness: (death cells are detected but 7-11 Ct later or 128x to 1000x less sensitive)
- Selectivity: No effect of the matrix tested was observed (PMA, DNA isolation and TaqMan)
- Reproducibility/Repeatability sddev 1 Ct value (Songhong and prof. Wei)
- Robustness (different equipment, and concentrations in comfort zone; incubation times and distance to the lamp are critical)

Outlook

- The current method is successful.
- However:
  - The method works in single tube format
  - Is not compatible with HTP-format of inspection services
  - The differentiation in the detection of viable and death cells could be improved

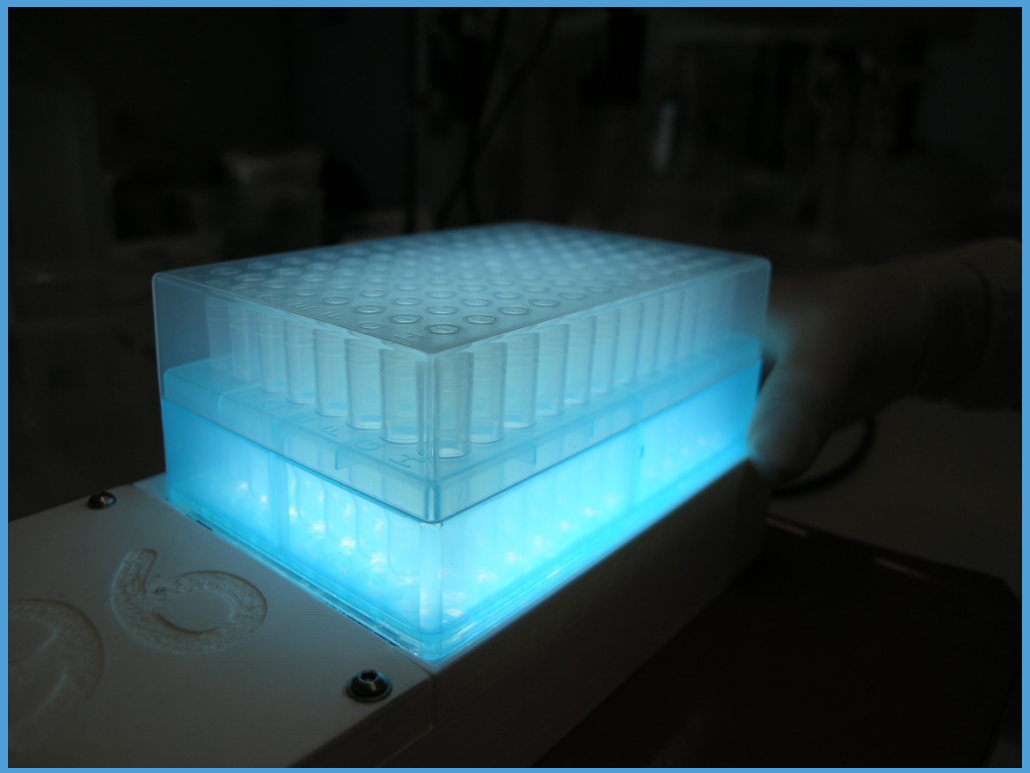
- Technical improvements
  - 96 well format
  - LED-light
  - Extraction procedure fit for automation/robotics

Protocol

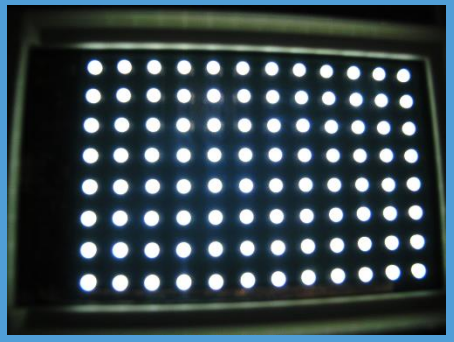
Xcc was grown for 48 hrs on TSA plates and incubated at 25°C. Colonies were transferred to liquid TSB medium and grown overnight after which 5 ml cell suspension was transferred to 45 ml fresh TSB and grown for 5hrs. A 10 ml suspension was made in Ringers. The OD measured and used was 0.32 (1.9x10^8 cfu/ml). Half of the suspension was killed by heating for 30 minutes at 70°C. 100% Living and dead cells were used in the PMA treatment.

PMA treatment

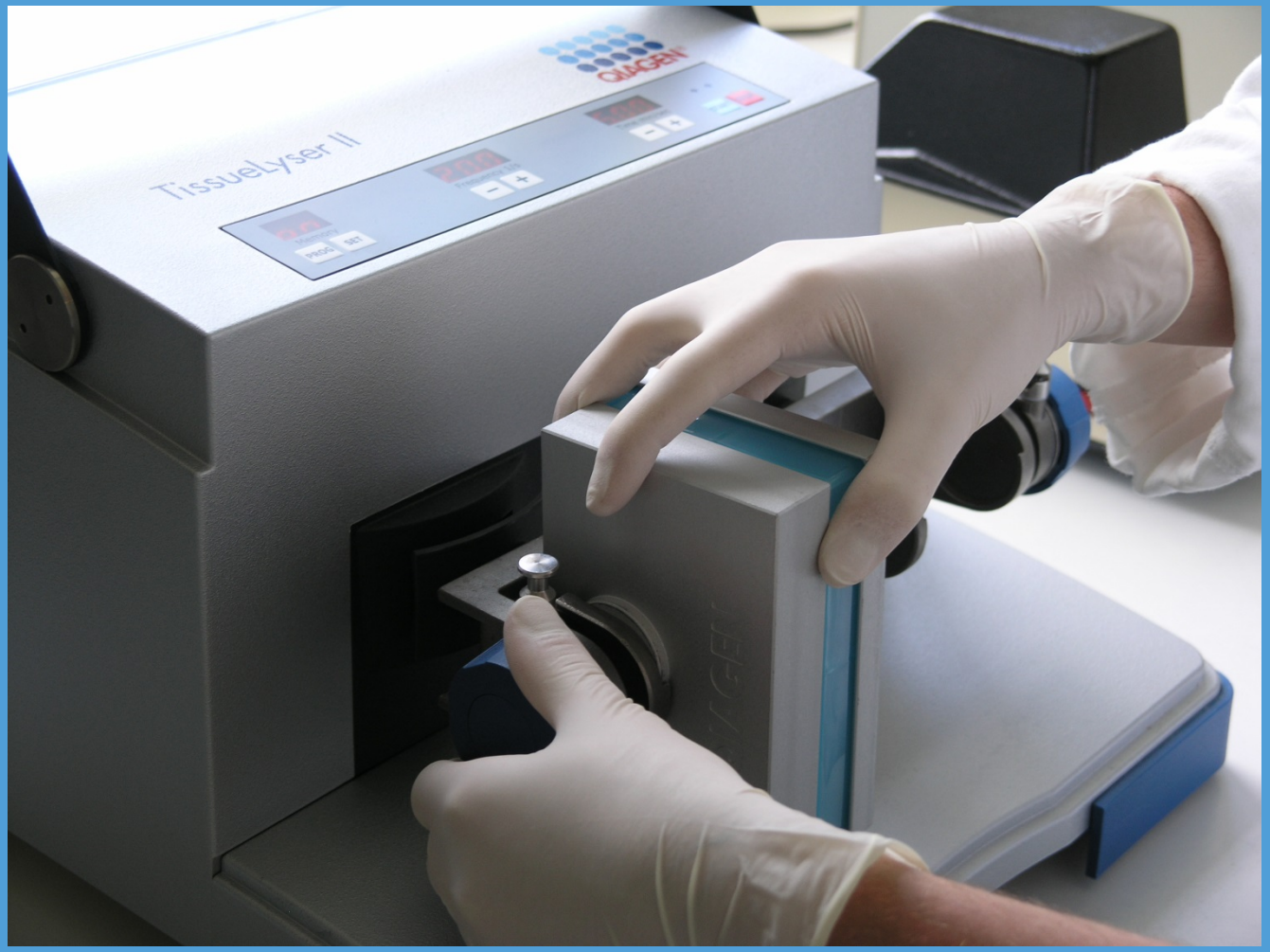
1. Transfer cells to a 96 well plate
2. Add PMA
3. Incubate for 15 minutes
4. Expose to 96 well LED light (customized design) for 15 minutes
5. Start the DNA isolation



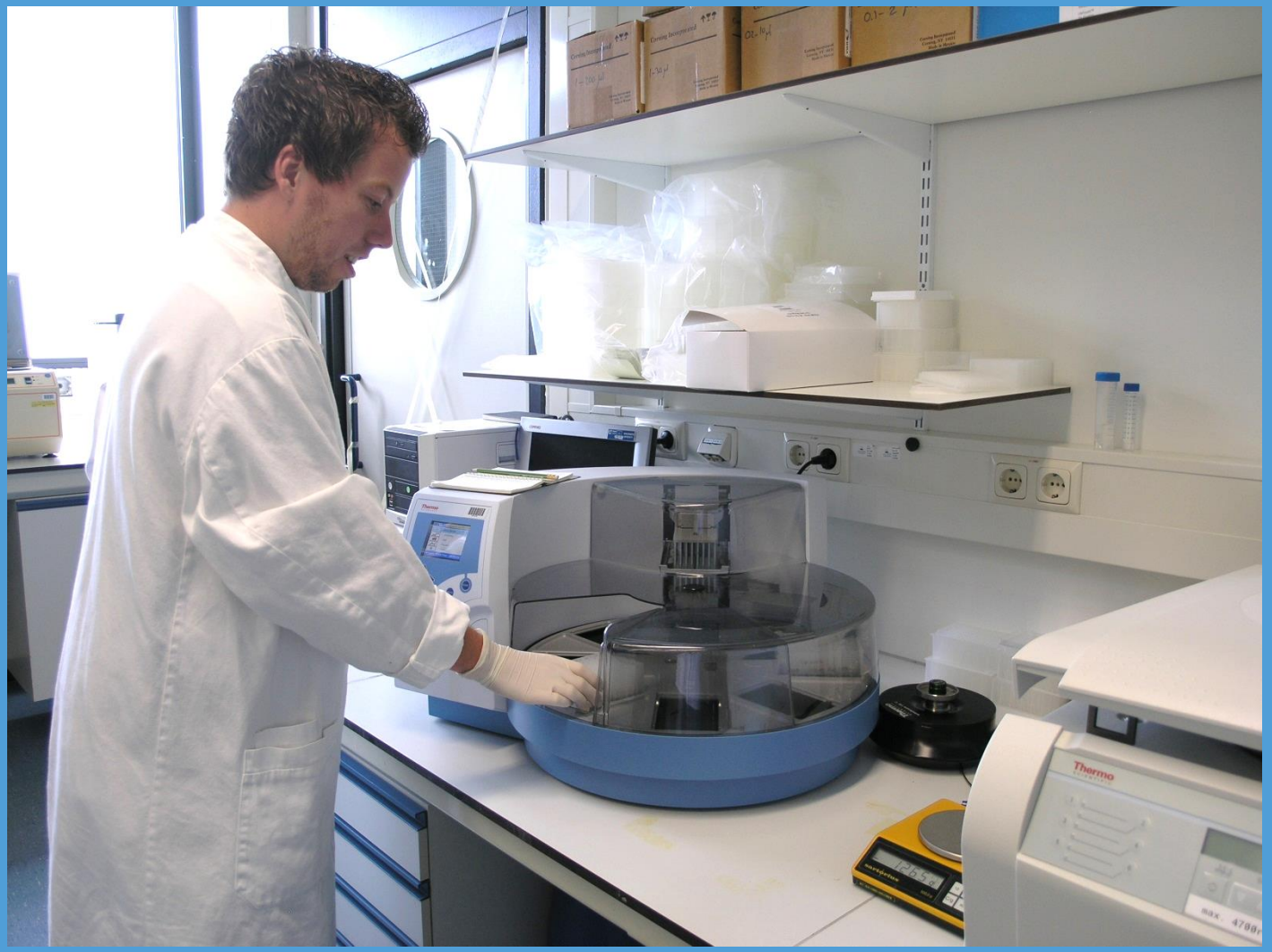
PMA treatment



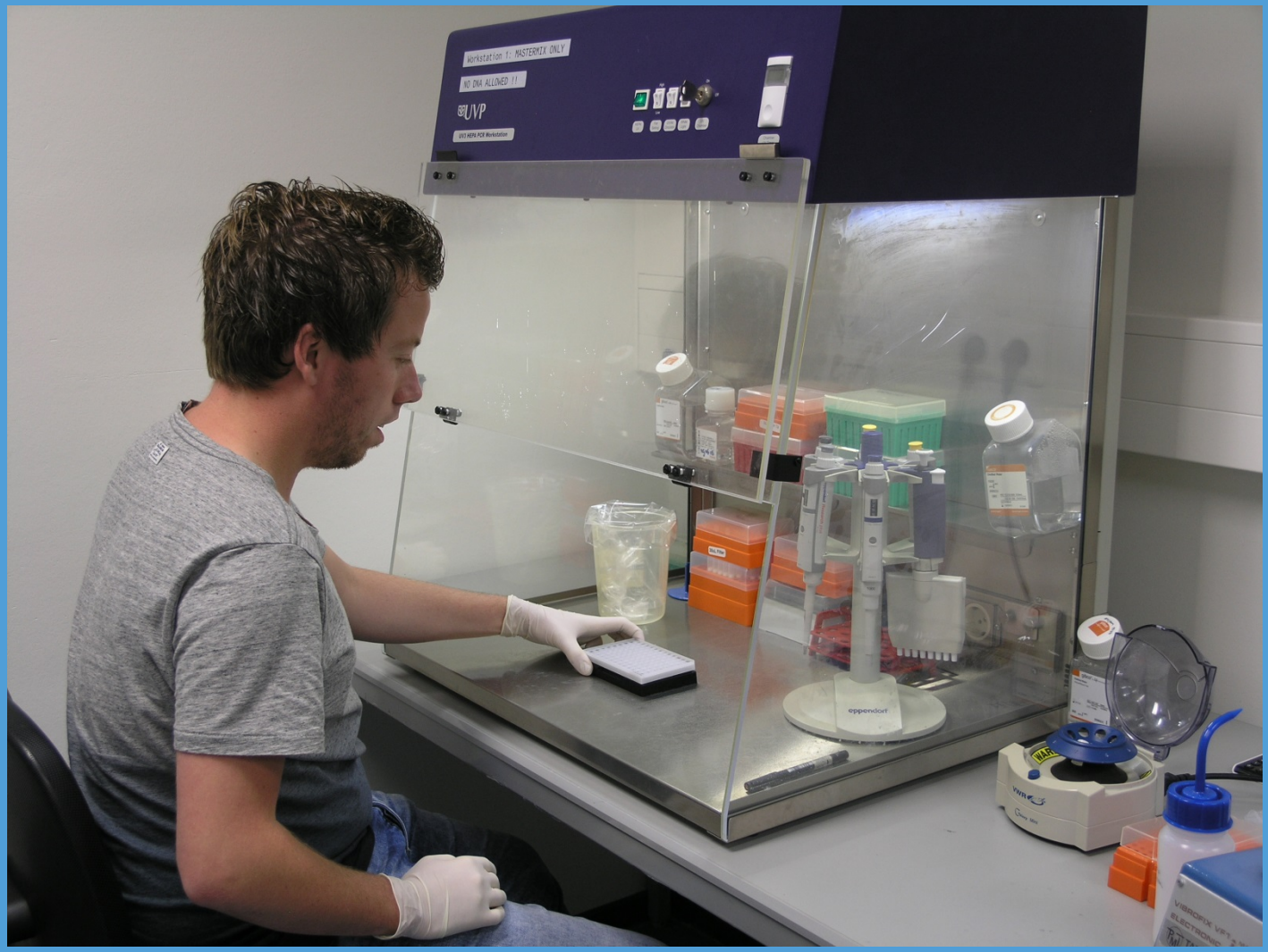
DNA isolation



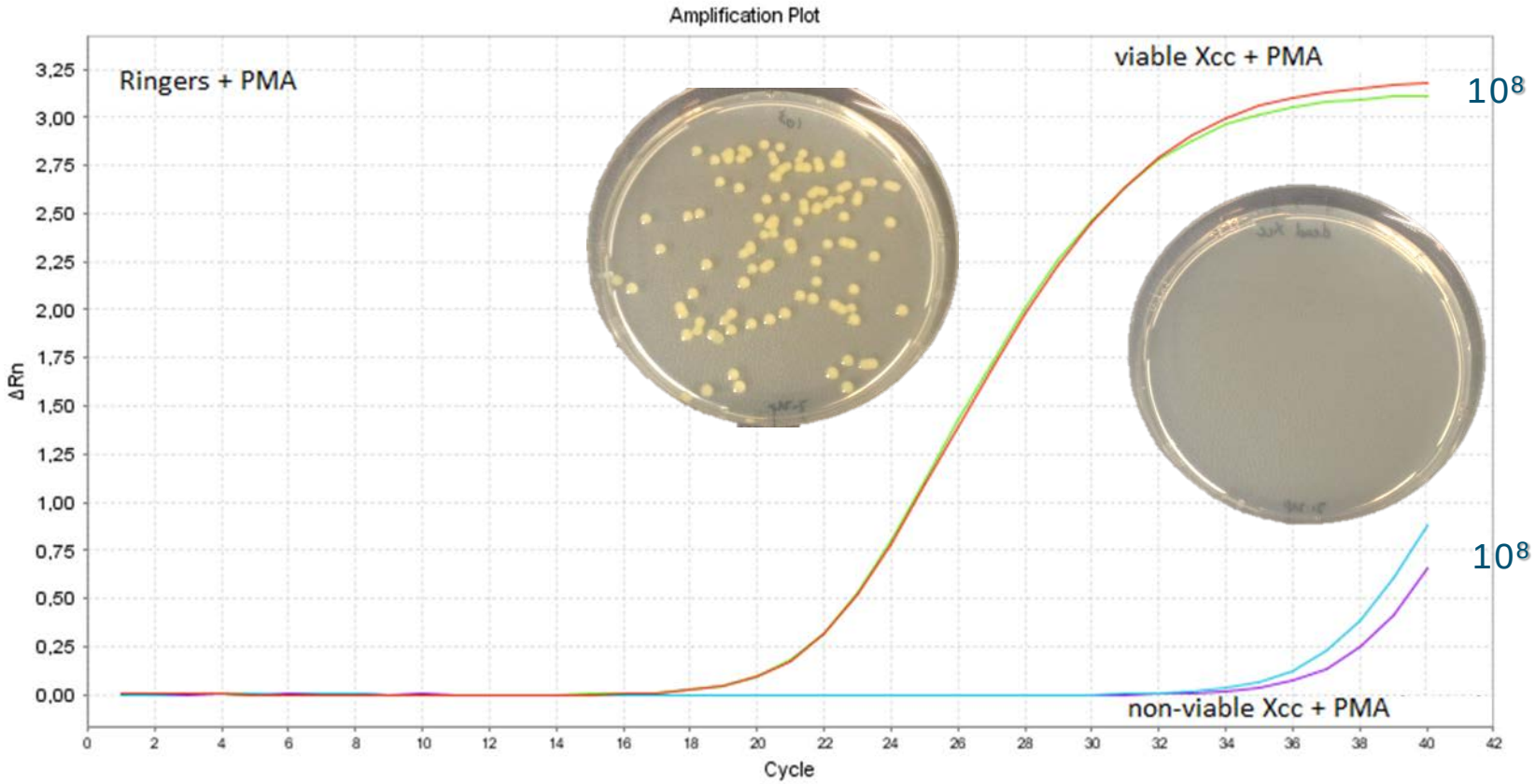
DNA isolation



TaqMan amplification

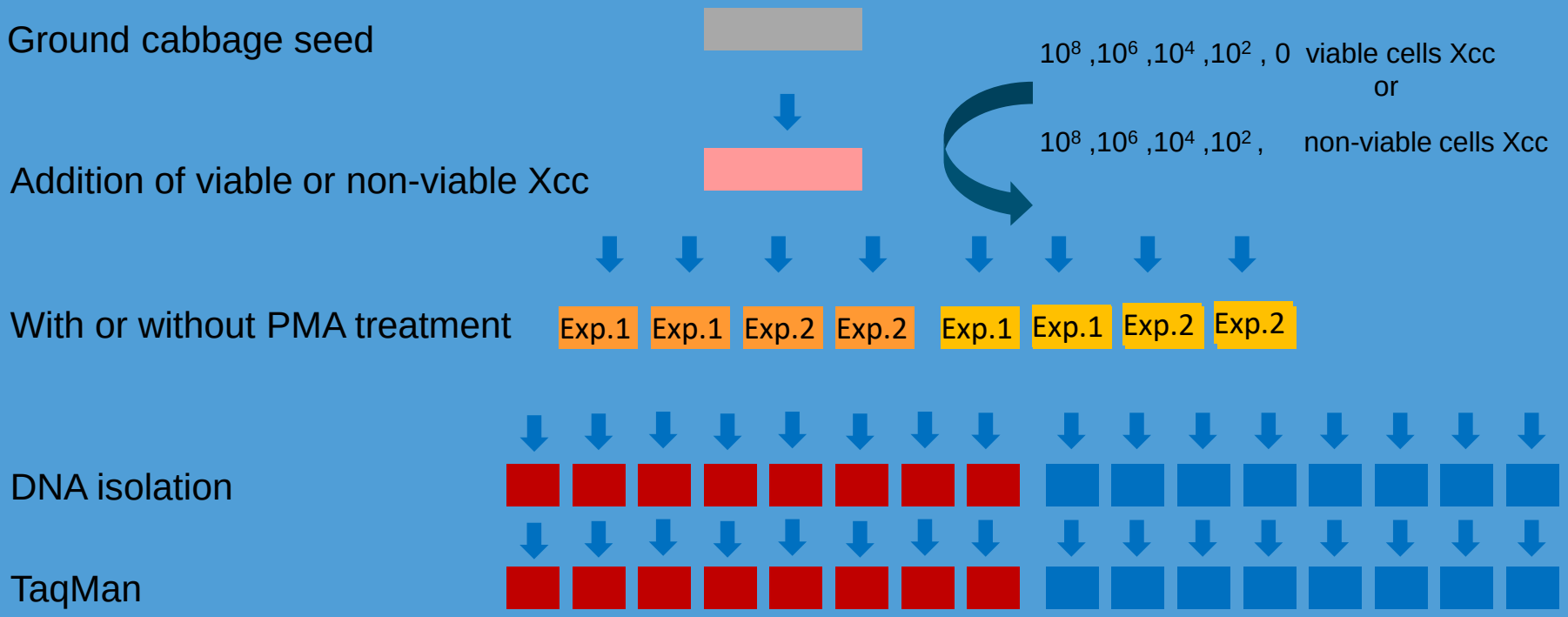


Customized LED PMA TaqMan for Xcc



Delta Ct 12 or more = >5000x

Differentiation between viable and non-viable Xcc by PMA



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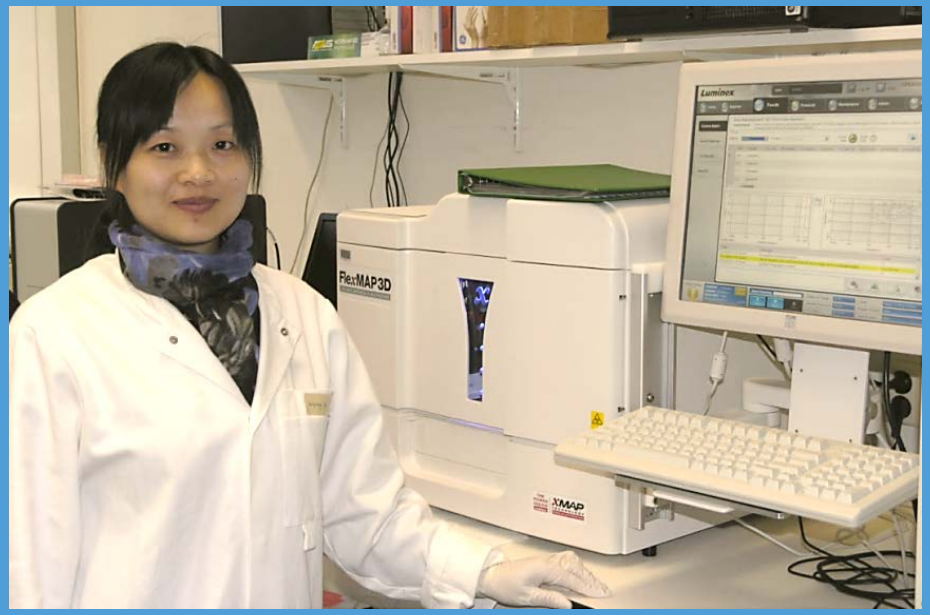
Summary and Outlook

- Validation
  - Specificity
  - Limit of detection
  - Detection range
  - Selectivity
  - Reproducibility
  - Repeatablility
  - Robustness
  - Justness

- Technical improvements
  - 96 well format LED-light
  - Extraction procedure fit for automation/robotics
- Other pathogens
- Other ways to die?

Acknowledgments



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Funding: EU TESTA, PPP death or alive?

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