

Quantitative multiplex detection of plant pathogens using PRI-lock probes and universal, ultra-high-throughput real-time PCR on OpenArrays™

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Introduction

- Detection and identification in disease management strategies
 - Fast
 - Accurate
 - Sensitive
 - **Multiplex**

- Current technologies
 - Low level of multiplexing
 - Low throughput
 - Laborious
 - **Not always quantitative**



Introduction

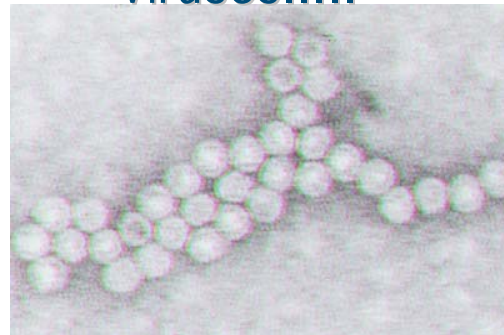
- Many targets to be detected simultaneously

- Pathogens

- Fungi
- Oomycetes
- Bacteria
- Nematodes
- “Viruses”

- Beneficial microorganisms

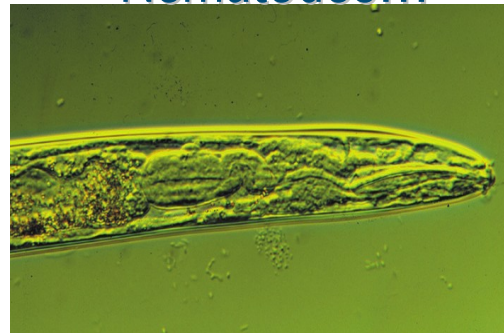
Viruses.....



Fungi.....



Nematodes...



Bacteria...

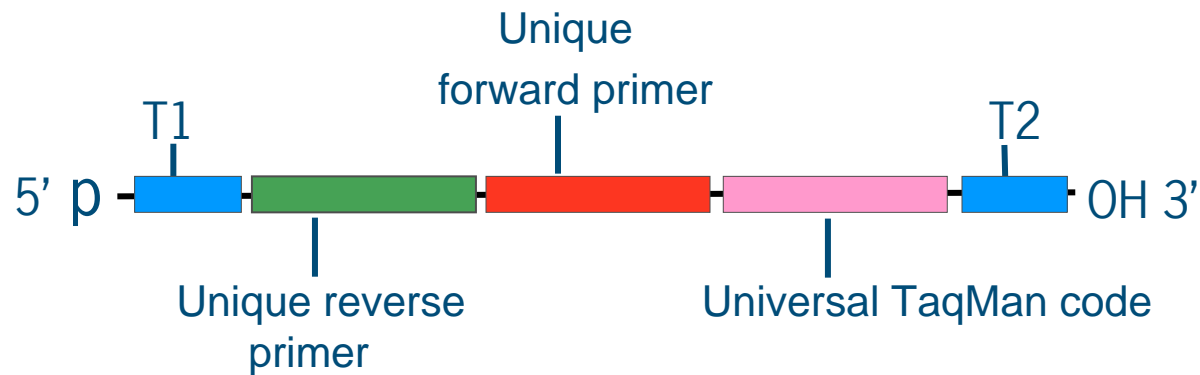


Introduction

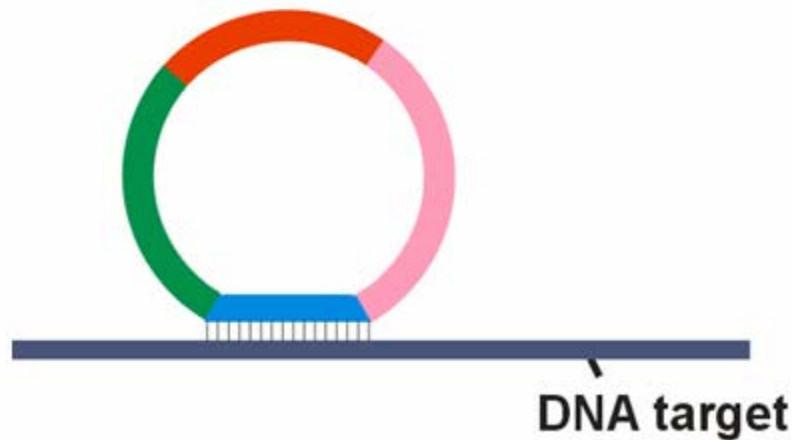
- With multiplex detection there is an increasing interest in quantification of the different organisms
- What are the possibilities for quantitative multiplex detection?
 - Sensitive DNA quantification only with qPCR
- Quantification in PCR for **multiple targets** is theoretically problematic



PRI-Lock Probe Principle



PRI-Lock



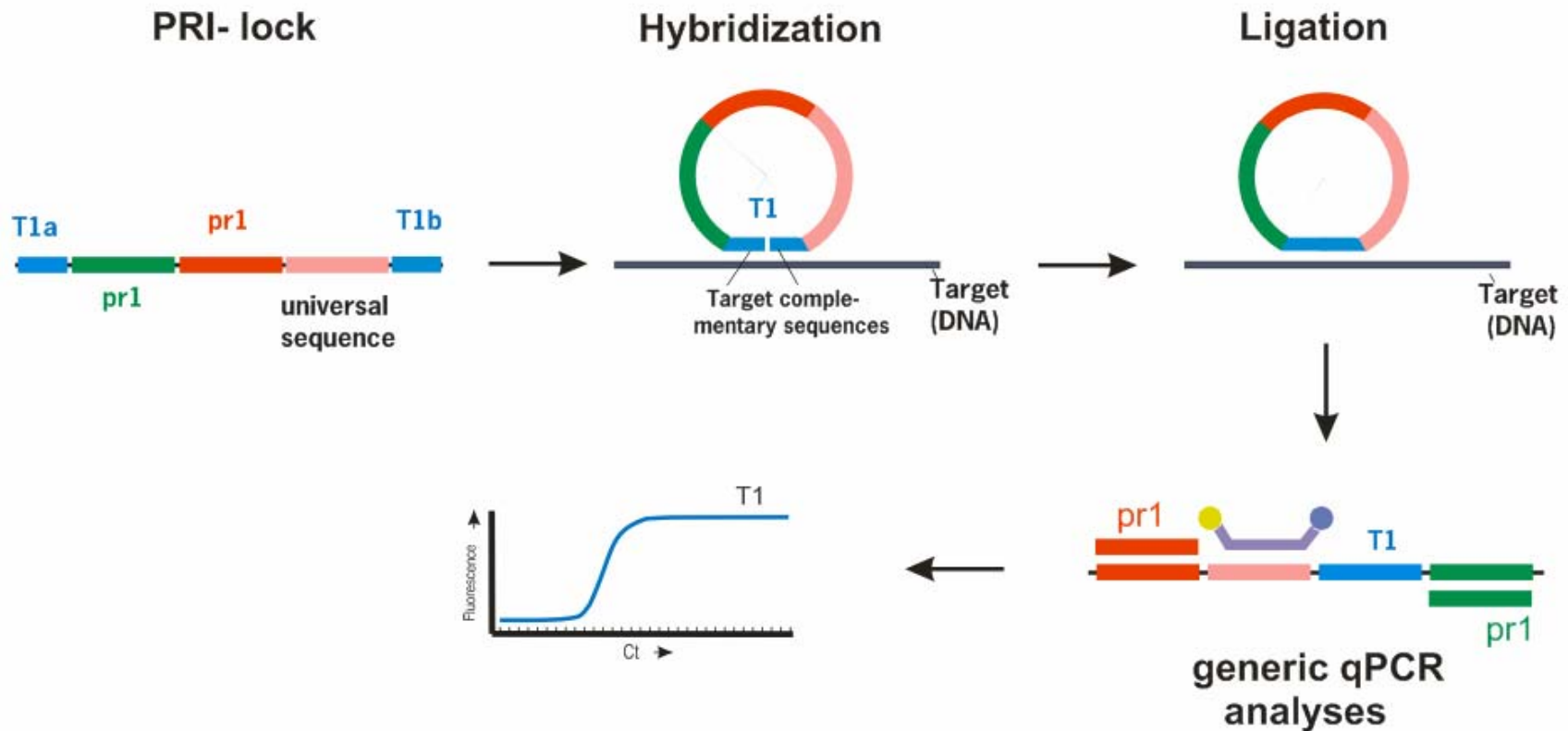
Hybridization
Ligation

Ligation Dependent



Quantitative Multiplex Target Detection

PRI-lock ligation followed by singleplex amplification



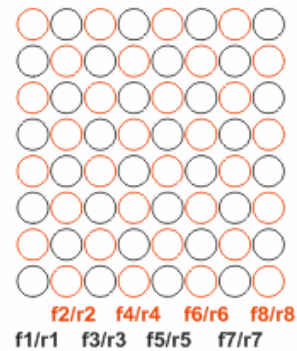
Quantitative Multiplex Target Detection

Multiplex PRI-lock ligation followed by **singleplex** amplification

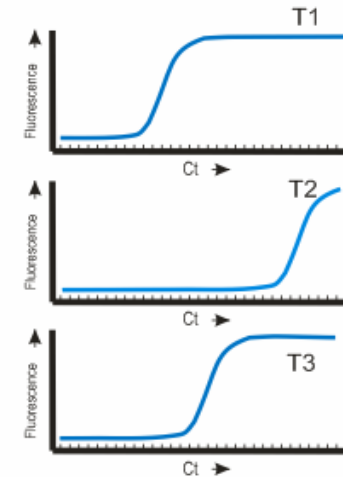
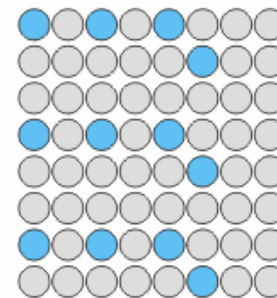
PRI-lock ligation product



Pre-spotted primer combinations



Universal qPCR analyses



Quantitative Multiplex Target Detection

- Testing PRI-lock/primer specificity in a multiplex (96 well) system.

Target template	PRI-lock	<i>Myr. ror.</i> primers	All <i>Phyt.</i> primers	<i>Phyt. inf.</i> primers
<i>Myr. ror.</i>	<i>Myr. ror.</i>	21	40	40
<i>Phyt. inf.</i>	All <i>Phyt.</i>	40	21	40
<i>Phyt. inf.</i>	<i>Phyt. inf.</i>	40	40	22
<i>Myr. ror.</i>	PRI -lock mixture	21	40	40
<i>Phyt. inf.</i>	PRI -lock mixture	40	21	22
<i>Myr. ror.</i> + <i>Phyt. inf.</i>	PRI -lock mixture	21	21	22
No target	PRI -lock mixture	40	40	40

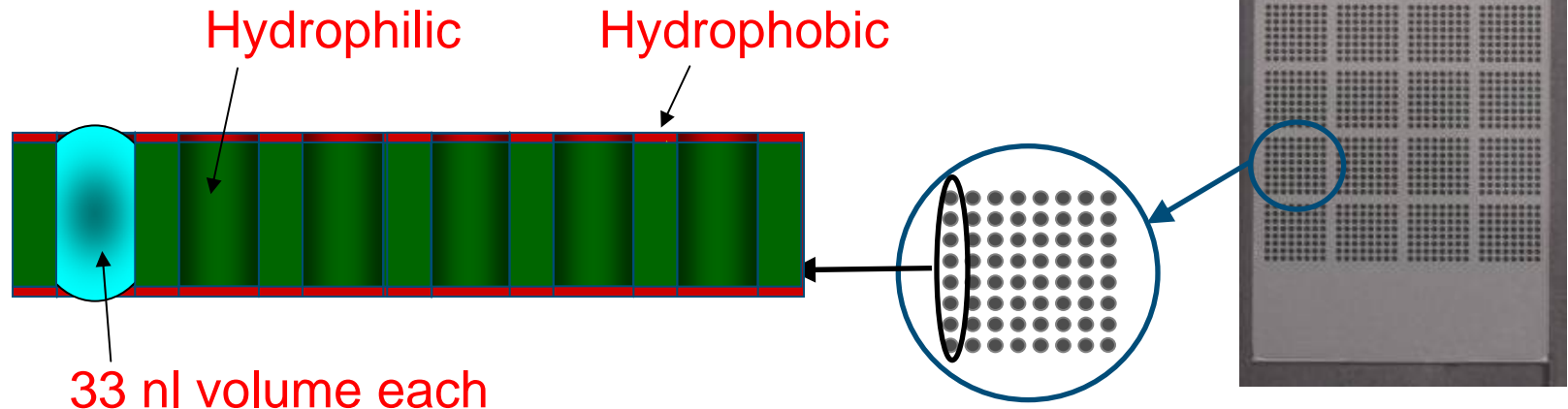
- PRI-lock/primer combinations are specific
- Ct values of the PRI-locks are not influenced by the presence of other templates and PRI-locks in the mixture

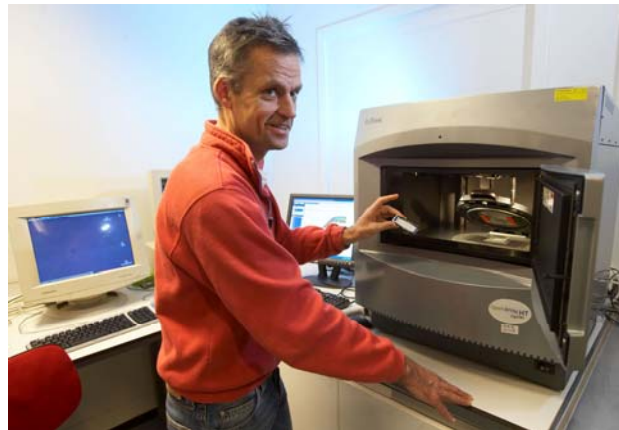
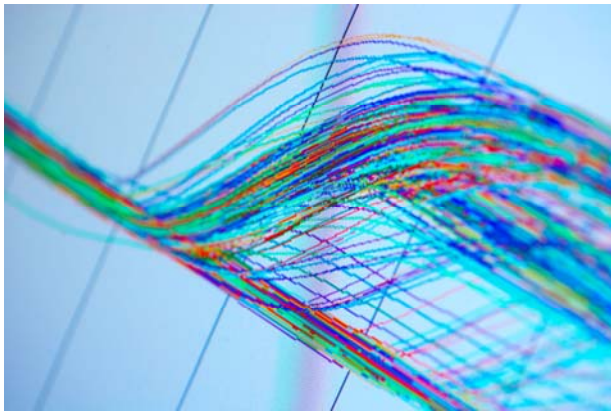
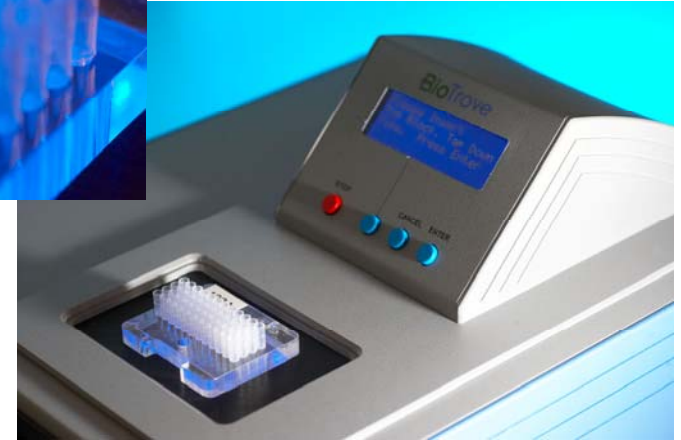
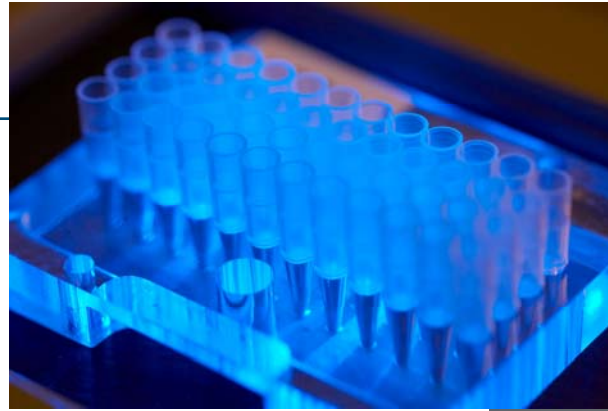
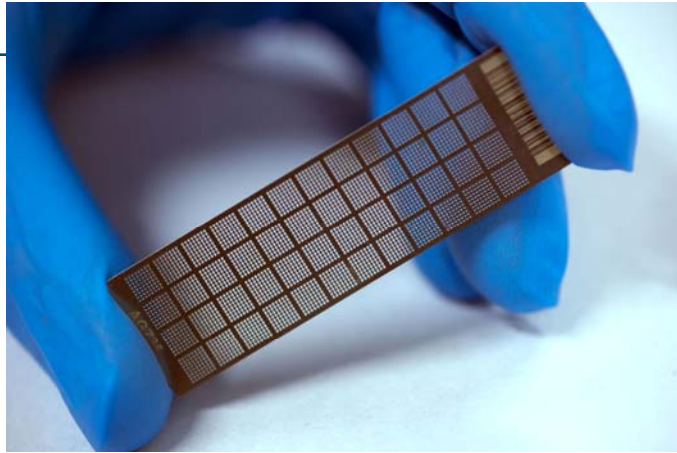


Quantitative Multiplex Target Detection

- 48 subarrays on each plate
- Each subarray consists of 64, 33 nL through-holes in an 8x8 pattern
- Primer pairs spotted in the through-holes
- 3072 reactions in each array

Through-hole cross-section



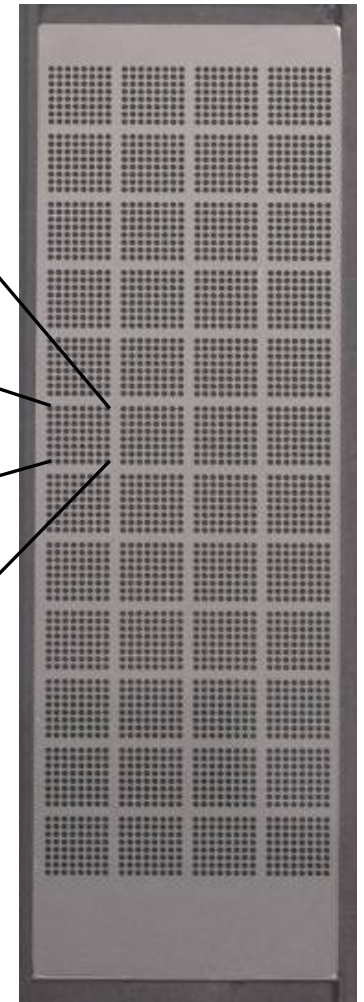


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Quantitative Multiplex Target Detection

<i>Myrothecium roridum</i>
<i>Phytophthora spp.</i>
<i>Phytophthora infestans</i>
<i>Agrobacterium tum.</i>
<i>Meloidogyne hapla</i>
PCR control
Internal Ligation Control ILC
<i>Verticillium dahliae</i>
<i>Verticillium albo-tric.</i>
<i>Rhizoctonia solani</i> AG 4-1
<i>Rhizoctonia solani</i> AG 2-2
<i>Gamma Proteobact.</i>
<i>Erwinia carot.</i>
<i>Rhizoctonia solani</i> AG 4-2
<i>Fusarium oxysporum</i>

	a	b	c	d	e	f	g	h
1	<i>P. inf</i>				<i>P. inf</i>			
2		<i>E. car</i>				<i>E. car</i>		
3	<i>Rh. 4-1</i>	<i>M. hap</i>	ILC		<i>Rh. 4-1</i>	<i>M. hap</i>	ILC	
4	<i>P. spp</i>			<i>V. dal</i>	<i>P. spp</i>			<i>V. dal</i>
5		<i>G.Prot</i>	<i>Rh. 4-2</i>			<i>G.Prot</i>	<i>Rh. 4-2</i>	
6	<i>V.alb-tri</i>	<i>A. tum</i>	PCR	<i>F. oxy</i>	<i>V.alb-tri</i>	<i>A. tum</i>	PCR	<i>F. oxy</i>
7								
8	<i>M. ror</i>	<i>Rh. 2-2</i>			<i>M. ror</i>	<i>Rh. 2-2</i>		



■ OpenArray Lay-Out

- 14 PRI-lock specific primer pairs and a PCR control primer pair are spotted in duplicate



Quantitative Multiplex Target Detection

- Testing PRI-lock/primer specificity in the Biotrove OpenArray platform

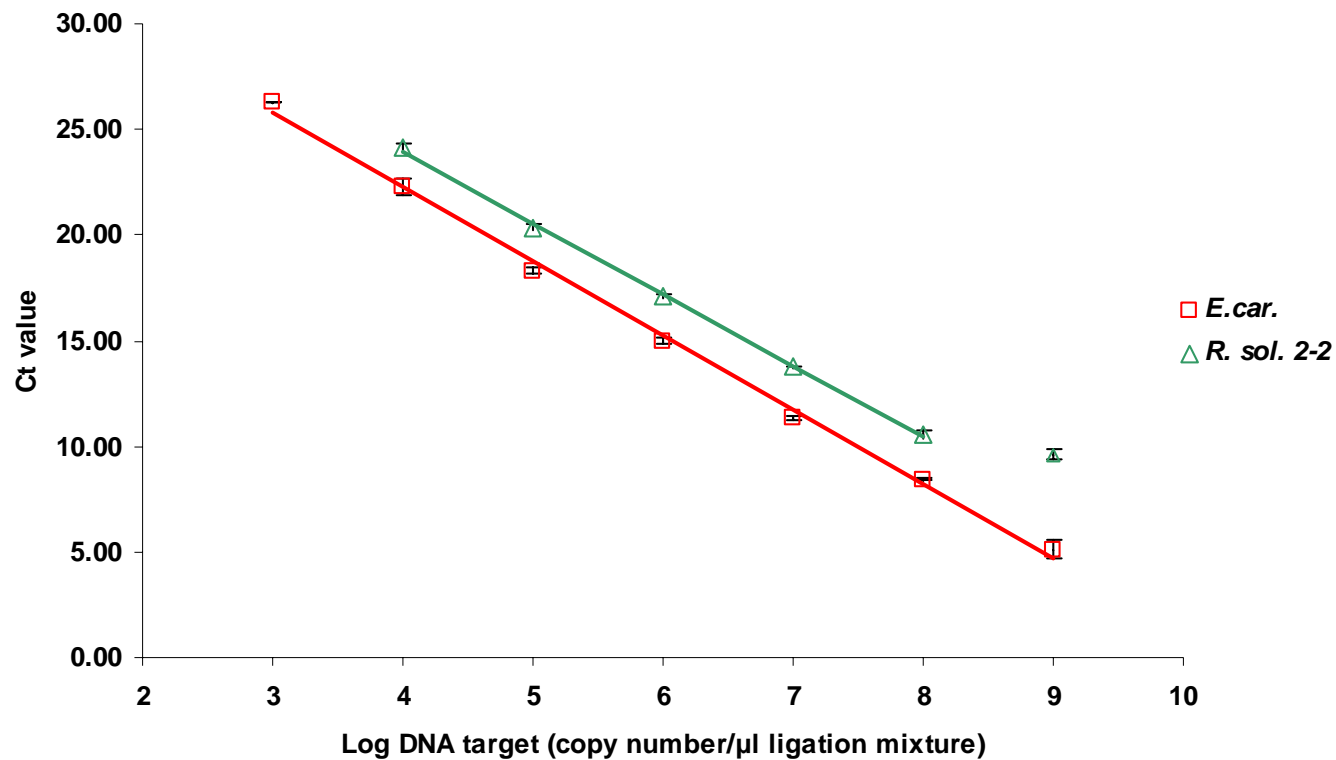
Primer pair	Phyt spp.	<i>P. inf</i>	<i>M. ror.</i>	<i>M. hap.</i>	<i>A.tum.</i>	<i>E. car.</i>	<i>F. oxy.</i>	<i>V. alb./ tri.</i>	<i>V. dah.</i>	<i>R. sol. 4-1</i>	<i>R. sol. 4-2</i>
Targets											
<i>P. infestans</i>	16.4	15.4	--	--	--	--	--	--	--	--	--
<i>A.tumefaciens</i>	--	--	--	--	16.2	--	--	--	--	--	--
<i>A. tum. / F. oxy. / V. dah.</i>	--	--	--	--	16.2	--	17.7	--	15.9	--	--
<i>P. inf. / M. ror. / M. hap. / A. tum. / E. car. / F. oxy. / V. alb. / V. dah. / R. sol. 4-1 / R. sol. 4-2</i>	15.9	15.0	17.0	16.9	15.9	15.4	17.3	18.0	15.8	14.4	18.8
Milli Q water	--	--	--	--	--	--	--	--	--	--	--

- PRI-lock/primer combinations are specific
- Ct values of the PRI-locks are not influenced by the presence of other templates in the mixture



Quantitative Multiplex Target Detection

- Testing the linear quantification range of the PRI-lock system



Quantitative Multiplex Target Detection

- Testing the linear quantification range of the PRI-lock system

PRI-lock probe	Calibration curve formula	R ² value	Linear quantification range (copy number/μl ligation mixture)
<i>Phytophthora</i> spp.	$y = -3.61x + 38.08$	0.995	10 ⁸ - 10 ⁴
<i>P. infestans</i>	$y = -3.31x + 35.74$	0.989	10 ⁸ - 10 ³
<i>R. solani</i> AG 2-2	$y = -3.49x + 37.90$	0.994	10 ⁸ - 10 ⁴
<i>R. solani</i> AG 4-1	$y = -3.49x + 35.78$	0.994	10 ⁹ - 10 ³
<i>R. solani</i> AG 4-2	$y = -3.35x + 39.16$	0.998	10 ⁸ - 10 ⁴
<i>F. oxysporum</i>	$y = -3.47x + 39.06$	0.990	10 ⁸ - 10 ⁴
<i>M. roridum</i>	$y = -3.15x + 36.60$	0.984	10 ⁹ - 10 ⁴
<i>V. dahliae</i>	$y = -3.42x + 36.27$	0.996	10 ⁸ - 10 ³
<i>V. alboatrum</i> / <i>V. tricornutum</i>	$y = -3.40x + 38.80$	0.991	10 ⁸ - 10 ⁴
<i>M. hapla</i>	$y = -3.39x + 38.06$	0.989	10 ⁸ - 10 ⁴
<i>E. carotovora carotovora</i>	$y = -3.51x + 36.32$	0.997	10 ⁹ - 10 ³
<i>A. tumefaciens</i>	$y = -3.36x + 36.51$	0.995	10 ⁸ - 10 ³
<i>G. Proteo</i> bacterial spp	$y = -3.51x + 38.10$	0.995	10 ⁸ - 10 ⁴

(x) = the Log copy number target input/μl ligation mixture. (y) = C_T value.

- The linear range of detection is between 5 and 7 log scales



Quantitative Multiplex Target Detection

■ Advantages:

- Quantitative
- High specificity
- Single and multiplex target detection independent
- Target recognition and amplification independent
- Universal TaqMan / SYBRGreen PCR conditions
- High-throughput
- Low background

■ Disadvantages:

- Low copy numbers of ligated PRI-locks in nanoliter wells



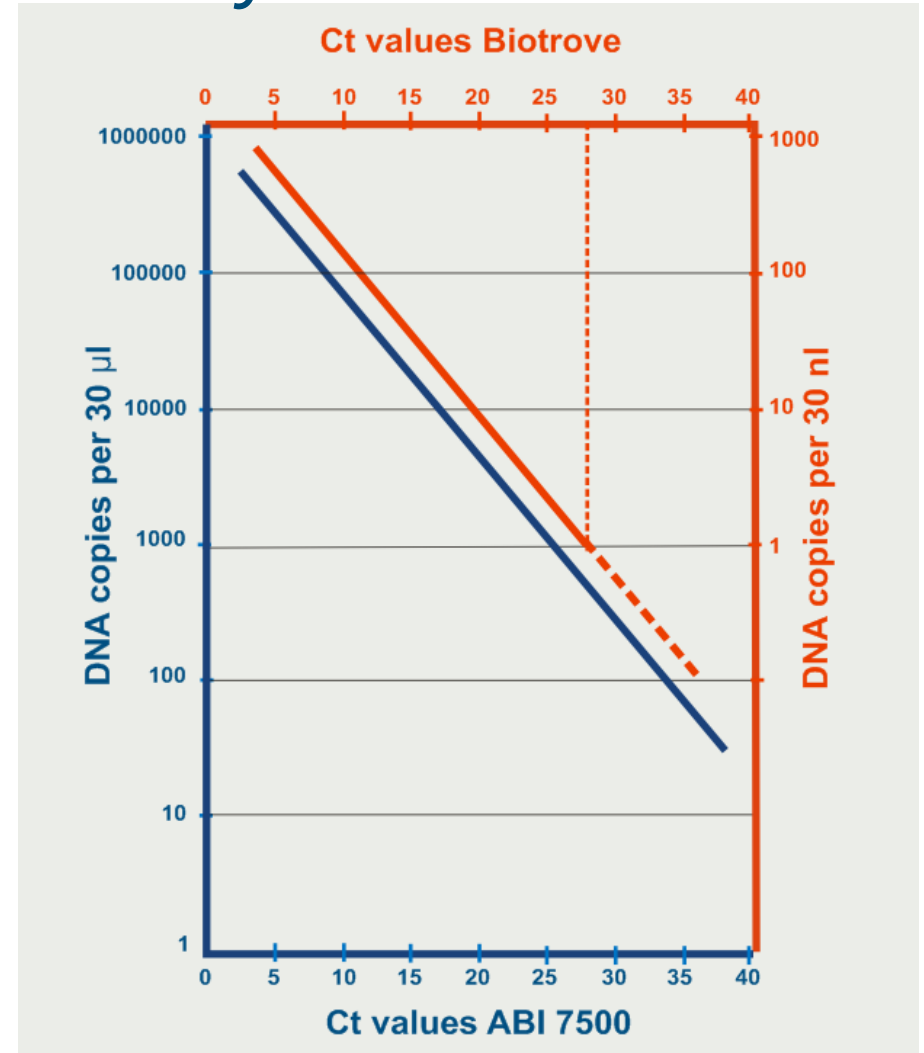
Comparison of qPCR sensitivity

Traditional (ABI 7500)

VS

Thru-hole Biotrove

30 μ L vs 30 nL



Current / Future Developments

- Current detection sensitivity 10^3 targets/ μ l ligation mixture

Only 0.04% of the total ligation mixture in each nanoliter PCR reaction

- To increase detection sensitivity, the PCR target input per nanoliter reaction should be increased

Strategy: Pre-amplification of the ligated PRI-lock probes

PCR based amplification leads to amplification biases, changing the ratios between the targets in the original biological sample

Non-PCR based, linear amplification strategies minimize amplification biases (Phi-29)



Quantitative Multiplex Target Detection

- Sensitive detection system
- Broader dynamic detection range

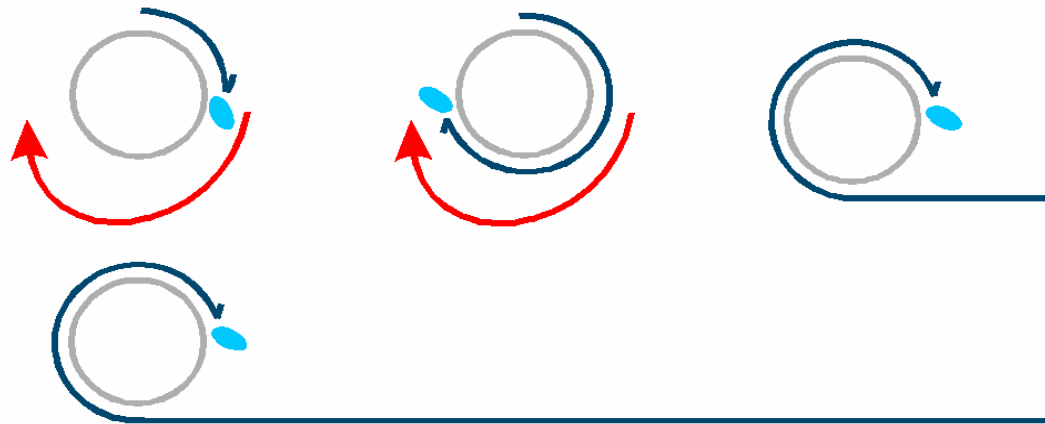
Development of a **linear** pre-amplification step to increase target input for Biotrove OpenArray



Current / Future Developments

- Rolling Circle Amplification (RCA)

RCA is used by bacteriophages for replication of their **circular** genome



One primer is targeted to a circular molecule

Linear amplification

Yield after RCA at 37°C with strand-displacing Phi-29 : $10^3 - 10^4$ copies



Conclusions

- Working PRI-lock probe multiplex detection system:
 - Currently 30 PRI-lock probes
- Padlock probes for linear pre-amplification
- Fields of application:
 - Multiplex quantitative target (pathogen) detection
 - Microbial community analysis
- Instrumentation:
 - Standard real-time PCR machines
 - Nanoliter 'PCR arrays' – OpenArray platform (BioTrove)



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Thank you for your attention

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