Quantitative multiplex detection of (plant) pathogens, including *Phytophthora* species, based on PRI-lock probe technology and the OpenArray platform

*Phytophthora, Pythium* workshop ICPP, August 2008
Peter Bonants, Ronald van Doorn, Odette Mendes, Richard van Hoof and Cor Schoen
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
Introduction

- For 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.
Padlock Probe Principle

Padlock probe

Hybridization

Ligation

DNA target

Amplified product

cZip-Code sequence

Hybridization to array

PCR amplification

Labeled primer

Non labeled primer

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Padlock Probe Principle

target A + Probe A → hybridization and ligation → match = circularized probe

target B + Probe A → mismatch = not circularized probe
Multiplex detection in recirculation water

- *Phytophthora* sp
- *P. nicotianae*
- *A. tumefaciens*
- *V. dahliae*
Phytophthora micro-array

- PLP for *Phytophthora* sp
- PLPs for different *Phytophthora* species
- ligation with mixture of PLPs
- amplification with generic primers
- detection on *Phytophthora* micro-array
PRI-lock Probe Principle

PRI-lock

Hybridization
Ligation
Ligation Dependent

5’ p

Unique reverse primer

Universal TaqMan code

OH 3’

T1

T2
Quantitative Multiplex Target Detection

PRI-lock ligation followed by singleplex amplification
Quantitative Multiplex Target Detection

Multiplex PRI-lock ligation followed by singleplex amplification

[Diagram showing PRI-lock ligation product, pre-spotted primer combinations, and universal qPCR analyses]
Quantitative Multiplex Target Detection

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

<table>
<thead>
<tr>
<th>Target template</th>
<th>PRI-lock probe</th>
<th>Myr. ror. primers</th>
<th>All Phyt. primers</th>
<th>Phytophthora primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myrothecium roridum</td>
<td>M. roridum</td>
<td>21</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Phytophthora infestans</td>
<td>All Phytophthora</td>
<td>40</td>
<td>21</td>
<td>40</td>
</tr>
<tr>
<td>P. Infestans</td>
<td>P. infestans</td>
<td>40</td>
<td>40</td>
<td>22</td>
</tr>
<tr>
<td>M. roridum</td>
<td>PRI-lock mixture</td>
<td>21</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>P. infestans</td>
<td>PRI-lock mixture</td>
<td>40</td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td>M. ror. + P. inf.</td>
<td>PRI-lock mixture</td>
<td>21</td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td>No target</td>
<td>PRI-lock mixture</td>
<td>40</td>
<td>40</td>
<td>40</td>
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</table>

- 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

Real-time quantification in a Biotrove ‘PCR array’
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
- Loading by capillary action

**Through-hole cross-section**

```
Hydrophilic

Hydrophobic
```

33 nl volume each
Quantitative Multiplex Target Detection

Loading three OpenArray plates to an OpenArray Cycler
Quantitative Multiplex Target Detection

Real-time quantification in a Biotrove ‘PCR array’

TABLE:

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>SS F</th>
<th>P. alf</th>
<th>M. asc</th>
<th>M. halo</th>
<th>C. spal</th>
<th>E. cor</th>
<th>R. rov 3</th>
<th>V. allier</th>
<th>V. diabl</th>
<th>R. rov 4</th>
<th>G. Protea</th>
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</thead>
<tbody>
<tr>
<td>Targets</td>
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<tr>
<td>P. alf M. asc</td>
<td>17.2</td>
<td>17.8</td>
<td>18.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>M. halo C. spal E. cor</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>15.2</td>
<td>15.4</td>
<td>17.4</td>
<td>ND</td>
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<tr>
<td>V. allier R. rov 4 R. rov 5 V. diabl</td>
<td>24.4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>17.3</td>
<td>19.4</td>
<td>17.4</td>
<td>19.2</td>
<td>ND</td>
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<tr>
<td>P. alf M. halo M. asc V. diabl V. allier V. diabl</td>
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<td>17.3</td>
<td>18.5</td>
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<td>18.1</td>
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# Quantitative Multiplex Target Detection

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

<table>
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<tr>
<td><strong>P. infestans</strong></td>
<td></td>
<td>16.4</td>
<td>15.4</td>
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<tr>
<td><strong>A. tumefaciens</strong></td>
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<td>--</td>
<td>--</td>
<td>--</td>
<td>16.2</td>
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</tr>
<tr>
<td><strong>A. tum. / F. oxy. / V. dah.</strong></td>
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<td>--</td>
<td>--</td>
<td>--</td>
<td>16.2</td>
<td>--</td>
<td>17.7</td>
<td>--</td>
<td>15.9</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td><strong>P. inf. / M. ror. / M. hap. / A. tum. / E. car. / F. oxy. / V. alb. / V. dah. / R. sol. 4-1 / R. sol. 4-2</strong></td>
<td>15.9</td>
<td>15.0</td>
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<td>17.3</td>
<td>18.0</td>
<td>15.8</td>
<td>14.4</td>
<td>18.8</td>
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<tr>
<td><strong>Milli Q water</strong></td>
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- Ct values of the PRI-locks are not influenced by the presence of other templates in the mixture
Quantitative Multiplex Target Detection

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

Disadvantages:
- Low copy numbers of ligated PRI-locks in nanoliter wells
Conclusions

- **Working PRI-lock probe multiplex detection system:**
  - Currently 30 PRI-lock probes

- **Fields of application:**
  - Multiplex quantitative target (pathogen) detection
  - Microbial community analysis
  - Multiplex quantitative gene expression analysis

- **Instrumentation:**
  - Standard real-time PCR machines
  - Nanoliter ‘PCR arrays’ – OpenArray platform (BioTrove)
Acknowledgements

- Plant Research International B.V.
  Wageningen, The Netherlands
  - Marianna Szemes
  - Carolien Zijlstra
  - Richard van Hoof
  - Ronald van Doorn
  - Els Nijhuis
  - Odette Mendez
  - Cor Schoen

- Isogen Life Science
  IJsselstein, The Netherlands
  - Nicole Wellens

- BioTrove Inc.
  Woburn, USA
  - Jonathan Schimmel
  - Elen Ortenberg

References

- Szemes et al. Nucleic Acids Research, 2005, Vol. 33, No. 8 e70
- Van Doorn et al. BMC Genomics, 2007, 8:276
Thank You For Your Attention