Improved Detection Techniques
for Food Borne Pathogens – Use
of Multi-Plex Detection Platforms

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Multiplex PCR
- Detect multiple target DNA sequences
- Multiple organism detection
- Reduction of false positives and false negatives
- Limited number of amplicons for simultaneous analysis
  - Electrophoresis limitation
  - Fluorophore pairs

Multiplex PCR primer and beacon binding sites in target genes from L. monocytogenes, Salmonella spp., and E. coli O157:H7

<table>
<thead>
<tr>
<th>Gene</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>spvC</td>
<td>190</td>
</tr>
<tr>
<td>sipC</td>
<td>190</td>
</tr>
<tr>
<td>invA</td>
<td>150</td>
</tr>
<tr>
<td>pafA</td>
<td>150</td>
</tr>
<tr>
<td>spa</td>
<td>150</td>
</tr>
<tr>
<td>ipaA</td>
<td>150</td>
</tr>
<tr>
<td>pfrA</td>
<td>150</td>
</tr>
<tr>
<td>eaeA</td>
<td>150</td>
</tr>
<tr>
<td>hlyA</td>
<td>150</td>
</tr>
<tr>
<td>rfbE</td>
<td>150</td>
</tr>
<tr>
<td>stxB</td>
<td>150</td>
</tr>
<tr>
<td>inlA</td>
<td>150</td>
</tr>
<tr>
<td>iap</td>
<td>150</td>
</tr>
<tr>
<td>hlyA</td>
<td>150</td>
</tr>
<tr>
<td>prfA</td>
<td>150</td>
</tr>
<tr>
<td>inlA</td>
<td>150</td>
</tr>
</tbody>
</table>

Analysis of multiplex PCR reactions
- Capillary Electrophoresis
- ROX standard
- JOE, FAM and NED tagged Primers for PCR

Multiplex PCR
- One reaction with 12 genes
- Three reactions with four genes for each pathogen

Capillary electrophoretic fragment analysis for multiplex PCR with approximately 4x10^3 copies of genomic DNA in 25 µL from E. coli O157:H7, S. Enteritidis, and L. monocytogenes.
Fragment analysis using capillary electrophoresis of a positive multiplex PCR from S. Enteritidis inoculated lettuce.

Multiplex Amplicon detection using Microarrays

Beacon Concept

Beacon structure and attachment chemistry
Results from 3 different microarrays hybridized with control (no DNA, top row), 1.0–1.5 ng/μL of DNA (1:50 dilution of the PCR products, middle row), and 5–10 ng/μL of DNA (1:10 dilution of PCR products, bottom row). Note that in the absence of target DNA, the microarray features appear red due to FRET signaling (B), and in the presence of target DNA the microarray features appear green due to the absence of FRET signaling (C).

<table>
<thead>
<tr>
<th>Target DNA concentration</th>
<th>eaeA</th>
<th>hlyC</th>
<th>rfbE</th>
<th>stxI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (none)</td>
<td>0.30 ± 0.03</td>
<td>0.10 ± 0.01</td>
<td>0.20 ± 0.02</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>1.0–1.5 ng/μL</td>
<td>1.9 ± 0.02</td>
<td>0.30 ± 0.03</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>5–10 ng/μL</td>
<td>12.8 ± 1.4</td>
<td>5.2 ± 0.6</td>
<td>7.3 ± 0.8</td>
<td>6.2 ± 0.7</td>
</tr>
</tbody>
</table>

Prototype Detector

The picture of the working biosensor prototype with the cover open. All components of the system are housed in a dark box. The green line in the picture is following the path of the green exciting laser (532 nm) which is hitting the target MB spot.


Green/red fluorescence ratios of beacon probes upon target DNA hybridization measured with prototype detection platform

<table>
<thead>
<tr>
<th>Target DNA concentration</th>
<th>Green/red fluorescence ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (none)</td>
<td>0.24 ± 0.03</td>
</tr>
<tr>
<td>0.5–1.0 ng/μL</td>
<td>0.23 ± 0.03 (0.96 ± 0.17)</td>
</tr>
<tr>
<td>5–10 ng/μL</td>
<td>0.53 ± 0.06 (2.21 ± 0.40)</td>
</tr>
<tr>
<td>50–100 ng/μL</td>
<td>0.86 ± 0.09 (3.58 ± 0.84)</td>
</tr>
</tbody>
</table>

In parentheses: degree of increase of the measured green/red fluorescence ratio compared to that of the control probe.

Hybridization Chamber PCR and Detection

(End-Point Detection)

Hyb Chamber contains:
1) Sample
2) PCR mix
3) Primers

95C (30 sec)
60C (30 sec)
72C (30 sec)
25C (1-2 min)

30 Cycles
Detect Fluorescence

Hybridization Chamber PCR and Detection

(Inter-Cycle Detection)

95C (30 sec)
60C (30 sec)
72C (30 sec)
25C (1 min)

30 Cycles
Detect Fluorescence

Hybridization Chamber PCR and Detection

Microarray & System can be re-used if QC is OK

Microscope slide sandwich with gasket

Emission/Fluorescence Detection

Hybridization Chamber (200 uL)

Peltier Block (thermocycles)

Gasket

Detect Fluorescence
Hybridization Chamber PCR and Detection

Use of mRNA to detect live cells
- mRNA’s high turnover rate makes it an ideal marker for the detection of viable cells.
- Transcription-PCR (RT-PCR) is currently being used to detect non-culturabable organisms such as viruses, and can also be used to detect presence of mRNA in prokaryotic and eukaryotic cells.
- RT-PCR can detect viable cells without enrichment and therefore eliminates the negative signal that would otherwise be considered positive when a bacterium has been induced into a viable but non-culturable state (VBNC) after being exposed to stressful conditions.

Live vs Dead cont.

To create a more robust RT-PCR reaction norepinephrine was incorporated in assays.

Live vs Dead cont.

<table>
<thead>
<tr>
<th>Ladder RNA</th>
<th>0</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
</table>

Cells exposed to increasing concentration of chlorine dioxide

Summary

Collective twelve gene mPCR was able to yield a detectable signal with 4E+03 copies of template chromosomal DNA.

Array detection of PCR products using the beacon approach was successful.

Prototype detection device has been completed and is currently being evaluated.

Currently developing peltier hybridization chamber for interfacing with detection platform (can be expanded into multiple formats).

mRNA may be problematic in the discrimination of live vs dead cells.

Acknowledgments

- Center for Food Safety Engineering, Purdue University
- Purdue core sequencing facility
- USDA/ARS cooperative agreement 1935-42000-035
Capture and detection of E. coli O157:H7 using polymer-immobilized phage

Quantification of Phage
- Serial dilutions of $\Phi V10\Delta recET::kan^8\text{cobA}$
- Plaque and lysogen assays

Lysogen Assay
- The assay consisted of mixing $\Phi V10\ text{kan:}\text{cobA1}$ with E. coli O157:H7 followed by incubation at room temperature for 1 hour
- Solutions were then plated on agar plates containing kanamycin and incubated overnight (18h) at 37°C
- Lysogens containing $\Phi V10\ text{kan:}\text{cobA1}$ were enumerated.
- A ratio of phage per cells was determined to provide an estimate of the detection limit and is shown

Lysogen Detection Assay

<table>
<thead>
<tr>
<th>Phage concentration (PFU/ml)</th>
<th>E.coli O157:H7 cell concentration (CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Phi V10\text{kan:}\text{cobA1}$ (plaque forming units)</td>
<td>$10^6$</td>
</tr>
<tr>
<td>$10^6$ Actual phage</td>
<td>positive</td>
</tr>
<tr>
<td>$10^5$ Actual phage</td>
<td>positive</td>
</tr>
<tr>
<td>$10^4$ Actual phage</td>
<td>positive</td>
</tr>
<tr>
<td>$10^3$ Actual phage</td>
<td>positive</td>
</tr>
</tbody>
</table>
Current status

- Formed and Incorporated Intelliphage
- Utility patent has been filed
- Secured funding to further develop and optimize prototype (Trask)