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

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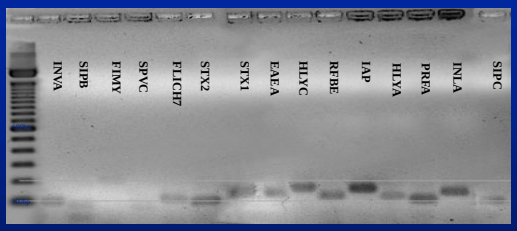
- **Multiplex PCR**
  - **Detect multiple target DNA sequences**
  - **Multiple organism detection**
  - **Reduction of false positives and false negatives**
  - **Limited number of amplicons for simultaneous analysis**
    - ***Electrophoresis limitation***
    - ***Fluorophore pairs***

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



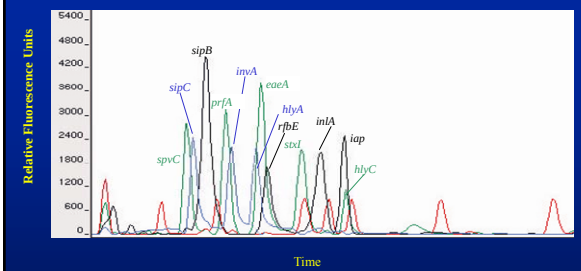
PCR of multiplex PCR primer pairs

Analysis of multiplex PCR reactions

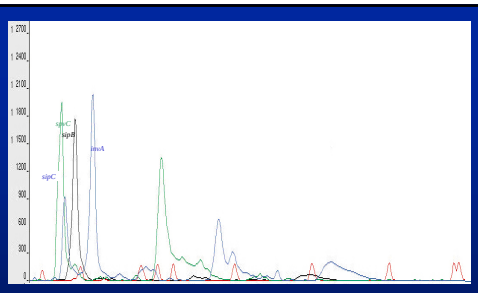
- **Capillary Electrophoresis**
- **ROX standard**
- **JOE, FAM and NED tagged Primers for PCR**

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* .



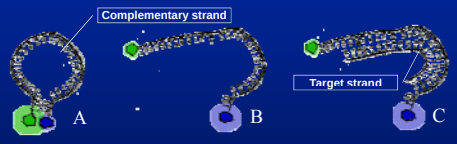
Fragment analysis using capillary electrophoresis of a positive multiplex PCR from *S.* Enteritidis inoculated lettuce.

mPCR results for *S. enteritidis* inoculated on lettuce

Repetition	Inoculation	Before Enrichment		After 6hr Enrichment	
		Plate count	mPCR	Plate count	mPCR
1	1E+06	1.0E+04	Negative	2.7E+07	Positive
	1E+05	1.3E+03	Negative	1.0E+06	Positive
	1E+04	5.0E+01	Negative	5.0E+04	Positive
	1E+03	0.0E+00	Negative	0.0E+00	Negative
	1E+02	0.0E+00	Negative	0.0E+00	Negative
	1E+01	0.0E+00	Negative	0.0E+00	Negative
	Control	0.0E+00	Negative	0.0E+00	Negative
2	1E+06	5.5E+05	Negative	1.8E+09	Positive
	1E+05	7.0E+03	Negative	2.0E+08	Positive
	1E+04	8.0E+03	Negative	7.7E+07	Positive
	1E+03	2.1E+03	Negative	1.1E+07	Positive
	1E+02	1.0E+02	Negative	9.4E+05	Positive
	1E+01	0.0E+00	Negative	3.1E+05	Positive
	Control	0.0E+00	Negative	0.0E+00	Negative
3	1E+06	7.5E+04	Negative	3.1E+08	Positive
	1E+05	8.5E+03	Negative	4.3E+07	Positive
	1E+04	8.5E+02	Negative	4.5E+06	Positive
	1E+03	0.0E+00	Negative	1.0E+05	Positive
	1E+02	0.0E+00	Negative	2.5E+04	Positive
	1E+01	0.0E+00	Negative	5.0E+03	Positive
	Control	0.0E+00	Negative	0.0E+00	Negative

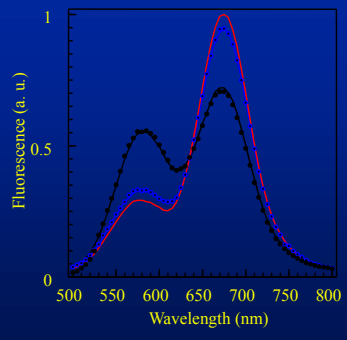
Multiplex Amplicon detection using Microarrays

Beacon Concept



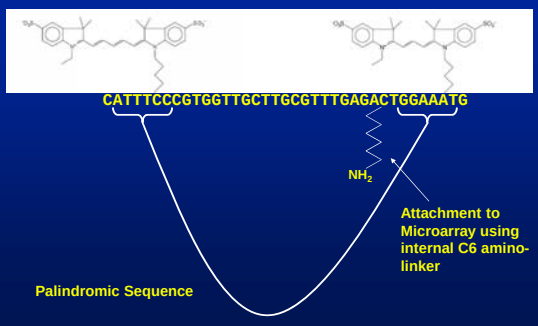
Molecular beacon closed (A), open (B), and hybridized (C).

Fluorescence of *iap* (Listeria) in solution with cy-3 and cy-5 dye labeled

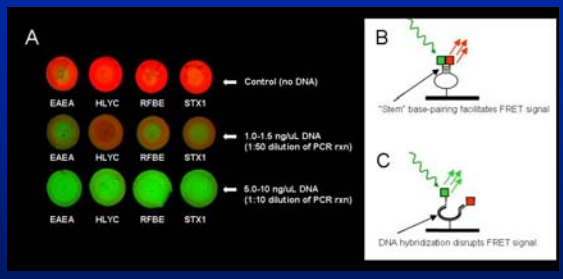


70 C (black, line) => 55 C (blue, line) => 40 C (red) => 55 C (blue, circle) => 70 C (black, circle)

Beacon structure and attachment chemistry



Multiplex Microarray



Microarray A. Results from 3 different microarrays hybridized with control (no DNA, top row), 1.0-1.5 ng/uL of DNA (1:50 dilution of the PCR products, middle row), and 5-10 ng/uL 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**).

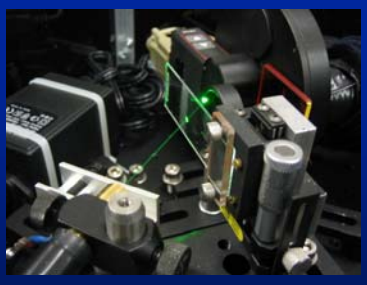
Green/red fluorescence ratios of beacon probes upon target DNA hybridization measured with microarray scanner

Target DNA concentration	*eaeA*	*hlyC*	*rfbE*	*stxI*
Control (none)	0.30 ± 0.03	0.10 ± 0.01	0.20 ± 0.02	0.20 ± 0.02
1.0–1.5 ng/µl	1.9 ± 0.02	0.30 ± 0.03	1.0 ± 0.1	1.0 ± 0.1
5–10 ng/µl	12.8 ± 1.4	5.2 ± 0.6	7.3 ± 0.8	6.2 ± 0.7

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.

Kim, et al. 2007. Biosensors and Bioelectronics. 22(6):1041-7.



The close view of the MB arrayed slide with the laser on. During the measurement, the neutral filters will be inserted at the output of the laser to adjust the intensity of the excitation. The temporary manual translation stage for the adjustment of the vertical height of the slide is also seen.

Kim, et al. 2007. Biosensors and Bioelectronics. 22(6):1041-7.

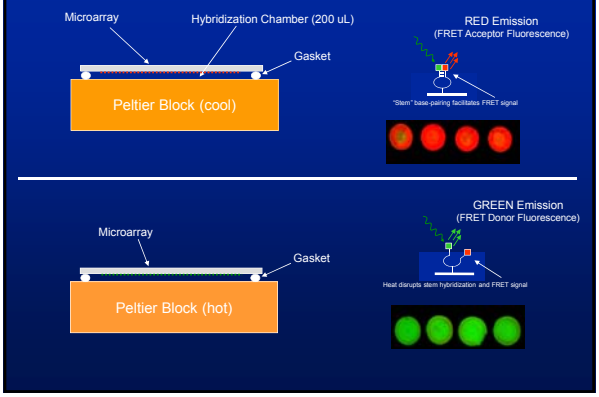
Green/red fluorescence ratios of *eaeA* sequenced beacon probes upon target DNA hybridization measured with prototype detection platform

Target DNA concentration	Green/red fluorescence ratio
Control (none)	0.24 ± 0.03
0.5–1.0 ng/µl	0.23 ± 0.03 (0.96 ± 0.17)
5–10 ng/µl	0.53 ± 0.06 (2.21 ± 0.40)
50–100 ng/µl	0.86 ± 0.09 (3.58 ± 0.64)

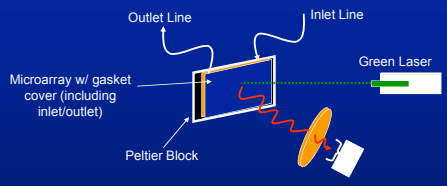
In parentheses: degree of increase of the measured green/red fluorescence ratio compared to that of the control probe.

Kim, et al. 2007. Biosensors and Bioelectronics. 22(6):1041-7.

Hybridization chamber prototype



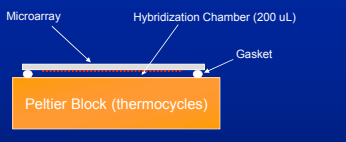
Online amplicon detection prototype



Bar Code Microarray Layout

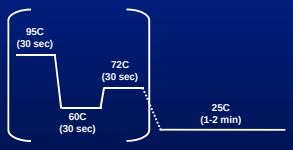
Each line (rather than a spot) is the FRET-probe, and its ID is derived from its location within the bar code pattern and/or the line "thickness".

Hybridization Chamber PCR and Detection (End-Point Detection)



Hyb Chamber contains:

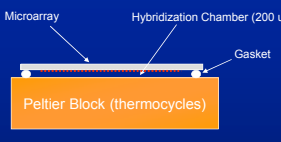
1) Sample
2) PCR mix
3) Primers

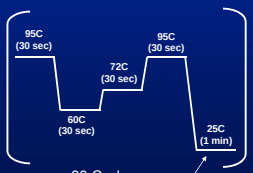


30 Cycles

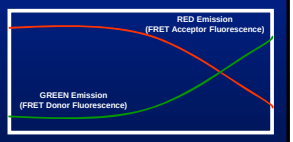
Detect Fluorescence

Hybridization Chamber PCR and Detection (Inter-Cycle Detection)





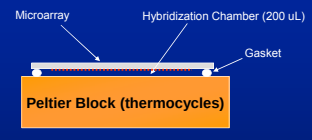
30 Cycles



Thermocycles

Detect Fluorescence

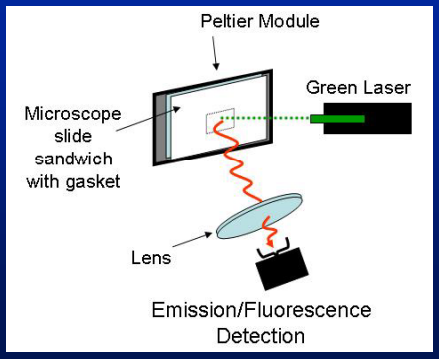
Hybridization Chamber PCR and Detection



Detect Fluorescence

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

Hybridization Chamber PCR and Detection

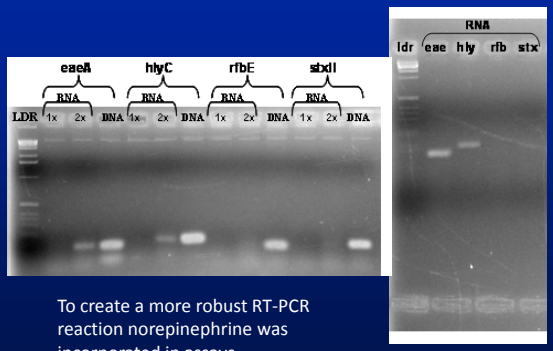


Hybridization Chamber PCR and Detection

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-culturable 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.

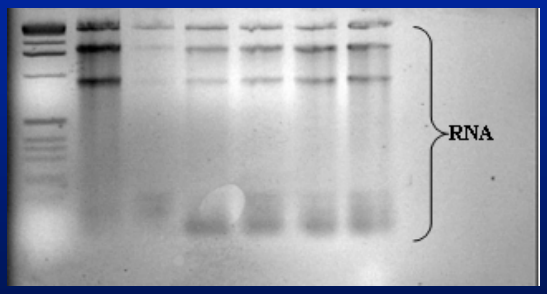


To create a more robust RT-PCR reaction norepinephrine was incorporated in assays.

Live vs Dead cont.

(chlorine dioxide ppm)

Ladder RNA 0 1 5 10 15



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 descrimiation of live vs dead cells

Acknowledgments

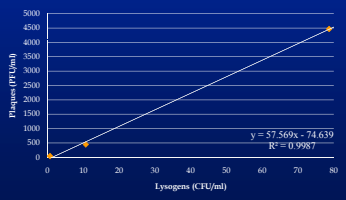
- **Center for Food Safety Engineering, Purdue University**
- **Purdue core sequencing facility**
- **USDA/ARS cooperative agreement 1935-42000-035**

Capture and detection of E. coli O157:H7 using polymer-immobilized phage

BACTERIA ANIMATION
DR. BRUCE APPLEGATE

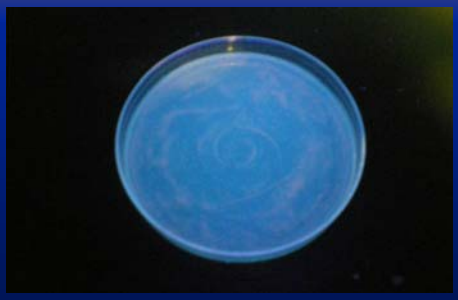
Quantification of Phage

- Serial dilutions of ϕV10Δ*recET*::kan^{R}*cobA*
- Plaque and lysogen assays



Lysogen Assay

- The assay consisted of mixing ΦV10 *kan:cobA*1 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 37ºC
- Lysogens containing ΦV10 *kan:cobA*1 were enumerated.
- A ratio of phage per cells was determined to provide an estimate of the detection limit and is shown



Lysogen Detection Assay

Phage concentration (PFU/ml)	*E.coli* O157:H7 cell concentration (CFU/ml)				
ΦV10 *kan:cobA* (plaque forming units)	**10^6**	**10^5**	**10^4**	**10^3**	**10^2**
10^7 Actual phage **10^6**	positive	positive	positive	positive	positive
10^6 Actual phage **10^5**	positive	positive	positive	positive	negative
10^5 Actual phage **10^4**	positive	positive	positive	negative	negative
10^4 Actual phage **10^3**	positive	positive	negative	negative	negative

Current status

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