Engineering of Biosystems for Detection of *Listeria monocytogenes* in Foods

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Outline

Introduction and Background: Goals

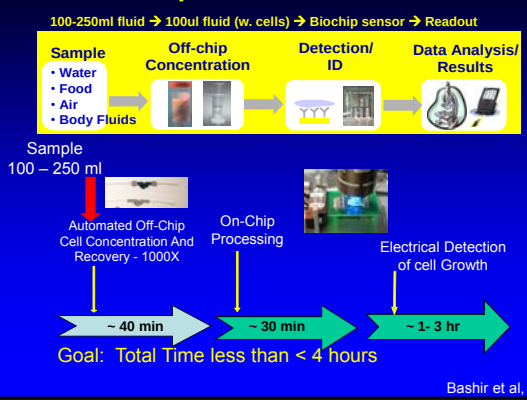
Rapid Cell Concentration and Recovery (CCR)
Membrane Systems: Fouling and bacterial capture

Mammalian cell receptor for capture of pathogens on biochip/biosensor surface

Microfluidics device design for pathogen detection: systems Integration of biochip functions

Conclusions/Next steps

Biochip Detection Process



Bashir et al, 2004

Goals

Detect low levels of foodborne pathogens
in complex and various foods
and in quick and precise way

Achieve rapid sample preparation
Scale-down of bioseparations
couple to specific and rapid detection

Biochip: Buffers, Receptors, Devices
Amplify, detect, identify pathogens
Sample volumes of 100 µL at 10 cell level

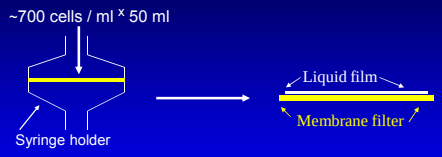
Benchmarks (Metrics)

Concentrate sample containing bacteria

- Final concentration of 10^3 to 10^4 cells / mL
- Final viable cell count on chip > 10 cells
- Concentrate cells in 30 min
- Process samples in 60 min
- Maintain cell viability

Introduce samples on chip, detect cells in 3 hr

Membrane Concentration Recognizing Role of Liquid Film

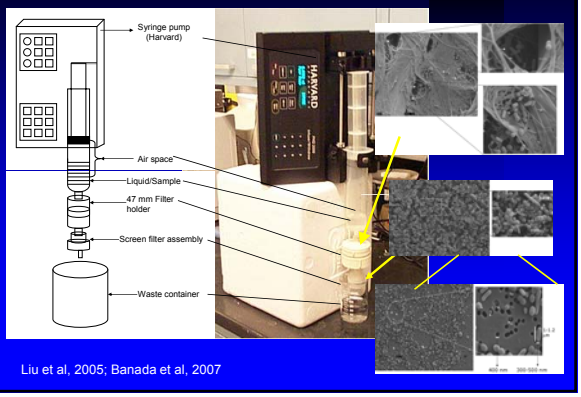


Assumption: 1mg=1 µl → Membrane retains 15 µl of liquid

Liquid film concentrates 10^4 cells into a volume of 15 µl of liquid.
Concentration factor equivalent to 6.7 x 10^6 cells / mL
Challenges: membrane fouling, recovering viable cells after concentration

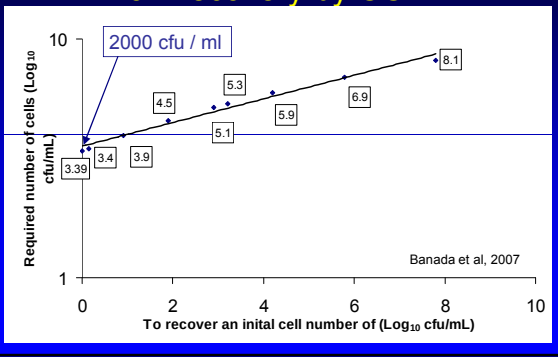
Chen et al, 2005

Flat Membrane CCR: 100 mL sample volume



Liu et al, 2005; Banada et al, 2007

Minimum Number of Cells in Sample for Recovery by CCR



Larger Volumes = Higher Sensitivity

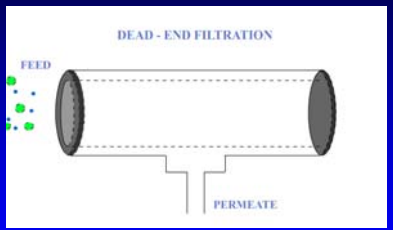
Flat membranes have limit of 120 mL before flow stops

Moderating loss of permeation rate (flux) and increasing throughput

1. Lipases and proteases may hydrolyze macromolecules believed to cause pore occlusion; improvement in permeability is small
2. High cross membrane fluid velocity
3. Low trans-membrane pressure drop

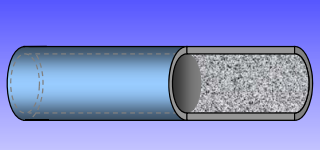
Cross flow membrane configurations: hollow fiber, flat membrane
Dead end filtration has limit of 120 mL. Cross flow an alternative option.

Cross Flow HF Microfiltration



- Liquid solution passes through the HF membrane. Particles retained on the inner HF membrane surface and module surface.
- Permeate flux decreases rapidly.
- A fouling layer build-up causes the system to plug up

Particle Transport



ϕ = Particle volume fraction
$\vec{u}$ = axial velocity
$\vec{v}$ = transverse velocity
x = axial position
y = transverse position
D = hydrodynamic diffusion coefficient

$$\underbrace{\frac{\partial(u\phi)}{\partial x}}_{\text{Axial Convection}} + \underbrace{\frac{\partial(v\phi)}{\partial y}}_{\text{Transverse Convection}} + \underbrace{\frac{\partial}{\partial y}\left(D\frac{\partial\phi}{dy}\right)}_{\text{Sheer Induced Hydrodynamic Diffusion}} = 0$$

Boundary Conditions

- Boundary Condition at Membrane Wall:
  - Zero Particle Transport
  - Transverse Fluid velocity is a function of trans-membrane pressure and the cake layer $\vec{v}$ = f(TMP,δ) $|_{membrane}$
  - This function is dependant on many system parameters and is not well characterized for many systems

Sheer Induced Diffusion

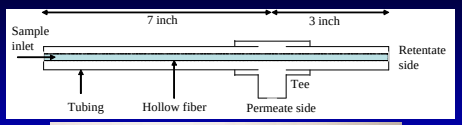
Use the Navier-Stokes Equations to solve for the flow and pressure field.

Balance particle transport to solve for particle concentration and cake layer

Particulates transport occurs via convection and Sheer induced diffusion

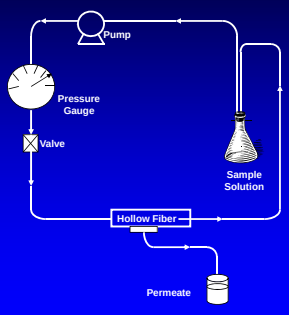
Equations coupled because flux is function of trans-membrane pressure and cake layer.

Hollow Fiber Membranes



Same flowrates, much smaller cross-sectional area

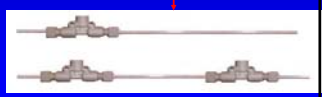
Cross Flow System



Bacterial Recovery

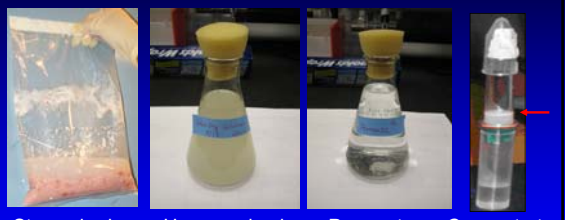
Initial test was done with *E. coli* GFP
Use of Hollow Fiber System (HST)
cross-flow, 0.45 µm pore size

Done with hot dog massage
250 ml starting volume



2 filtration steps
prior to concentration
50 to 100% recovery
test milk, vegetables

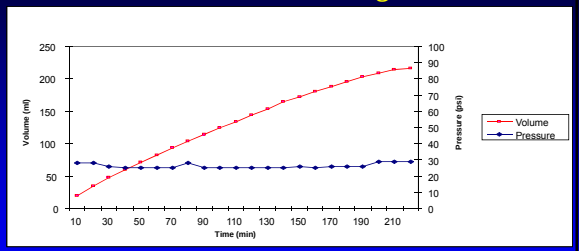
Processing of Stomached Hot Dog



Stomached Hot dog | Homogenized Hot Dog | Permeate | Concentrate

Liu et al, 2007

Pressure, Permeate Volume vs. Time 220 mL Stomached Hot Dog Processed



Initial Cell Concentration (cells/mL)	% Cell change (cells/mL)	Pressure (PSI)	Leakage	Recovery viable cells	Total Captured Cells	% recovered *E. Coli*
190	13 x 10^3	25-29	0.0	610	53 x 10^3	114

Cross flow hollow fiber

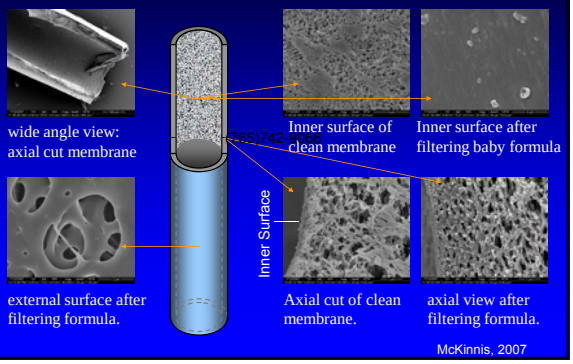
Able to process 250 mL or more
- Homogenized hot dog
- Dry milk
- Vegetables (leafy matter)

Mechanisms being studied

Testing being carried out

McKinnis, Rodriguez et al, 2007

SEM Photos of Membrane Fouling: with Baby Formula (contains fat)



McKinnis, 2007

Stainless Steel Membrane

- Stainless Steel Construction
- Smooth Inner Surface
- Low Adhesion
- High Chemical Resistance
- Small Pore Size 0. 1µm

Summary on Cell Concentration

Cell Concentration and recovery should result in 1000 To 10,000 Cells / mL

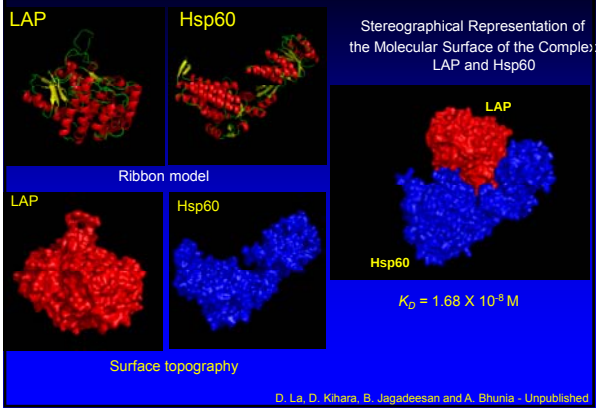
CCR for a single large volume is preferred over replicates of smaller proportions of the same volume

Cross flow membranes are able to process homogenized or stomached samples that block flat membranes

Mammalian cell receptor for capture of pathogens on sensor surface

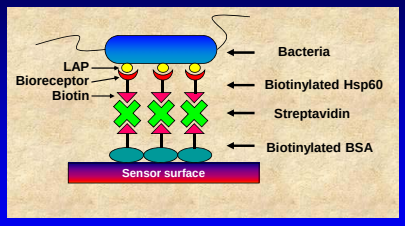
Goal: to investigate if mammalian cell receptor can be used as a capture molecule for biosensor application

- LAP (~94 kDa), a membrane bound alcohol acetaldehyde dehydrogenase enzyme responsible for adhesion to mammalian cells
- Bi-functional protein: (i) Enzyme (ii) Adhesion
- N-terminal part is ALDH (acetaldehyde dehydrogenase)
- C-terminal end is ADH (alcohol dehydrogenase)
- Interacts with eukaryotic Hsp60 (chaperone protein)
- Hsp60 is present on mammalian cell surface

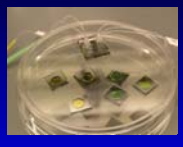


D. La, D. Kihara, B. Jagadeesan and A. Bhunia - Unpublished

Hsp60 (receptor) immobilization



Silicon dioxide chips with microfluidic set up

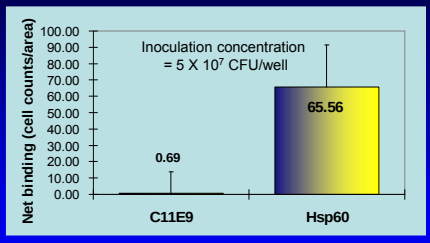


PDMS
Well area=23.039 mm^2

- Bacteria added: 5x10^7 cfu/well
- Incubate for 2 h at RT
- Wash and stain with propidium iodide
- Count under microscope (area: 130 x 98 µm)

Koo et al (unpublished)

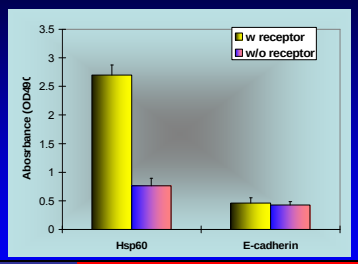
Comparison of MAb C11E9 and Hsp60-mediated capture of *L. monocytogenes* cells on silicon dioxide surface

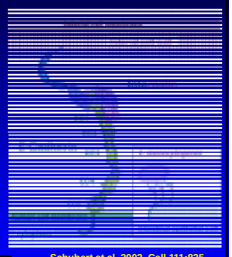


Receptor	NET binding (Cell number/image)	Estimated total cell numbers/well
Hsp60	65.56 ± 25.94	1.19E+05 ± 4.69E+04
MAb C11E9	0.69 ± 13.16	1.25E+03 ± 2.38E+04

Koo et al (unpublished)

E-cadherin (InlA)-mediated capture of *L. monocytogenes* cells in microtiter plate



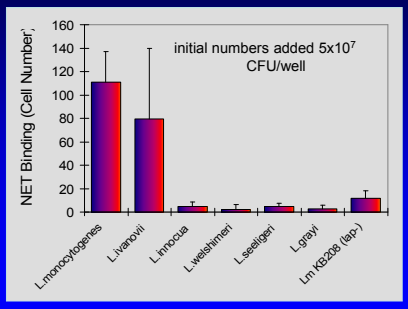


Schubert et al. 2002. Cell 111:825

	Lm counts with receptor	Lm counts without receptor
Hsp60	2.699±0.175	0.764 ± 0.130
E-cadherin	0.463 ± 0.092	0.427 ± 0.058

Koo et al (unpublished)

Specificity of Hsp60 in capturing *Listeria* cells with Hsp60 coated SiO_2 surface



Koo et al (unpublished)

Captured *Listeria* cells on Hsp60 coated SiO_2 chip

Koo et al (unpublished)

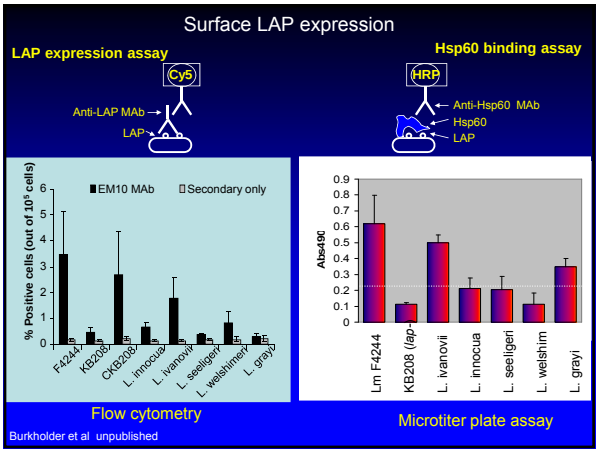
Hsp60 mediated capture profile of *Listeria* cells on SiO_2 surface

Bacteria	NET binding (counts/image[a])	Initial CFU/well	Estimated total cells/well	% Capture
L. monocytogenes	110.93 ± 26.29	5.80E+07	2.01E+05 ± 4.75E+04	0.401 ± 0.095
L. ivanovii	79.46 ± 60.11	4.10E+07	1.44E+05 ± 1.09E+05	0.287 ± 0.217
L. Innocua	4.92 ± 3.44	3.30E+07	8.89E+03 ± 6.22E+03	0.018 ± 0.012
L. welshimeri	1.92 ± 4.27	2.20E+07	3.46E+03 ± 7.71E+03	0.007 ± 0.015
L. seeligeri	4.72 ± 2.96	3.30E+07	8.52E+03 ± 5.35E+03	0.017 ± 0.011
L. grayi	2.52 ± 3.12	2.50E+07	4.55E+03 ± 5.65E+03	0.009 ± 0.011
Lm KB208 (*lap-*)	12 ± 6.25	4.40E+07	2.17E+04 ± 1.13E+04	0.043 ± 0.023

[a] The area for each image was 130 x 98μm.

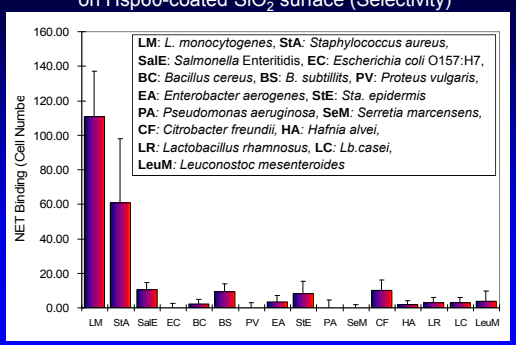
Koo et al (unpublished)

Surface LAP expression



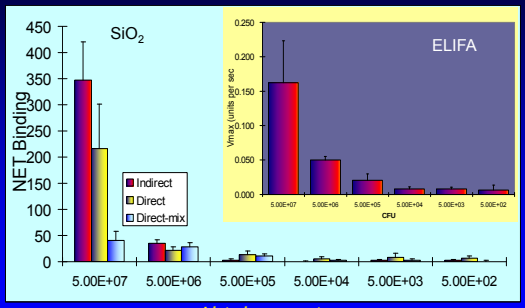
Burkholder et al unpublished

Capture profile of various food-associated microorganisms on Hsp60-coated SiO_2 surface (Selectivity)



(Inoculation concentration was ~10^7 CFU/well)

Estimation of limit of detection (sensitivity) for Hsp60 using biochip and ELIFA (inset)



Listeria monocytogenes

ELIFA: enzyme: alkaline phosphatase, substrate: 4-methylumbelliferyl phosphate (MUP); measurement (Ex/Em): 360/440nm

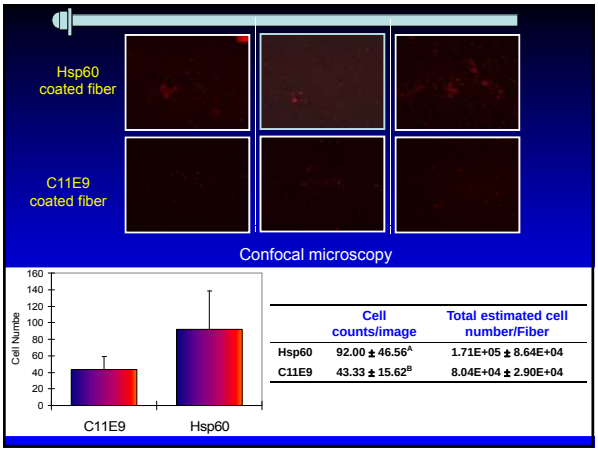
Selective capture of *L. monocytogenes* in the presence of other bacteria by Hsp60 on Chip

LM	SE	EC	Total Counts	LM Counts	% capture (LM)
1	1	1	15.42 ± 12.95	11.33 ± 10.3	73.48
1	1	0	18.9 ± 11.8	11 ± 6.29	58.2
1	0	1	17.2 ± 11.13	13.2 ± 10.86	76.74
0	1	1	5.8 ± 1.48		

LM	K-12	LbA	Total Counts	LM Counts	% capture (LM)
1	1	1	18 ± 7.38	10.18 ± 6.66	56.56
1	1	0	18.6 ± 6.36	11.6 ± 5.27	62.37
1	0	1	14.1 ± 4.01	9.2 ± 3.77	65.25
0	1	1	12 ± 5.93	--	--

LM: L. monocytogenes, SE: Sal. Enteritidis, EC: E. coli O157:H7; K-12: E. coli K-12, LbA: Lb. acidophilus

Application of Hsp60 for capture of *Listeria* on optical waveguide (Fiber Optic)



	Cell counts/image	Total estimated cell number/Fiber
Hsp60	92.00 ± 46.56[A]	1.71E+05 ± 8.64E+04
C11E9	43.33 ± 15.62[B]	8.04E+04 ± 2.90E+04

Next steps with Hsp60

- Determine capture efficiency on microfluidic chip with or without DEP
- Determine capture efficiency on SPR
- Determine capture efficiency with magnetic beads
- AFM to examine the binding strength/patterns
- Determine the interaction domains for LAP and Hsp60

Microfluidics device design for pathogen detection: systems integration of biochip functions

Overview of Biochip-based Detection Process

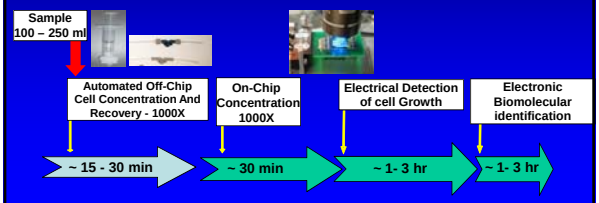
100-250ml fluid → 100ul fluid (w. cells) → Biochip sensor → Readout

Sample
- Water
- Food
- Air
- Body Fluids

Off-chip Concentration

Detection/ ID

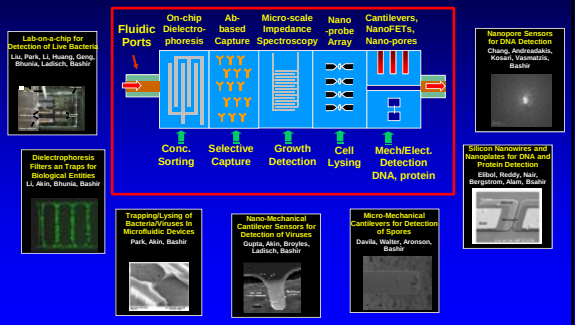
Data Analysis/ Results



Outline

1. Brief review of prior work
2. PCR in the "Petri Dish on a Chip"
   a. Optical Detection
   b. Label-Free Electrical Detection
3. Future Work

Integrated BioChips for Study of Microorganisms and Cells

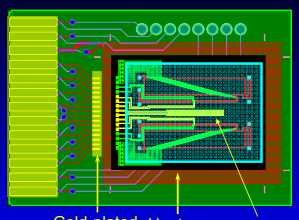


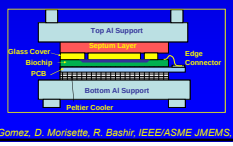
"Lab on a Chip" with microfluidics and micro/nanosensors

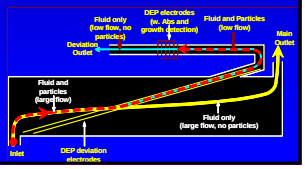
Outline of "PCR in Petri Dish" Current Work

- PCR reaction with a 508 bp *Listeria monocytogenes* prfA gene.
- Calibration of the thin film temperature sensor on chip using LabView data acquisition.
- Design and realization of automatic thermal cycling on chip at low average power values.
- Development of a real time PCR protocol for *Listeria monocytogenes* on chip
- Direct electrical detection of PCR products

On Chip PCR

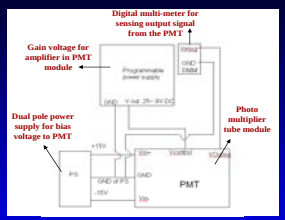






R. Gomez, D. Morisette, R. Bashir, IEEE/ASME JMEMS, 2005

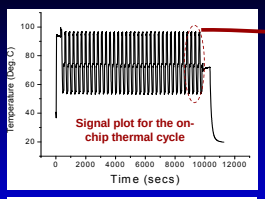
System and PCR Details

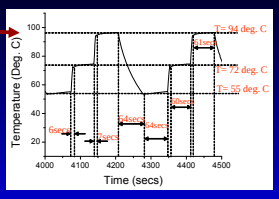


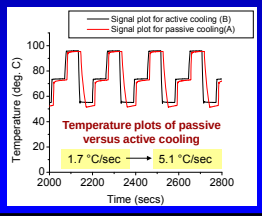
- Integration of a PMT (photomultiplier tube) module for fluorescence detection
- 0.5µl/min flow rate at a 20Vpp DEP voltage with frequency 100 KHz in LCGM
- PCR reagents introduced, cells lysed at 95C, and PCR cycling initiated
  - SYBR Green based PCR Assay
  - prfA 508 bp segment target for specfic detection of LM
  - 5'CGGGATAAAACCAAAACAATTT3' and R-5'TGAGCTATGTGCGATGCCACTT3'

Bhattacharya, et al. 2007, Submitted

PCR Thermal Cycling



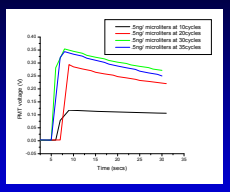


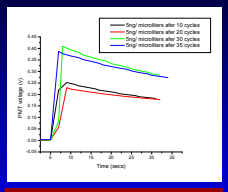


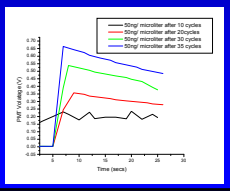
- Average power 2.5 W
- Total time 112 min for active cooling
- Times at each temperature can be reduced

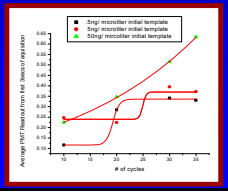
Bhattacharya, et al. 2007, Submitted

Calibration of signal acquisition using SYBR green based PCR mix

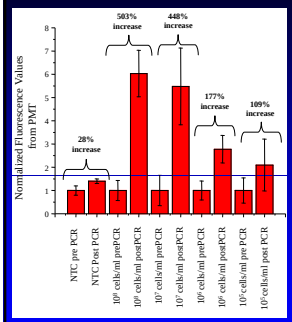






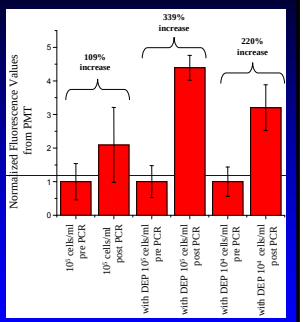


End Point PCR Detection Sensitivity



Pre and post PCR summary for various cell concentrations without DEP concentration.

10^5 cells/ml, 0.5ul/min for 1.2min, → 60 cells

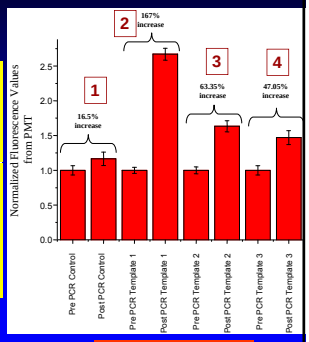


Pre and post PCR summary for various cell concentrations with DEP concentration.

10^4 cells/ml, 0.5ul/min for 12min, → 60 cells !

Specificity Trials Without DEP

Trial #	Sample details	Pre PCR	Post PCR	% increase
1	*Listeria Innocua + E. coli* (control)	1	1.16	16
2	10^6 cells/ml (*Listeria Innocua + E. coli + Listeria Monocytogenes V7*)	1	2.67	167
3	10^7 cells/ml (*Listeria Innocua + E. coli + Listeria Monocytogenes V7*)	1	1.63	63
4	10^6 cells/ml (*Listeria Innocua + E. coli + Listeria Monocytogenes V7*)	1	1.47	47



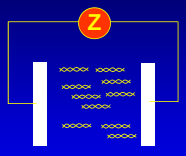
0.5ul/min for 1.2min, 10^6 cells/ml → 600 cells

Bhattacharya, et al. 2007, Submitted

Towards Label Free Electrical Detection of PCR Products

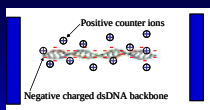
- Polymerase Chain Reaction
  - Target molecule doubles every cycle

Cycle #	# of Molecules	Conc. (#/ul)
1	2	
2	4	
3	8	
4	16	
5	32	
10	1024 (10^3)	10^3 #/ul
20	1048576 (10^6)	10^6 #/ul
30	1073741824 (10^9)	10^9 #/ul
40	1099511627776 (10^{12})	10^{12} #/ul

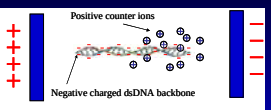


- What is the minimum concentration of dsDNA molecules (e.g. 500bp) that can be directly detected in solution using impedance measurements ???

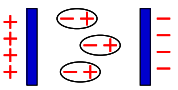
Electrical Nature of DNA Molecules



Before applying electrical field



Induced dipole when DNA molecules are probed electrically in solution



$E_{effective} = E - E_{polarization} = \frac{\sigma}{k\varepsilon_0}$

$Cap. = \frac{k\varepsilon_0 A}{d}$

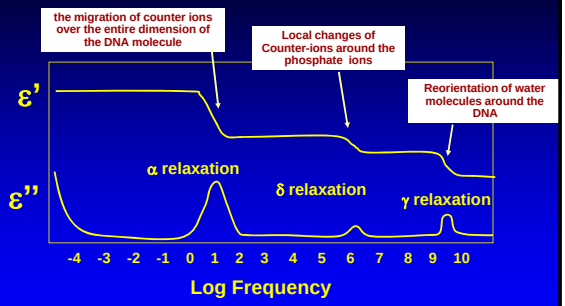
- DNA polarization (dipole effect)
- Dielectric relaxation (Debye relaxation) :

$$\varepsilon = \varepsilon_\infty + \frac{\Delta\varepsilon}{1 + j\omega\tau}$$ ↑ with L(or #)↑

⇒ Cap. ↑ Z ↓

- Counter Ion movement: Rsol ↓ Z ↓

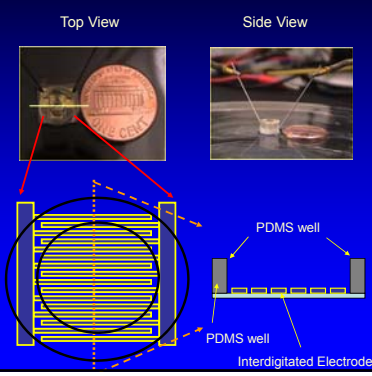
Dielectric Relaxation of DNA in Solution



(ref: "Electrical Properties and Dielectric Relaxation of DNA in Solution", James Baker-Jarvis, Chriss A. Jones, Bill Riddle, NIST Technical Note 1509, 1998.)

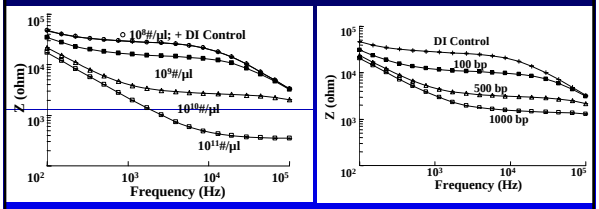
Proof of Concept Experiments

Experiment set-up, side and top-view of device. (Measurement equipments include a LCR meter (Agilent 4284A), a data acquisition system (Agilent 34905A), micro-manipulators (Micromanipulator M-450, M-550), a computer with self-developed labview control software.)



Label free DNA detection in DI water

500 bp dsDNA | 10^9 #/µl dsDNA

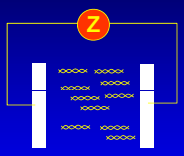


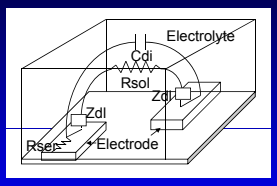
ε increases with # or L, C increases, Z decreases

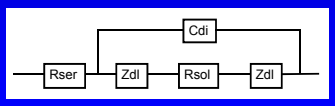
Detection limit in DI Water for 500bp DNA = 1e9 #/µl (1.33 nM)

DNA prepared by QIAquick Gel Extraction Kit, QIAGEN, Valencia, CA

Circuit Model and Parameter Extraction

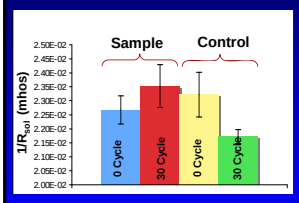


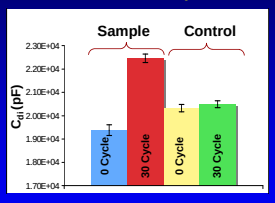




Label free DNA detection in PCR Solution

Extracted Solution Conductance | Extracted Solution Capacitance

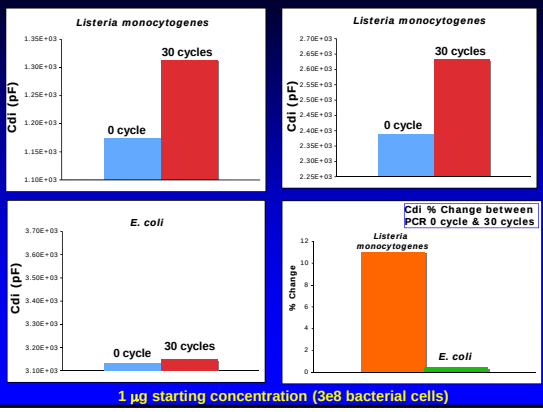




sample (PCR mix plus DNA template)
control (PCR mix only)

1 µg starting concentration (3e8 bacterial cells)

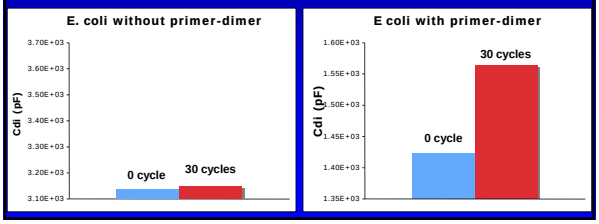
Selectivity Measurements



1 µg starting concentration (3e8 bacterial cells)

Selectivity Experiment Summary (L. m. & E. coli):

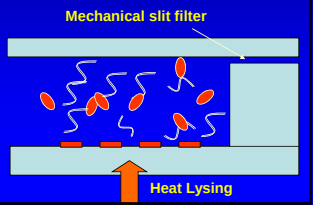
- 10 - 12 % increase in Cdi with L. m. template and L. m. prfA gene primer.
- 0.5 % increase in Cdi with *E. coli* template and L. m. prfA gene primer.
- If primer-dimers formed, the increase in Cdi is about 9% in Cdi.
  - We have to avoid primer-dimers and unspecific amplifications !
  - The detection is real, i.e. Cdi will change whenever there are significant DNA molecules.



Detection Limits

- We used 1 µg of initial genomic DNA →3e8 bacterial cells (we used 25µl solution) → ~1e7 #/µl in PCR mix
- 1e7 #/µl → after 30 cycle PCR → ***1e16 #/µl***
- 1e7 #/µl → ***1e4 #/nl*** before 30 cycle PCR

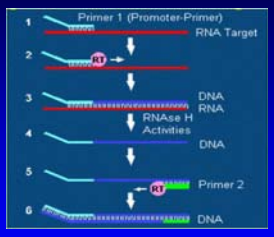
- Use 1000 cells in 0.1nl
- Mechanical Filter
- On-Chip PCR and direct electrical detection



Next Steps

- Optimize real time PCR with fluorescence detection for reduced time
  - Also demonstrate for *Escherichia coli* (in progress) and *Salmonella*
- On-chip label-free electrical detection of PCR product in microfluidic device
- Move to RNA detection so as to
  - lower the limit of detection
  - have the possibility of live/deal information about Bacterium.
- Isothermal RNA (TMA) fluorescence detection off and on chip
- Isothermal RNA amplification (TMA) electrical detection on chip
  - To obviate the need for thermal cycling requirement
  - To eliminate time loss in ramp up/down

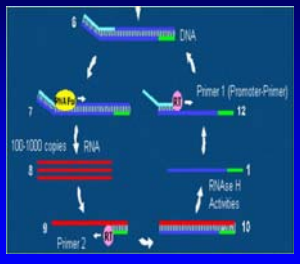
Isothermal RNA Amplification



- Promoter primer binds to rRNA target.
- Reverse transcriptase creates a DNA copy of the rRNA target.
- The RNA - DNA duplex.
- RNAse H activity of the reverse transcriptase degrades the rRNA.
- Primer 2 binds to the DNA and reverse transcriptase creates a new DNA copy.
- Double stranded DNA template with a promoter sequence.

(figure from the Gen-Probe Inc)

Isothermal RNA Amplification



- RNA polymerase initiates transcription of RNA from the DNA template.
- 100 to 1000 copies of RNA amplicon are produced.
- Promoter primer binds to each RNA amplicon and reverse transcriptase creates a DNA copy.
- RNA - DNA duplex.
- RNase H activity of the reverse transcriptase degrades the rRNA.

(figure from the Gen-Probe Inc)

Final Conclusions

- Cell concentration and recovery using large volumes demonstrated
- Processing of complex samples (baby milk, vegetables) begun
- Very promising receptor (hsp-60) identified for capture of *Listeria monocytogenes*
- Capture demonstrated on biochips and fiber optic biosensor
- PCR on a Chip with fluorescence detection of 60 cells
- Label-free electrical detection of PCR product on chip initiated