



Cultivation-independent DNA Microarray Based Approach for Monitoring Microorganisms in Soil

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Goal:

Detection and identification of microorganisms in environmental samples

- pathogenic
- genetically engineered
- biotechnological relevant

 Fast and without cultivation

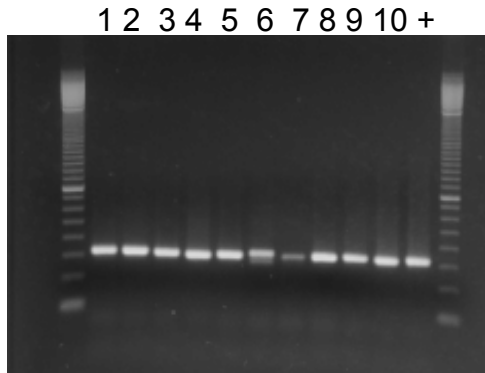
Approach:

Microarray detection of unique 16S rRNA genes in complex samples



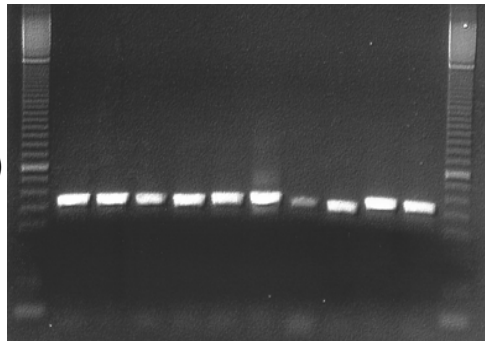
Test of Universal PCR Primersets

16S
(S1)



1 2 3 4 5 6 7 8 9 10

16S
(S1+S2)

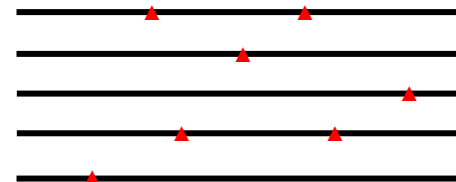


Expected Sizes of Fragments:

16S (S1): ~ 330 bp

16S (S1-S2): ~ 540 bp

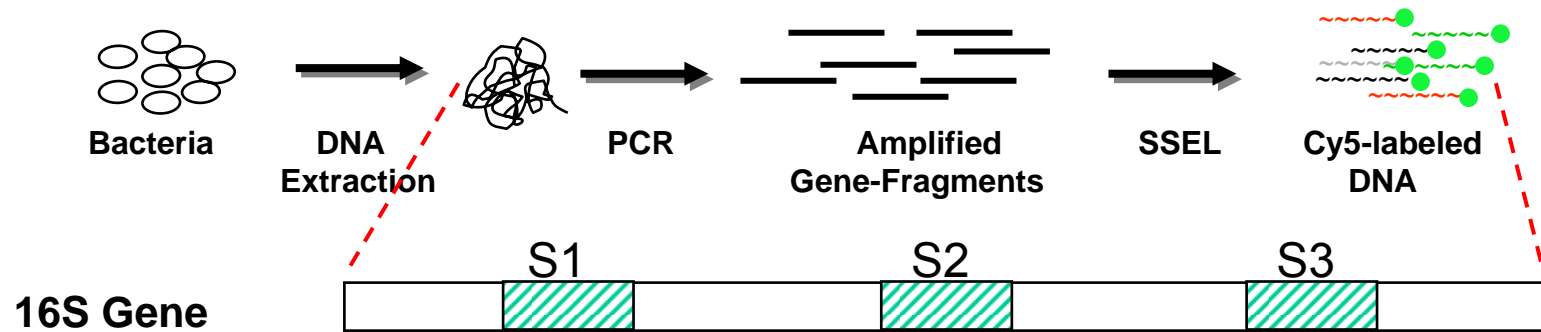
	16S (S1)	16S (S2)	16S (S3)	16S (S1+S2)
1. <i>B. subtilis</i>	+	+	+	+
2. <i>S. aureus</i>	+	+	+	+
3. <i>L. pneumophila</i>	+	+	+	+
4. <i>P. aeruginosa</i>	+	+	+	+
5. <i>E. coli</i>	+	+	+	+
6. <i>P. polymyxa</i>	+	+	+	+
7. <i>M. kansasii</i>	(+)	(+)	+	(+)
8. <i>C. jejuni</i>	+	+	+	+
9. <i>S. pneumoniae</i>	+	+	+	+
10. <i>S. enterica</i>	+	+	+	+



16S amplicons
with few differences
in DNA sequence

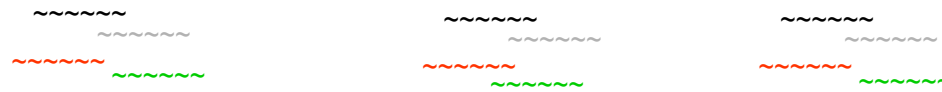


Sequence Specific End-labeling of DNA



Unsp. Amplification: 5' ————— 3'

Spec. Oligos:



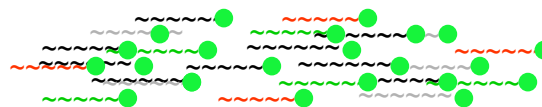
+

Sequence specific
end-labeling:

ddCTP* + ThermoSequenase



**Cy5-labeled DNA
(Targets)**

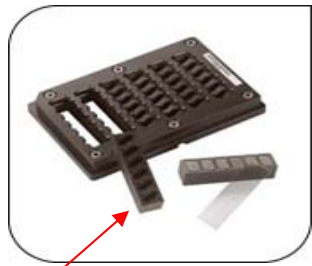


→ Elongation of oligo by a single labeled ddCTP

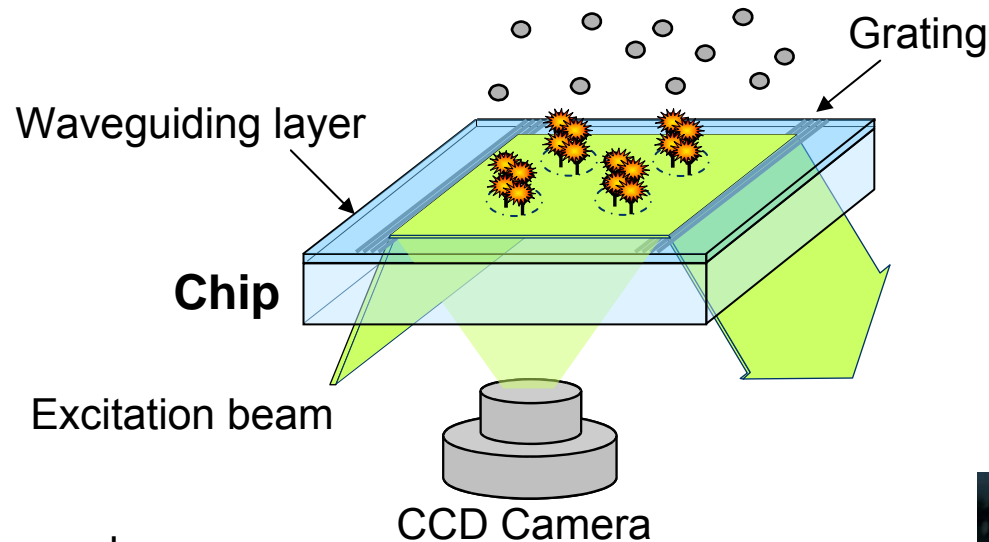
(Rudi and Holck, 2003)



Zeptosens Platform: Planar Waveguide Technology (PWG)



Flowcell and Carrier



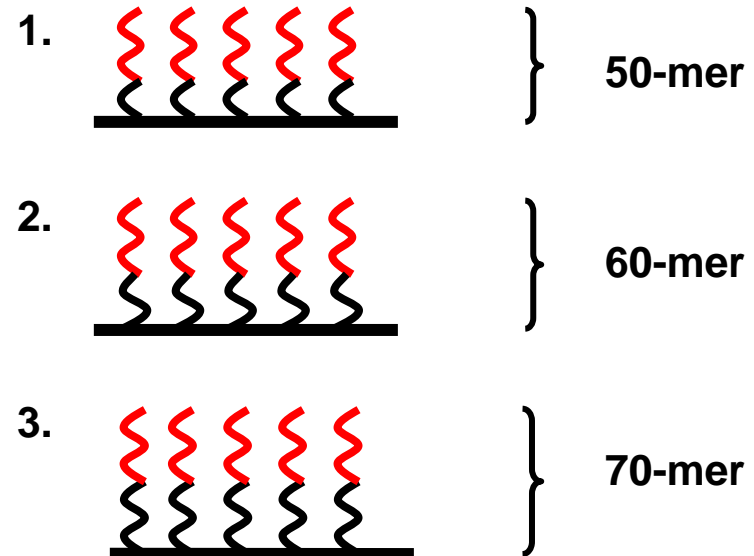
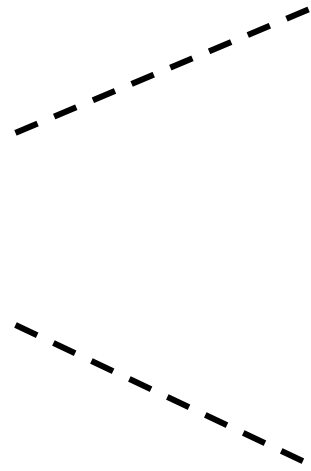
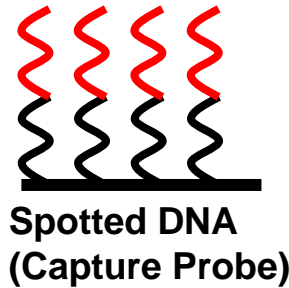
- Reduced background
- Improved S/N ratio
- Image acquisition “in solution” - reduced light scattering effects and photo bleaching



ZeptoReader



Pilot-Array: Construction

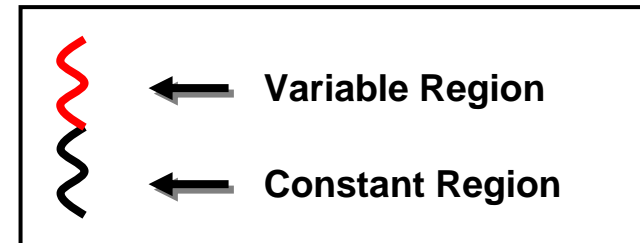


Variable Region:

1. 24-mer of 16S Gen of *P. aeruginosa*
2. 33-mer of 16S Gen of *S. aureus*:
1 Mismatch (MM), 3 MMs und 5 MMs

Constant Region:

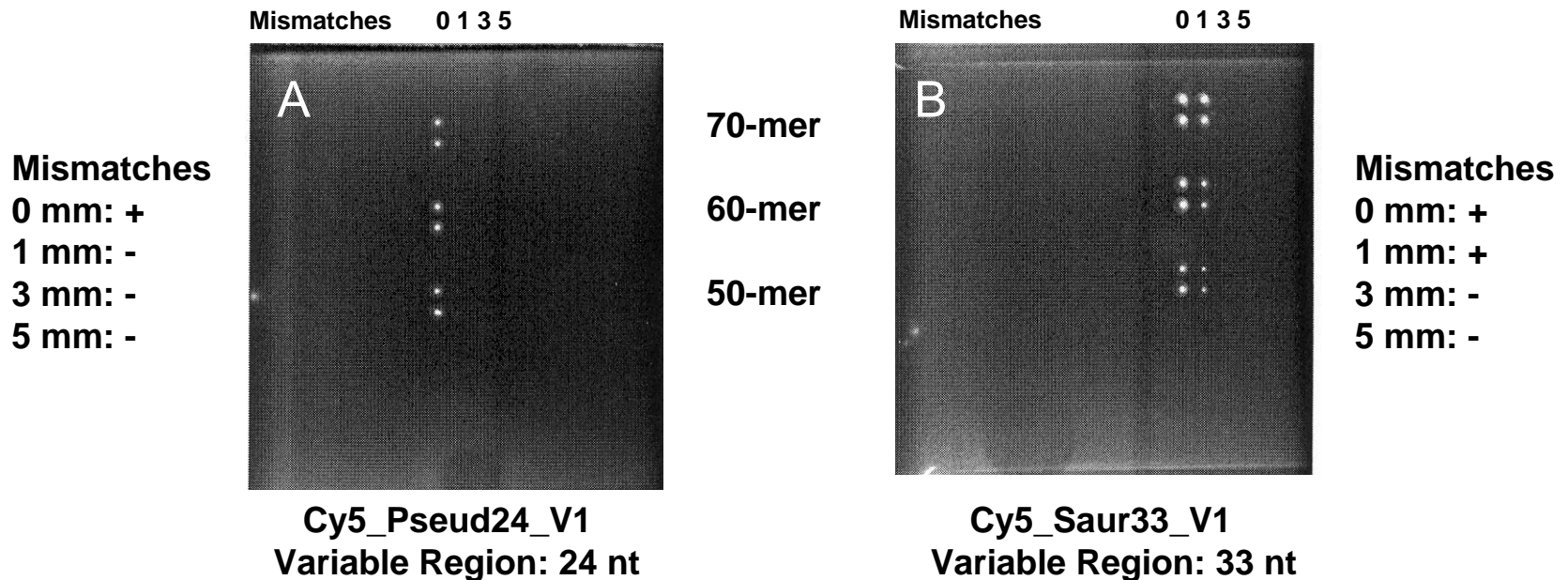
26 nt, 36 nt, 46 nt or 17 nt, 27 nt, 37 nt





Pilot-Array: Results

Hybridization at 55°C, 15 % Formamide



- ▶ Length of capture probes not essential
- ▶ Length of variable region influences potential to discriminate mismatches



Requirements for Probe Design

- ▶ Probes were constructed using 16S rRNA database (NCBI) and aligned with closely related species (MultAlign)
- ▶ If possible: more than 1 MM to closely related species
- ▶ Similar melting temperature (targeted 60 °C)
- ▶ Length of variable region between 24 and 33 nucleotides
- ▶ Each capture probe needs C-terminal guanosine for sequence specific end-labeling of target oligonucleotide



Microarray Manufacture and Processing

I. Manufacture

A. Probes:

- 50-mer oligonucleotides with N-terminal constant region
- 27 probes 16S specific (S1+S2 region)
- Differentiation of 13 genera and 27 species

B. Chips:

- Proprietary surface chemistry optimized for non-covalent immobilization
- 340 probes/ array (170 different probes)
- 6 arrays/ slide → 1020 different probes/ chip

C. Technology:

- Planar Waveguide Technology (Zeptosens)

II. Processing

- Labeling of probes: Sequence specific end-labeling (SSEL)
- Hybridization at 55 °C o/n, 15% Formamide



Probe Set Validation with Pure Culture and Mixed Culture: Experimental Design

- A. 1. One Oligo – 1 matching bacterial DNA**
- 2. One Oligo + mix of 15 different bacterial DNAs**

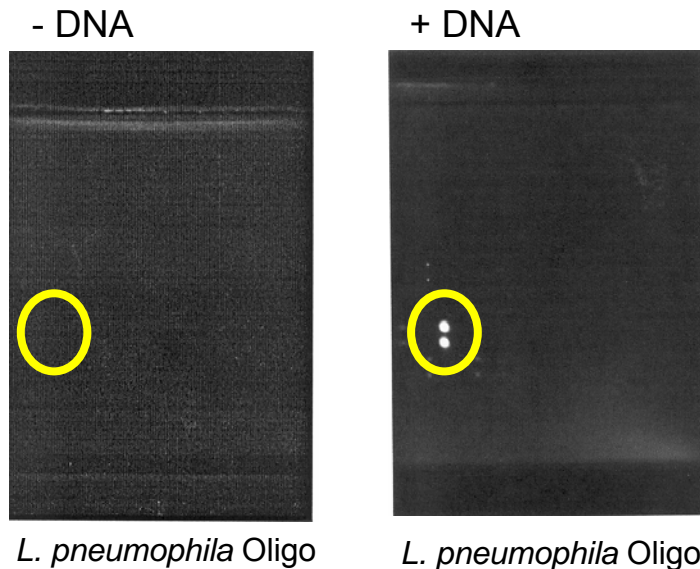
- B. 1. Multiplexing: Three 16S Oligo-mixtures + 1 DNA**
 - 1.1. Mixture of 14 Oligos (gram negative set)
 - 1.2. Mixture of 13 Oligos (gram positive set)
 - 1.3. Mixture of 5 Oligos (gram + and gram -), which show the best results for single hybridization

- C. 1. 27 Oligos + mix of 10 different bacterial DNAs**



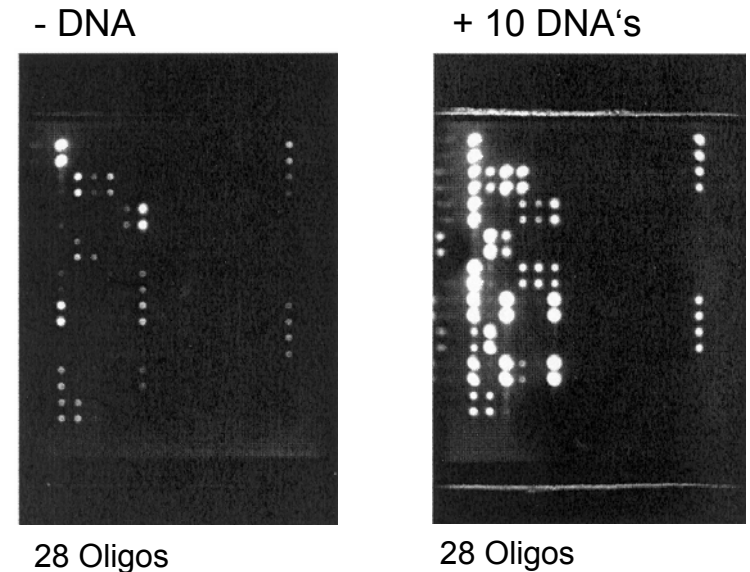
Probe Set Validation

Specific Labeling – Specific Hybridization



DNA: *L. pneumophila*
Oligo: *L. pneumophila* specific
→ **Specific hybridization**

Multiplexing: Mixture of 27 Oligos + Mixture of 10 DNAs



DNA: 10 bacterial DNAs
Oligo: Mixture of 27 16S-specific oligos
→ **Specific + unspecific labeling + hybridization**



Probe Set Validation: 1 Oligo + Mix of 15 bacterial DNAs

Targeted	16S region	Labeled oligos																										
		332	333	335	336	337	339	340	341	342	344	346	347	348	350	352	353	354	356	358	359	361	362	363	368	370	371	373
C. jejuni	S1	+++																										
C. upsaliensis	S2		+++																									
S. aureus	S1			+++																								
S. epidermidis	S2				+++																							
S. haemolyticus	S1					+++																						
S. salivarius	S1						+																					
S. pyogenes	S1							+																				
E. coli	S1								+++																			
E. vulneris	S2									+																		
L. pneumophila	S1										+++																	
L. hackeliae	S2											+++																
Mpl. pneumoniae	S1												+															
Mpl. genitalum	S2													+														
P. aeruginosa	S1														+++													
P. putida	S2		+++																									
H. influenzae	S2															+++												
H. parainfluenzae	S1																++											
H. ducreyi	S2																	+++										
G. vaginalis	S1																		+++									
M. tuberculosis	S2																			++								
M. avium	S1																				+++							
Y. pestis	S1										+																	
Y. enterocolitica	S2																											
B. anthracis	S2																											
B. thuringiensis	S1																											
P. polymyxa	S1																										+++	
P. macerans	S2																											+++

Positive: Species-specific
 Positive: Genus-specific
 False negative / positive

SNR 10-50: +
 SNR 50-200: ++
 SNR > 200: +++



- 19/25 positive: 76 %
- 6/25 false negative: 24 %
- 3/25 false positive: 12 %



Probe Set Validation: Mix of 27 Oligos + Mix of 10 Bacterial DNAs

Targeted	16S region	Labeled oligos																										
		332	333	335	336	337	339	340	341	342	344	346	347	348	350	352	353	354	356	358	359	361	362	363	368	370	371	373
C. jejuni	S1	+++																										
C. upsaliensis	S2		+++																									
S. aureus	S1			+++																								
S. epidermidis	S2				+++																							
S. haemolyticus	S1					+																						
S. salivarius	S1						++																					
S. pyogenes	S1																											
E. coli	S1																											
E. vulneris	S2																											
L. pneumophila	S1																											
L. hackeliae	S2																											
Mpl. pneumoniae	S1																											
Mpl. genitalum	S2																											
P. aeruginosa	S1																											
P. putida	S2																											
H. influenzae	S2																											
H. parainfluenzae	S1																											
H. ducreyi	S2																											
G. vaginalis	S1																											
M. tuberculosis	S2																											
M. avium	S1																											
Y. pestis	S1																											
Y. enterocolitica	S2																											
B. anthracis	S2																											
B. thuringiensis	S1																											
P. polymyxa	S1																											
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Positive: Species-specific
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 False negative / positive

SNR 10-50: +
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 SNR > 200: +++

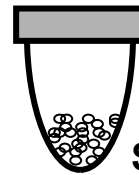


- 16/19 positive: 84.2 %
- 3/19 false negative: 15.8 %
- 5 false positives



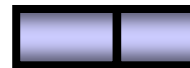
Spiking of Soil with 4 Different Bacteria and Extraction of Bacterial Genomic DNA

Spiking A Bacteria (cfu)	A	Spiking B Bacteria (cfu)	B
	C. jejuni (3.8×10^5) + L. pneumophila (1.2×10^2) + P. aeruginosa (4.6×10^4) + 0. No spiking 1. S. aureus (4.6×10^5) 2. S. aureus (4.6×10^4) 3. S. aureus (4.6×10^3) 4. S. aureus (4.6×10^2)		C. jejuni (3.8×10^5) + L. pneumophila (1.2×10^2) + S. aureus (4.6×10^4) + 0. No spiking 5. P. aeruginosa (4.6×10^5) 6. P. aeruginosa (4.6×10^4) 7. P. aeruginosa (4.6×10^3) 8. P. aeruginosa (4.6×10^2)



Soil + Glass Beads

99 °C, 10 min



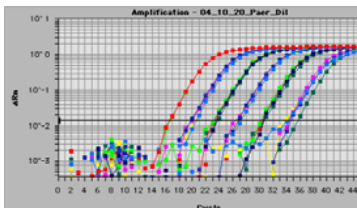
Bead beater, 1 min

M&N DNA
Extraction Kit



Genomic DNA

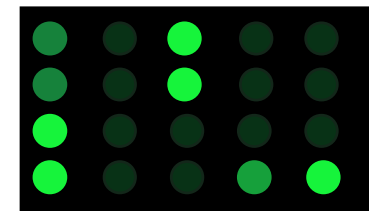
TaqMan PCR



2.5 %

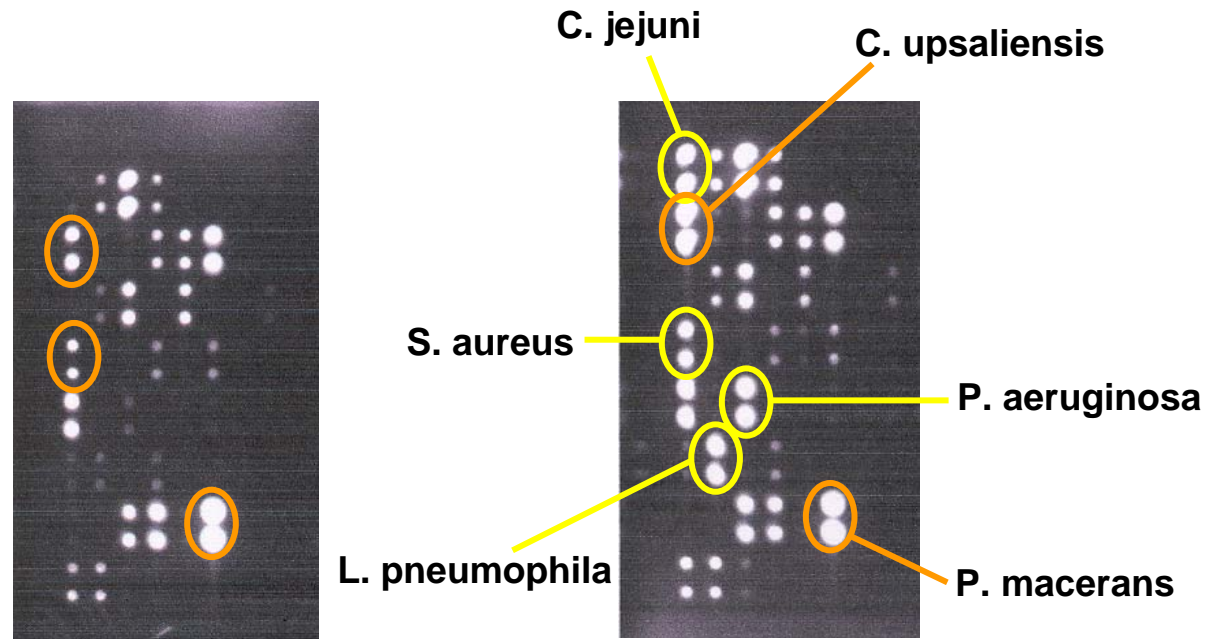
1.25 %

DNA Array





Detection of Bacteria in Soil Samples Using DNA Microarray



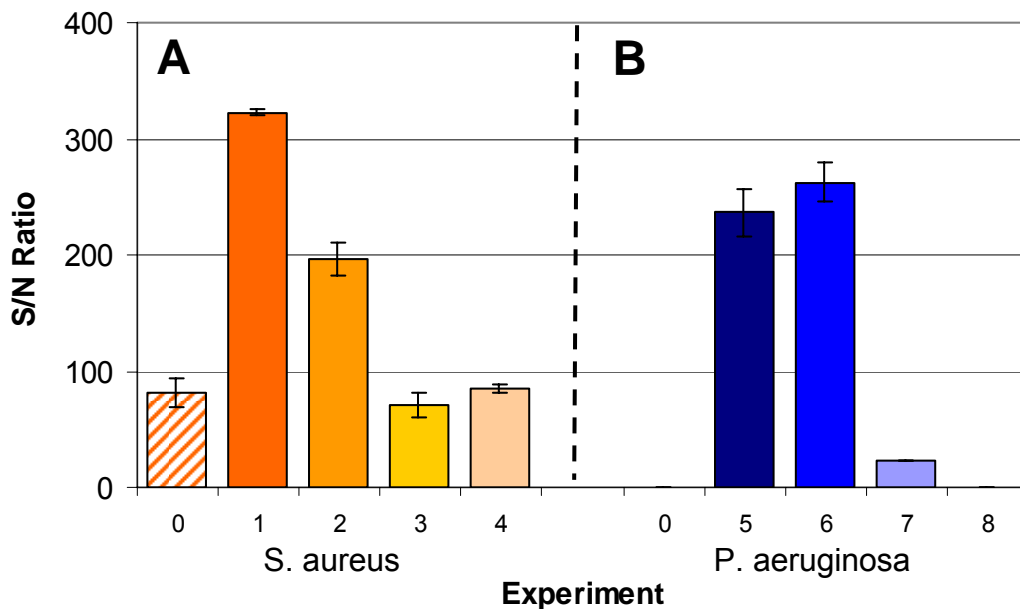
Soil only

Soil + C. jejuni
+ P. aeruginosa
+ S. aureus
+ L. pneumophila

Multiplex labeling and hybridization
with mix of 27 oligos



Detection of Spiked Bacteria in Soil Samples Using DNA Microarray



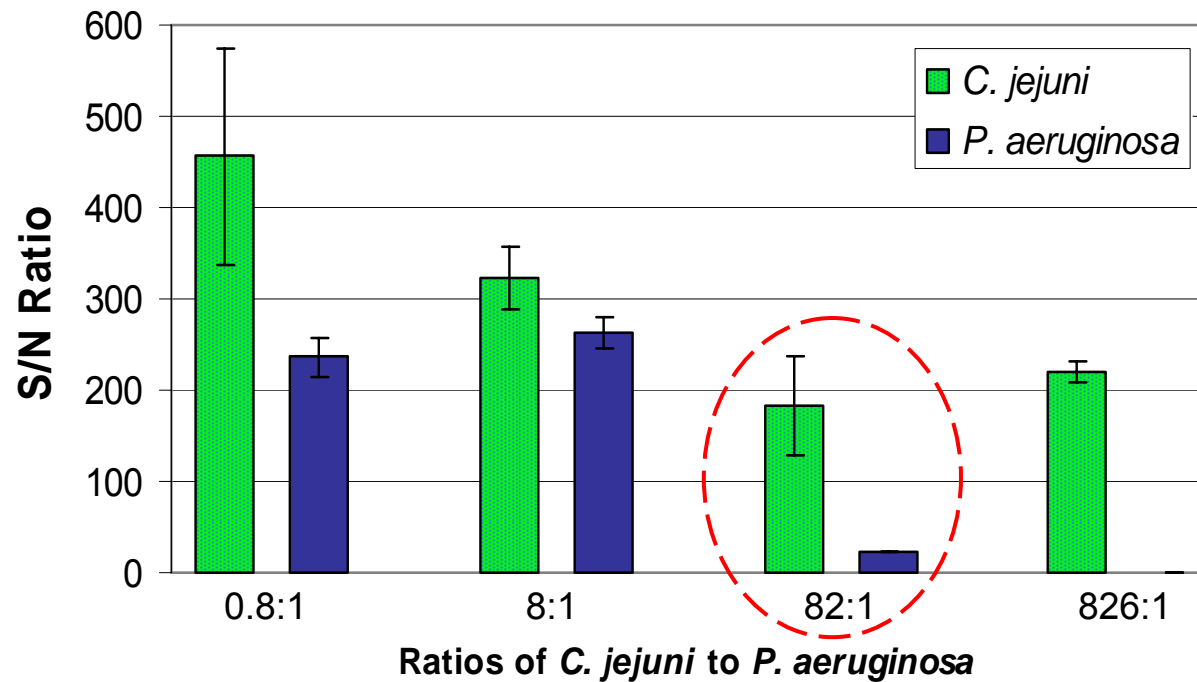
Spiking A Bacteria (cfu)	A	Spiking B Bacteria (cfu)	B
C. jejuni (3.8×10^5) + L. pneumophila (1.2×10^2) + P. aeruginosa (4.6×10^4) +		C. jejuni (3.8×10^5) + L. pneumophila (1.2×10^2) + S. aureus (4.6×10^4) +	
0. No spiking		0. No spiking	
1. S. aureus (4.6×10^5)		5. P. aeruginosa (4.6×10^5)	
2. S. aureus (4.6×10^4)		6. P. aeruginosa (4.6×10^4)	
3. S. aureus (4.6×10^3)		7. P. aeruginosa (4.6×10^3)	
4. S. aureus (4.6×10^2)		8. P. aeruginosa (4.6×10^2)	

Detection limit in soil:

- A. S. aureus: ~ 2000 cfu equivalents
- B. P. aeruginosa: ~ 200 cfu equivalents



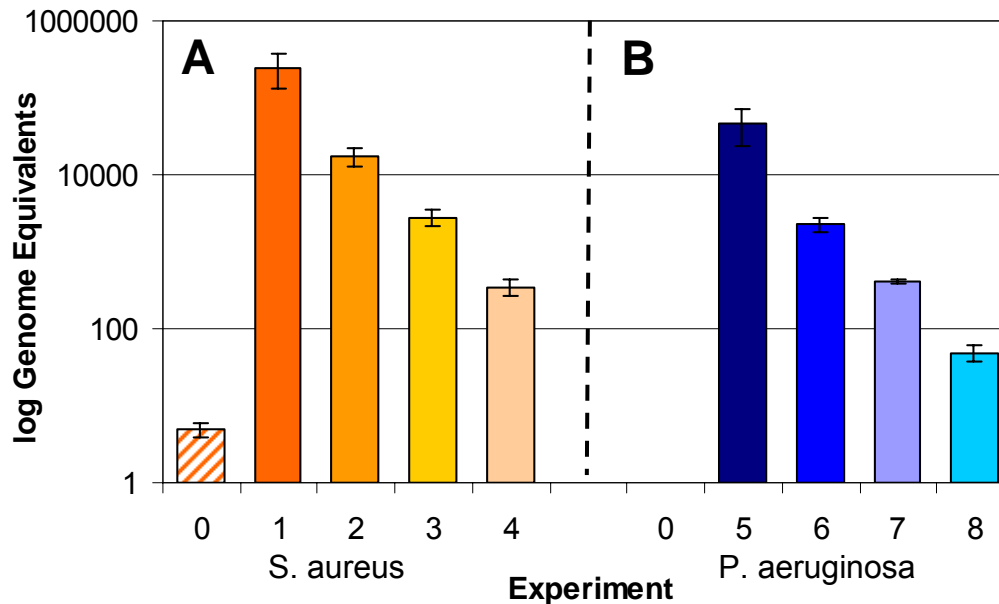
Detection Sensitivity of Spiked *P. aeruginosa* in Soil Samples Using DNA Microarray



Minimum relative abundance of *P. aeruginosa* in soil: 1.2 %



Detection of Spiked Bacteria in Soil Samples Using TaqMan PCR



Spiking A Bacteria (cfu)	A	Spiking B Bacteria (cfu)	B
C. jejuni (3.8×10^5) + L. pneumophila (1.2×10^2) + P. aeruginosa (4.6×10^4) +		C. jejuni (3.8×10^5) + L. pneumophila (1.2×10^2) + S. aureus (4.6×10^4) +	
0. No spiking		0. No spiking	
1. S. aureus (4.6×10^5)		5. P. aeruginosa (4.6×10^5)	
2. S. aureus (4.6×10^4)		6. P. aeruginosa (4.6×10^4)	
3. S. aureus (4.6×10^3)		7. P. aeruginosa (4.6×10^3)	
4. S. aureus (4.6×10^2)		8. P. aeruginosa (4.6×10^2)	

Detection limit in soil:

A. S. aureus: ~ 4 cfu equivalents

B. P. aeruginosa: ~ 4 cfu equivalents



DNA-Microarray and TaqMan PCR: A Comparison

	DNA-Microarray	TaqMan PCR
Advantage	<ul style="list-style-type: none">• Simultaneous detection of many targets (170 with Zeptosens platform)	<ul style="list-style-type: none">• Sensitivity is 50 – 500x better• Specificity is better due to single, highly specific reactions• Quantitative results
Disadvantage	<ul style="list-style-type: none">• Specificity is inferior• Data are only semi-quantitative	<ul style="list-style-type: none">• Costs are higher from a number of 20 targets or more compared to DNA-Array



Conclusions

- ▶ DNA Microarray is a suitable method for parallel detection and identification of low abundance bacteria within a complex microbial community considering limitations in sensitivity and specificity
- ▶ *In silico* prediction of probe specificity needs further improvement (expansion of NCBI database, collection of hybridization results)
- ▶ The detection limit of TaqMan PCR is lower, but costs are higher for large numbers of targets




KLBS 

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Hans Hosbach





Detection Limit of Bacterial DNA in Microarray and TaqMan PCR

A. DNA Microarray

S. aureus and *P. aeruginosa*: Dilution of DNA

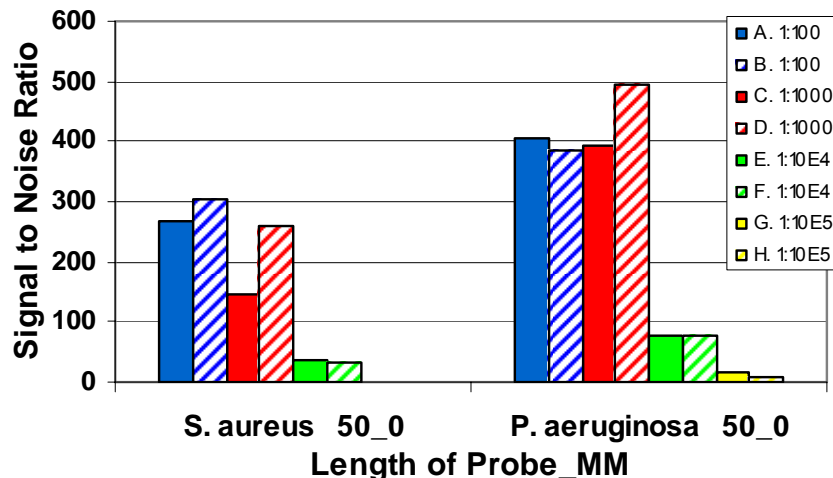


Figure shows 1 out of 4 experiments. MM: Mismatch

B. TaqMan PCR vs. DNA Microarray

	Genome Copies	
	Microarray	TaqMan PCR
<i>S. aureus</i>	50-800*	10-20
<i>P. aeruginosa</i>	80-400*	10-20

* Range of results from four different experiments

Experiment

Genomic DNA of 9 different bacteria + DNA dilutions of *S. aureus* or *P. aeruginosa* ($1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$). Hybridization after amplification and SSEL

Detection limit

S. aureus: $1:10^4$

P. aeruginosa: $1:10^5$

Detection limits for *S. aureus* and *P. aeruginosa* in DNA Microarray is only up to 40x higher compared to TaqMan PCR



Limitations of Microbial Diagnostic Microarrays (MDM) in Environmental Monitoring

- ▶ Microbial RNA is scarce in environmental samples → use of DNA
- ▶ PCR amplification of target DNA is necessary
 - enzymatically induced signal alterations or bias
 - quantification of samples not possible (rel. abundance)
- ▶ Conserved marker gene makes probe design more difficult
- ▶ Cross-reactivity can not be completely excluded for closely related bacteria
- ▶ Detection of DNA from dead bacteria can not be excluded
- ▶ Detection limit is higher than TaqMan PCR