HOPE(s) for Qantitative Environmental Microbiological Studies

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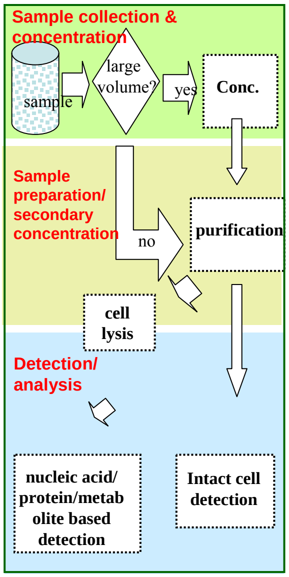
Division of Environmental Science & Engineering

Molecular Biological Tools (MBT)



Sample collection & concentration

sample

large volume?

yes

Conc.

Sample preparation/ secondary concentration

no

purification

cell lysis

Detection/ analysis

nucleic acid/ protein/metabolite based detection

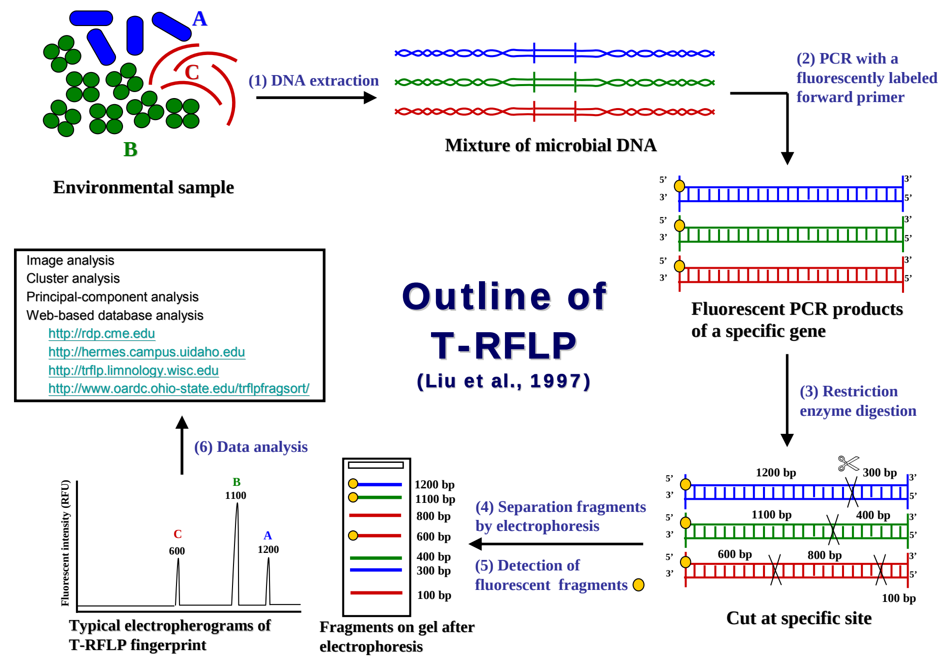
Intact cell detection

Nucleic Acid based	Whole cell based
- DNA/RNA extraction - DNA/RNA quantification	- Cell fixation
- Quantitation • q-PCR • q-rt PCR	- FISH • NA-based • immuno-assay
- Fingerprinting • DGGE • T-RFLP • LH-PCR • microarray	- MAR - SIP
- Sequencing • Clone library • Metagenomics	- Detection System • Optical microscope • RAMAN scope • Flow Cytometry

Other “Omics”

Challenges

- types of targets
- QA/QC
  - Reproducibility
  - Reliability
- Ease of use
- Sensitivity/specificity
  - lowest amount/conc.
  - 1-bp
- Quantitation
- Resolution (phylogeny)
- Time
- Cost
- Safety
- lab use v.s. on-site tools
  - clone library v.s. fingerprinting methods



Outline of T-RFLP

(Liu et al., 1997)

Image analysis
Cluster analysis
Principal-component analysis
Web-based database analysis
- http://rdp.cme.edu
- http://hermes.campus.uidaho.edu
- http://trflp.limnology.wisc.edu
- http://www.oardc.ohio-state.edu/trflpfragsort/

Constraints of current molecular quantitative techniques

The difficulty to conduct multiplexing analysis is the most severe bottleneck for launching large-scale investigation of microbial abundances.

Novel quantitative approach

- Easy
- Fast
- Multiplexing

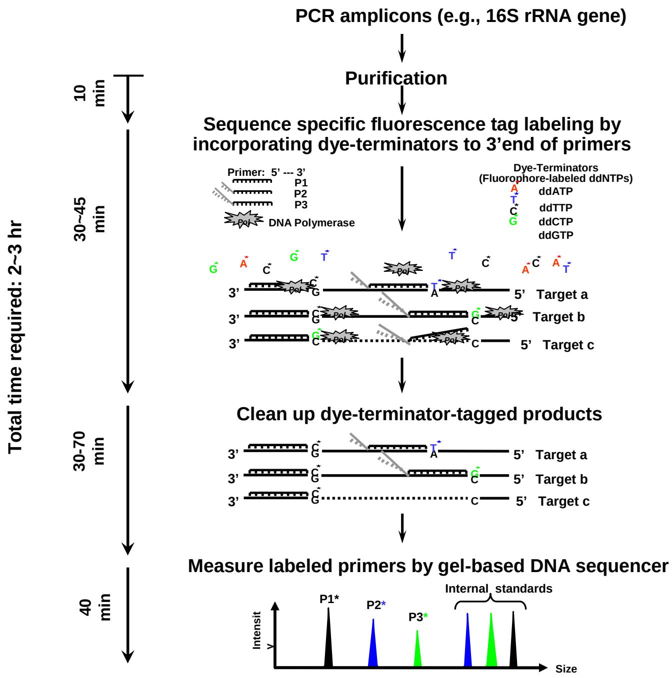
Hierarchical Oligonucleotide Primer Extension (HOPE) (Wu and Liu, NAR, 2007)

Acknowledgement

- Wu Jer-Horng
- Hong Pei-Ying
- NUS funding

Outline

- concept
- hierarchical primer design
- specificity
- sensitivity
- multiplexing



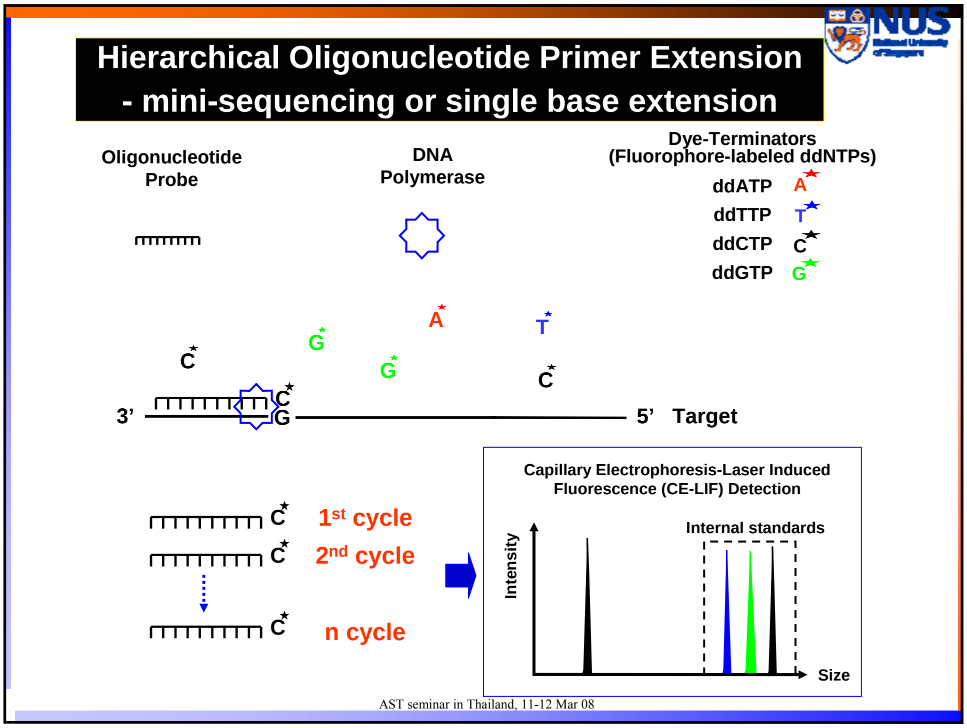
PCR amplicons (e.g., 16S rRNA gene)

Purification

Sequence specific fluorescence tag labeling by incorporating dye-terminators to 3'end of primers

Clean up dye-terminator-tagged products

Measure labeled primers by gel-based DNA sequencer



Hierarchical Oligonucleotide Primer Extension - mini-sequencing or single base extension

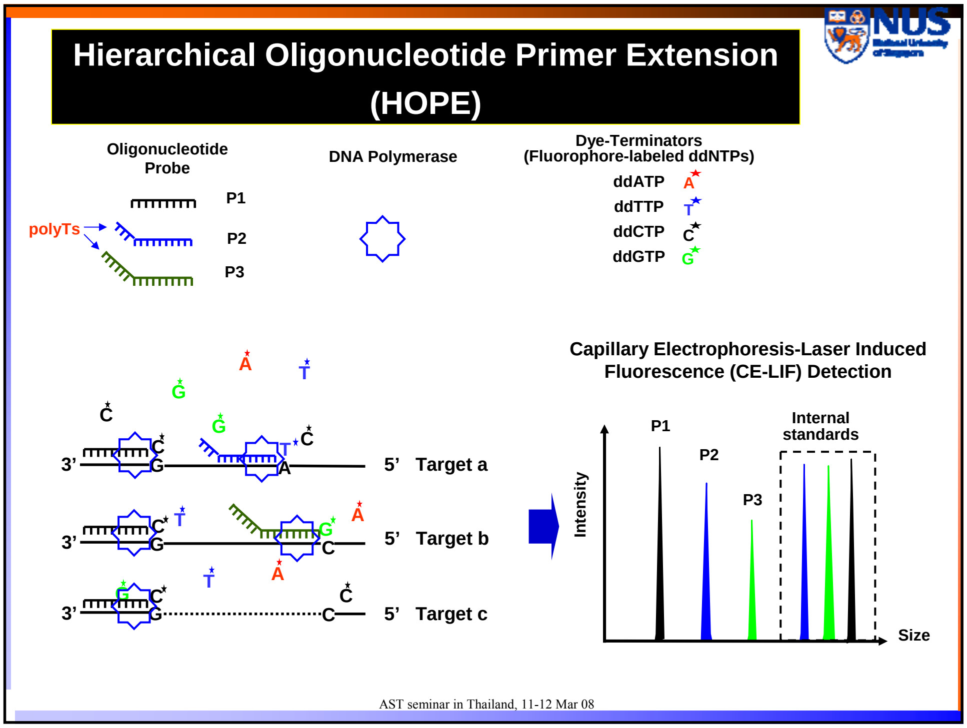
Oligonucleotide Probe

DNA Polymerase

Dye-Terminators (Fluorophore-labeled ddNTPs)

ddATP A*
ddTTP T*
ddCTP C*
ddGTP G*

Capillary Electrophoresis-Laser Induced Fluorescence (CE-LIF) Detection



Hierarchical Oligonucleotide Primer Extension (HOPE)

Oligonucleotide Probe

DNA Polymerase

Dye-Terminators (Fluorophore-labeled ddNTPs)

ddATP A*
ddTTP T*
ddCTP C*
ddGTP G*

Capillary Electrophoresis-Laser Induced Fluorescence (CE-LIF) Detection

Principle of HOPE

$C_{p1} = C_{o,\,p1} \times E_1 \times n$

$C_{p2} = C_{o,\,p2} \times E_2 \times n$

$C_{p3} = C_{o,\,p3} \times E_3 \times n$

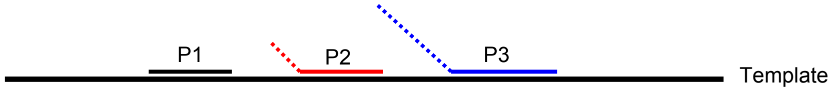
C, final concentration of the extended primer,
C$_o$, the initial concentration of target template
E, the extension efficiency, a constant
n, the number of thermal cycles

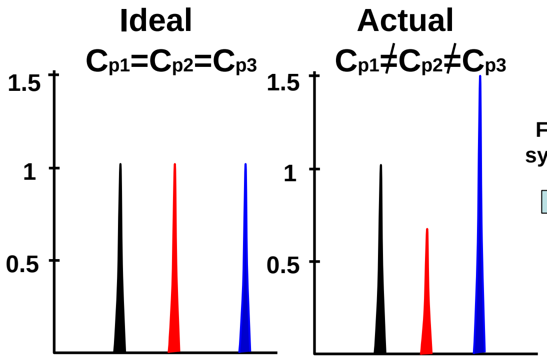
E_1, E_2, E_3, constant, but **$E_1 \neq E_2 \neq E_3$**

$E_2 / E_1 = a$
$E_3 / E_1 = b$ ➡

Calibrated
$C_{p1} = aC_{p2} = bC_{p3}$

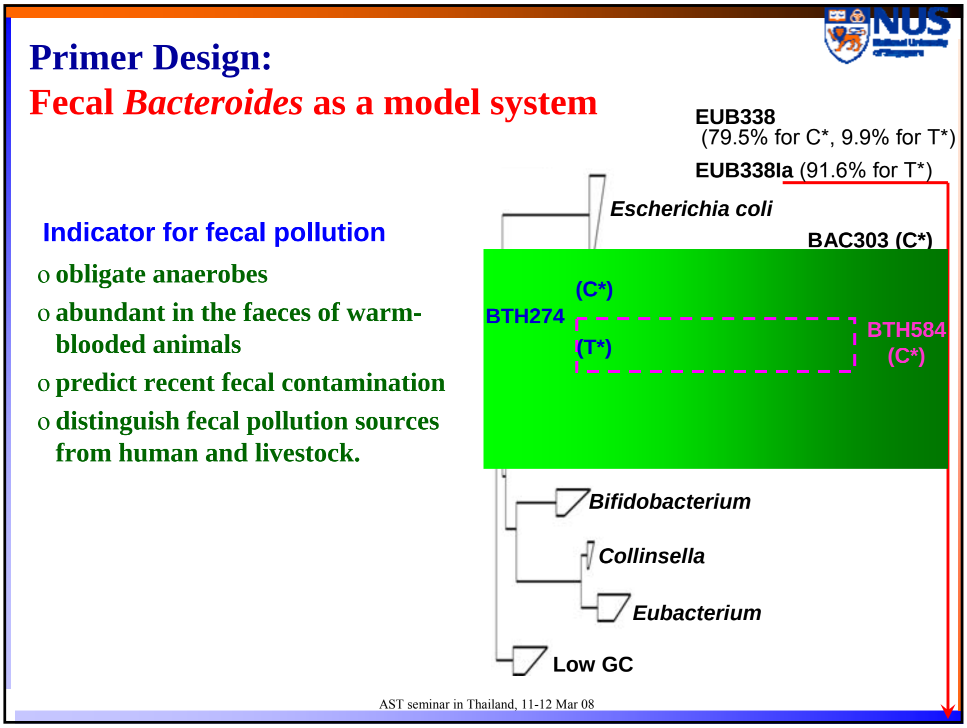
Hierarchical probes





Factors to synchronize ➡

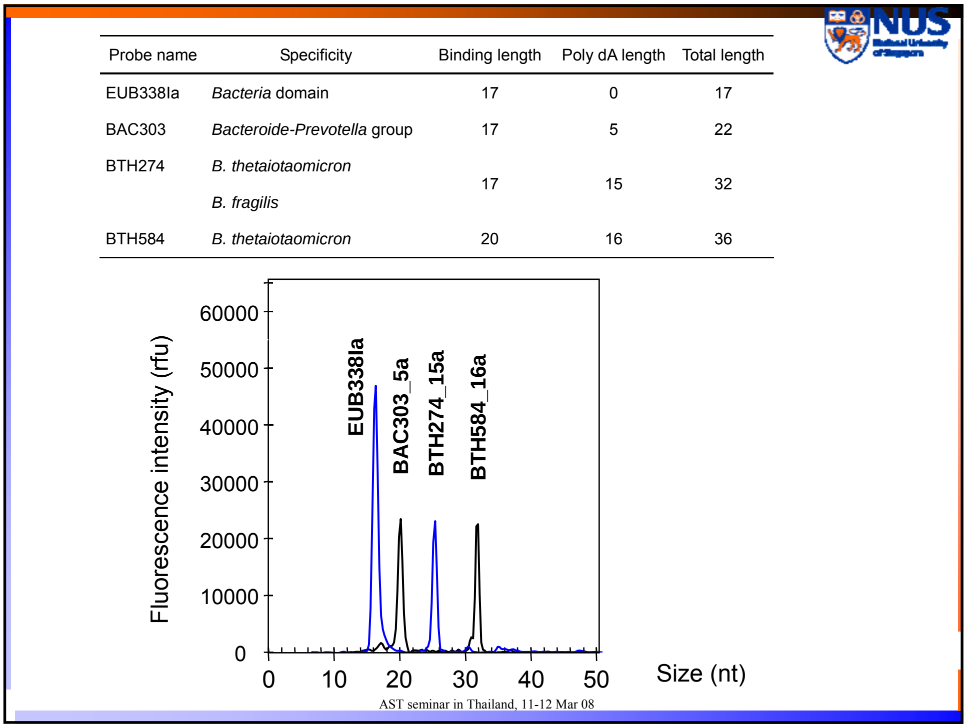
Calibrated
$C_{p1}=aC_{p2}=bC_{p3}$



Primer Design:
Fecal *Bacteroides* as a model system

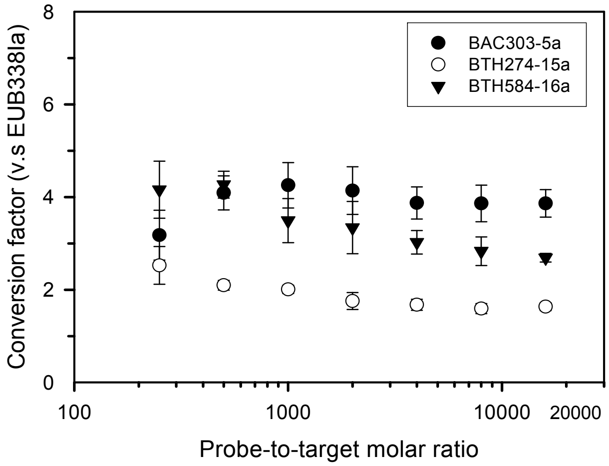
Indicator for fecal pollution

- obligate anaerobes
- abundant in the faeces of warm-blooded animals
- predict recent fecal contamination
- distinguish fecal pollution sources from human and livestock.



Probe name	Specificity	Binding length	Poly dA length	Total length
EUB338Ia	*Bacteria* domain	17	0	17
BAC303	*Bacteroide-Prevotella* group	17	5	22
BTH274	*B. thetaiotaomicron* *B. fragilis*	17	15	32
BTH584	*B. thetaiotaomicron*	20	16	36

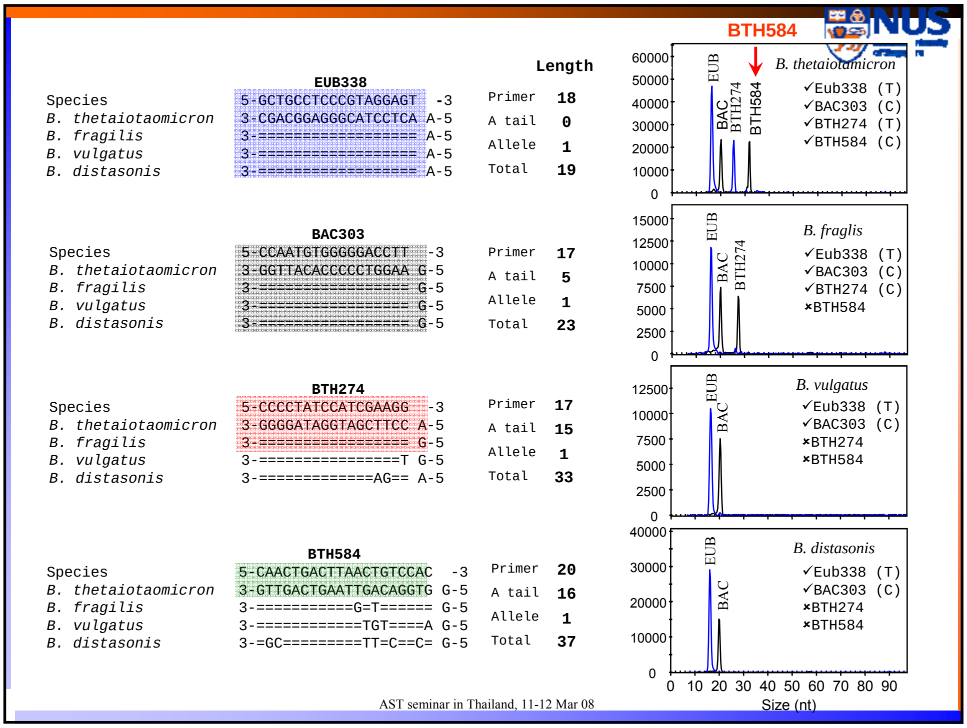
Conversion factor = constant?



(Fixed probe concentration but varied template concentrations)

Specificity

- Target vs. non-target



Length

EUB338

```
Species                  5-GCTGCCTCCCGTAGGAGT  -3     Primer   18
B. thetaiotaomicron      3-CGACGGAGGGCATCCTCA A-5     A tail    0
B. fragilis              3-================== A-5     Allele    1
B. vulgatus              3-================== A-5     Total    19
B. distasonis            3-================== A-5
```

BAC303

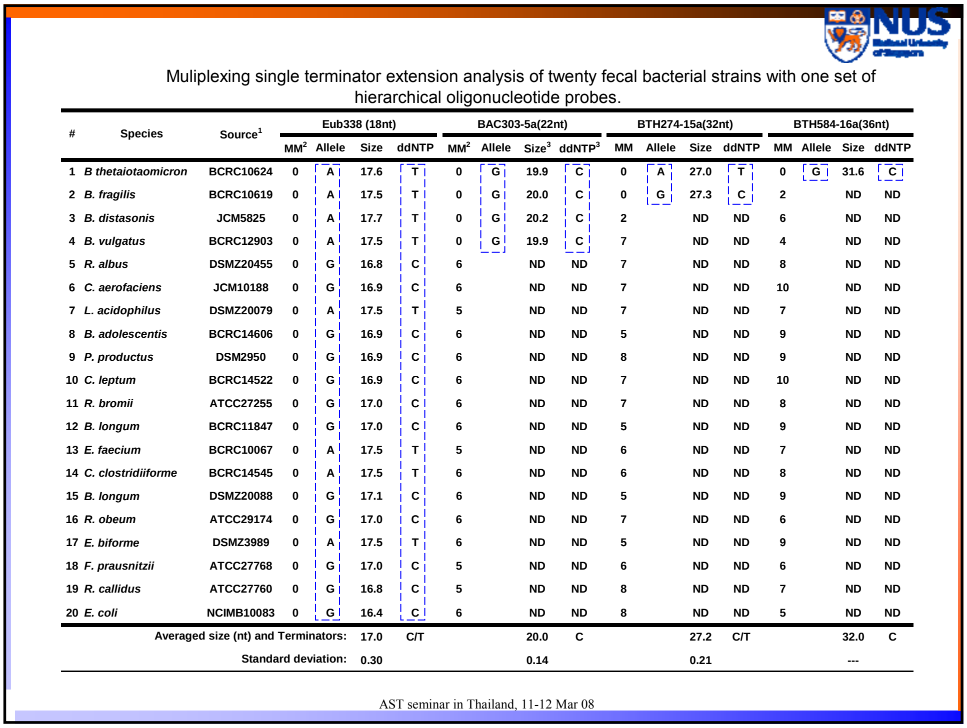
```
Species                  5-CCAATGTGGGGGACCTT  -3      Primer   17
B. thetaiotaomicron      3-GGTTACACCCCCTGGAA G-5      A tail    5
B. fragilis              3-================= G-5      Allele    1
B. vulgatus              3-================= G-5      Total    23
B. distasonis            3-================= G-5
```

BTH274

```
Species                  5-CCCCTATCCATCGAAGG  -3      Primer   17
B. thetaiotaomicron      3-GGGGATAGGTAGCTTCC A-5      A tail   15
B. fragilis              3-================= G-5      Allele    1
B. vulgatus              3-================T G-5      Total    33
B. distasonis            3-=============AG== A-5
```

BTH584

```
Species                  5-CAACTGACTTAACTGTCCAC  -3     Primer   20
B. thetaiotaomicron      3-GTTGACTGAATTGACAGGTG G-5     A tail   16
B. fragilis              3-===========G=T====== G-5     Allele    1
B. vulgatus              3-============TGT====A G-5     Total    37
B. distasonis            3-=GC=========TT=C==C= G-5
```



Muliplexing single terminator extension analysis of twenty fecal bacterial strains with one set of hierarchical oligonucleotide probes.

#	Species	Source[1]	Eub338 (18nt)				BAC303-5a(22nt)				BTH274-15a(32nt)				BTH584-16a(36nt)			
			MM[2]	Allele	Size	ddNTP	MM[2]	Allele	Size[3]	ddNTP[3]	MM	Allele	Size	ddNTP	MM	Allele	Size	ddNTP
1	*B thetaiotaomicron*	BCRC10624	0	A	17.6	T	0	G	19.9	C	0	A	27.0	T	0	G	31.6	C
2	*B. fragilis*	BCRC10619	0	A	17.5	T	0	G	20.0	C	0	G	27.3	C	2		ND	ND
3	*B. distasonis*	JCM5825	0	A	17.7	T	0	G	20.2	C	2		ND	ND	6		ND	ND
4	*B. vulgatus*	BCRC12903	0	A	17.5	T	0	G	19.9	C	7		ND	ND	4		ND	ND
5	*R. albus*	DSMZ20455	0	G	16.8	C	6		ND	ND	7		ND	ND	8		ND	ND
6	*C. aerofaciens*	JCM10188	0	G	16.9	C	6		ND	ND	7		ND	ND	10		ND	ND
7	*L. acidophilus*	DSMZ20079	0	A	17.5	T	5		ND	ND	7		ND	ND	7		ND	ND
8	*B. adolescentis*	BCRC14606	0	G	16.9	C	6		ND	ND	5		ND	ND	9		ND	ND
9	*P. productus*	DSM2950	0	G	16.9	C	6		ND	ND	8		ND	ND	9		ND	ND
10	*C. leptum*	BCRC14522	0	G	16.9	C	6		ND	ND	7		ND	ND	10		ND	ND
11	*R. bromii*	ATCC27255	0	G	17.0	C	6		ND	ND	7		ND	ND	8		ND	ND
12	*B. longum*	BCRC11847	0	G	17.0	C	6		ND	ND	5		ND	ND	9		ND	ND
13	*E. faecium*	BCRC10067	0	A	17.5	T	5		ND	ND	6		ND	ND	7		ND	ND
14	*C. clostridiiforme*	BCRC14545	0	A	17.5	T	6		ND	ND	6		ND	ND	8		ND	ND
15	*B. longum*	DSMZ20088	0	G	17.1	C	6		ND	ND	5		ND	ND	9		ND	ND
16	*R. obeum*	ATCC29174	0	G	17.0	C	6		ND	ND	7		ND	ND	6		ND	ND
17	*E. biforme*	DSMZ3989	0	A	17.5	T	6		ND	ND	5		ND	ND	9		ND	ND
18	*F. prausnitzii*	ATCC27768	0	G	17.0	C	5		ND	ND	6		ND	ND	6		ND	ND
19	*R. callidus*	ATCC27760	0	G	16.8	C	5		ND	ND	8		ND	ND	7		ND	ND
20	*E. coli*	NCIMB10083	0	G	16.4	C	6		ND	ND	8		ND	ND	5		ND	ND
	Averaged size (nt) and Terminators:				17.0	C/T			20.0	C			27.2	C/T			32.0	C
	Standard deviation:				0.30				0.14				0.21				---	

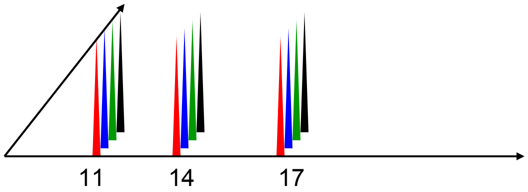
Multiplexing

- 4 Plexing
- 6 Plexing
- 7 Plexing
- Multiple tube approach

Multiplexing – theoretical

n (length type) x m (fluorescence type)

ATGCGGGCGC A	ATGCGGGCGCGCC A	ATGCGGGCGATCTT A
TTGCATGCCG T	TTGCATGCCGTTA T	TTGCATGCCGGGCG T
TCCATGCGGG G	TCCATGCGGGTGG G	TCCATGCGGGAATT G
AGTGCGCGCG C	AGTGCGCGCGAAT C	AGTGCGCGCGCCAT C



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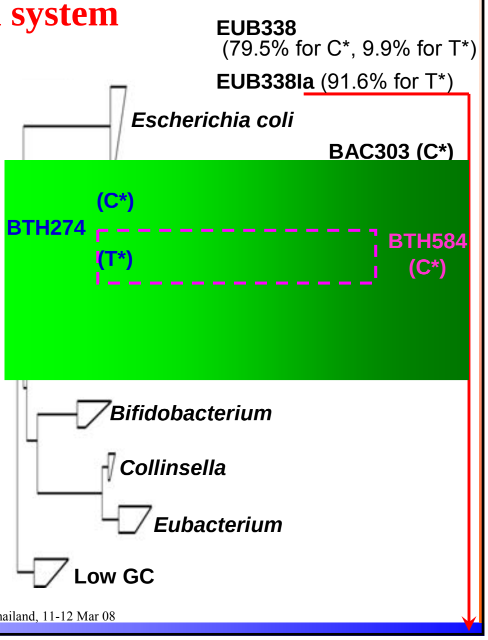


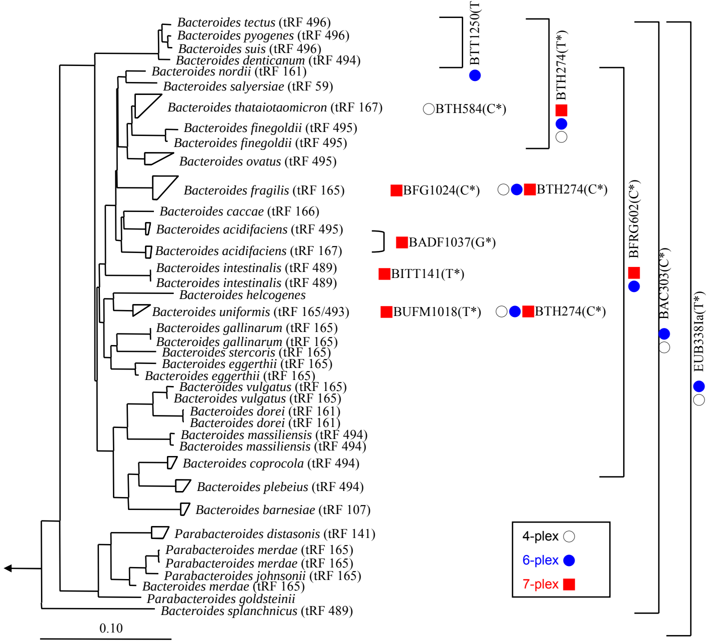
Model microbial communities.

Artificial communities	% of the probes for specific groups with respect to probe EUB338Ia for domain *Bacteria* (mean ± sd, n=6)					
	BAC303-5a (for *Bacteroides*)		**BTH274-15a** (for *B. thetaiotaomicron*)		**BTH584-16a** (for *B. thetaiotaomicron*)	
	Theoretical	Observed	Theoretical	Observed	Theoretical	Observed
[1]AC1	72.7	77.0 ± 3.9	22.1	18.4 ± 0.7	22.1	18.5 ± 1.2
[2]AC2	46.2	49.2 ± 3.6	21.8	17.6 ± 0.7	21.8	18.1 ± 1.4
[3]AC3	21.5	14.8 ± 1.0	21.5	14.4 ± 0.9	21.5	14.9 ± 1.0
AC2 + *E. coli* (15.8 fmol/µl)	11.1	9.7 ± 0.8	5.2	2.8 ± 0.3	5.2	3.4 ± 1.0
AC2 + *M. barkeri* (12.8 fmol/µl)	46.2	53.1 ± 2.9	21.8	17.4 ± 0.4	21.8	15.3 ± 1.2

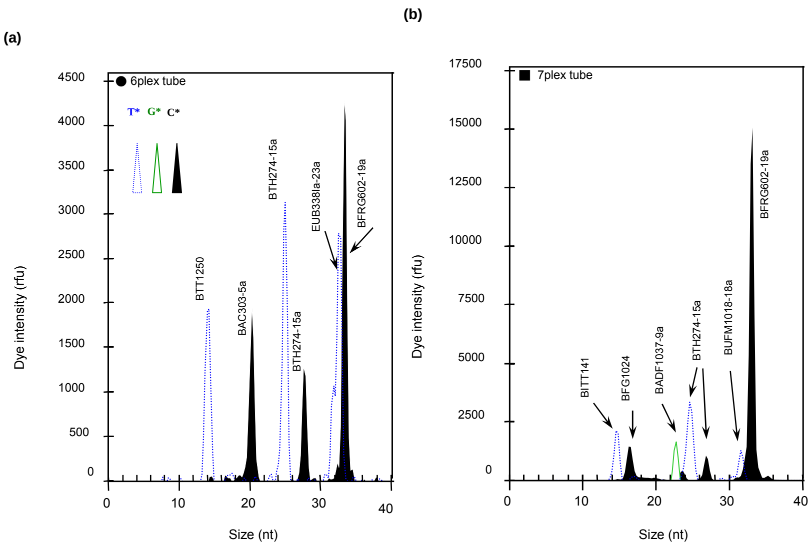
[1] AC1 contained 16S rRNA gene amplicons of ***B. thetaiotaomicron* (2.2 fmol/µl), *B. distasonis* (2.5 fmol/µl), *B. vulgatus* (2.5 fmol/µl) and *L. acidophilus* (2.7 fmol/µl).**

[2] AC2 contained 16S rRNA gene amplicons of *B. thetaiotaomicron* (2.2 fmol/µl), *B. distasonis* (2.5 fmol/µl), *L. acidophilus* (2.7 fmol/µl) and *E. faecium* (2.7 fmol/µl).

[3] AC3 contained 16S rRNA gene amplicons of *B. thetaiotaomicron* (2.2 fmol/µl), *L. acidophilus* (2.7 fmol/µl) and *E. faecium* (2.7 fmol/µl), *P. productus* (2.7 fmol/µl).



AST seminar in Thailand, 11-12 Mar 08



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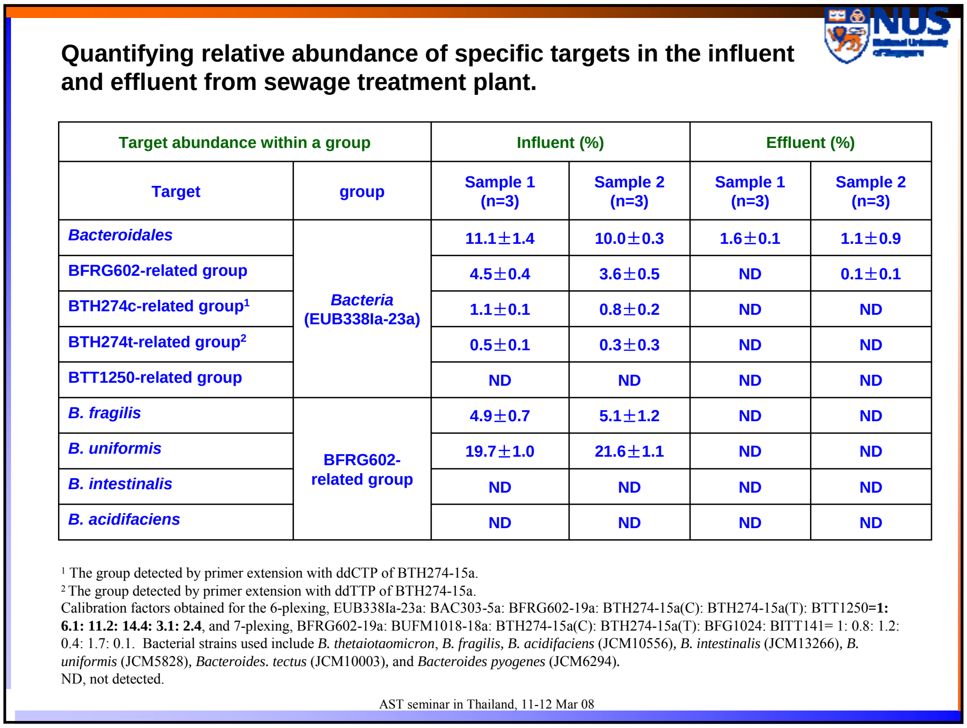


Quantifying relative abundance of specific targets in the influent and effluent from sewage treatment plant.

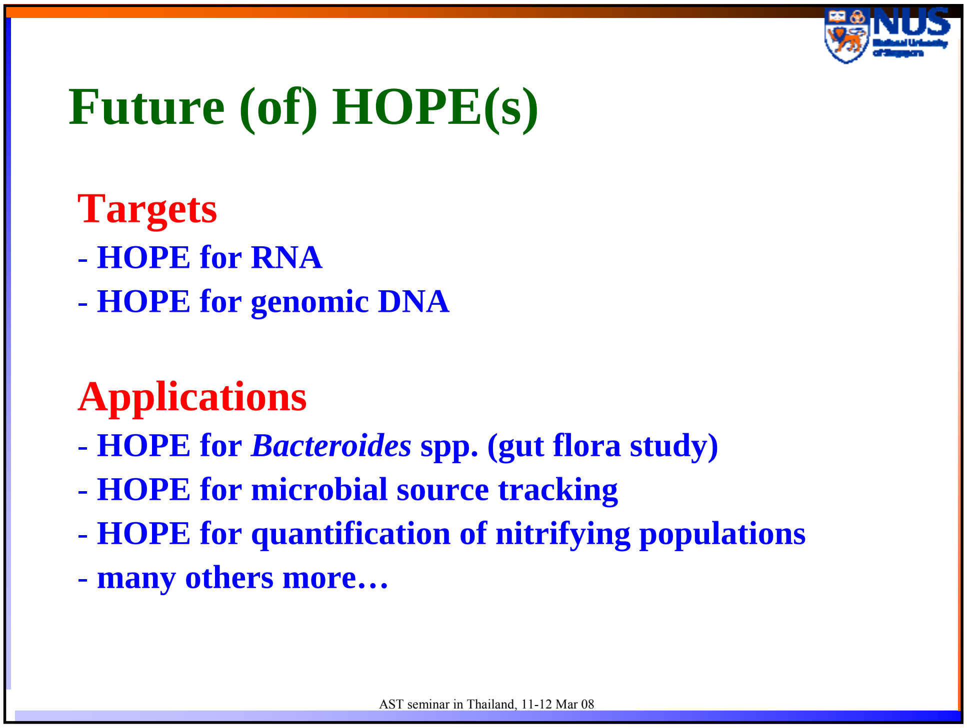
Target abundance within a group		Influent (%)		Effluent (%)	
Target	group	Sample 1 (n=3)	Sample 2 (n=3)	Sample 1 (n=3)	Sample 2 (n=3)
Bacteroidales	*Bacteria* (EUB338Ia-23a)	11.1±1.4	10.0±0.3	1.6±0.1	1.1±0.9
BFRG602-related group		4.5±0.4	3.6±0.5	ND	0.1±0.1
BTH274c-related group[1]		1.1±0.1	0.8±0.2	ND	ND
BTH274t-related group[2]		0.5±0.1	0.3±0.3	ND	ND
BTT1250-related group		ND	ND	ND	ND
B. fragilis	BFRG602-related group	4.9±0.7	5.1±1.2	ND	ND
B. uniformis		19.7±1.0	21.6±1.1	ND	ND
B. intestinalis		ND	ND	ND	ND
B. acidifaciens		ND	ND	ND	ND

[1] The group detected by primer extension with ddCTP of BTH274-15a.

[2] The group detected by primer extension with ddTTP of BTH274-15a.

Calibration factors obtained for the 6-plexing, EUB338Ia-23a: BAC303-5a: BFRG602-19a: BTH274-15a(C): BTH274-15a(T): BTT1250=**1: 6.1: 11.2: 14.4: 3.1: 2.4**, and 7-plexing, BFRG602-19a: BUFM1018-18a: BTH274-15a(C): BTH274-15a(T): BFG1024: BITT141= 1: 0.8: 1.2: 0.4: 1.7: 0.1. Bacterial strains used include *B. thetaiotaomicron*, *B. fragilis, B. acidifaciens* (JCM10556), *B. intestinalis* (JCM13266), *B. uniformis* (JCM5828), *Bacteroides. tectus* (JCM10003), and *Bacteroides pyogenes* (JCM6294).

ND, not detected.



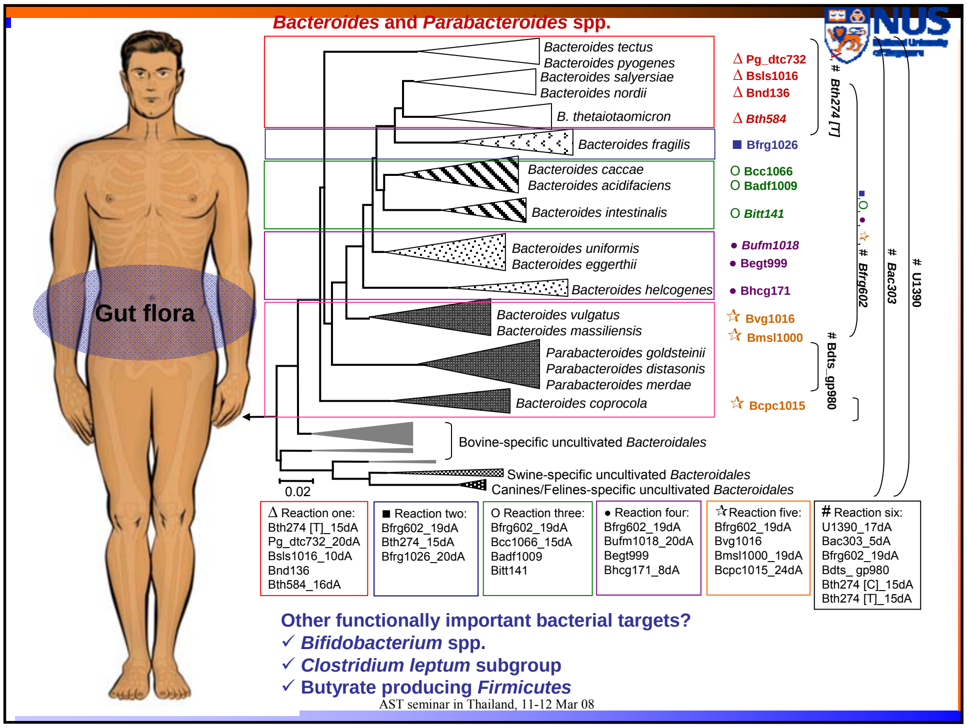
Future (of) HOPE(s)

Targets

- HOPE for RNA
- HOPE for genomic DNA

Applications

- HOPE for *Bacteroides* spp. (gut flora study)
- HOPE for microbial source tracking
- HOPE for quantification of nitrifying populations
- many others more…



Bacteroides and *Parabacteroides* spp.

Δ Reaction one:
Bth274 [T]_15dA
Pg_dtc732_20dA
Bsls1016_10dA
Bnd136
Bth584_16dA

■ Reaction two:
Bfrg602_19dA
Bth274_15dA
Bfrg1026_20dA

O Reaction three:
Bfrg602_19dA
Bcc1066_15dA
Badf1009
Bitt141

● Reaction four:
Bfrg602_19dA
Bufm1018_20dA
Begt999
Bhcg171_8dA

☆ Reaction five:
Bfrg602_19dA
Bvg1016
Bmsl1000_19dA
Bcpc1015_24dA

Reaction six:
U1390_17dA
Bac303_5dA
Bfrg602_19dA
Bdts_ gp980
Bth274 [C]_15dA
Bth274 [T]_15dA

Other functionally important bacterial targets?
- ✓ ***Bifidobacterium* spp.**
- ✓ ***Clostridium leptum* subgroup**
- ✓ **Butyrate producing *Firmicutes***

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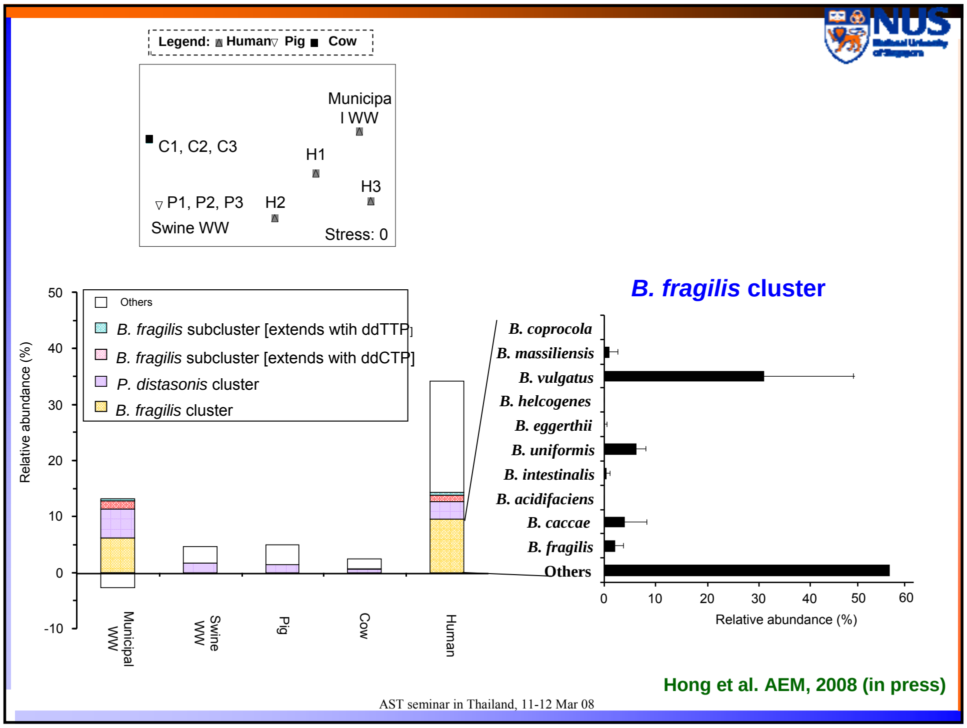

***B. fragilis* cluster**

Hong et al. AEM, 2008 (in press)

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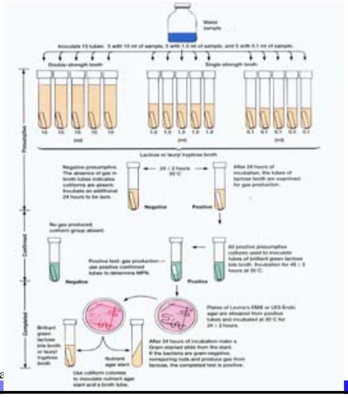
Fecal source tracking

Fecal contamination in drinking water sources and recreational bodies

- ○Adverse health effects and liability issues

Conventional monitoring and detection

- ○24 - 48h FC test
- ○Cultivation bias
- ○Not able to pinpoint the fecal origin



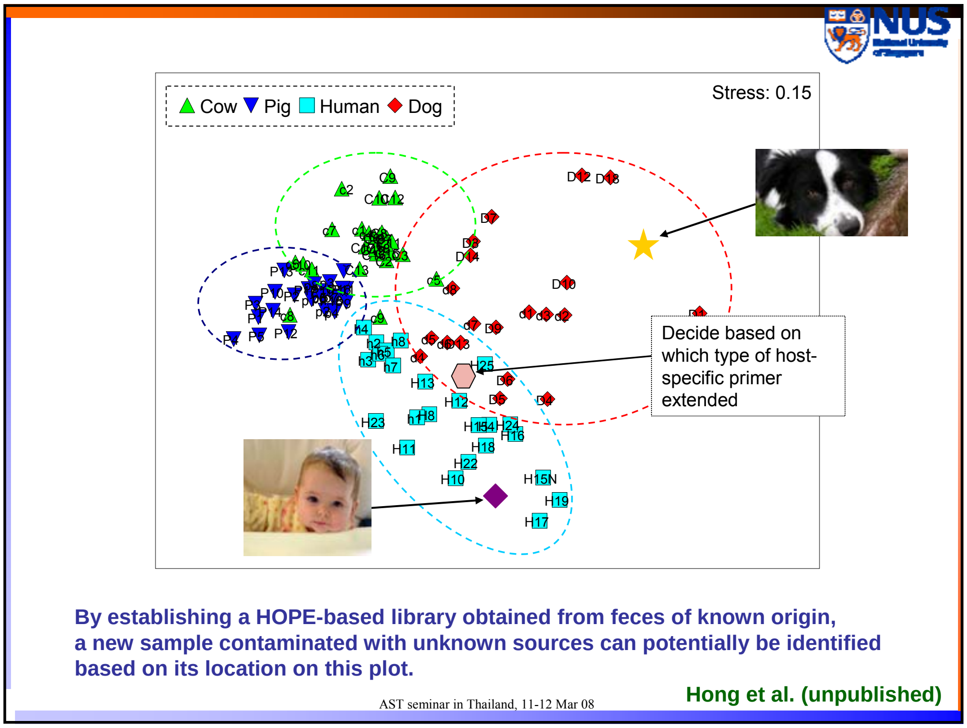
HOPE for microbial source tracking

Human? **Cow?** **Pig?** **Dog?**

Host-specific primers

Predominantly in human feces	Bovine-specific	Swine-specific	Canine-specific
Bacteroides caccae (Bcc1066)	Uncultivated bovine-specific *Bacteroidales* (C367)	Uncultivated swine-specific *Bacteroidales* (P163)	Uncultivated canine-specific *Bacteroidales* (D475)
Bacteroides fragilis (Bfrg1028)			
Bacteroides vulgatus (Bvg1016)			
Bacteroides uniformis (Bufm1018)			

1. **Presence and absence of extended host-specific primer (library-independent)**
2. **Relative abundance of each microbial target (library-dependent)**



Decide based on which type of host-specific primer extended

By establishing a HOPE-based library obtained from feces of known origin, a new sample contaminated with unknown sources can potentially be identified based on its location on this plot.

Hong et al. (unpublished)

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Thank You!

More HOPEs…