

# Microbial Multiplexing for Fun and Profit

A Full-Workflow Case Study on a Collection of Diverse Isolates

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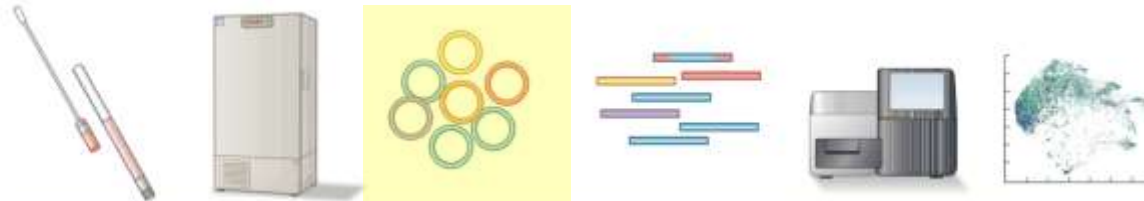
Pacific Biosciences User Group Meeting

9/20/2018



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# Sources of Error/Bias in Microbiome Sequencing



Sample processing step

Technical sources of error and bias

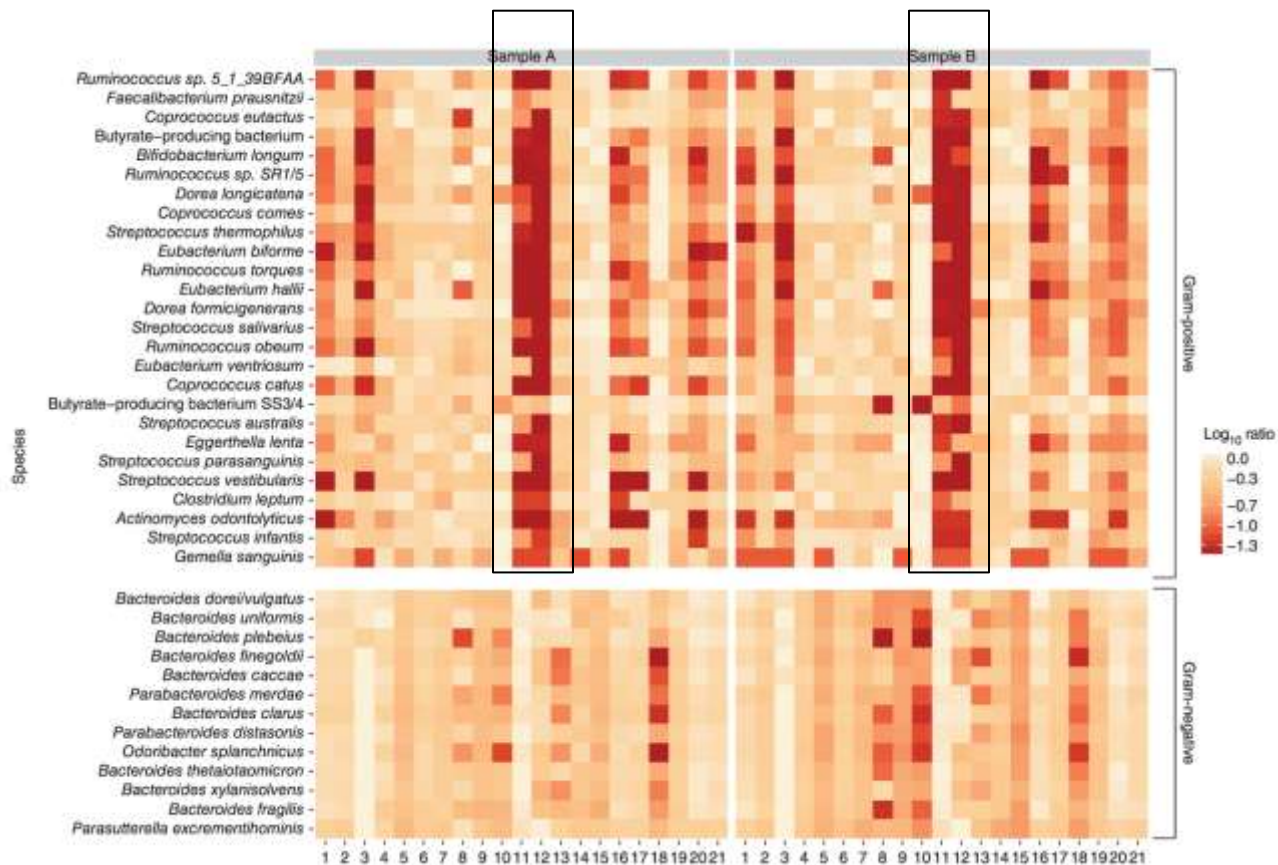
Sample processing step	Sample collection	Sample storage	DNA extraction	Sequencing library preparation	DNA sequencing	Computational analysis
Technical sources of error and bias	<ul style="list-style-type: none"> <li>Inadequate sampling</li> <li>Incomplete sample stabilization</li> <li>Sampling kit contamination</li> <li>Mislabeling of samples</li> </ul>	<ul style="list-style-type: none"> <li>Change in community structure due to differential growth</li> <li>Degradation of DNA during freeze-thaw cycles</li> </ul>	<ul style="list-style-type: none"> <li>Differential recovery of DNA from different strains</li> <li>Extraction kit contamination</li> <li>Sample swaps during transfer</li> <li>Sample cross-contamination</li> </ul>	<ul style="list-style-type: none"> <li>Quantitative amplification bias (PCR efficiency)</li> <li>Qualitative amplification bias (primer mismatches)</li> <li>Amplification errors (PCR chimeras, substitution errors)</li> <li>Reagent contamination</li> <li>Sample cross-contamination</li> </ul>	<ul style="list-style-type: none"> <li>Sequencing errors</li> <li>Run-to-run carryover</li> <li>Barcode swapping</li> <li>Demultiplexing errors</li> </ul>	<ul style="list-style-type: none"> <li>Suboptimal quality control or filtering</li> <li>Alignment errors</li> <li>Database errors</li> <li>Database bias</li> <li>Batch effects</li> <li>Failure to flag contaminants</li> </ul>

- **Mock communities**
  - Zymo
  - HMP Even/Staggered
  - ATCC
  - ABRF
  - Others
- **Synthetic standards**
  - Sequins
  - AIST (Toulouse) 16S spike-in

Gohl, Daryl M. "The ecological landscape of microbiome science." *Nature biotechnology* 35.11 (2017): 1047.

# DNA Extraction

Gram Positive = Under-represented



Costea, Paul I., et al. "Towards standards for human fecal sample processing in metagenomic studies." *Nature biotechnology* 35.11 (2017): 1069.



# Xenobiologic Microbial Standards



Cell-based standard of microbes not observed in human samples



*In development*

Organism	Gram stain	GC content	Genome Size (Mb)	Environment
<i>Flavobacterium pectinovorum</i>	Gram -	34%	5.61	Soil
<i>Nonlabens xylanidelens</i>	Gram -	35%	3.28	Aquatic, Marine
<i>Niastella koreensis</i>	Gram -	45%	9.03	Soil
<i>Algoriphagus terrigena</i>	Gram -	48%	5.78	Soil
<i>Saccharomonospora viridis</i>	Gram -	67%	4.31	Bog, Wetland
<i>Bordetella bronchiseptica</i>	Gram -	68%	5.34	Host pathogen
<i>Granulicatella balaenopterae</i>	Gram+	34%	2.14	Unknown, isolated from minke whale
<i>Staphylococcus condimenti</i>	Gram+	35%	2.60	Fermentation
<i>Jeotgalibacillus marinus</i>	Gram+	44%	4.10	Sediment
<i>Lactobacillus zeae</i>	Gram+	48%	3.11	Dairy isolates, Food factory water, Intestinal tract
<i>Gordonia polyisoprenivorans</i>	Gram+	67%	5.84	Soil
<i>Serinibacter salmonus</i>	Gram+	71%	3.27	Host (Fish)



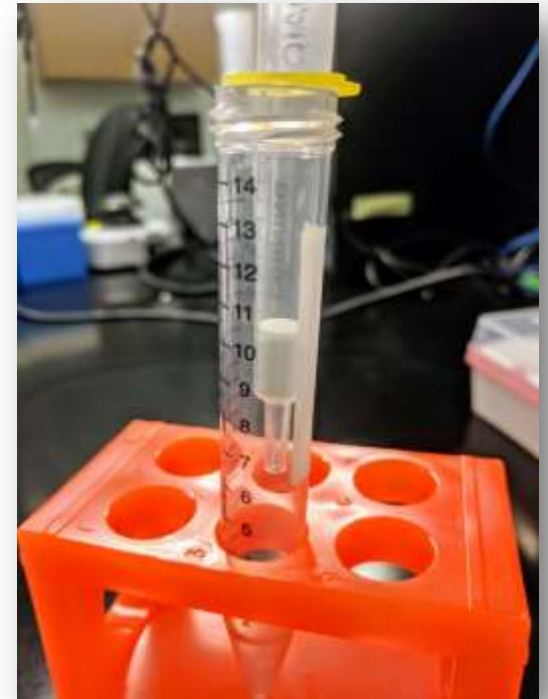
# Extraction

## Qiagen Genomic Tip 20/G

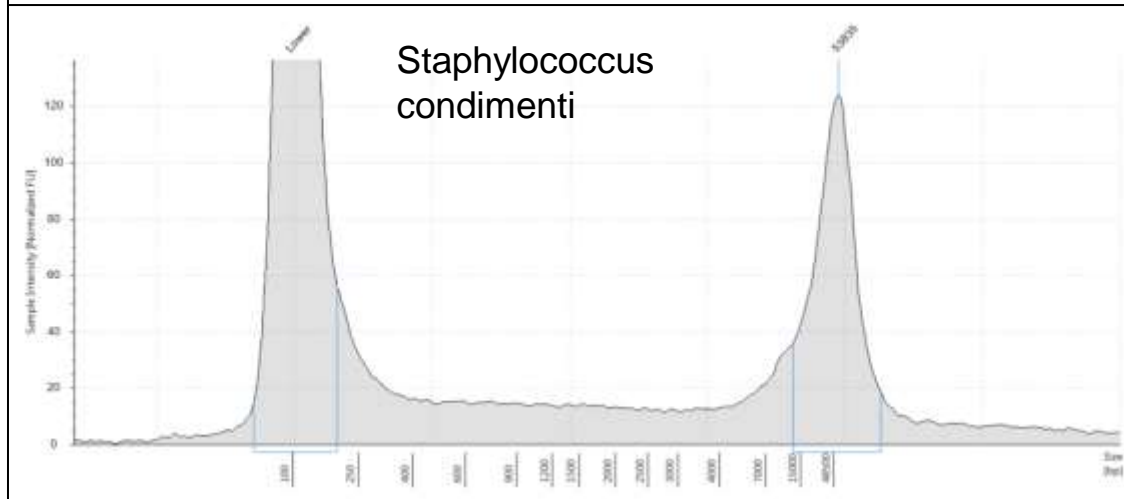
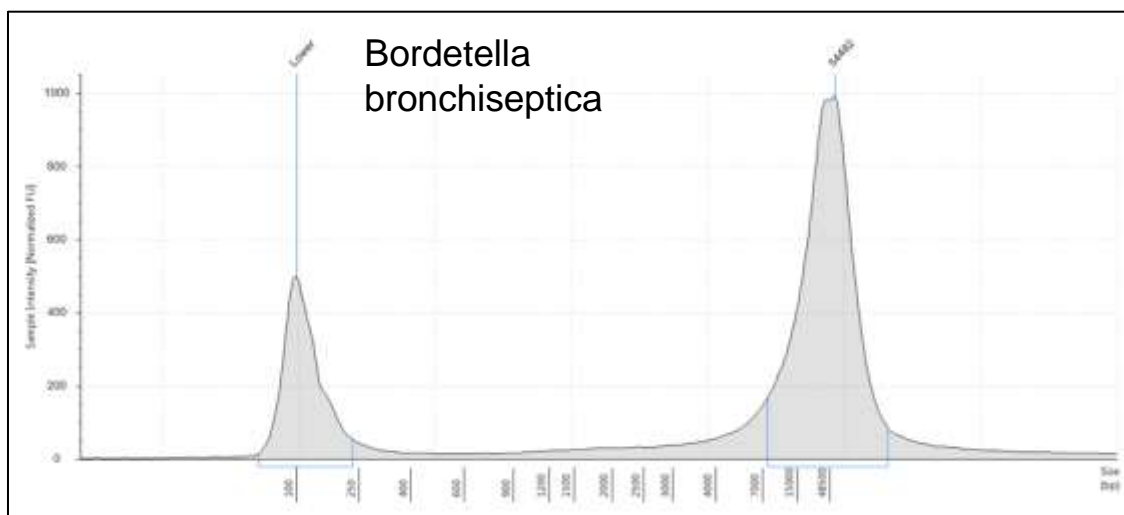
Enzymatic/Chemical lysis

Gravity column purification

Organism	Gram stain	Cells	Mass (ng)
Flavobacterium pectinovorum	Gram -	3.60E+09	<b>4400</b>
Nonlabens xylanidelens	Gram -	3.60E+09	<b>1836</b>
Algoriphagus terrigena	Gram -	3.00E+09	<b>7600</b>
Bordetella bronchiseptica	Gram -	3.60E+09	<b>4480</b>
Staphylococcus condimenti	Gram+	4.50E+09	<b>1032</b>
Jeotgalibacillus marinus	Gram+	3.00E+09	<b>4160</b>
Gordonia polyisoprenivorans	Gram+	8.85E+08	<b>2900</b>
Serinibacter salmoneus	Gram+	2.40E+09	<b>1280</b>



Qiagen Genomic Tip 20/G



Gram negatives



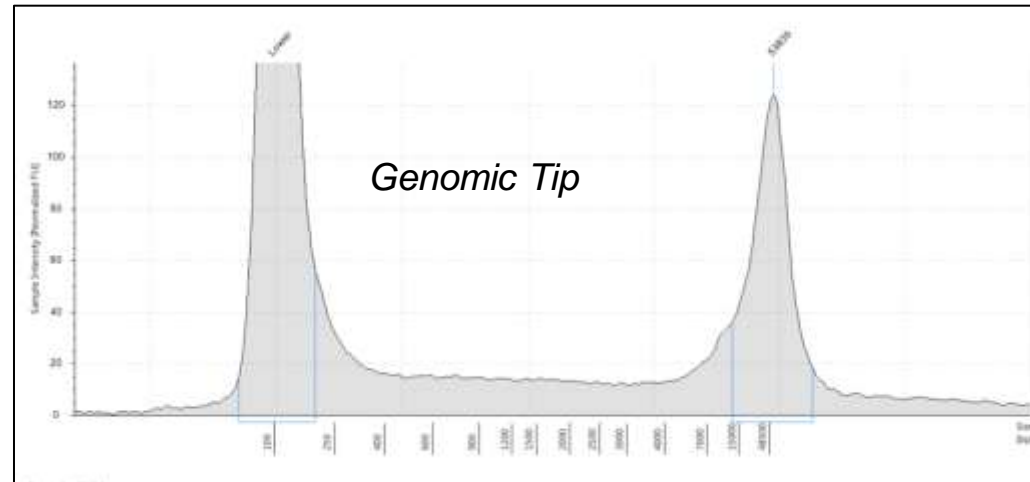
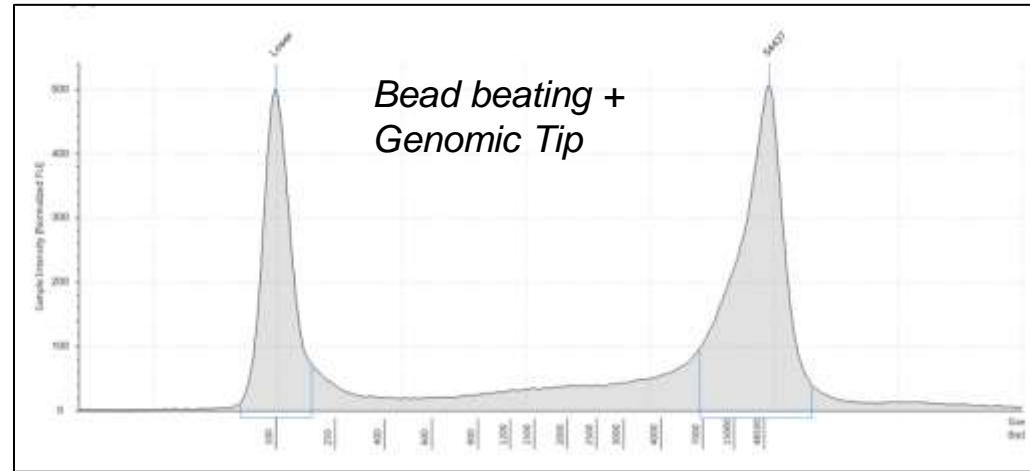
Gram positives



## Hybrid Extraction: Genomic Tip + Beads

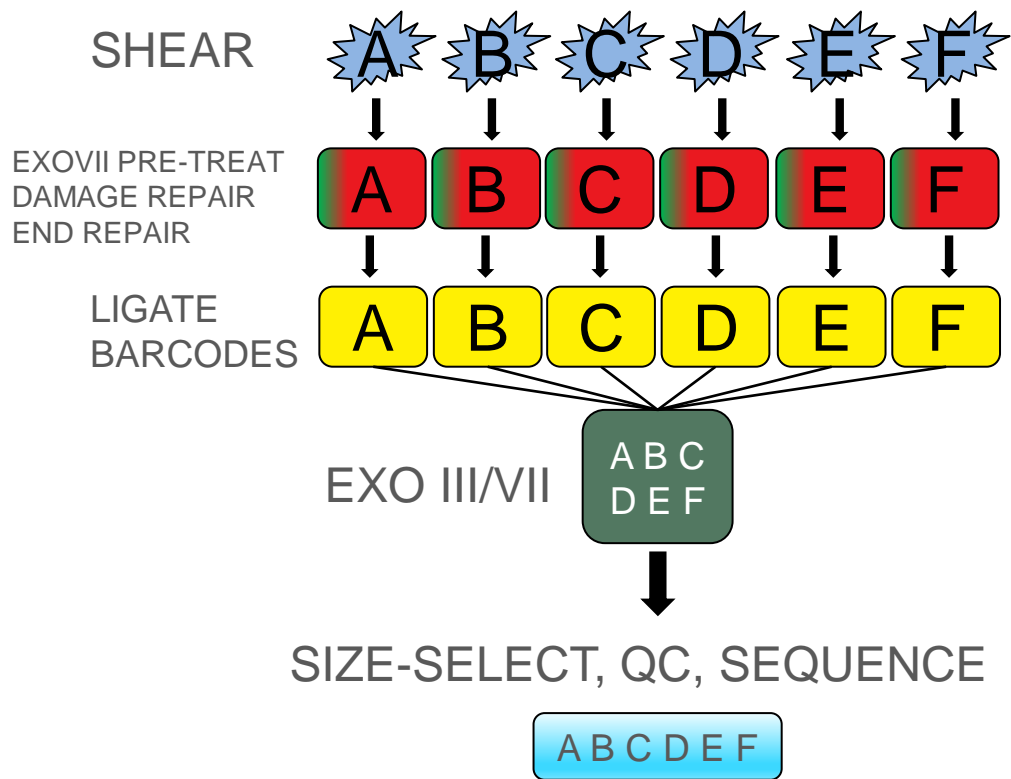
Organism	Gram stain	Mass (ng)	
		Genomic Tip	w/ Beads
<i>Nonlabens xylanidellens</i>	Gram -	1836	6483
<i>Staphylococcus condimentii</i>	Gram+	1032	12520
<i>Serinibacter salmoneus</i>	Gram+	1280	58

- Genomic DNA is not excessively sheared by conservative bead-beating
- Yields far superior *in some cases*





# Microbial Multiplexing: 2018 Update



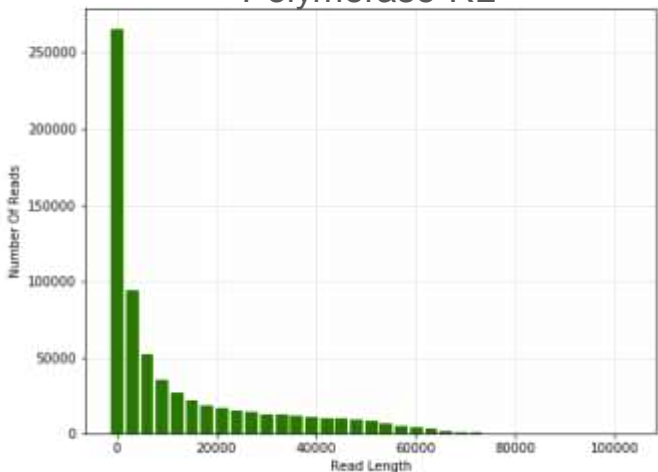
## What's new?

- More mass carried into prep (supports size selection)
- End-repair moved out of ligation
- Adapter optimization
- Improved tools/calculator

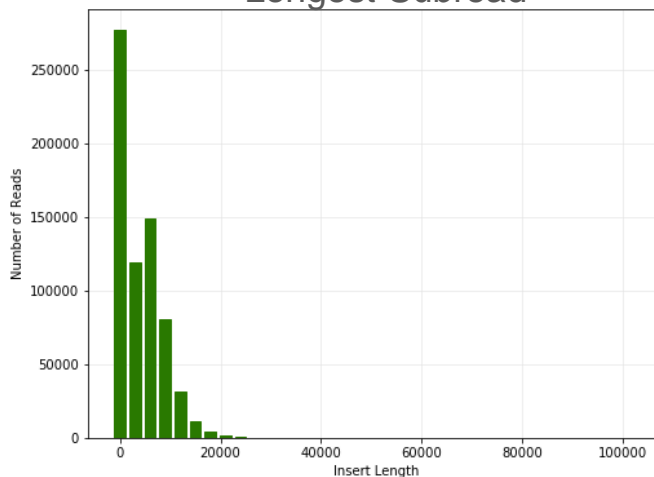
# Sequencing

Sample	Size selection	Loading conc	Polymerase read length		Longest subread			P0	P1	P2
			Total Bases (Gb)	Mean	N50	Mean	N50			
Library A	>6kb	7 pM	9.13	13,428	35,750	5,323	8,750	22%	67%	11%
Library B	>7kb	6 pM	8.47	13,783	37,750	5,973	9,750	30%	61%	9%
Library C	>6kb	6 pM	9.84	16,786	42,250	6,427	9,750	36%	58%	6%

Polymerase RL



Longest Subread

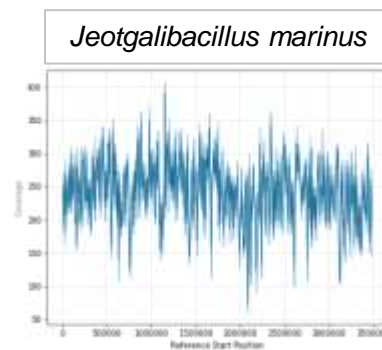
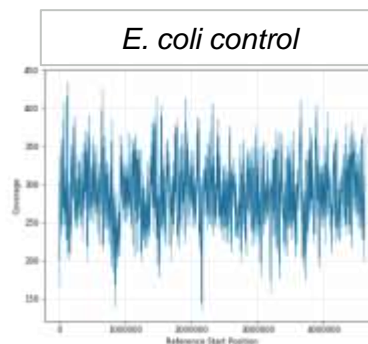
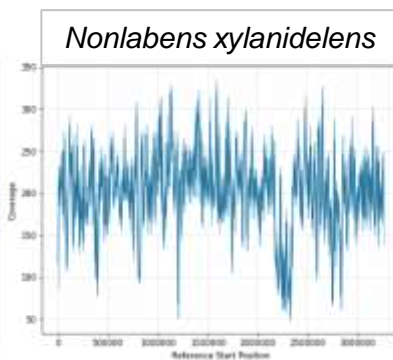
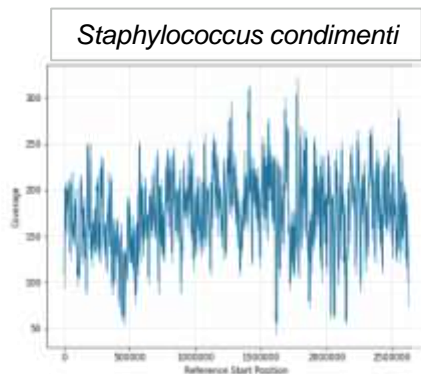


2.1 Chemistry  
120m Pre-Extension  
10h Movie



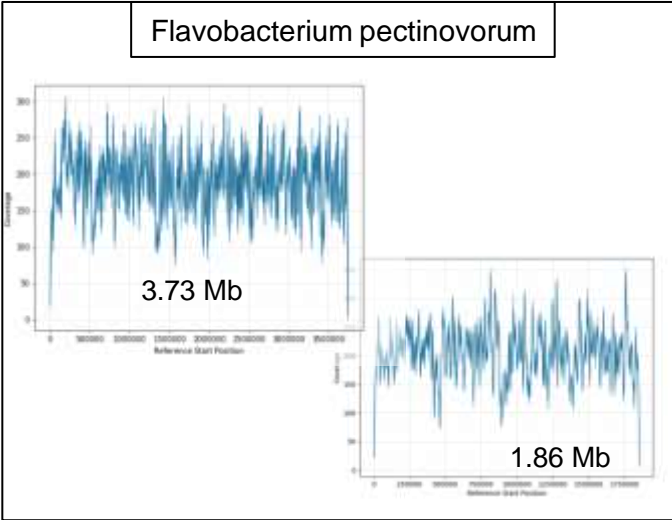
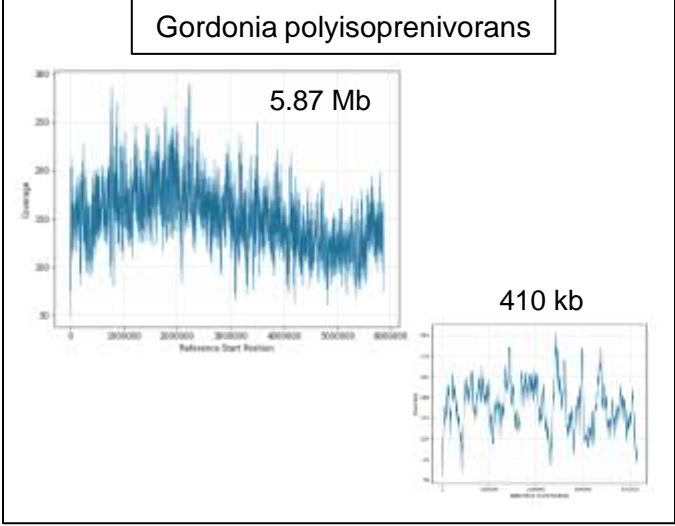
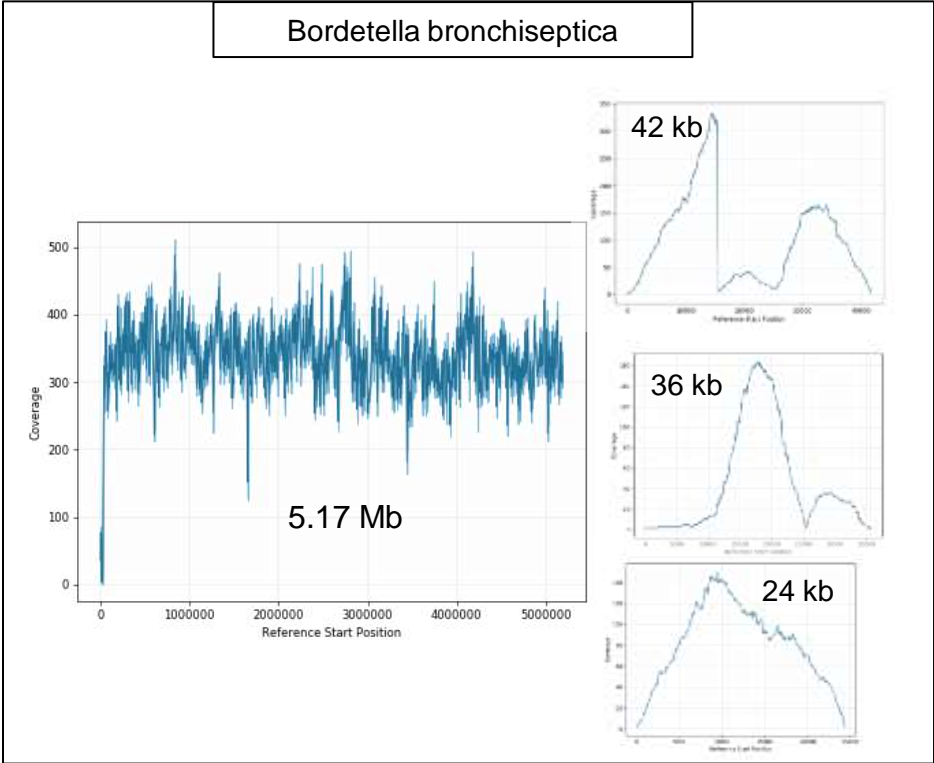
# Assembly

Microbe	Extraction	Reported Size (Mb)	HGAP4		Canu		Avg Coverage (X)	Gram	GC content
			Assembled Size (Mb)	Contigs	Assembled Size (Mb)	Contigs			
Flavobacterium pectinovorum	Genomic Tip	5.61	5.66	2	5.64	5	194	Gram-	34%
Nonlabens xylanidelens	Genomic Tip w/ Beads	3.28	3.29	1	3.29	5	202	Gram-	35%
Bordetella bronchiseptica	Genomic Tip	5.34	5.31	4	5.21	1	332	Gram-	68%
E. coli control	PacBio Supplied	4.64	4.64	1	4.65	1	288	Gram-	50%
Staphylococcus condimenti	Genomic Tip w/ Beads	2.60	2.65	1	2.67	4	175	Gram+	35%
Jeotgalibacillus marinus	Genomic Tip	4.10	3.51	1	3.51	1	242	Gram+	44%
Gordonia polyisoprenivorans	Genomic Tip	5.84	6.32	2	6.34	7	150	Gram+	67%



HGAP4 assemblies

# Multi-contig assemblies in HGAP4



*Plots not to scale of contig/genome*

# Costs

- Consumables @ 8-plex, per microbe  
Library prep: \$140 each  
Sequencing: \$110 each  
= **\$250 per 5Mb microbe**
- Labor, Depreciation, Extraction and Bioinformatics extra
- Future cost reductions: Sequel 8M



# Summary

- **Diverse microbes across GC% and genome size can be multiplexed on Sequel and result in single or single-digit contigs**
- Lower input with multiplexing is more forgiving of low extraction yields, but high enough to support size selection
- Bead-beating in combination with gentle gravity-column purification can yield suitable DNA
- Stay tuned for additional updates on xenobiologic community



# Acknowledgements



n=36!

Get in touch with our team!  
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Brian Beck





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