

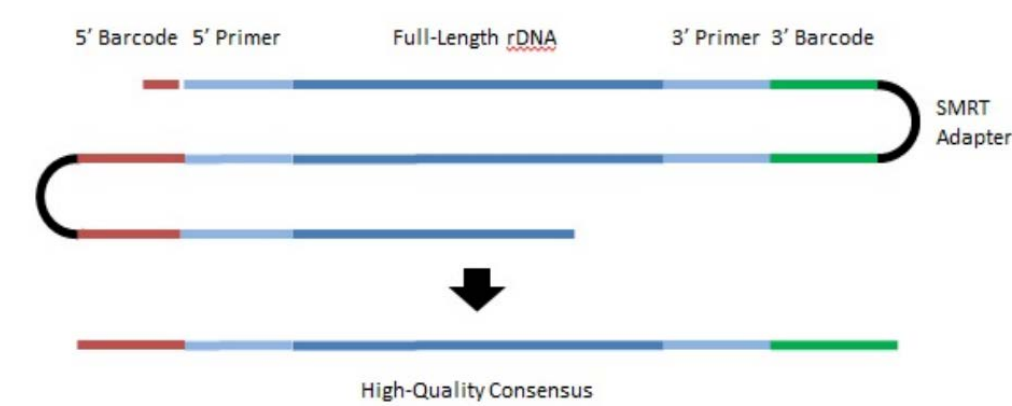
Introduction

- High-throughput sequencing of the complete 16S rRNA gene has become a valuable tool for characterizing microbial communities.
- However, the short reads produced by second-generation sequencing cannot provide taxonomic classification below the genus level.
- In this study, we demonstrate the capability of PacBio's Single Molecule, Real-Time (SMRT®) Sequencing to generate community profiles using mock microbial community samples from BEI Resources.
- We also evaluate multiplexing capabilities using PacBio® barcodes on pooled samples comprising heterogeneous 16S amplicon populations representing soil, fecal, and mock communities.

CCS Sequencing and Analysis Workflow

Circular Consensus Sequence (CCS) Reads

- PacBio's long reads and circularized templates provide high-quality consensus from multiple passes over the same molecule

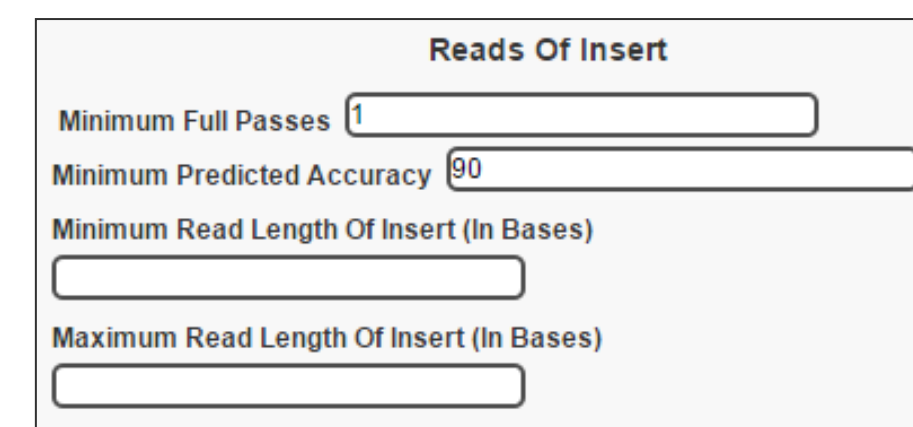


Analysis Workflow

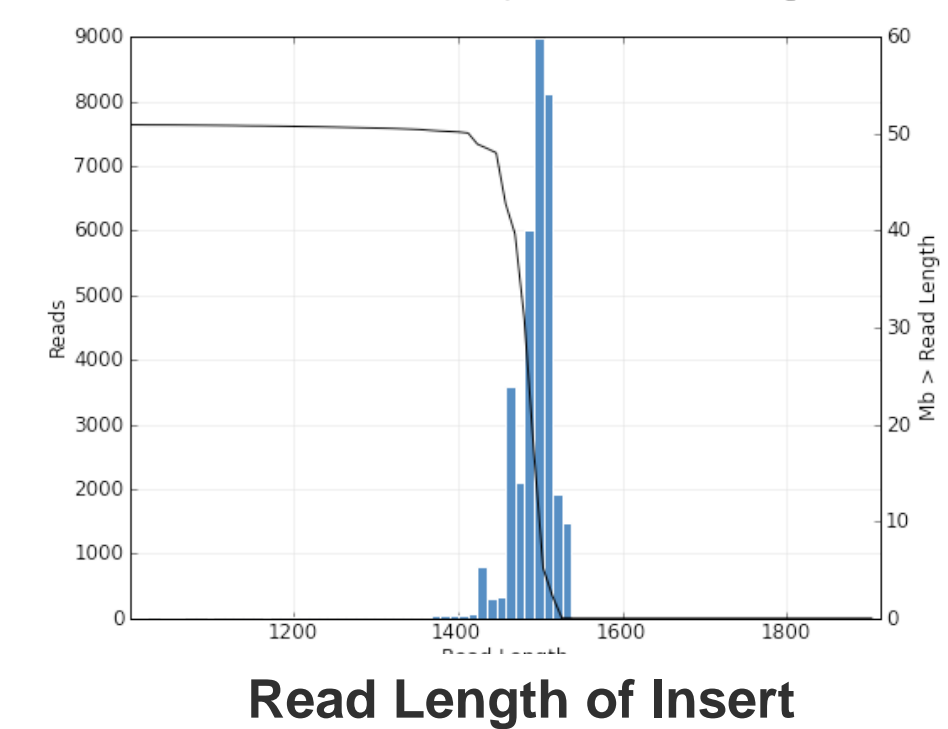
- CCS reads are exported, quality filtered, and where required binned by barcode using the "Reads of Insert" program in SMRT Analysis version 2.3.0.p4.
- Further 16S analysis is carried out using PacBio's rDNA tools pipeline, described at right. CCS sequences undergo further filtering and clustering to generate sequences for classification.
- Output sequences may be classified using a number of available databases.

Accurate Single Molecule 16S Sequences

The PacBio **SMRT Portal Reads of Insert protocol** generates highly accurate CCS reads. Filtering parameters are adjustable according to project needs.

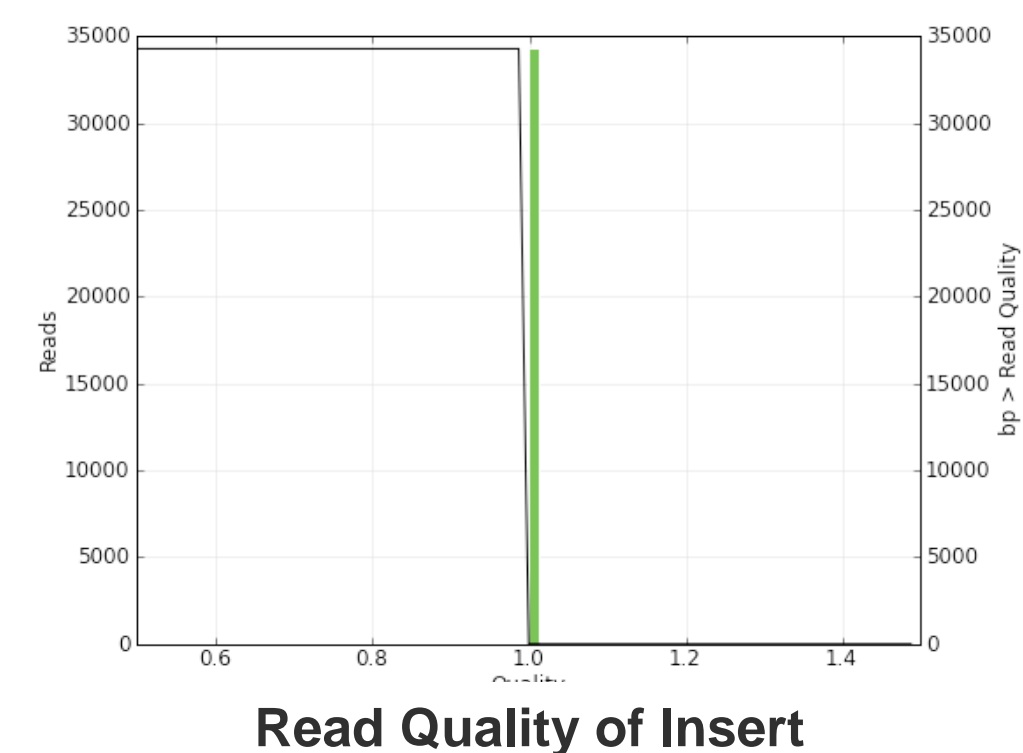


Output of 99.9% predicted accuracy filtering



CCS yield depends on filtering criteria

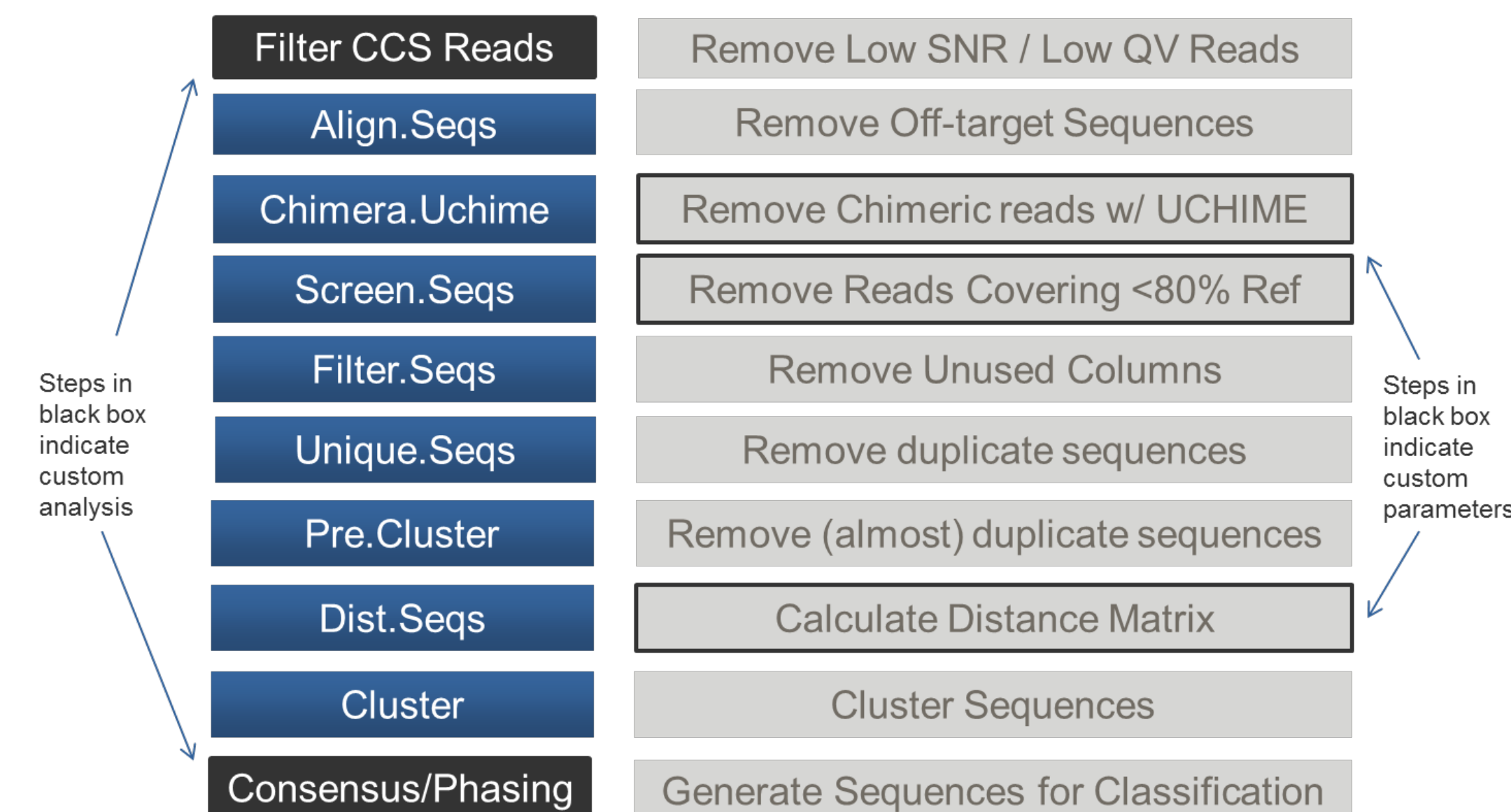
Predicted CCS Accuracy	Full-Length 16S Reads per SMRT Cell
90.0 %	50K
99.0 %	25K
99.9 %	10K



rDNA Tools Pipeline

Analysis of 16S CCS sequences may be carried out with standard tools from the Mothur package (<http://www.mothur.org>)² combined with Python custom scripts. The complete rDNA tools analysis pipeline with custom scripts is available for download on GitHub:

<https://github.com/PacificBiosciences/rDnaTools>

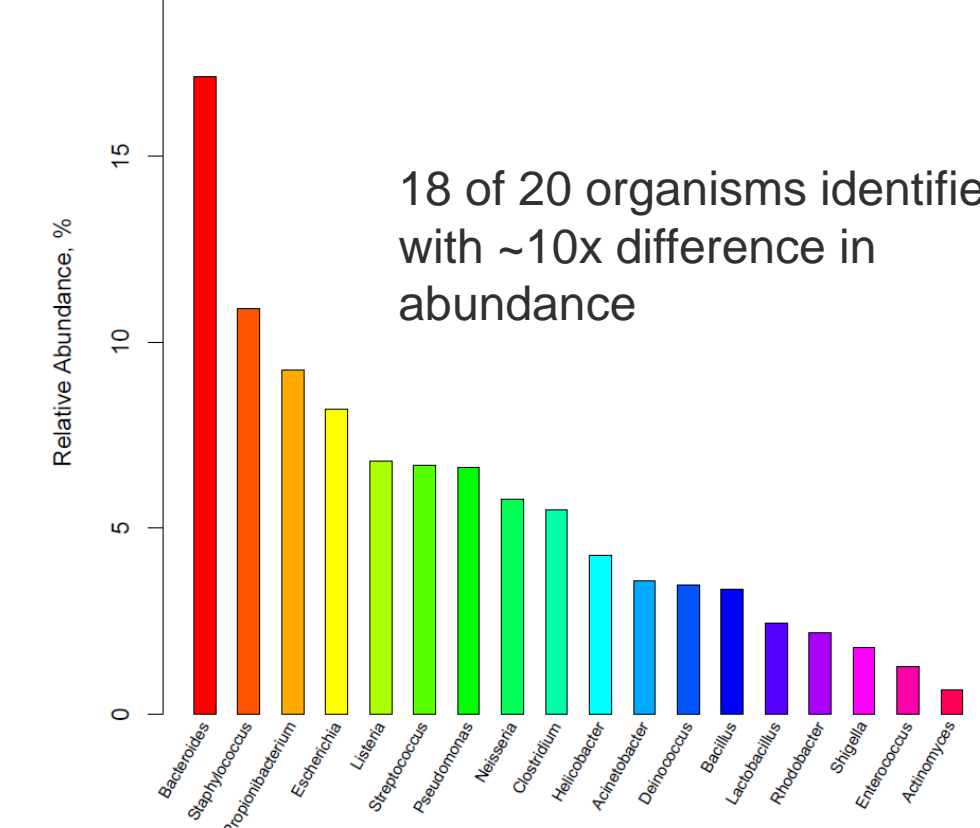


16S Analysis of BEI Mock Communities

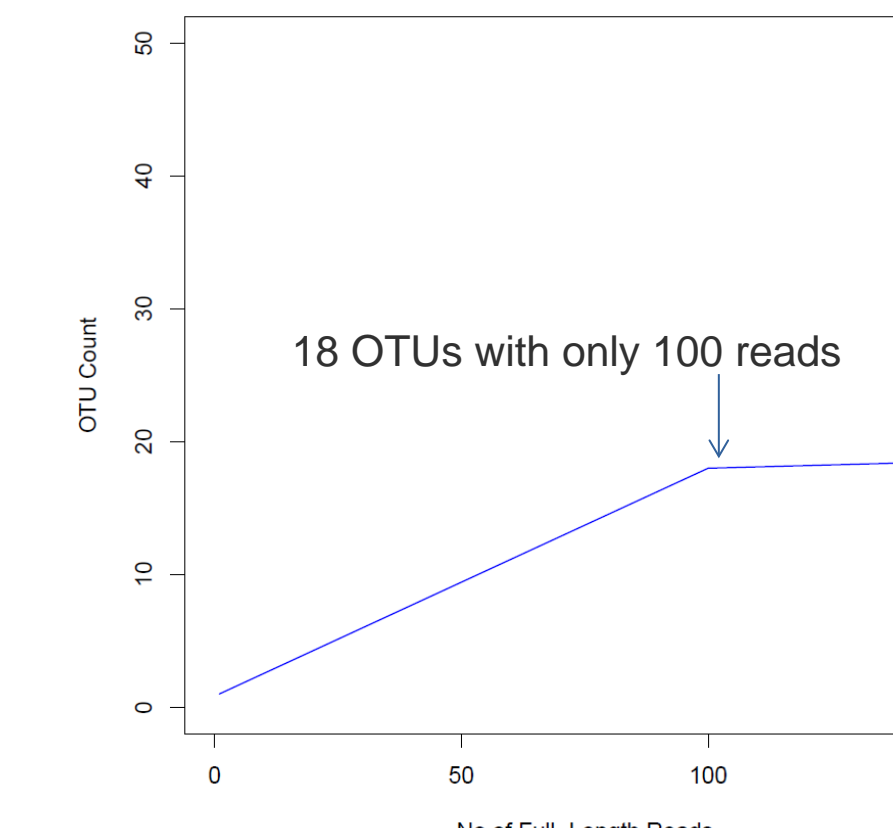
- Full-length 16S amplification was performed on two mock microbial community samples from BEI Resources.
 - Microbial Mock Community B, Even, containing DNA from 20 bacterial strains at ~equimolar concentrations
 - Microbial Mock Community B, Staggered, containing DNA from 20 bacterial strains at up to 4 orders of magnitude difference in concentrations
- SMRTbell™ Libraries were prepared following standard protocols.
- A single SMRT Cell was run for each BEI Mock Community using standard P6-C4 chemistry and protocols, with a three-hour collection time.

BEI Even Mock Community Analysis from 1 SMRT Cell

Relative Abundance

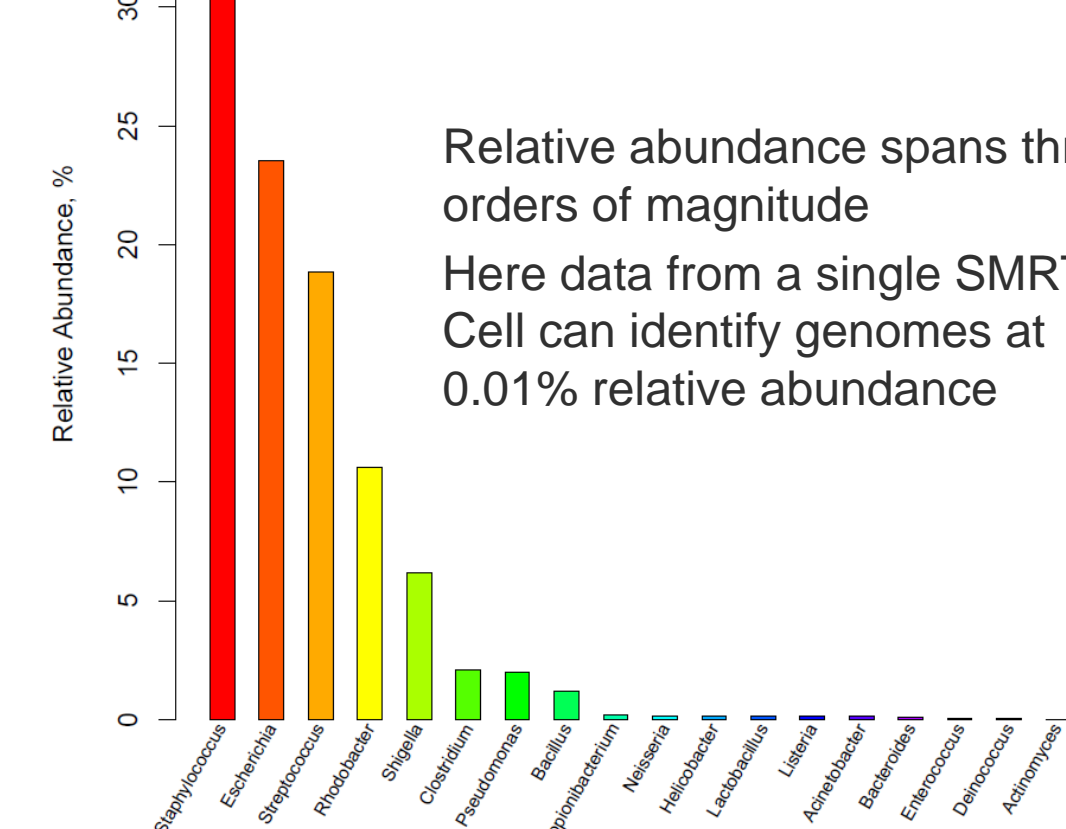


Rarefaction Curve

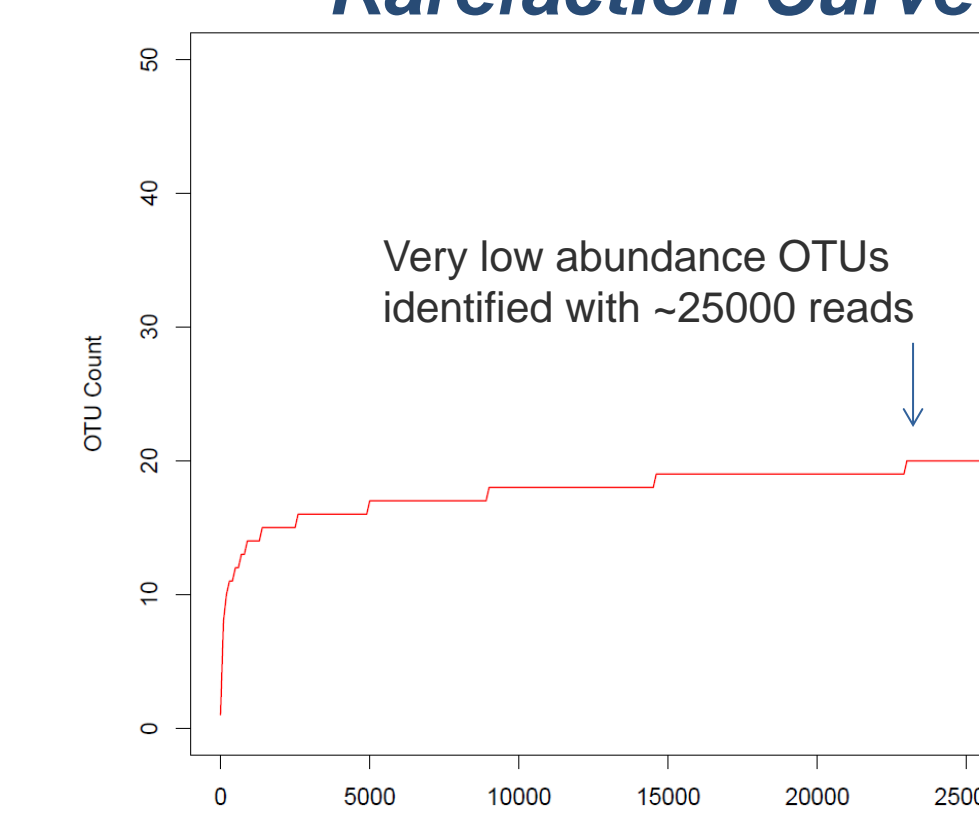


BEI Staggered Mock Community Analysis from One SMRT Cell

Relative Abundance



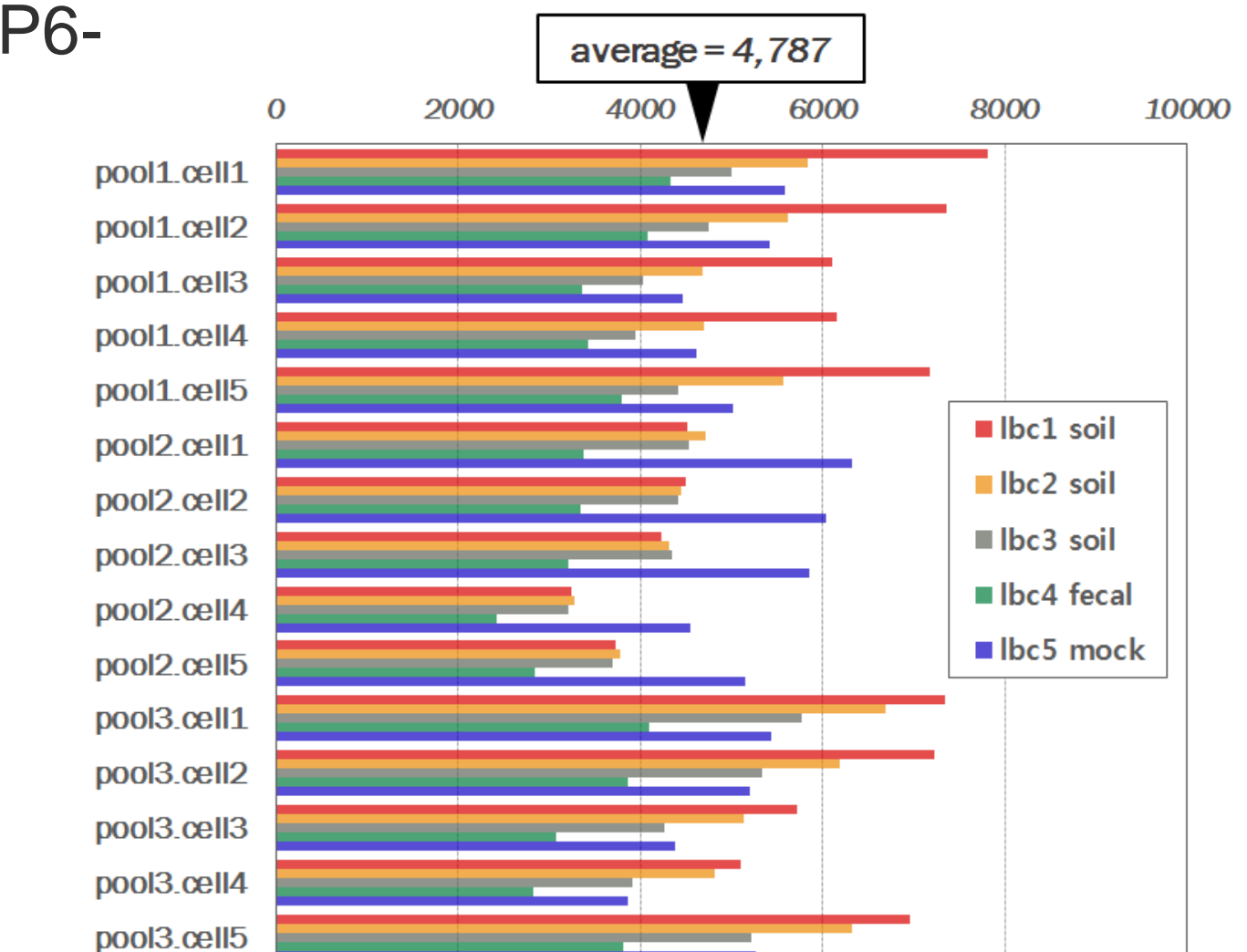
Rarefaction Curve



Multiplexing 16S Samples with Barcodes

- 16S amplification was carried out on five metagenomic samples, three soil, one fecal, and one mock community, using PCR primers tailed with PacBio barcodes.
- Equimolar amounts of the five barcoded amplicons were pooled for SMRTbell library preparation using standard protocols.
- Five SMRT Cells were run for each library using standard P6-C4 chemistry and protocols, with 3-hour collection times.

Results of demultiplexing using SMRT Portal Reads of Insert protocol. All samples were represented by >2000 reads/cell with stringent filtering criteria.



Conclusions

- SMRT Sequencing provides accurate, full-length 16S sequences for identification of community constituents to the species level.
- PacBio provides tools for 16S data analysis: Reads of Insert analysis for generation of accurate single-molecule sequences and barcode demultiplexing, and rDNA tools pipeline for 16S-specific filtering and clustering.
- With the PacBio RS II system, genomes at 0.01% relative abundance can be accurately identified with a single SMRT Cell.
- Multiplexing with PacBio barcodes offers cost-effective solutions for 16S sequencing.

References

- rDnaTools - <https://github.com/PacificBiosciences/rDnaTools>
- Schloss, P.D., *et al.*, Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol*, 2009. 75(23):7537-41 (<http://www.mothur.org>).
- Bowman, *et al.*, [2013] [Analysis of Full Length Metagenomic 16S Genes by SMRT Sequencing](https://doi.org/10.1038/nbt.2700).

Acknowledgements

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