Technical Strategies for Oxford Nanopore Sequencing and Rapid Pathogen Detection and Biosurveillance

Scott Tighe

University of Vermont Cancer Center
Advanced Genomics Core

ABRF Metagenomics Research Group

Speaker Info

- Steering Committee of IMMSA
- Chair ABRF Metagenomics Research Group
- Leader Extreme Microbiome Project (50 members)
- Member MetaSub International Consortium
- Member Genomics Standards Consortium

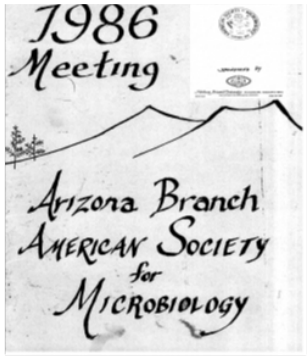
Background:

Microbiologist, Mycologist, Phycologist, Molecular Biologist
Next generation sequencing facility manager
Oxford Nanopore, Illumina HiSeq

Cultured and identified tens of thousands of bacteria, fungi, parasites using growth, CFA, Biolog, MIS-MIDI, microscopy, ect

Started working in Microbiome analysis in 1984 at Northern Arizona Univ

Contact Info scott.tighe@uvm.edu



Antibiotic Modification of the Bacterial Population of the Digestive System of the Bruchid Beetle, Acanthoscelides obtectus. Brenneman, Kristine, and Harold Speidel. Dept. of Biol. Sciences, Nor. Ariz. Univ., Flagstaff, Ariz.

Modification of Juniper Toxins by Microbial Isolates of the Woodrat Digestive System. Scott, Tighe, Ali Hekmati, and Harold Speidel. Dept. of Biol. Sciences,

The ABRF Metagenomics Research is a team devoted to study and improve methods and consumables used in metagenomics research

The XMP is a proving ground for studying consumables on the most challenging of sample types

As well as collect shotgun data on novel sample sites

Oxford Nanopore Sequencing and Rapid Pathogen Detection and Biosurveillance

- Rapid Pathogen Screening has its Challenges
- Many sample types have limited DNA
- Require some amplification
  Swabs, drinking water, food wash, ect
- Bacteria are difficult to lyse uniformly
- Lysis slurries often inhibit PCR reactions and other synthesis reactions

Recent Innovations Enable Rapid Lysis and Amplification

- Metapolyzyme
- PrepMan Ultra™
- Unreactive beater beads-Diamond
- High performance Titanium Taq Polymerase
- Modified SKQ-RAB201 Oxford Nanopore Protocol

Originally designed for rapid screening of *Legionella pneumophilia*

The Goal: Swab to Sequence in 60 minutes

Swab Technique modified from *Mason et al* MetaSub International Metagenomics Consortium

Workflow for Rapid Bacterial Screening

100ul PBS pH7.5 with diamond Beads

Swab surface

Add 4ul MetaPolyzyme 35C 20 min

Add 10-100ul of Prepman Ultra™

100C 10 min -Vortex

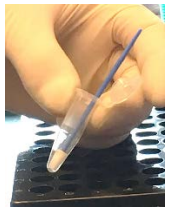
1-4ul input 16s PCR

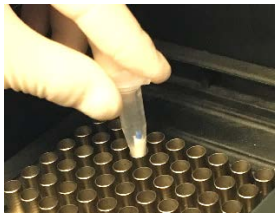
Use Clontech/Takara Titanium Taq

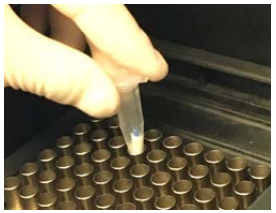
25 cycles

150 ng input to SQK-RAB201

ONT

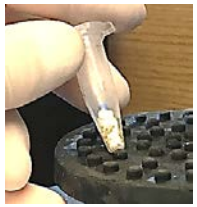






35C 20m

100C 10m



4ul

50ul Titanium Taq PCR

95/1:00

95/30

54/30 25cyc

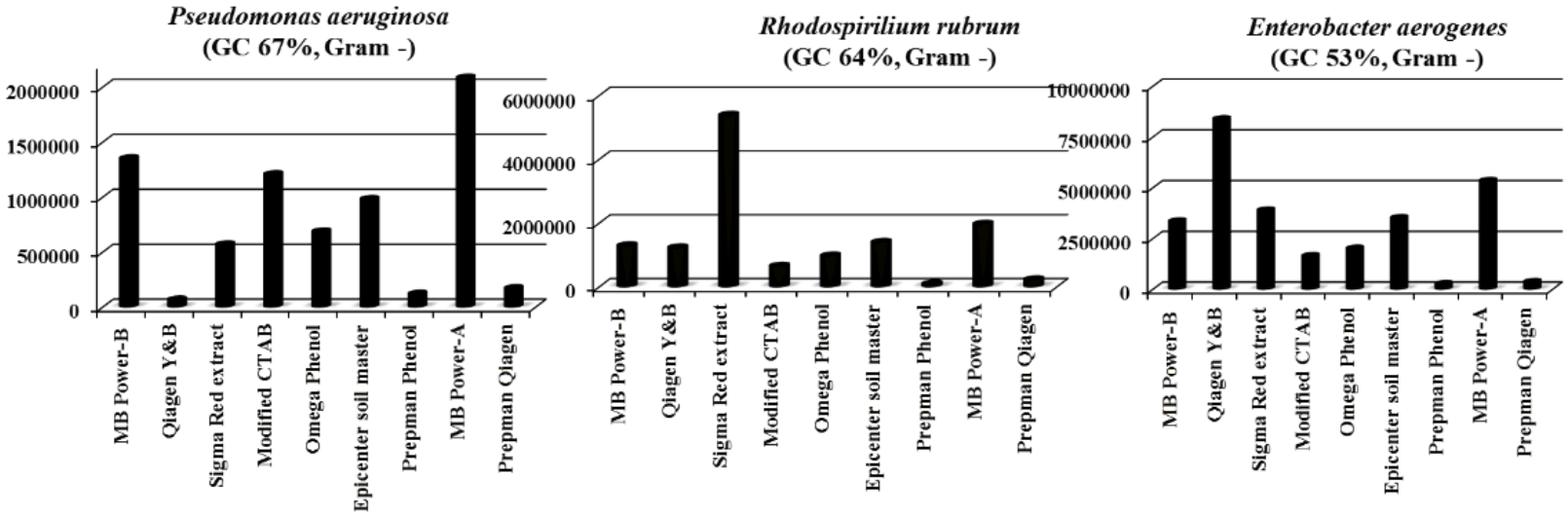
68/1:30

68/3:00

Ampure Beads

Bacteria and Fungi have a Cell Wall and are Difficult to Lyse

- DNA extraction efficiency studies
- DNA extraction Study with 12 bacteria at known concentration by the ABRF Nucleic Acids Research group in 2011.
- Whole cell microbial standard -ETOH fixed
- Microscopically counted

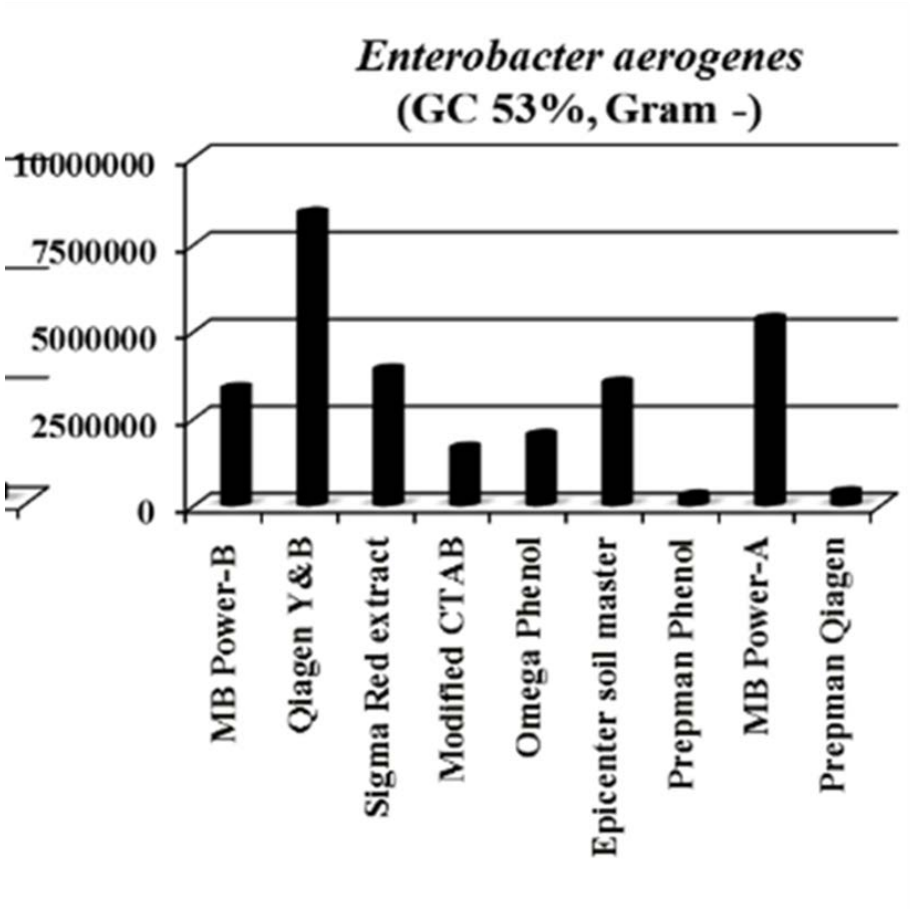


ABRF Nucleic Acids Research Group 2012-2013 Study

Evaluating DNA Extraction Methods for Metagenomic Analysis

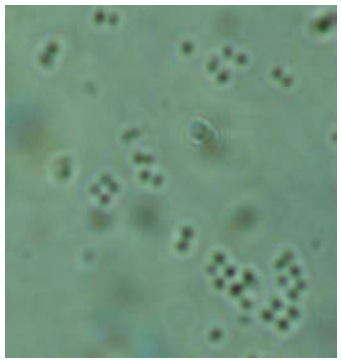
V. Nadella[1], J. Holbrook[2], R. Carmical[3], M. Robinson[4], C. Rosato[5], H. Auer[6], N. Beckloff[7], Z. Herbert[8], S. Chittur[9], A. Perera[10] , W. Trimble[11], S. Tighe[12]

[1]Ohio University, [2]Nemours/A.I. DuPont Hospital for Children, [3]University of Texas Medical Branch, [4] University of Zurich, Switzerland, [5]Oregon State University, [6]Institute for Research in Biomedicine, Barcellona, Spain, [7]Case Western Reserve University, [8]Dana Farber Cancer Institute, [9]University at Albany-SUNY, [10]Stowers Institute for Medical Research, [11]Argonne National Laboratory, [12]University of Vermont.

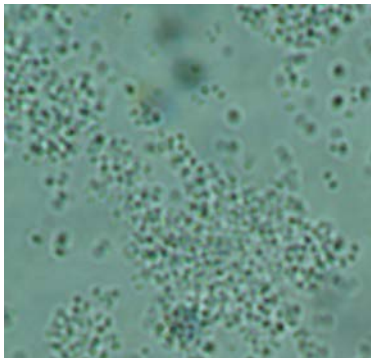

Enterobacter aerogenes
(GC 53%, Gram -)
10000000
7500000
5000000
2500000
0
MB Power-B
Qiagen Y&B
Sigma Red extract
Modified CTAB
Omega Phenol
Epicenter soil master
Prepman Phenol
MB Power-A
Prepman Qiagen

Chemical and beads alone don’t lyse all cells

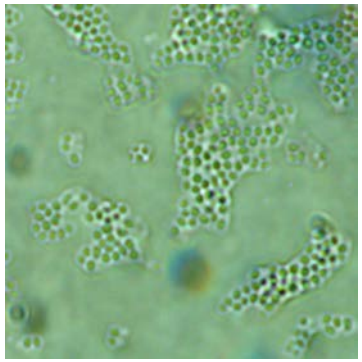
Micrococcus luteus lysis vs chemical and Beater Beads



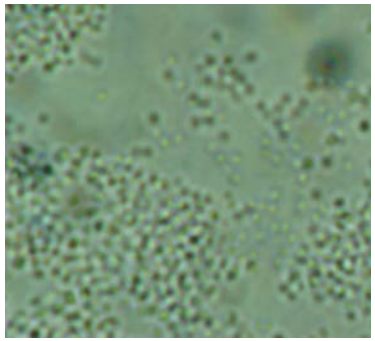
Control



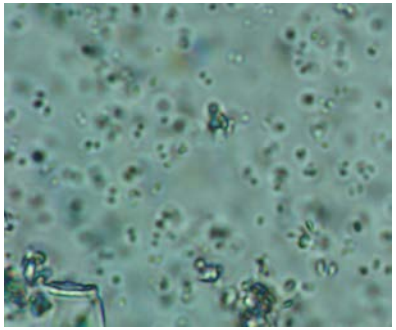
CTAB



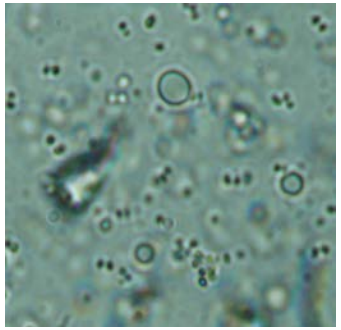
Phenol



SDS



CTAB+Beater Beads



CTAB+Phenol+Beader

New Innovations=New Approaches

Metapolyzyme
PrepMan Ultra
Titanium Taq
Oxford Nanopore sequencer
Rapid Analysis Software

Metapolyzyme

35C pH 7.5

Use alone or combined with Lysozyme

REQUIRES PBS pH 7.5 and NO EDTA

Designed specifically for Bacterial, Fungi, and Yeast

Originally formulated in 2004

Tested in 2016 by ABRF MGRG

Commercialized in 2017 by Millipore Sigma

Why Develop Metapolyzyme?

Lysis without beater beads or aggressive vortexing. If you can sphearoplast all the cells, Proteinase K and Detergent can do the rest.

SIGMA-ALDRICH®

sigma-aldrich.com

3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

MetaPolyzme
Multilytic Enzyme Mix

Catalog Number **MAC4L**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

Metagenomics is a rapidly expanding field of basic and applied research which looks at all DNA that has been isolated directly from given single samples (e.g. environmental samples, biological organisms).[1,2] Metagenomics allows for the investigation of microbes that exist in extreme environments, and which have been historically difficult to isolate, culture, and study.[3] Metagenomics has revealed the existence of novel microbial species.[4] Applications of metagenomic studies include public health data analysis,[5,6] discovery of novel proteins, enzymes and natural products,[7,8] environmental studies,[9,10] and agricultural investigations.[11,12]

This product was evaluated and developed in consultation and collaboration with the Association of Biomolecular Resource Facilities (ABRF) Metagenomics Research Group (MGRG).[13-16] The enzymes in MetaPolyzme are:

- Mutanolysin
- Achromopeptidase
- Lyticase
- Chitinase
- Lysostaphin
- Lysozyme

Mutanolysin (from *Streptomyces globisporus*):

Sphearoplasting is Key

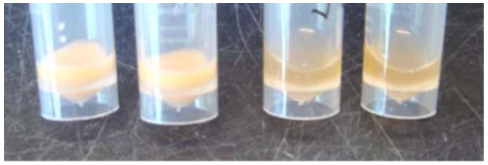
- Multi-lytic Enzyme Mix for Digestion of Cell Walls

  MetaPolyzyme

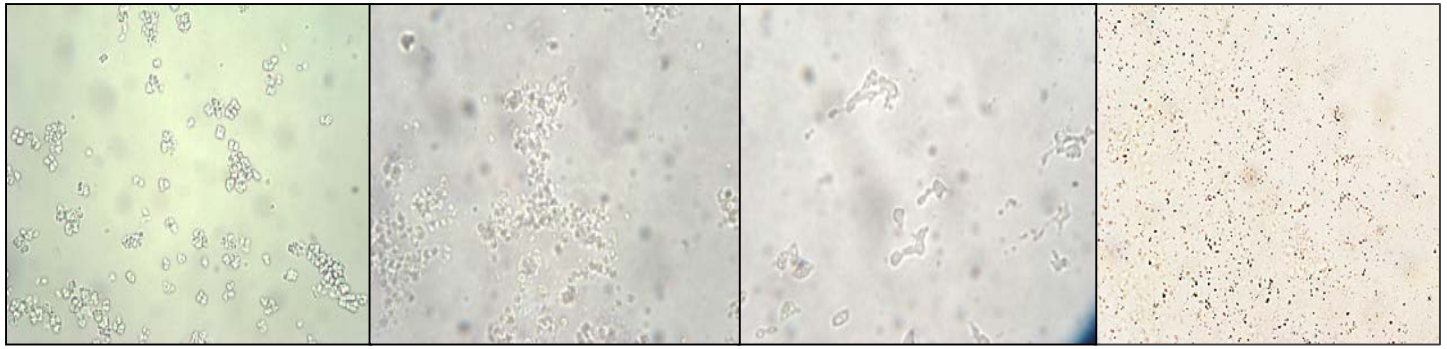
  Bacteria, yeast, fungi cell walls

  Phylozyme (3 additional enzymes for Plant and Algae)

  Testing Started 11/2016



No Enzyme **Enzyme**

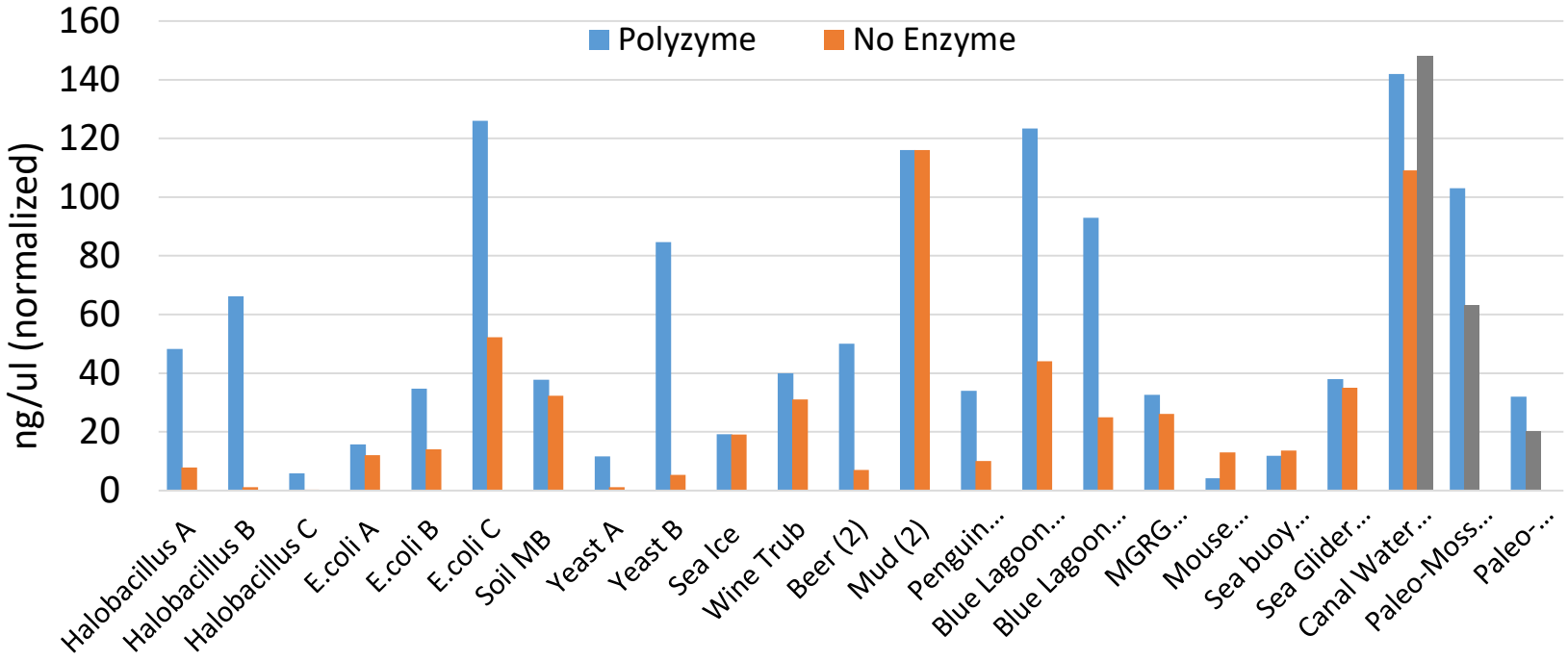


Micrococcus luteus

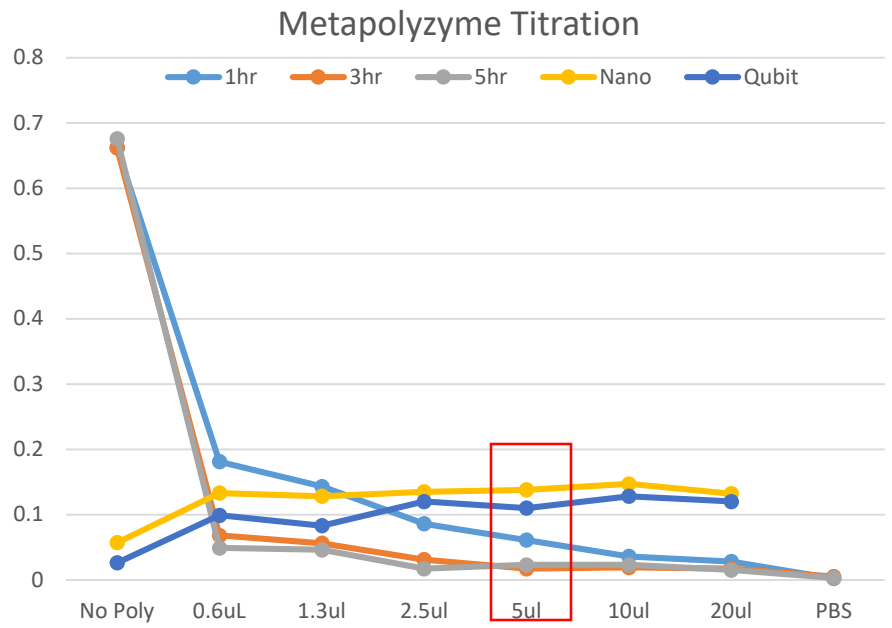
Sphearoplasting vs Exposure time and 0.1% SDS

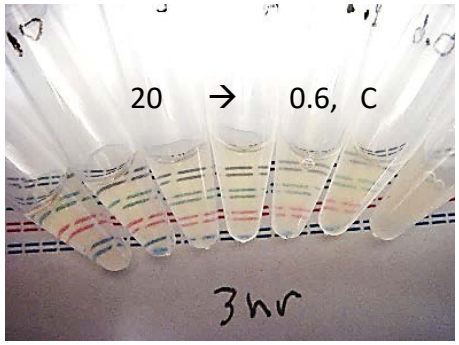
Beta Test Results

- Over 150 sample trials (Polyzyme, PBS only, Polyzyme only)
- 3 trials with Lysozyme alone-Need more data
- 6 labs , 17 matrices
- Any kits



How Low and Fast can Sphearoplasting Occur?





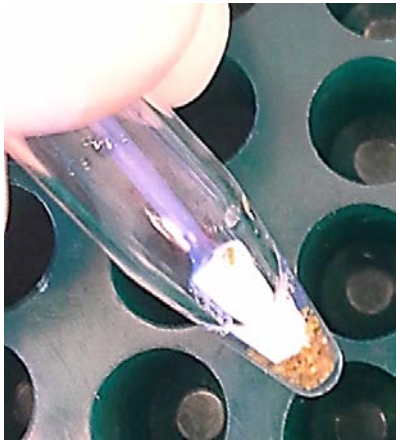
PrepMan Ultra™

Rapid cell lysis reagent
100c 10min
Used for ABI's MicroSeq ID system
Bacteria and fungi
Very little inhibition on downstream reactions

2-Butoxyethanol
Sodium metasilicate
Citric Acid
Buffers

Diamond Beater Beads

- Beater Beads Fragment DNA
- Not ideal for Oxford Nanopore but ok for full length 16s
- Not desirable when using long read sequencers
- Mild use for 16s full amplicon-1492bp
- Synthetic Diamond is unreactive
- Limited vortexing



Workflow for Rapid Bacterial Screening

100ul PBS pH7.5 with diamond Beads

Swab surface

Add 4ul MetaPolyzyme 35C 20 min

Add 10-100ul of Prepman Ultra™

100C 10 min -Vortex

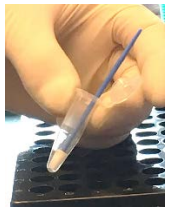
1-4ul input 16s PCR

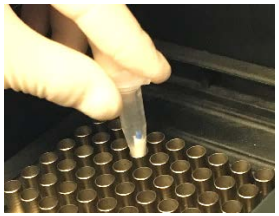
Use Clontech/Takara Titanium Taq

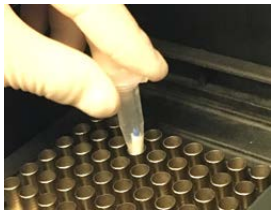
25 cycles

150 ng input to SQK-RAB201

ONT

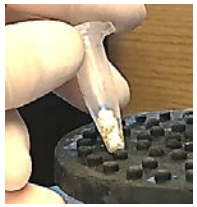






35C 20m

100C 10m



4ul

50ul Titanium Taq PCR

95/1:00

95/30

54/30 25cyc

68/1:30

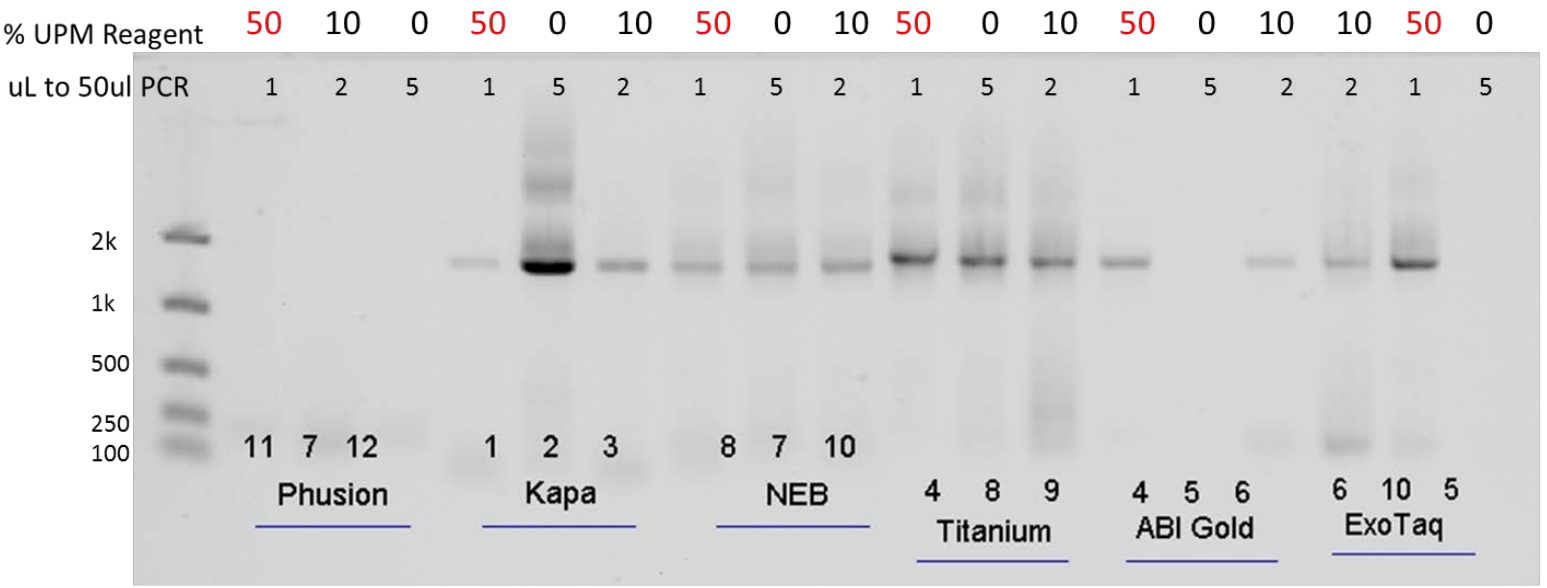
68/3:00

Ampure Beads

Successful PCR is the Key

- Tested 6 high performance Taq polymerases
  - Life Phusion
  - NEB Q5
  - KAPA HIFI
  - Takara Ex-Taq
  - ABI Gold MM
  - Takara Titanium
- Mixed with different amounts of lysis material
- Different input of samples
- Used Oxford Nanopore 16s Barcode primers.
  - 1492 rev
  - 27 Fwd -Single degenerate-Not optimal

Comparison of Taq Polymerase in the Presence of MetaPolyzyme and Prepman Ultra



16s Full Length PCR on Rapid Sample
Oxford Nanopore Primers –SQK-RAB201

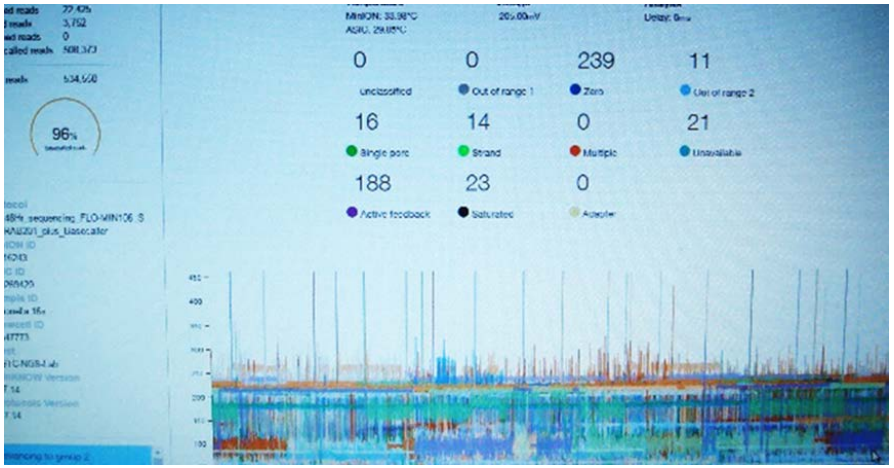
Oxford Nanopore Sequencing

Use of the 16s Rapid Barcoding Kit (SQK RAB201)

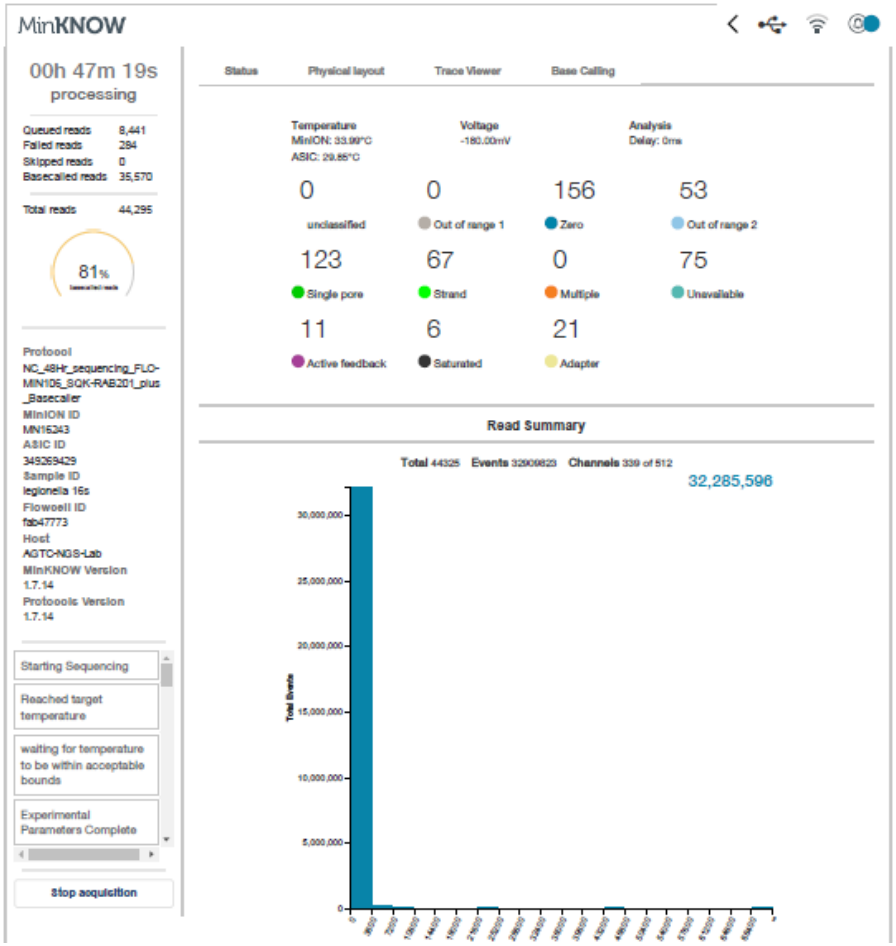
- 10 ng input gDNA (normally)
- 27f (1d) and 1492r PCR step
- Barcoding of 12 samples
- Modified PCR step using Clontech Titatium Taq (639209)
- 25 cycles
- Requires 100 ng input of full length 16s Amplicon library into the sequencer

ONT Sequencer Stats

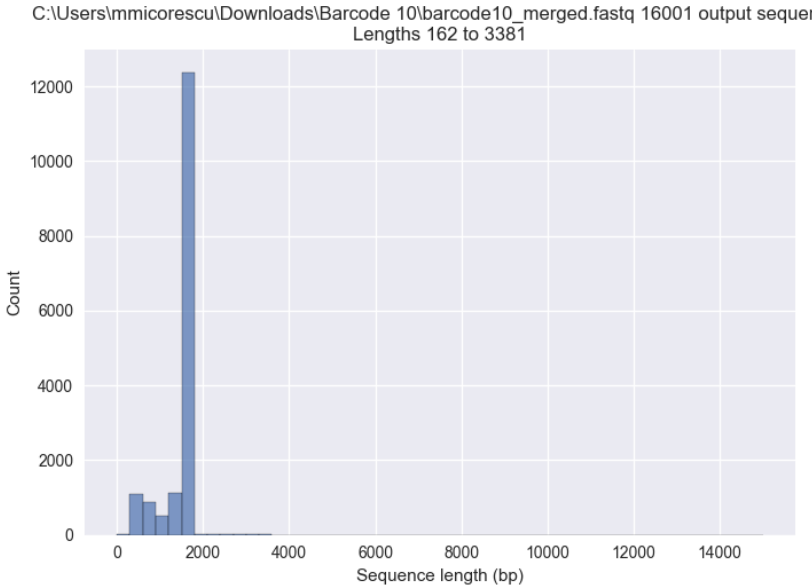
- R9.4 (893 pores)**
- 35,000 reads in 50 minutes
- 150 ng input library
- 500,000 reads in 14 hrs

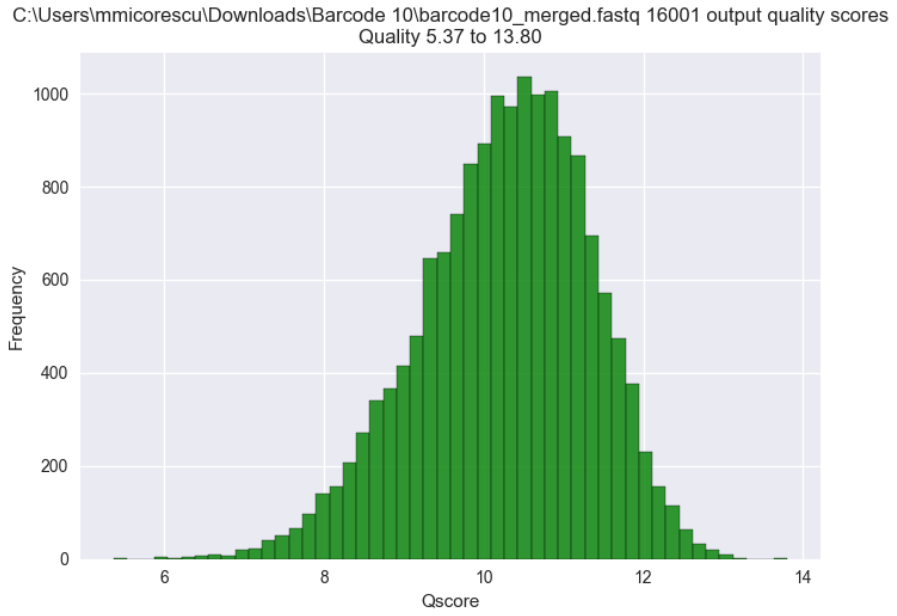


** Flow cell traveled to Antarctica and back and was 6 months old



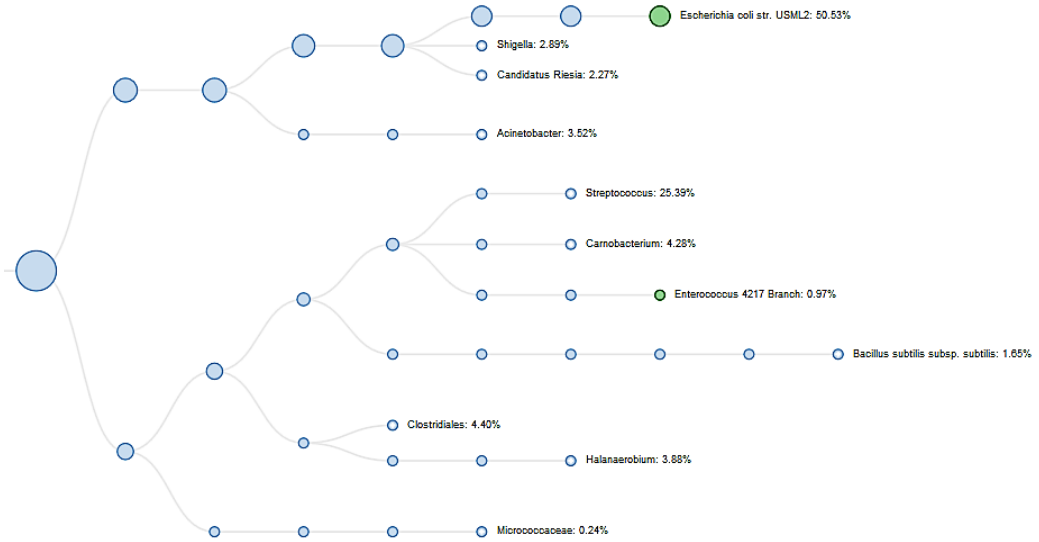
QC Output for Oxford RAB201-TiTaq



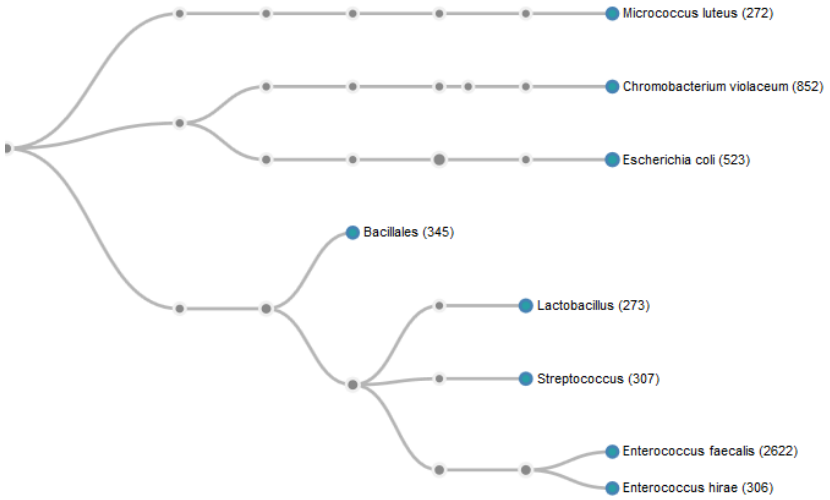


Results from Rapid Analysis Software

COSMOSID®



ONE CODEX



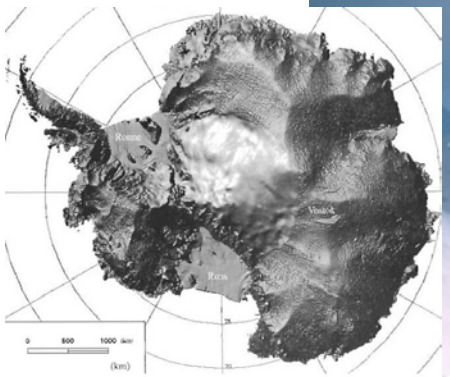
What’s Next

- Live vs Dead
- Modified Nextera/Transposase WGS
- Unk-ome or dark matter

Acknowledgements

- The ABRF MGRG Team
- The Extreme Microbiome Team
- Michael Micorescu Oxford Nanopore
- Sarah Johnson Georgetown
- Sam Greenfield-UVM
- Applied Biosystems
- Chris Mason and Noah Alexander-Weill Cornell
- Scott Jackson and Jason Kralj -NIST
- Cosmos ID- Rita, Manoj, Nur, Huai
- One Codex-Nick Greenfield

Rapid Field Sequencing in Antarctica using the Oxford Nanopore Sequencer



NSF EAGER grant: Single-Molecule Sequencing of Antarctic Paleolakes

JBT JOURNAL OF BIOMOLECULAR TECHNIQUES

J Biomol Tech. 2017 Apr ; jbt.17-2801-009. PMCID: PMC5362188
Published online 2017 Mar 22. doi: 10.7171/jbt.17-2801-009

Real-Time DNA Sequencing in the Antarctic Dry Valleys Using the Oxford Nanopore Sequencer

Sarah S. Johnson,[1,2,*] Elena Zaikova,[1] David S. Goerlitz,[3] Yu Bai,[1] and Scott W. Tighe[4]

Author information ▶ Copyright and License information ▶

Abstract

Go to:

The ability to sequence DNA outside of the laboratory setting has enabled novel research questions to be addressed in the field in diverse areas, ranging from environmental microbiology to viral epidemics. Here, we demonstrate the application of offline DNA sequencing of environmental samples using a hand-held nanopore sequencer in a remote field location: the McMurdo Dry Valleys, Antarctica. Sequencing was performed using a MK1B MinION sequencer from Oxford Nanopore Technologies (ONT; Oxford, United Kingdom) that was equipped with software to operate without internet connectivity. One-direction (1D) genomic libraries were prepared using portable field techniques on DNA isolated from desiccated microbial mats. By adequately insulating the sequencer and laptop, it was possible to run the sequencing protocol for up to 2½ h under arduous conditions.

Keywords: Antarctica, extremophiles, MinION, Nanopore sequencing

The Sarah Johnson team (AKA G062M)

Sarah Johnson (PI), Angela Bai, David Goerlitz, Scott Tighe, Elena Zaikova

Antarctica Dry Valleys

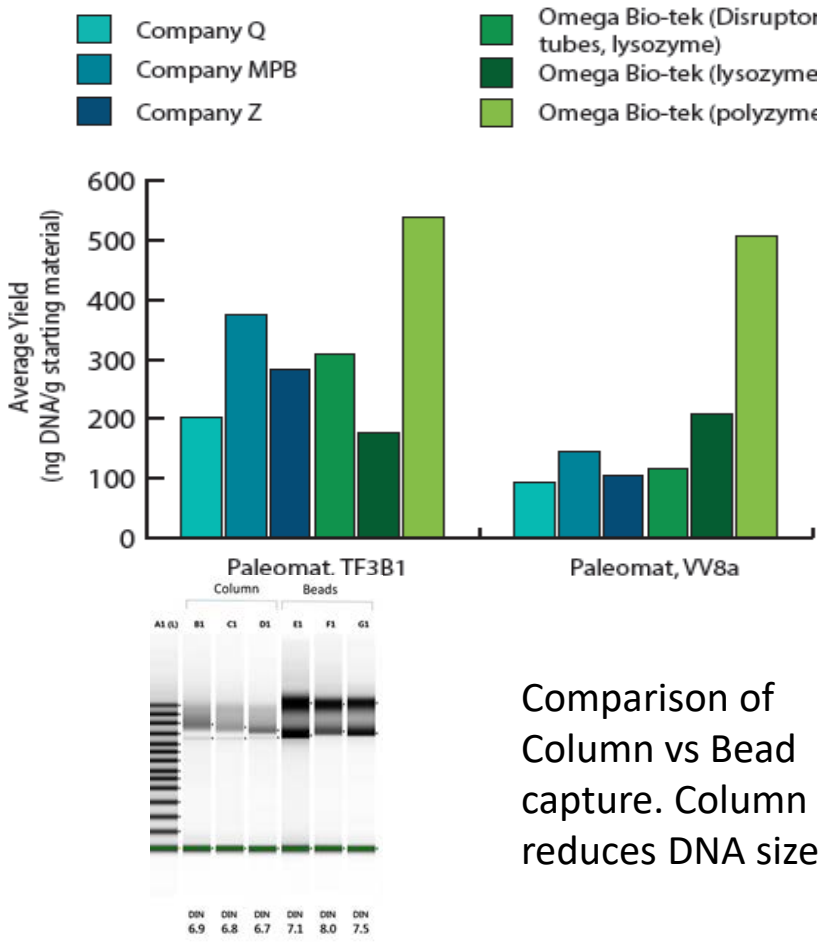
Glacial Lake Victoria

Long gone but microbes remain that we can sequence

Collecting The Ancient Microbial Biofilms using Nucleic acid free sampling

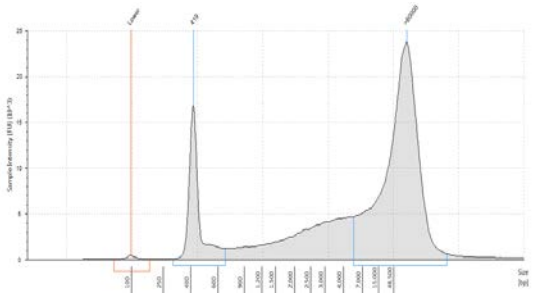
DNA Extraction Results

Average Yield from Victoria land samples



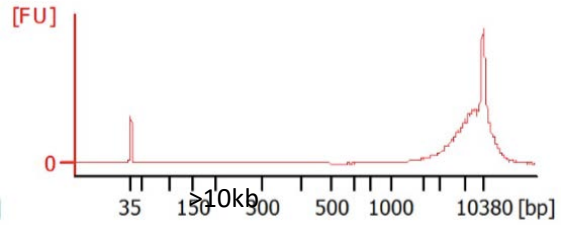
Comparison of Column vs Bead capture. Column reduces DNA size

Agilent 2200 genomic tape

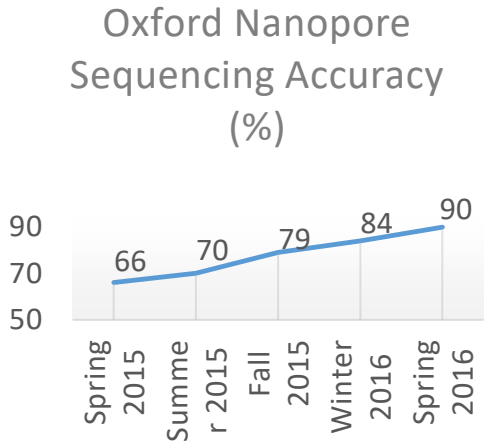


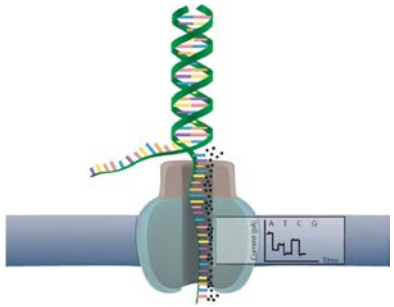
15-60kb

Agilent BA2100 HS Chip



Field Deployable Oxford Nanopore Minion Sequencers

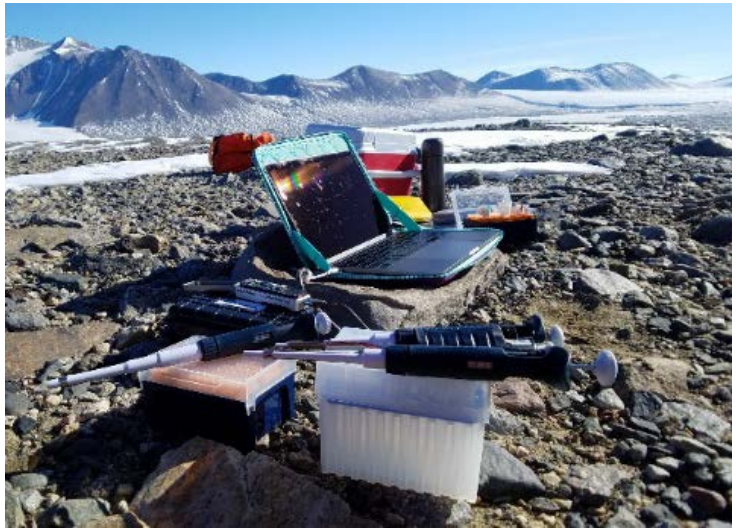


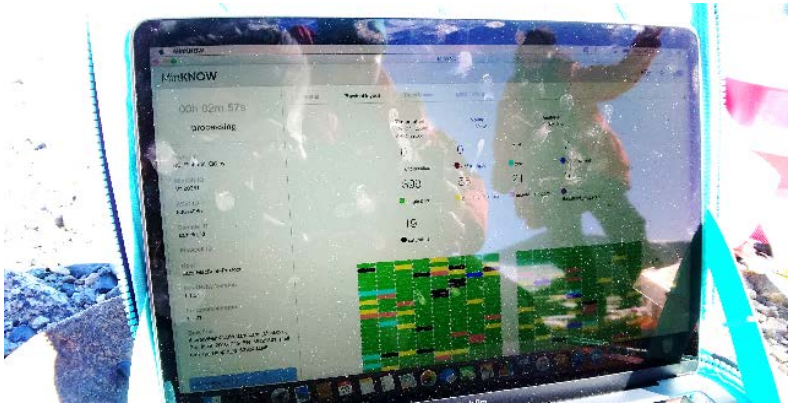


DNA Sequencing in the Field

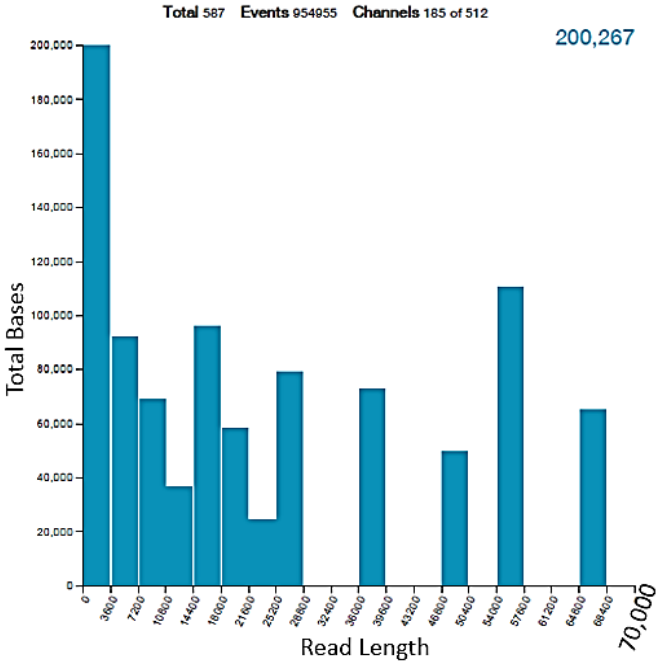
- Library prep in the field with hot water incubator
- Sequencer kept warm with hand warmers
- Proof of principle for Exoplanet grant
- The dry valleys are the perfect Mars analog site

Field Sequencing

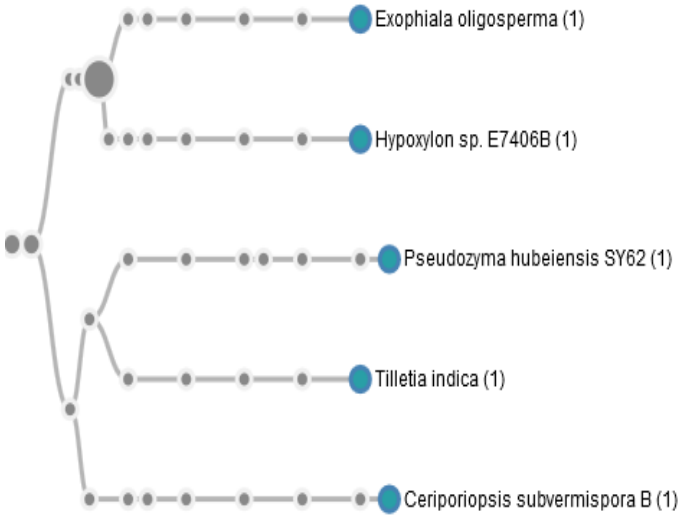




First Field : Taylor Valley Antarctica



Read length and nucleotide distribution for the MK1B MinION during field tests in the Taylor Valley, Antarctica.



Partial classification of reads collected in the field analyzed using One Codex Software. Interesting but questionable

Compiled Runs Results

Metadata and ID sequencing run performance for the Antarctic tests

Sample	Description	Sequencing date	Ambient T	Conditions	Sequencing location	Longest pass read, bp	Mean pass read length, bp	Duration of run
Control	lambda Phage DNA	Nov. 23, 2016	RT	S. S. Johnson lab	Georgetown University	79,414	7763	3 h
Control	lambda Phage DNA	Feb. 24, 2017	4°C	S. S. Johnson lab	Georgetown University	15,922	3764	50 min
Calibration	Configuration cell	Dec. 10, 2016	−5°C	Light snow, overcast	Hut Point	N/A	N/A	N/A
Environmental	Paleomat, Lake Fryxell	Dec. 15, 2016	RT	Crary lab	McMurdo Station	90,183	562	10 h
Environmental	Modern mat, Lake Vida	Dec. 17, 2016	RT	Crary lab	McMurdo Station	171,106	3874	8 h
Environmental	Paleomat, Lake Vanda	Dec. 19, 2016	−1°C	Sunny, slight wind	Taylor Valley	22,128	777	50 min[a]
Environmental	Paleomat, Lake Vida	Dec. 20, 2016	2°C	Sunny	Taylor Valley	21,357	3449	2.5 h

[a]Run terminated for evacuation in advance of impeding weather.

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- One Codex-Nick Greenfield