Metagenomic NGS for Pathogen Detection: Quality Control Issues and Strategies

Steve Miller MD PhD

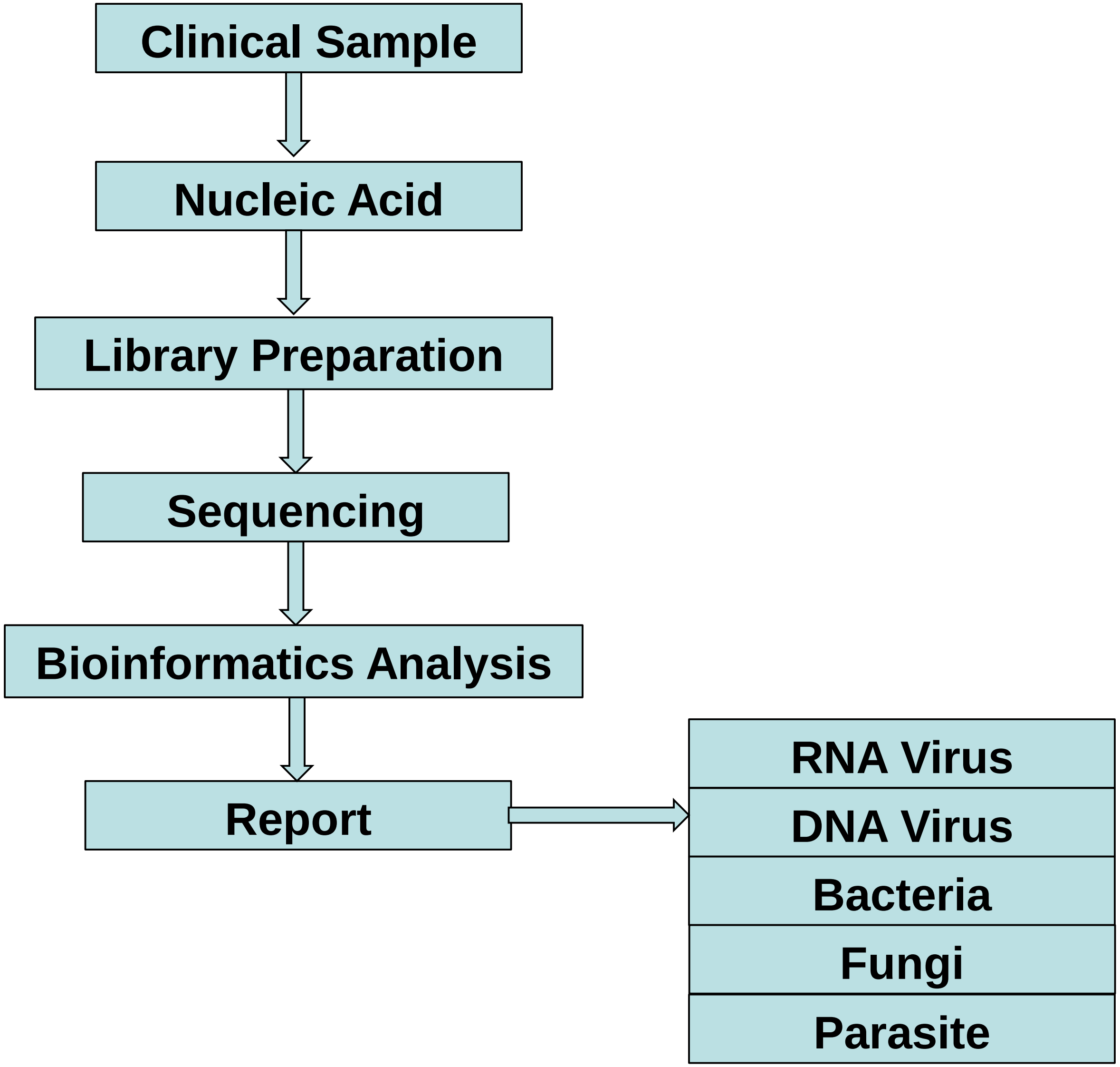
Department of Laboratory Medicine

University of California San Francisco

Disclosures

- Major funding provided by:
  - CA Initiative to Advance Precision Medicine
  - Sandler and Bowes Foundations
  - Schwab Foundation
  - Marcus Foundation

mNGS Pathogen Detection



Clinical Sample

Nucleic Acid

Library Preparation

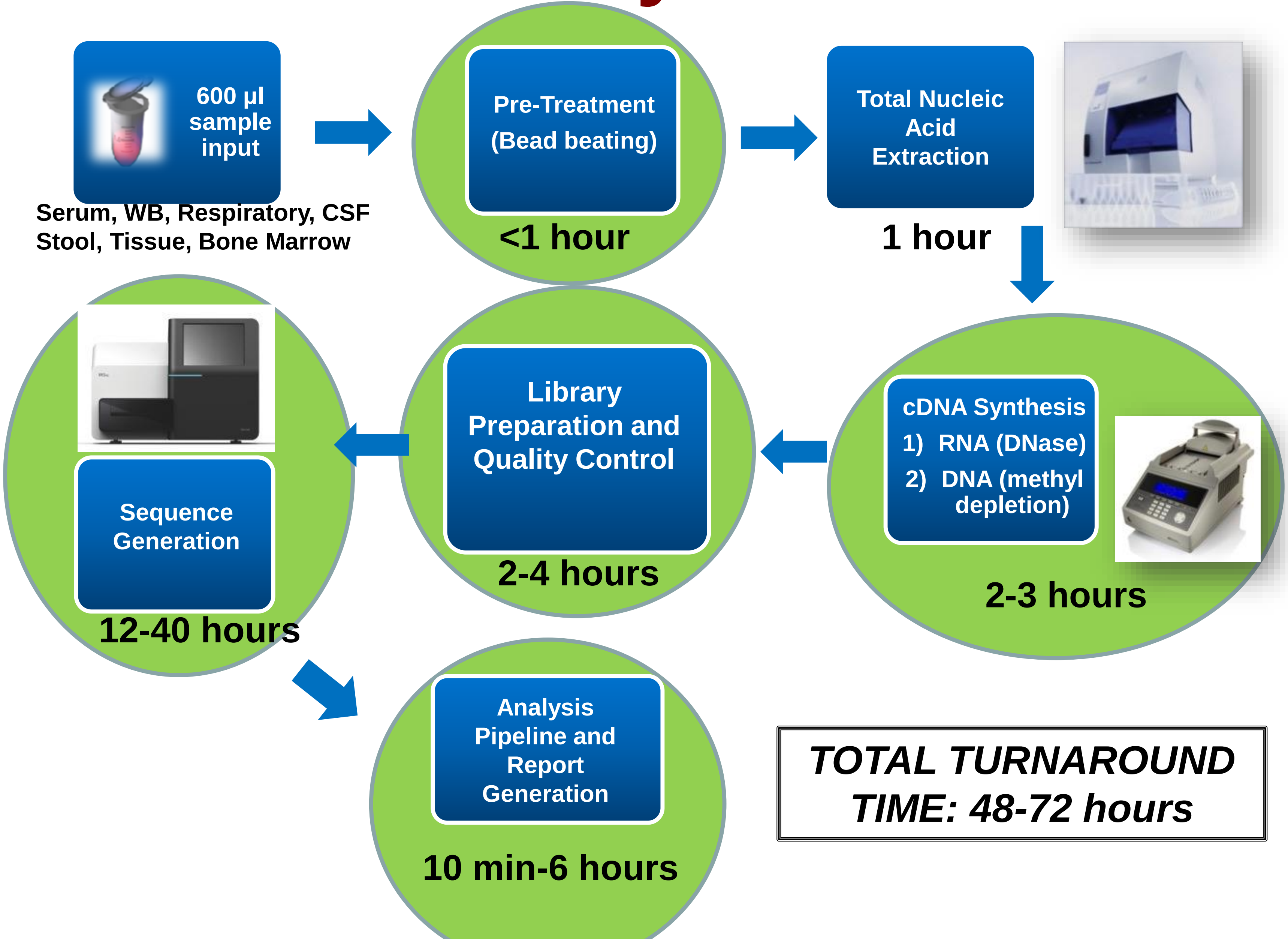
Sequencing

Bioinformatics Analysis

Report

- RNA Virus
- DNA Virus
- Bacteria
- Fungi
- Parasite

mNGS Assay Workflow



600 µl sample input

Serum, WB, Respiratory, CSF
Stool, Tissue, Bone Marrow

Pre-Treatment
(Bead beating)

<1 hour

Total Nucleic Acid Extraction

1 hour

cDNA Synthesis
1) RNA (DNase)
2) DNA (methyl depletion)

2-3 hours

Library Preparation and Quality Control

2-4 hours

Sequence Generation

12-40 hours

Analysis Pipeline and Report Generation

10 min-6 hours

TOTAL TURNAROUND TIME: 48-72 hours

CLIA Implementation of mNGS

- Required for patient testing
  - Laboratory Developed Test (LDT)
- Protocol Development
- Assay Validation
- Quality Control / Quality Assurance
- Staff Training / Competency
- Proficiency Testing

Quality Control Approach

- Adapted from CLIA Regulations
  - QA/QC monitoring
- Failure Mode Analysis
  - Likely points of assay failure
  - Develop method to detect failure
- Assay Monitoring
  - Define expected performance
  - Monitor changes over time

Quality Control Methods

- Reagent Quality Control
- External Controls
- Internal Controls
- Process Controls / Checkpoints
- Contamination Control

Reagent Quality Control

- What reagents need QC?
- How to perform?
- What are acceptible criteria?

Reagent QC

- Define “Critical” Reagents
  - Enzymes
  - Major assay steps
  - Confirm performance before putting into use
    - External control mNGS or surrogate assays acceptable
- Non-critical reagents
  - Manufacturer QC acceptable
  - Confirm acceptability using external control results

Critical Reagent Log

(Very partial list)

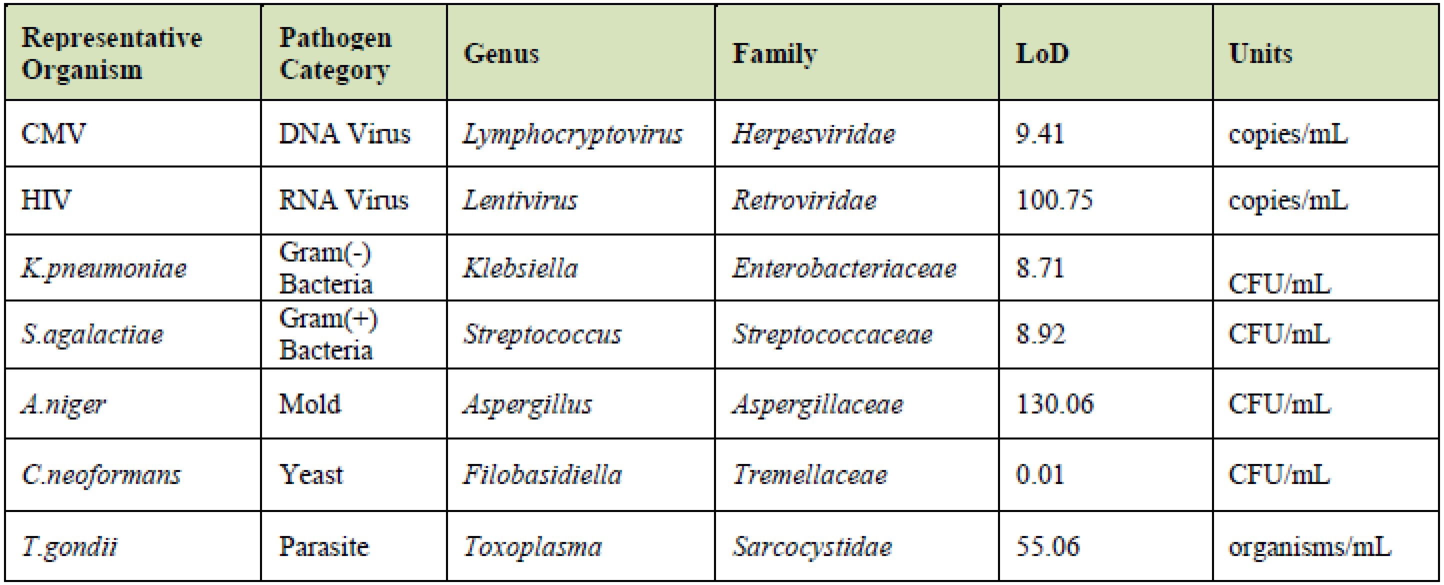
Baseline DNase	Epicentre - Illumina
C.neoformans	American Type Culture Collection
DNA Clean & Concentrator Kit -5 Capped Columns	Zymo Research
dNTPs	Thermo Fisher - Invitrogen
EZ1 Virus Mini Kit v.2.0	QIAGEN
HiSeq Rapid SR Cluster Kit v2 Flowcell	Illumina
HiSeq Rapid SR Cluster Kit v2 Rapid SR Cluster Kit	Illumina
HiSeq Rapid SR Cluster Kit v2 Flowcell	Illumina
K.pneumoniae	American Type Culture Collection
Linear acrylamide	Thermo Fisher - Life Technologies
Lysing Matrix B Tube (2mL)	MP Biomedicals
MS2 Phage	American Type Culture Collection
NEBNext Microbiome DNA Enrichment Kit	NEBNext (New England Biolabs)
Nextera Index Kit v2 Set A (96 indexes, 384 samples)	Illumina
Phusion High-Fidelity PCR Kit	Thermo Fisher - Life Technologies
SEQUENASE VERSION 2.0 DNA POLYMERASE - UNDILUTED	Affymetrix
Superscript III enzyme	Thermo Fisher - Invitrogen
Synthetic CSF Matrix 100 mL	Golden West Biologicals
T1 Phage	American Type Culture Collection
Turbo Dnase Kit	Thermo Fisher - Ambion
Water, (DNASE, RNASE free), Fisher BioReagents	Fisher Scientific
Buffer EB	Qiagen
Water, (DNASE, RNASE free), Fisher BioReagents	Fisher Scientific

Include:
Enzyme kits
Control organisms
Sterile materials

QC metric:
Prior to use
Activity
Contamination

External Controls

- Positive Control



Representative Organism	Pathogen Category	Genus	Family	LoD	Units
CMV	DNA Virus	*Lymphocryptovirus*	*Herpesviridae*	9.41	copies/mL
HIV	RNA Virus	*Lentivirus*	*Retroviridae*	100.75	copies/mL
K.pneumoniae	Gram(-) Bacteria	*Klebsiella*	*Enterobacteriaceae*	8.71	CFU/mL
S.agalactiae	Gram(+) Bacteria	*Streptococcus*	*Streptococcaceae*	8.92	CFU/mL
A.niger	Mold	*Aspergillus*	*Aspergillaceae*	130.06	CFU/mL
C.neoformans	Yeast	*Filobasidiella*	*Tremellaceae*	0.01	CFU/mL
T.gondii	Parasite	*Toxoplasma*	*Sarcocystidae*	55.06	organisms/mL

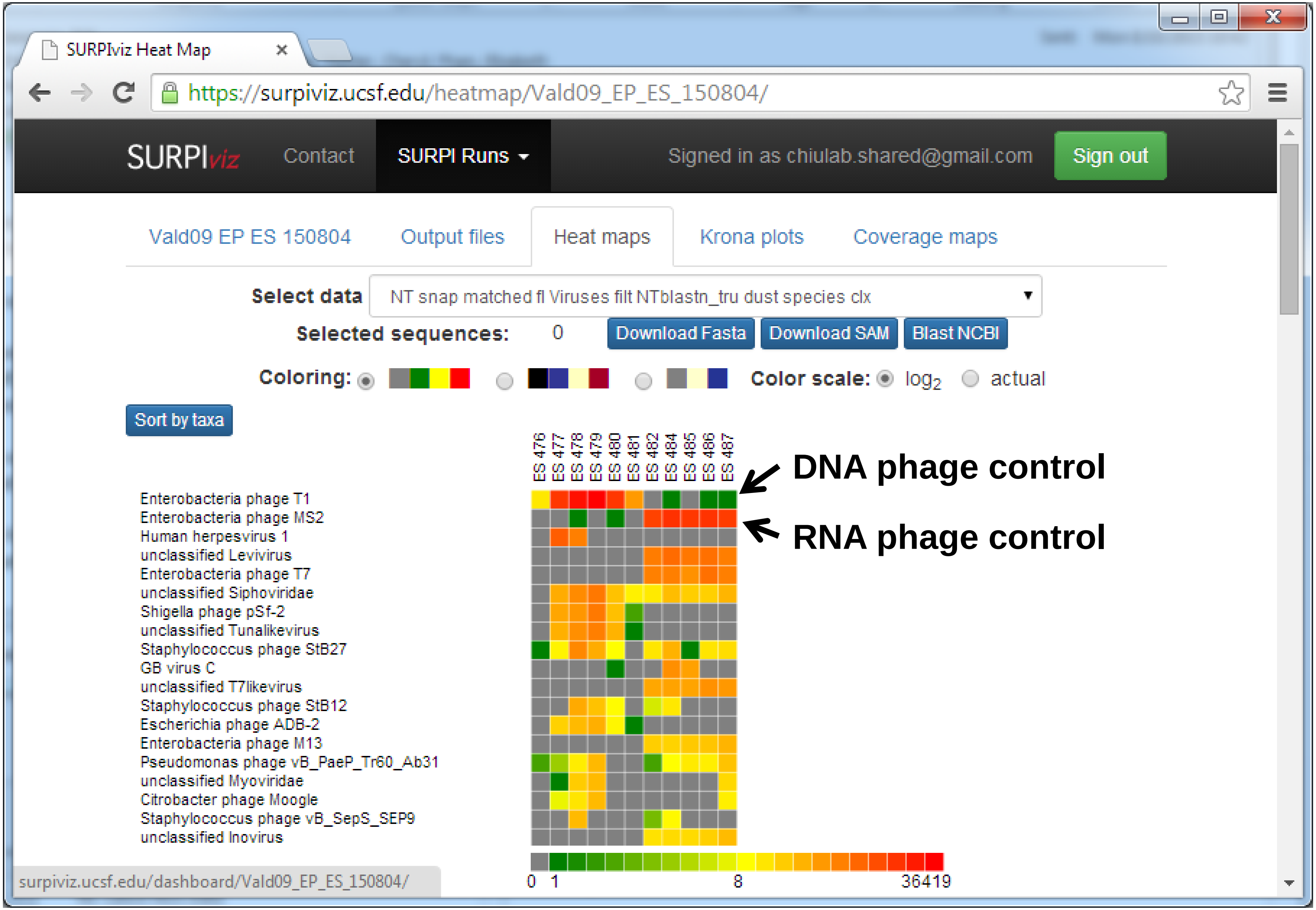
- Negative Control
  - Elution buffer (negative extract)
  - Also used as background normalizer

Internal Controls

Control	Concentration	Average % reads	CV % reads	Threshold number of reads
MS2 phage (RNA)	10^3 cp/ml	0.011	49.5%	>= 100
T1 phage (DNA)	10^5 cp/ml	0.022	29.8%	>= 100

Controls for entire test process

Low number of IC reads in high background samples (decreased sensitivity)



Select data NT snap matched fl Viruses filt NTblastn_tru dust species clx

Selected sequences: 0

Coloring: ● 🟫🟩🟨🟥 ○ ⬛🟦⬜🟥 ○ 🟫⬜🟦 **Color scale:** ● $\log_2$ ○ actual

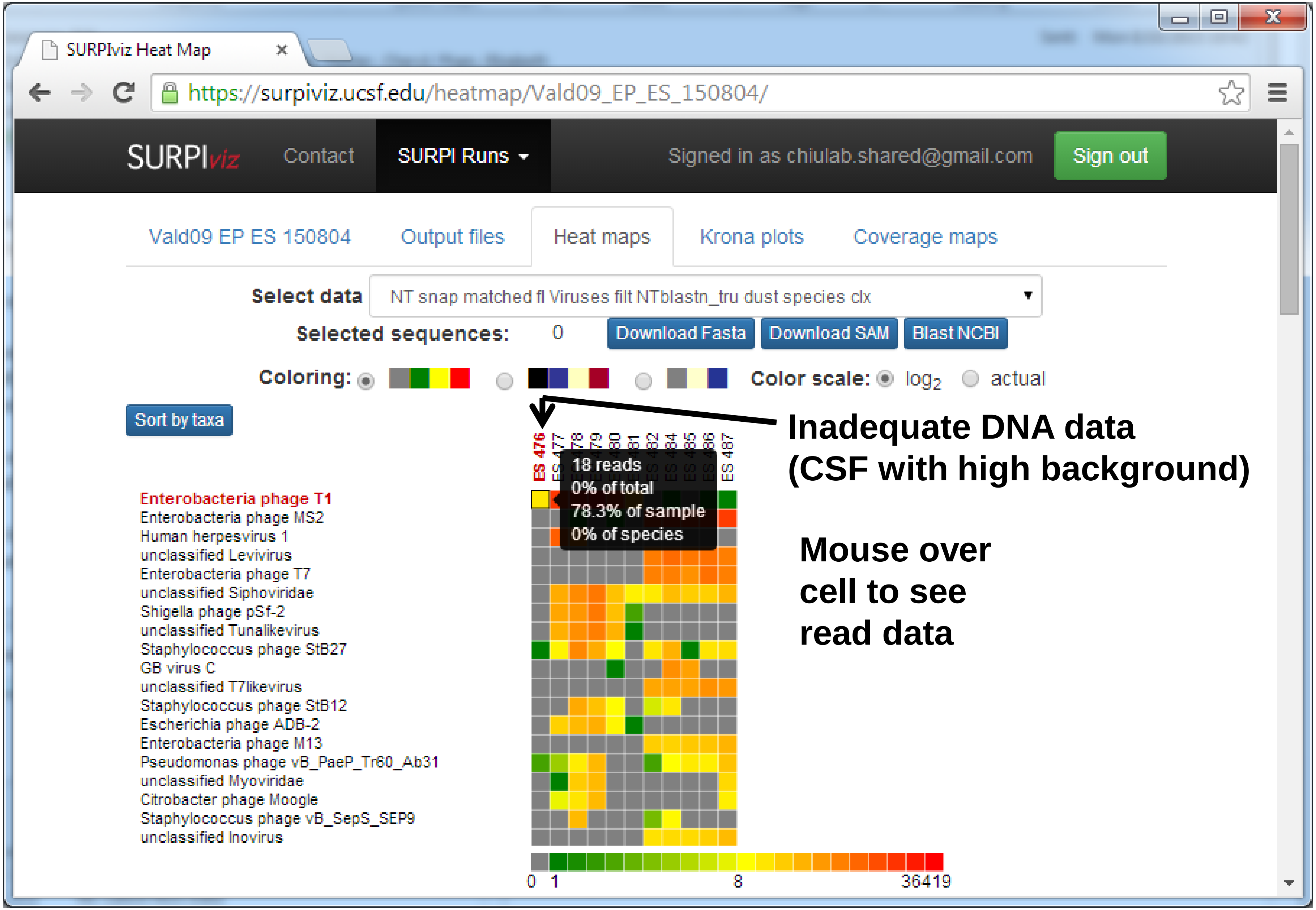
ES 476, ES 477, ES 478, ES 479, ES 480, ES 481, ES 482, ES 484, ES 485, ES 486, ES 487

Enterobacteria phage T1
Enterobacteria phage MS2
Human herpesvirus 1
unclassified Levivirus
Enterobacteria phage T7
unclassified Siphoviridae
Shigella phage pSf-2
unclassified Tunalikevirus
Staphylococcus phage StB27
GB virus C
unclassified T7likevirus
Staphylococcus phage StB12
Escherichia phage ADB-2
Enterobacteria phage M13
Pseudomonas phage vB_PaeP_Tr60_Ab31
unclassified Myoviridae
Citrobacter phage Moogle
Staphylococcus phage vB_SepS_SEP9
unclassified Inovirus

0 1 8 36419

DNA phage control

RNA phage control



Signed in as chiulab.shared@gmail.com

Select data NT snap matched fl Viruses filt NTblastn_tru dust species clx

Selected sequences: 0

Coloring: ● ○ ○ **Color scale:** ● $\log_2$ ○ actual

Enterobacteria phage T1
Enterobacteria phage MS2
Human herpesvirus 1
unclassified Levivirus
Enterobacteria phage T7
unclassified Siphoviridae
Shigella phage pSf-2
unclassified Tunalikevirus
Staphylococcus phage StB27
GB virus C
unclassified T7likevirus
Staphylococcus phage StB12
Escherichia phage ADB-2
Enterobacteria phage M13
Pseudomonas phage vB_PaeP_Tr60_Ab31
unclassified Myoviridae
Citrobacter phage Moogle
Staphylococcus phage vB_SepS_SEP9
unclassified Inovirus

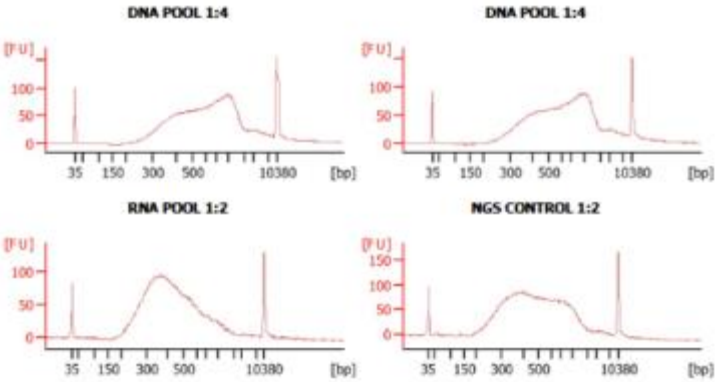
18 reads
0% of total
78.3% of sample
0% of species

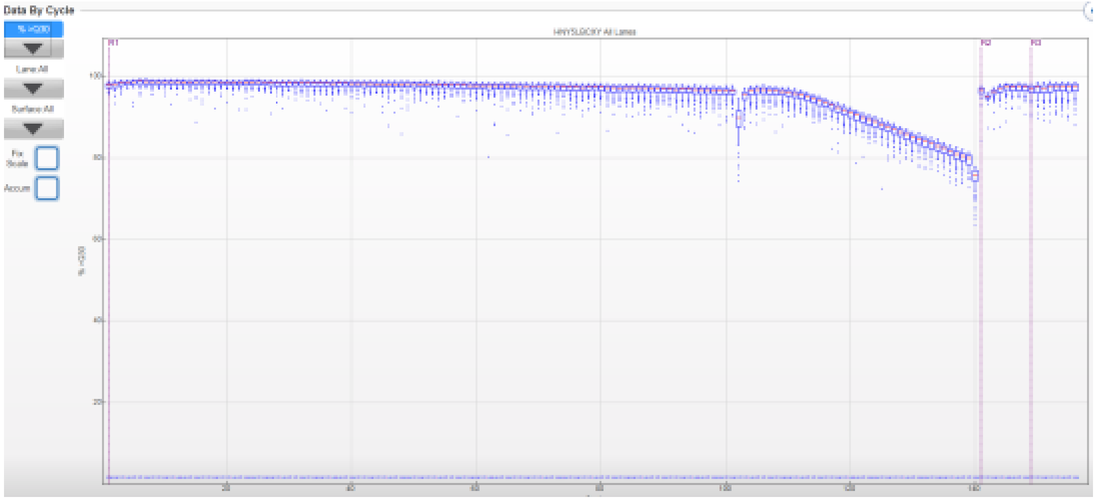
Inadequate DNA data (CSF with high background)

Mouse over cell to see read data

Process Controls

- Library Quality
  - Library Size
  - Library Concentration
- Sequencing Quality
  - Cluster Density
  - Q score > 30



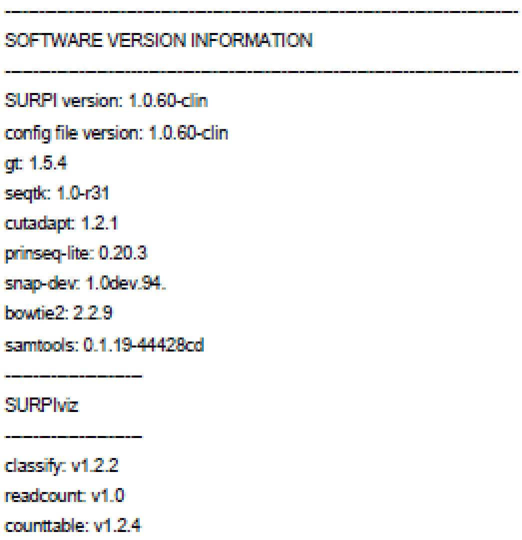


Analyzer Controls

- Concentration controls (Qubit)
  - Stock libraries
- Bioanalyzer controls
  - Stock libraries
- Develop metrics for QC of library analyzers
  - Concentration range
  - Size range

Periodic Monitoring

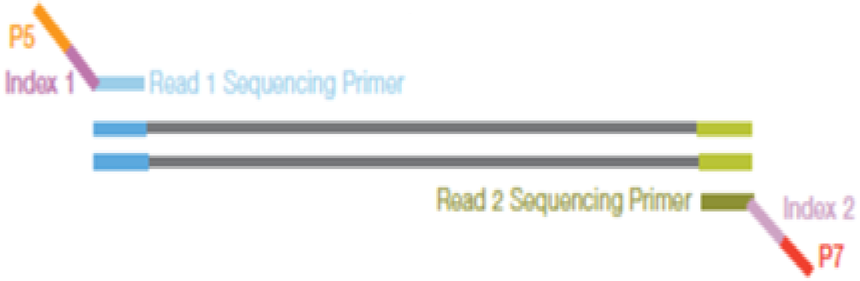
- Positive threshold verification
  - Verify detection at 1:10 dilution of positive control
- Software run log
  - Document versions
- Instrument repair log
- Troubleshooting log



```
------------------------------------------------------------
SOFTWARE VERSION INFORMATION
------------------------------------------------------------
SURPI version: 1.0.60-clin
config file version: 1.0.60-clin
gt: 1.5.4
seqtk: 1.0-r31
cutadapt: 1.2.1
prinseq-lite: 0.20.3
snap-dev: 1.0dev.94.
bowtie2: 2.2.9
samtools: 0.1.19-44428cd
----------------
SURPIviz
----------------
classify: v1.2.2
readcount: v1.0
counttable: v1.2.4
```

Contamination Control

- NGS assay extremely sensitive to contamination
  - Initial sample
  - Library preparation
  - Instrument carryover



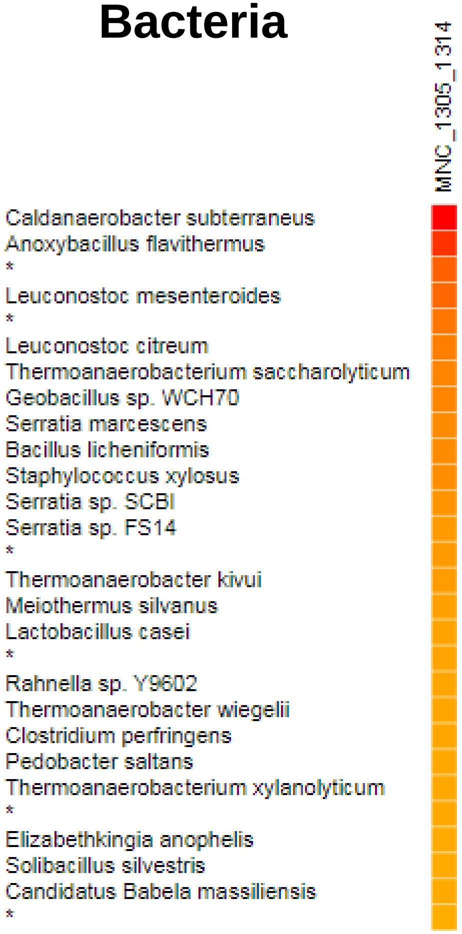
- Double barcoded adaptors
- Need caution interpreting significance of a few sequence reads when high positive sample is on same or recent run
- Bacterial background reads complicate detection of low levels of bacteria in clinical samples
- Use ratio of bacteria, fungal and parasite reads to NTC
  - Threshold of 10x reads in sample compared to NTC

Contamination Control

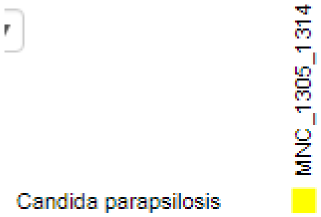
- Swipe Tests
  - Background laboratory contamination
    - Amplicon
    - Organisms
- What is successful swipe test?
  - Assess flora
  - Unusual detections
  - Pathogenic organisms

Swipe Test Results

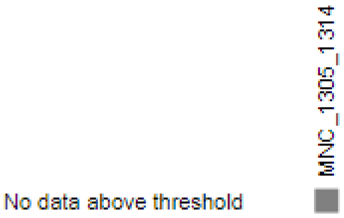
Bacteria



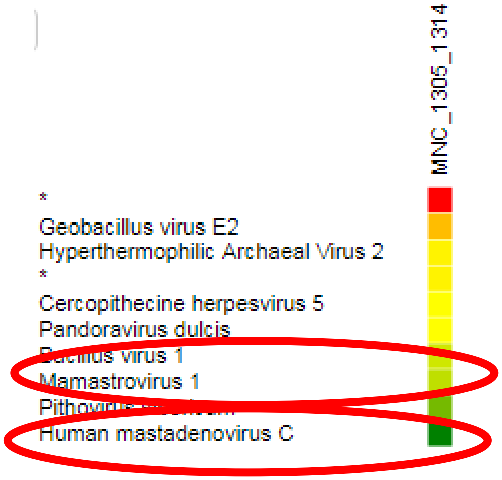
Fungi



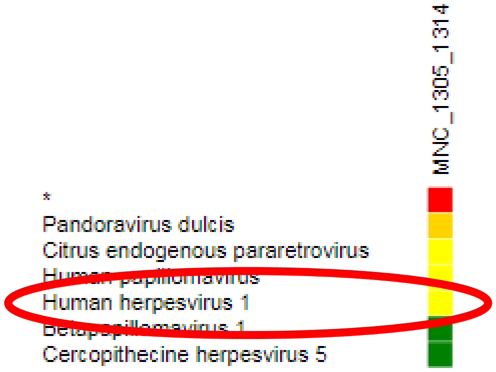
Parasite



DNA Virus



RNA Virus

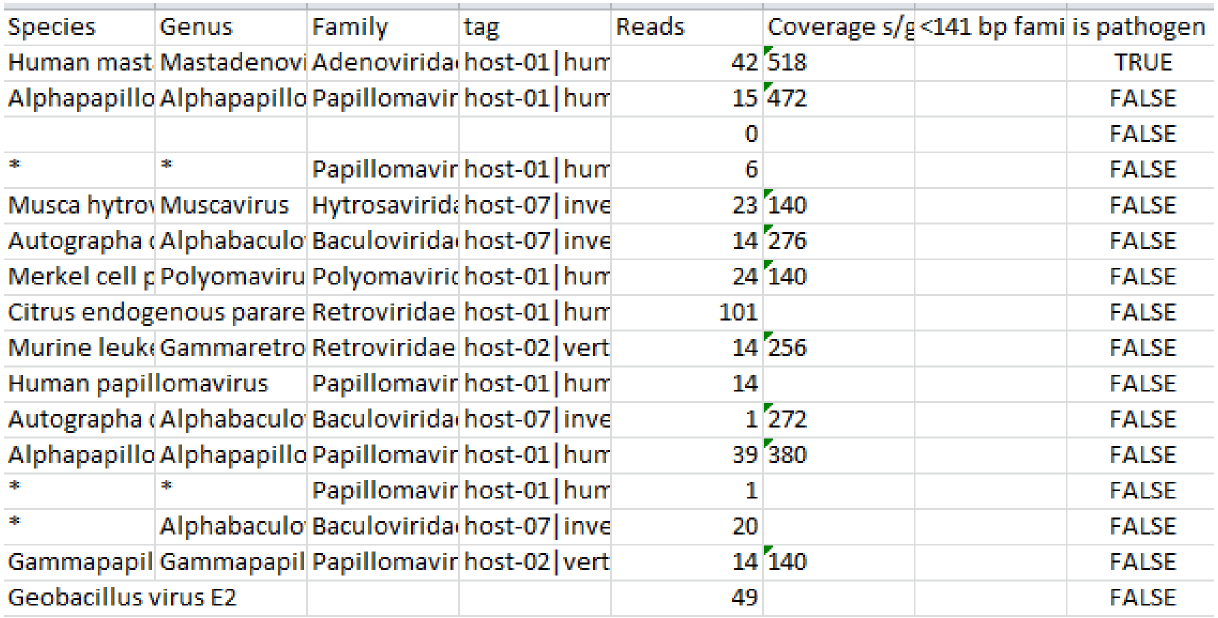


Environmental bacterial / fungal flora - acceptable
Few reads to potentially pathogenic viruses – clean and repeat

Contaminant Database

- Track contamination over time
  - NTC
  - PC
  - Samples
  - Swipe tests

NTC Contaminants

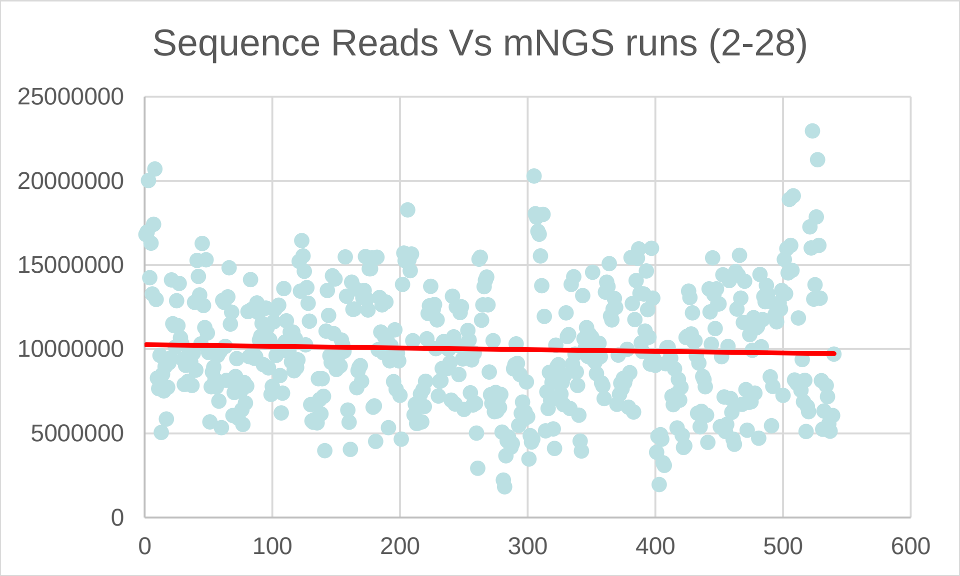


Species	Genus	Family	tag	Reads	Coverage s/g	<141 bp fami	is pathogen
Human mast	Mastadenovi	Adenovirida	host-01|hum	42	518		TRUE
Alphapapillo	Alphapapillo	Papillomavir	host-01|hum	15	472		FALSE
				0			FALSE
*	*	Papillomavir	host-01|hum	6			FALSE
Musca hytrov	Muscavirus	Hytrosavirida	host-07|inve	23	140		FALSE
Autographa c	Alphabaculo	Baculovirida	host-07|inve	14	276		FALSE
Merkel cell p	Polyomaviru	Polyomavirid	host-01|hum	24	140		FALSE
Citrus endogenous parare		Retroviridae	host-01|hum	101			FALSE
Murine leuke	Gammaretro	Retroviridae	host-02|vert	14	256		FALSE
Human papillomavirus		Papillomavir	host-01|hum	14			FALSE
Autographa c	Alphabaculo	Baculovirida	host-07|inve	1	272		FALSE
Alphapapillo	Alphapapillo	Papillomavir	host-01|hum	39	380		FALSE
*	*	Papillomavir	host-01|hum	1			FALSE
*	Alphabaculo	Baculovirida	host-07|inve	20			FALSE
Gammapapil	Gammapapil	Papillomavir	host-02|vert	14	140		FALSE
Geobacillus virus E2				49			FALSE

Troubleshooting Example

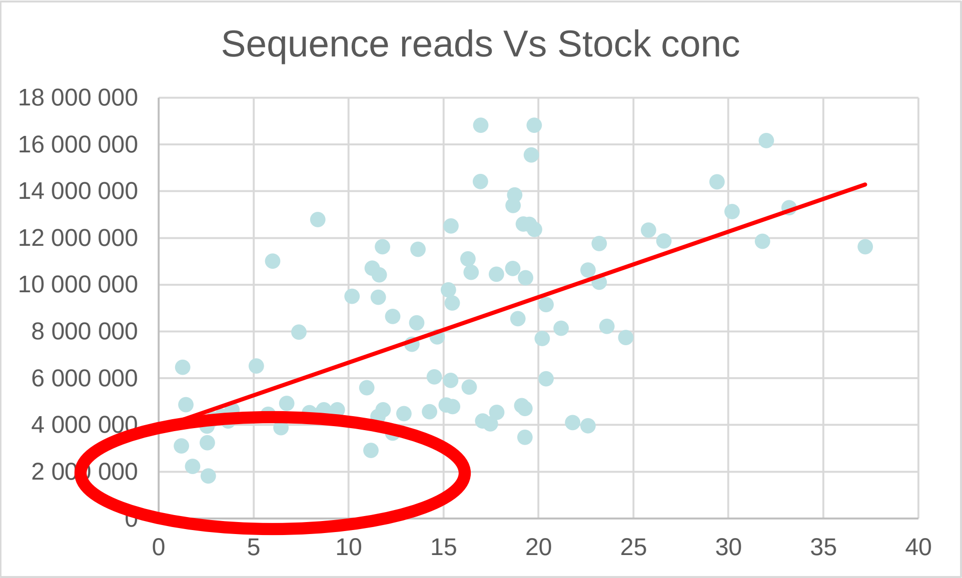
- Failure to achieve sufficient reads per library
  - 5 million required for adequate analysis
  - Interpreted with comment that sensitivity may be decreased
- Why are some libraries not providing enough sequence?

Sequencing Instrument



Total number of reads per run fairly constant

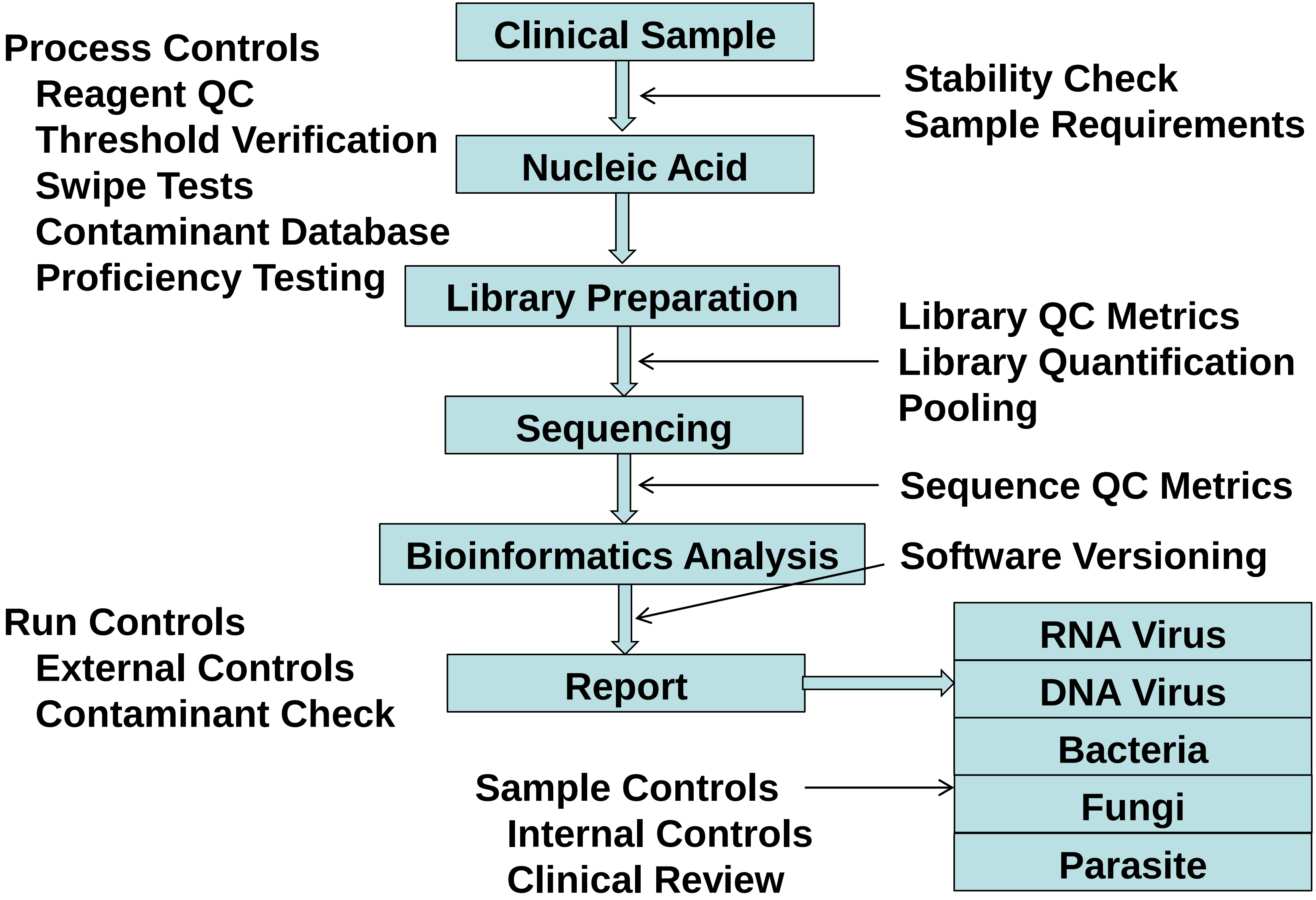
Library Quantification



Low number of reads from libraries with lower final concentration
Even after correcting for this in the pool

Low input libraries tend to have non-adaptor DNA present
Alternative normalization strategies investigated
  Add more library to pool
  Quantify with kappa PCR or MiSeq run

Overview – mNGS QC



Process Controls
  Reagent QC
  Threshold Verification
  Swipe Tests
  Contaminant Database
  Proficiency Testing

Clinical Sample

Stability Check
Sample Requirements

Nucleic Acid

Library Preparation

Library QC Metrics
Library Quantification
Pooling

Sequencing

Sequence QC Metrics

Bioinformatics Analysis

Software Versioning

Run Controls
  External Controls
  Contaminant Check

Report

RNA Virus
DNA Virus
Bacteria
Fungi
Parasite

Sample Controls
  Internal Controls
  Clinical Review

Summary – mNGS QA

- Incorporate standard QC and Risk-Based approaches
- Many QC criteria / checkpoints
  - External controls
  - Internal controls
  - Checkpoint Metrics
  - Contamination Control
- Process controls
  - Workflow
  - Contaminant databases
  - Reagent QC
  - Swipe Tests
- Assay maintenance
  - Results correlation
  - Proficiency Testing
  - Investigate usual results / QC trends

Acknowledgements

UCSF
University of California
San Francisco

UCSF Chiu Lab and VDDC
Charles Chiu, MD/PhD
Erik Samayoa, CLS
Shaun Arevalo, CLS
Becky Fung, CLS
Hannah Sample, BS
Samia Naccache, PhD
Scot Federman, BS
Doug Stryke, BS
Joseph DeRisi, PhD
Michael Wilson, MD
Jeffrey Gelfand, MD
Michael Geschwind, MD

UC DAVIS
UNIVERSITY OF CALIFORNIA

UCLA

Berkeley
UNIVERSITY OF CALIFORNIA

Children's Hospital
LOS ANGELES
We Treat Kids Better

UCLA
Jeffrey Klausner, MD/MPH
Romney Humphries, PhD (ABMM)

University of California, Berkeley
Brent Fulton, PhD/MBA

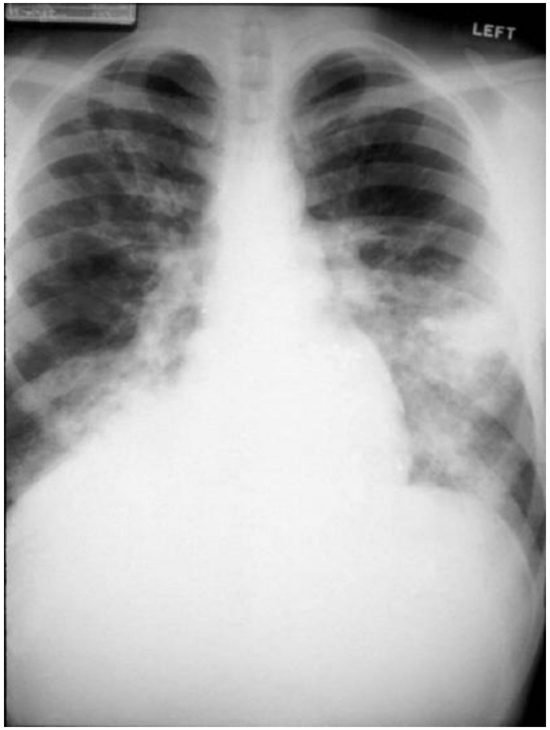
UCDavis
Chris Polage, MD
Stuart Cohen, MD

Children's Hospital Los Angeles
Jeffrey Bender, MD
Jennifer Dien-Bard, MD

Children's National Medical Center
Brittany Goldberg, MD
Joseph Campos, MD

St. Jude's Medical Center
Randall Hayden, MD/PhD

Many Acute Infectious Diseases Remain Undiagnosed



Pneumonia

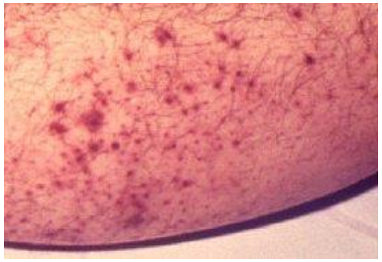
15 – 25%
unknown cause

Diarrheal Disease

Up to 50%
unknown cause

Meningitis / Encephalitis

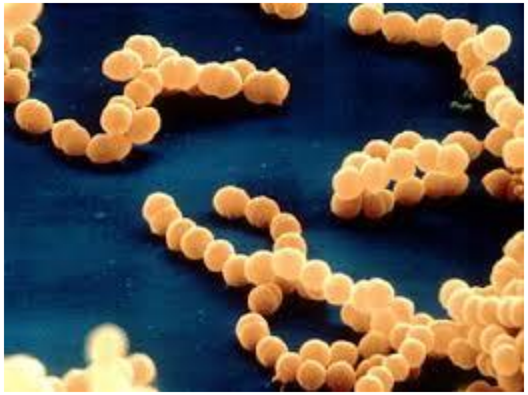
60-80%
unknown cause



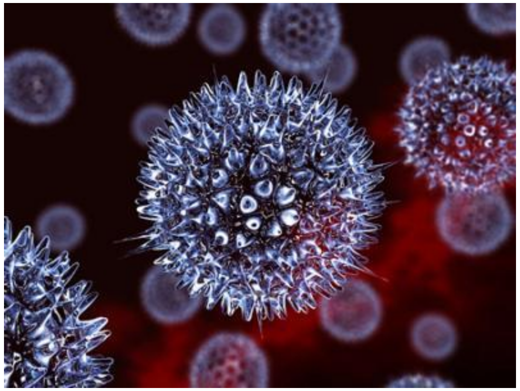
Fever / Sepsis

~20% unknown
cause

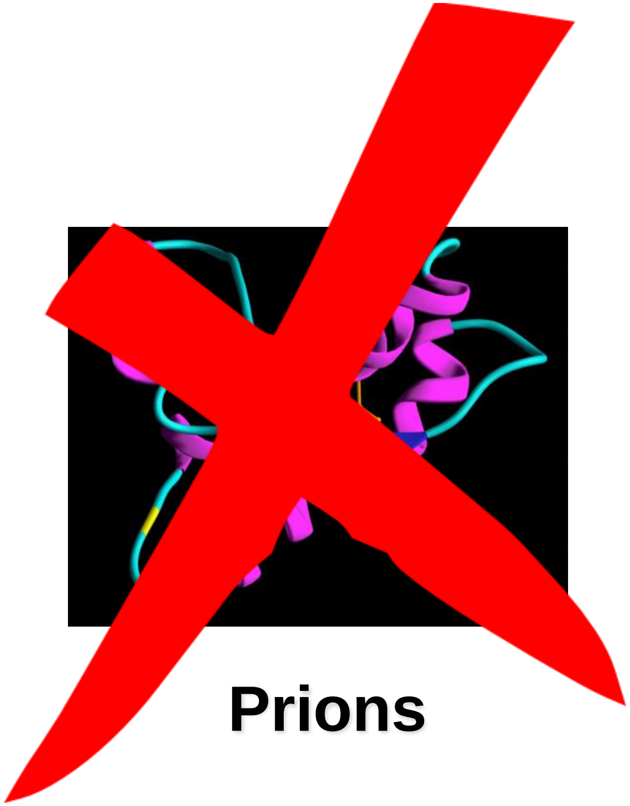
Nearly All Agents that Cause Infectious Diseases Contain Nucleic Acid (DNA/RNA)



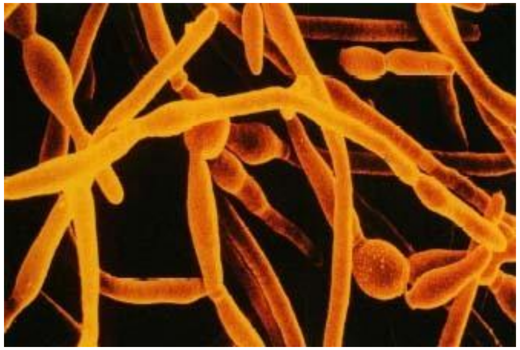
Bacteria



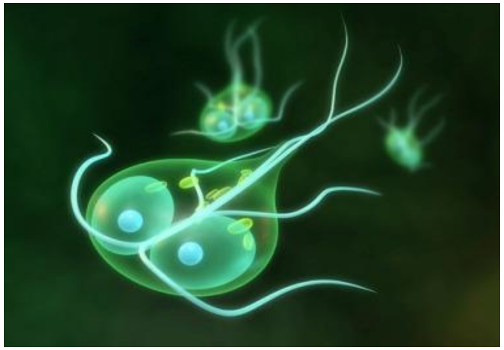
Viruses



Prions



Fungi



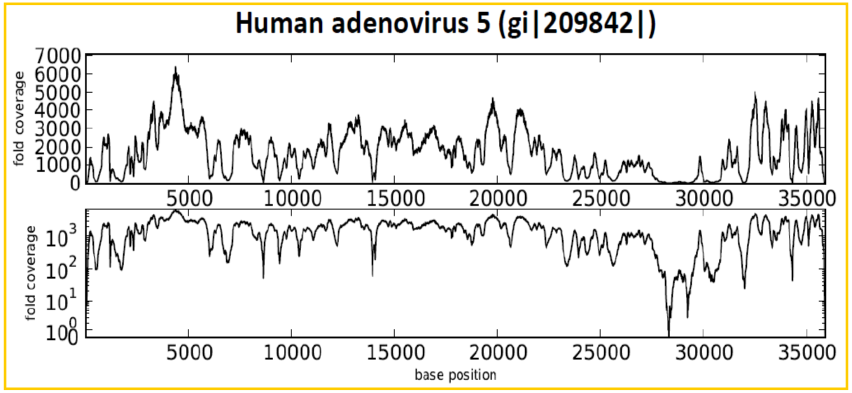
Parasites

NGS Pathogen Detection Allows For

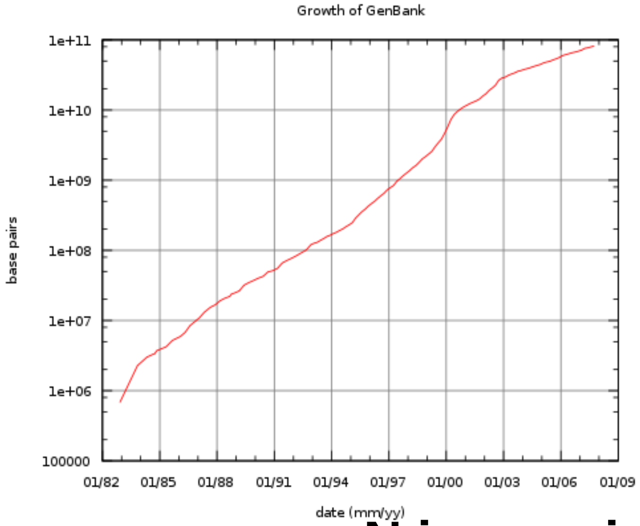
- Diagnosis
  - Treatment optimization
- Pathogen Discovery
- Public Health Investigation
  - Unknown outbreak

Bioinformatics Challenges

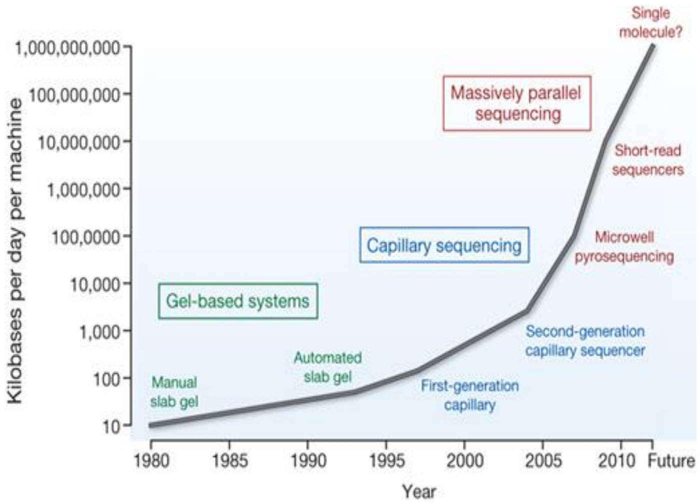
```
GCTTTTTTGAGTTTTATAAAGGA-
TAAATGGAGCGAAGAAACCCATCT
GAGCGGGGGGTACCTGCTGGATT
TTCTGGCCATGCATCTGTGGAGAG
CGGTGGTGAGACACAAGAATCGCC
TGCTACTGTTGTCTTCCGTCCGCC
CGGCAATAATACCGACGGAGGAG
CAACAGCAGGAGGAAGCCAGGCG
GCGGCGGCGGCAGGAGCAGAGCC
```



GenBank Size

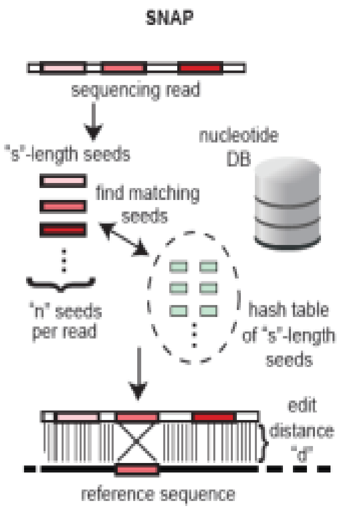


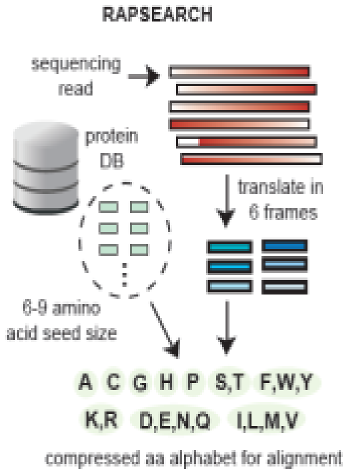
Rate of DNA Sequencing



N is growing at an exponential rate!

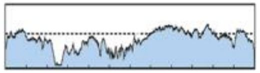
Sequence Based Ultra-Rapid Pathogen Identification (SURPI)



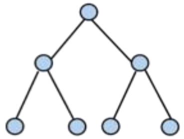


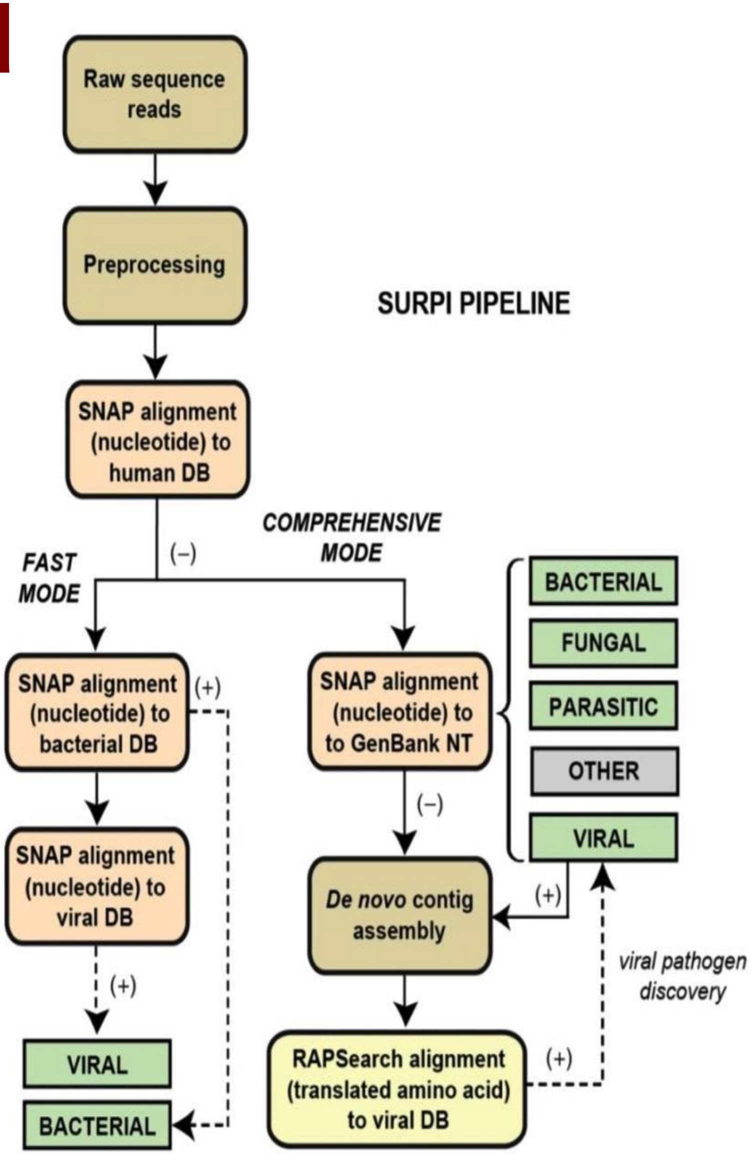
Interpretative report

Coverage map

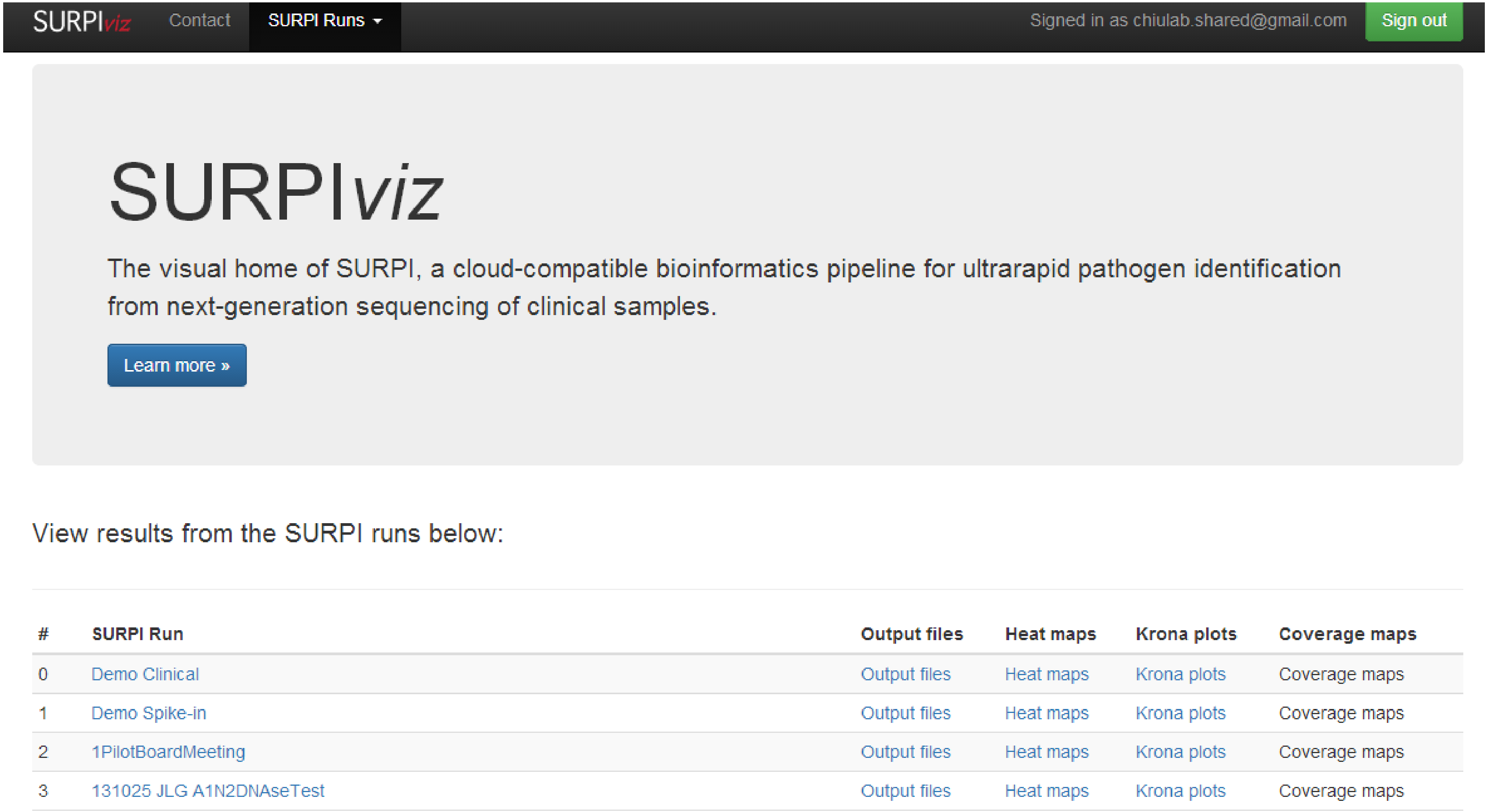


Taxonomic classification





SURPI visualization tool

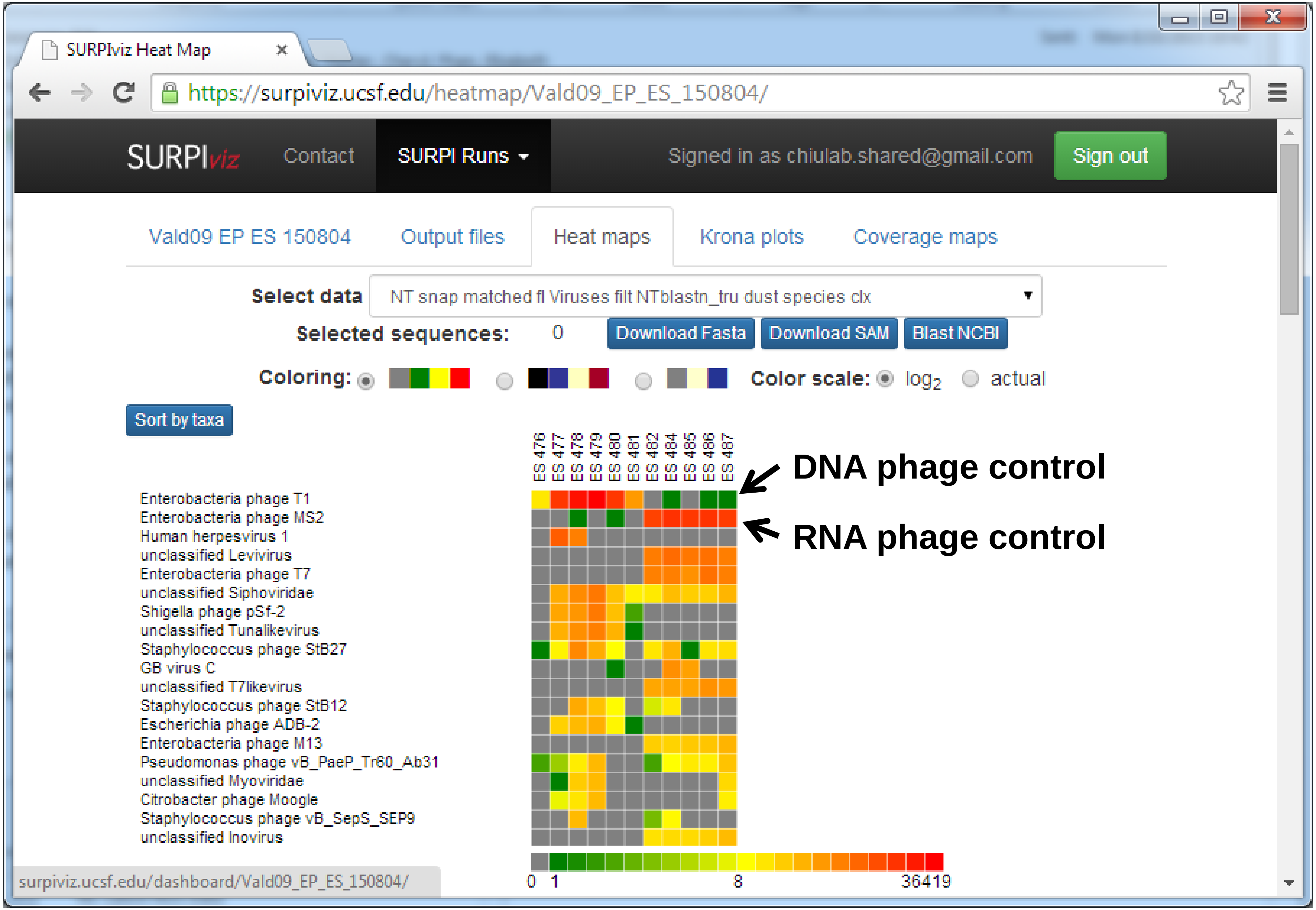


SURPI*viz*

The visual home of SURPI, a cloud-compatible bioinformatics pipeline for ultrarapid pathogen identification from next-generation sequencing of clinical samples.

View results from the SURPI runs below:

#	SURPI Run	Output files	Heat maps	Krona plots	Coverage maps
0	Demo Clinical	Output files	Heat maps	Krona plots	Coverage maps
1	Demo Spike-in	Output files	Heat maps	Krona plots	Coverage maps
2	1PilotBoardMeeting	Output files	Heat maps	Krona plots	Coverage maps
3	131025 JLG A1N2DNAseTest	Output files	Heat maps	Krona plots	Coverage maps



https://surpiviz.ucsf.edu/heatmap/Vald09_EP_ES_150804/

Signed in as chiulab.shared@gmail.com

Select data NT snap matched fl Viruses filt NTblastn_tru dust species clx

Selected sequences: 0

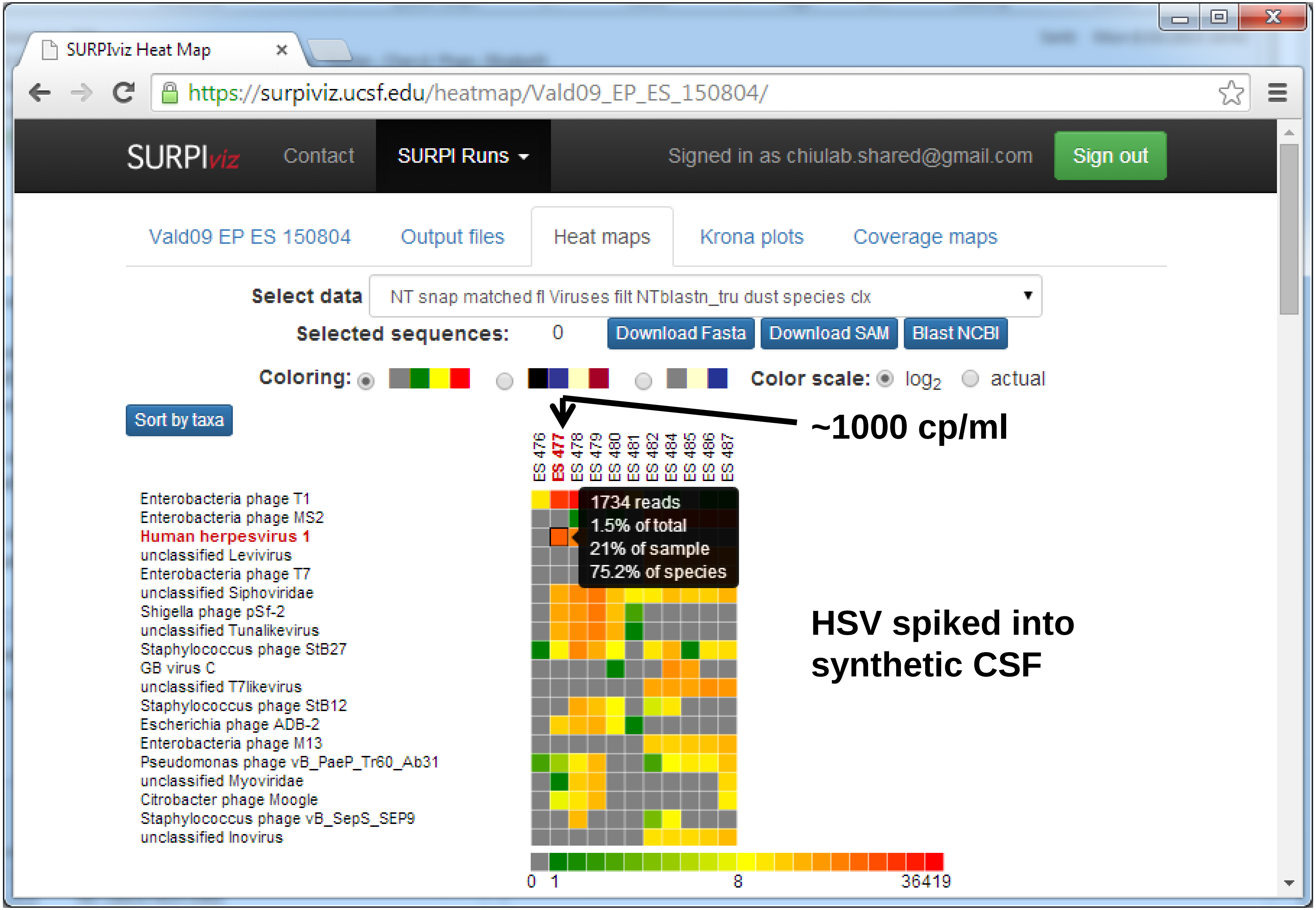
Coloring: ● 🟫🟩🟨🟥 ○ ⬛🟦⬜🟥 ○ 🟫⬜🟦

Color scale: ● $\log_2$ ○ actual

surpiviz.ucsf.edu/dashboard/Vald09_EP_ES_150804/

DNA phage control

RNA phage control



Select data NT snap matched fl Viruses filt NTblastn_tru dust species clx

Selected sequences: 0

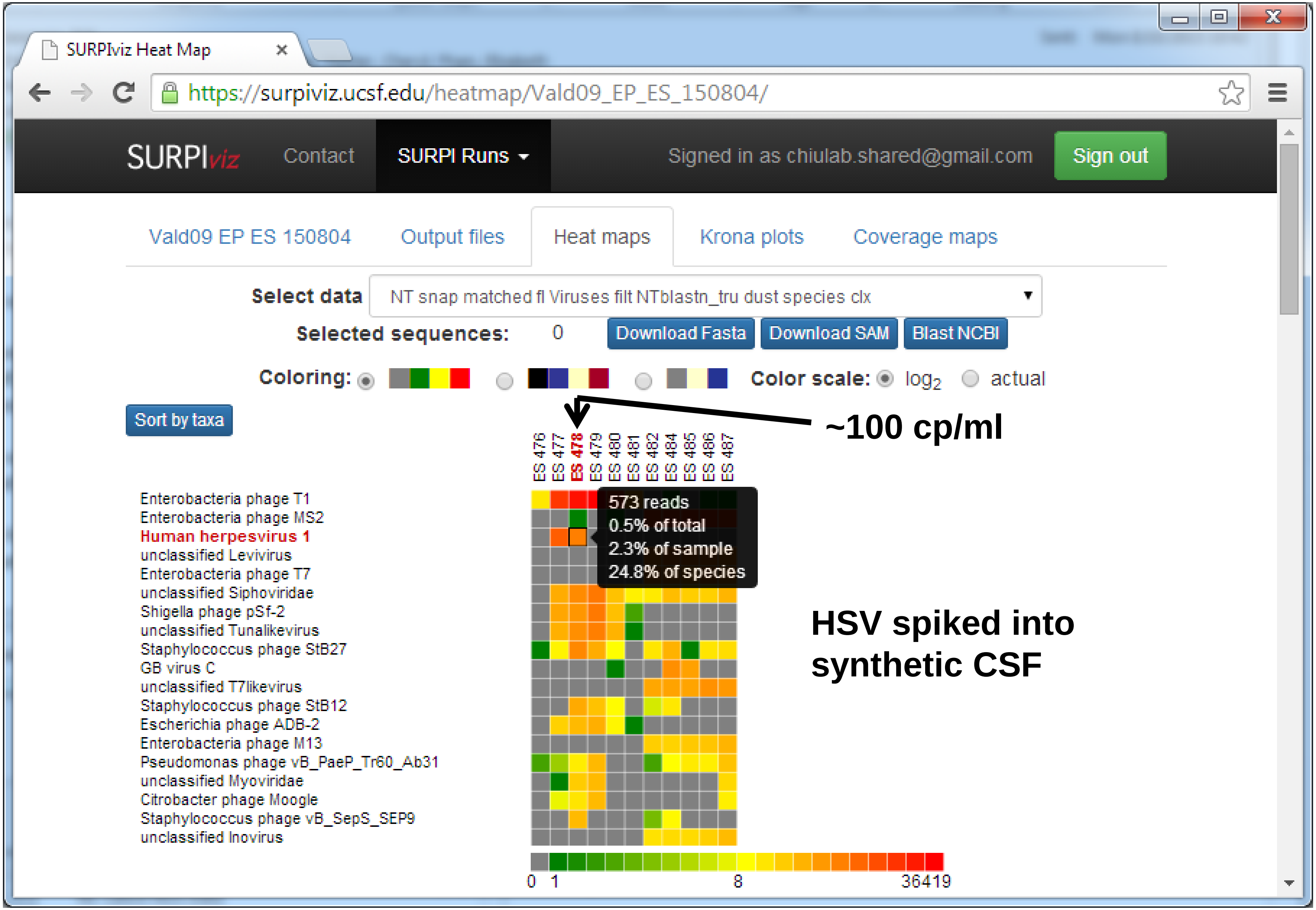
Coloring: ● ▨🟩🟨🟥 ○ ■🟦🟨🟥 ○ ▨🟨🟦 **Color scale:** ● $\log_2$ ○ actual

1734 reads
1.5% of total
21% of sample
75.2% of species

Enterobacteria phage T1
Enterobacteria phage MS2
Human herpesvirus 1
unclassified Levivirus
Enterobacteria phage T7
unclassified Siphoviridae
Shigella phage pSf-2
unclassified Tunalikevirus
Staphylococcus phage StB27
GB virus C
unclassified T7likevirus
Staphylococcus phage StB12
Escherichia phage ADB-2
Enterobacteria phage M13
Pseudomonas phage vB_PaeP_Tr60_Ab31
unclassified Myoviridae
Citrobacter phage Moogle
Staphylococcus phage vB_SepS_SEP9
unclassified Inovirus

~1000 cp/ml

HSV spiked into synthetic CSF



Signed in as chiulab.shared@gmail.com

Select data NT snap matched fl Viruses filt NTblastn_tru dust species clx

Selected sequences: 0

Coloring: ● ○ ○ **Color scale:** ● $\log_2$ ○ actual

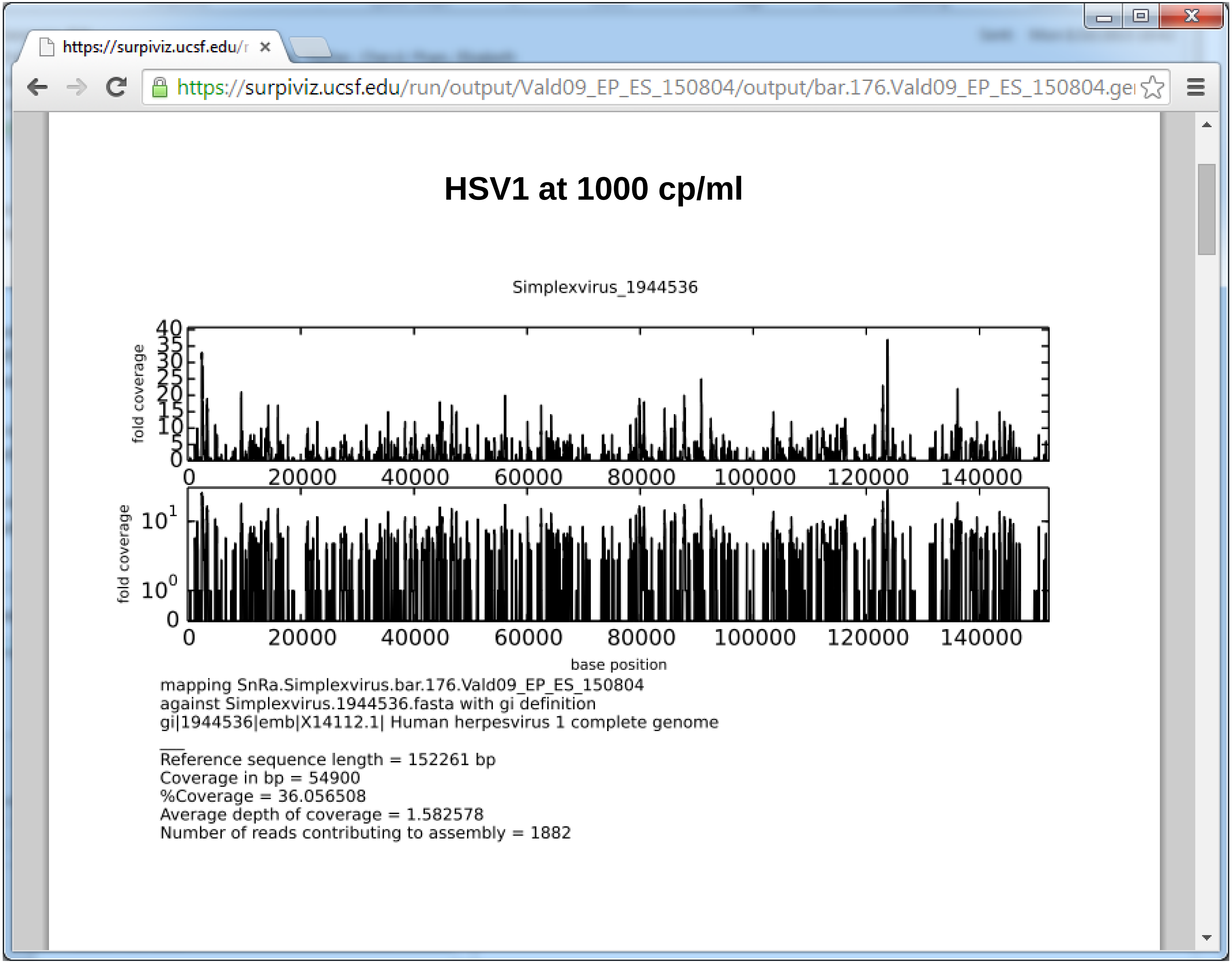
Enterobacteria phage T1
Enterobacteria phage MS2
Human herpesvirus 1
unclassified Levivirus
Enterobacteria phage T7
unclassified Siphoviridae
Shigella phage pSf-2
unclassified Tunalikevirus
Staphylococcus phage StB27
GB virus C
unclassified T7likevirus
Staphylococcus phage StB12
Escherichia phage ADB-2
Enterobacteria phage M13
Pseudomonas phage vB_PaeP_Tr60_Ab31
unclassified Myoviridae
Citrobacter phage Moogle
Staphylococcus phage vB_SepS_SEP9
unclassified Inovirus

573 reads
0.5% of total
2.3% of sample
24.8% of species

~100 cp/ml

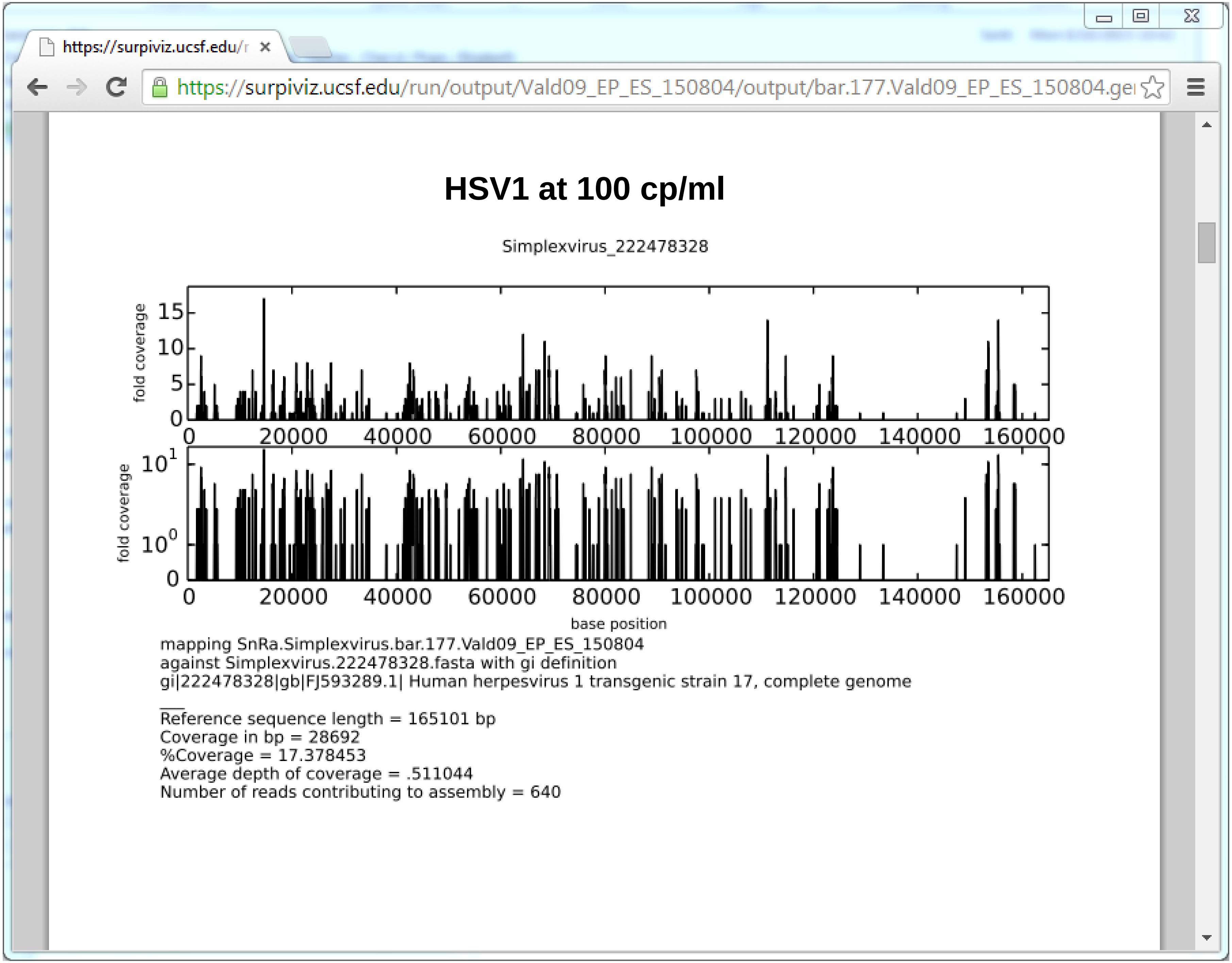
HSV spiked into synthetic CSF

HSV1 at 1000 cp/ml



mapping SnRa.Simplexvirus.bar.176.Vald09_EP_ES_150804
against Simplexvirus.1944536.fasta with gi definition
gi|1944536|emb|X14112.1| Human herpesvirus 1 complete genome

___
Reference sequence length = 152261 bp
Coverage in bp = 54900
%Coverage = 36.056508
Average depth of coverage = 1.582578
Number of reads contributing to assembly = 1882

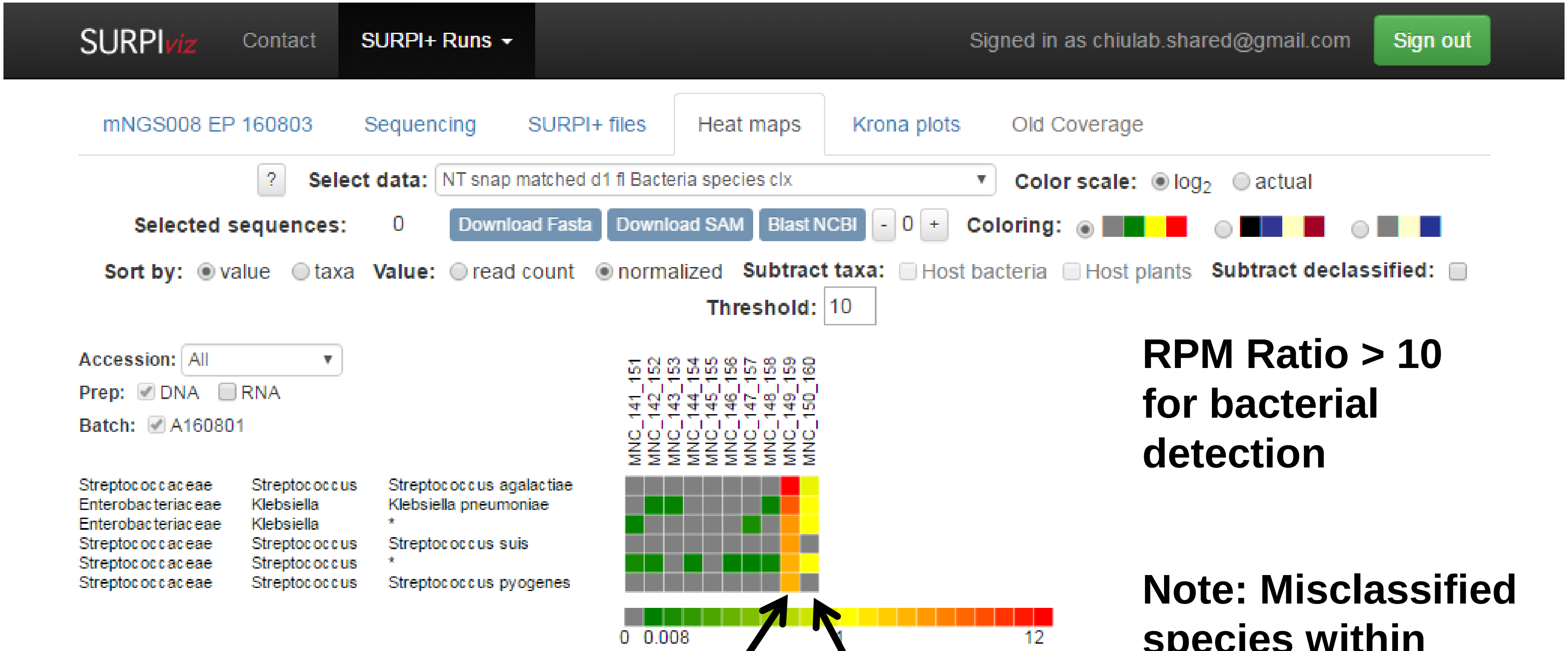


HSV1 at 100 cp/ml

Simplexvirus_222478328

mapping SnRa.Simplexvirus.bar.177.Vald09_EP_ES_150804
against Simplexvirus.222478328.fasta with gi definition
gi|222478328|gb|FJ593289.1| Human herpesvirus 1 transgenic strain 17, complete genome

Reference sequence length = 165101 bp
Coverage in bp = 28692
%Coverage = 17.378453
Average depth of coverage = .511044
Number of reads contributing to assembly = 640



Signed in as chiulab.shared@gmail.com

Select data: NT snap matched d1 fl Bacteria species clx

Color scale: ● $\log_2$ ○ actual

Selected sequences: 0

- 0 +

Coloring: ● ○ ○

Sort by: ● value ○ taxa **Value:** ○ read count ● normalized **Subtract taxa:** ☐ Host bacteria ☐ Host plants **Subtract declassified:** ☐

Threshold: 10

Accession: All

Prep: ☑ DNA ☐ RNA

Batch: ☑ A160801

Streptococcaceae	Streptococcus	Streptococcus agalactiae
Enterobacteriaceae	Klebsiella	Klebsiella pneumoniae
Enterobacteriaceae	Klebsiella	*
Streptococcaceae	Streptococcus	Streptococcus suis
Streptococcaceae	Streptococcus	*
Streptococcaceae	Streptococcus	Streptococcus pyogenes

RPM Ratio > 10 for bacterial detection

Positive control

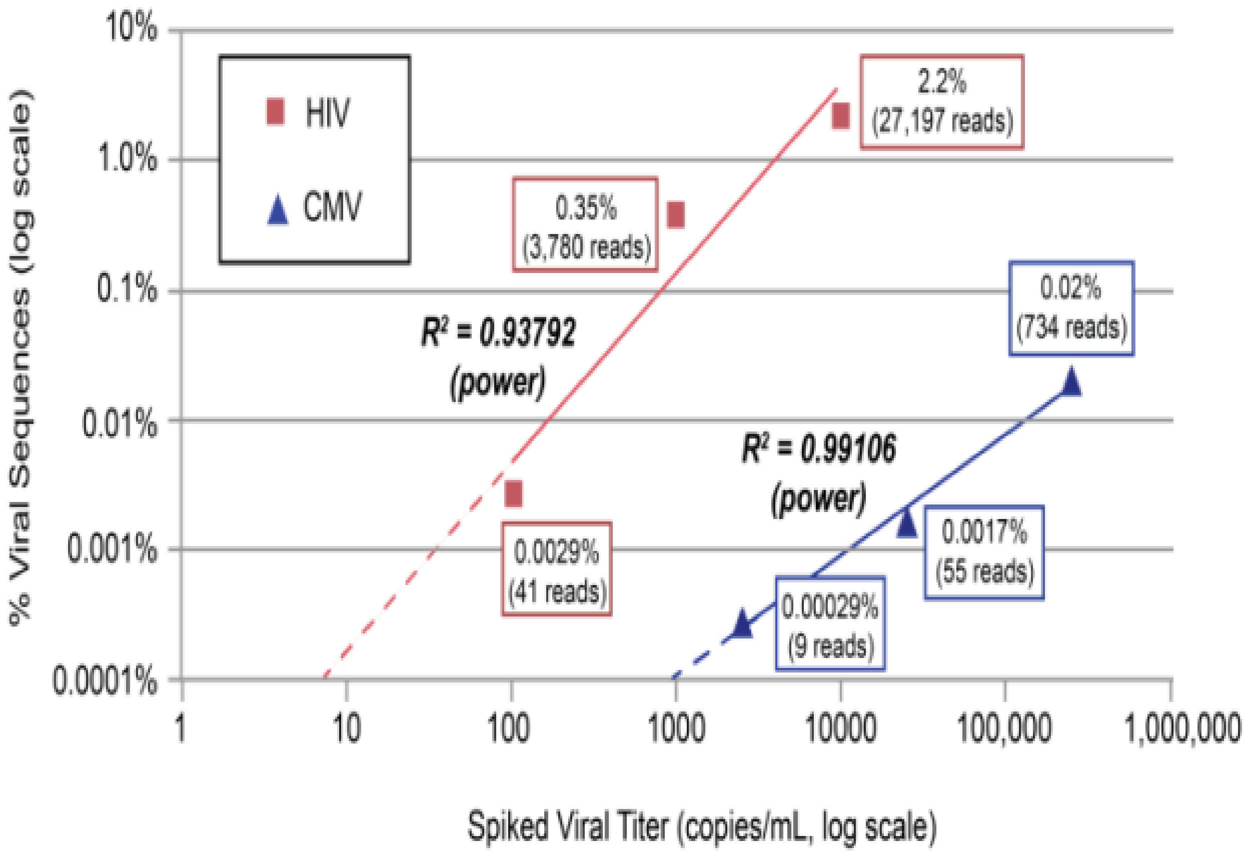
No Template control

Note: Misclassified species within same genus due to incomplete database

Validation of NGS

- Development of Controls
- Assay Performance Characteristics
- Contamination Control
- Clinical Study – Precision Diagnosis of Acute Infectious Disease
  - Interesting Case Reports & Discussion

Assay Sensitivity



RNA viruses detected to <100 copies/ml

DNA viruses detected to ~1000 copies/ml

Accuracy

Results-based accuracy (after discrepancy testing)

		ClinMicro Reference Lab			
		Pos	Neg	Untested	Total
mNGS	Pos	68	7	22	97
	Neg	11	327		338
	Total	79	334	22	

Sensitivity TPR	TP/(TP+FN)	86.1
Specificity TNR	TN/(TN+FP)	97.9

Contamination Control

- NGS assay extremely sensitive to contamination
  - Initial sample
  - Library preparation
  - Instrument carryover

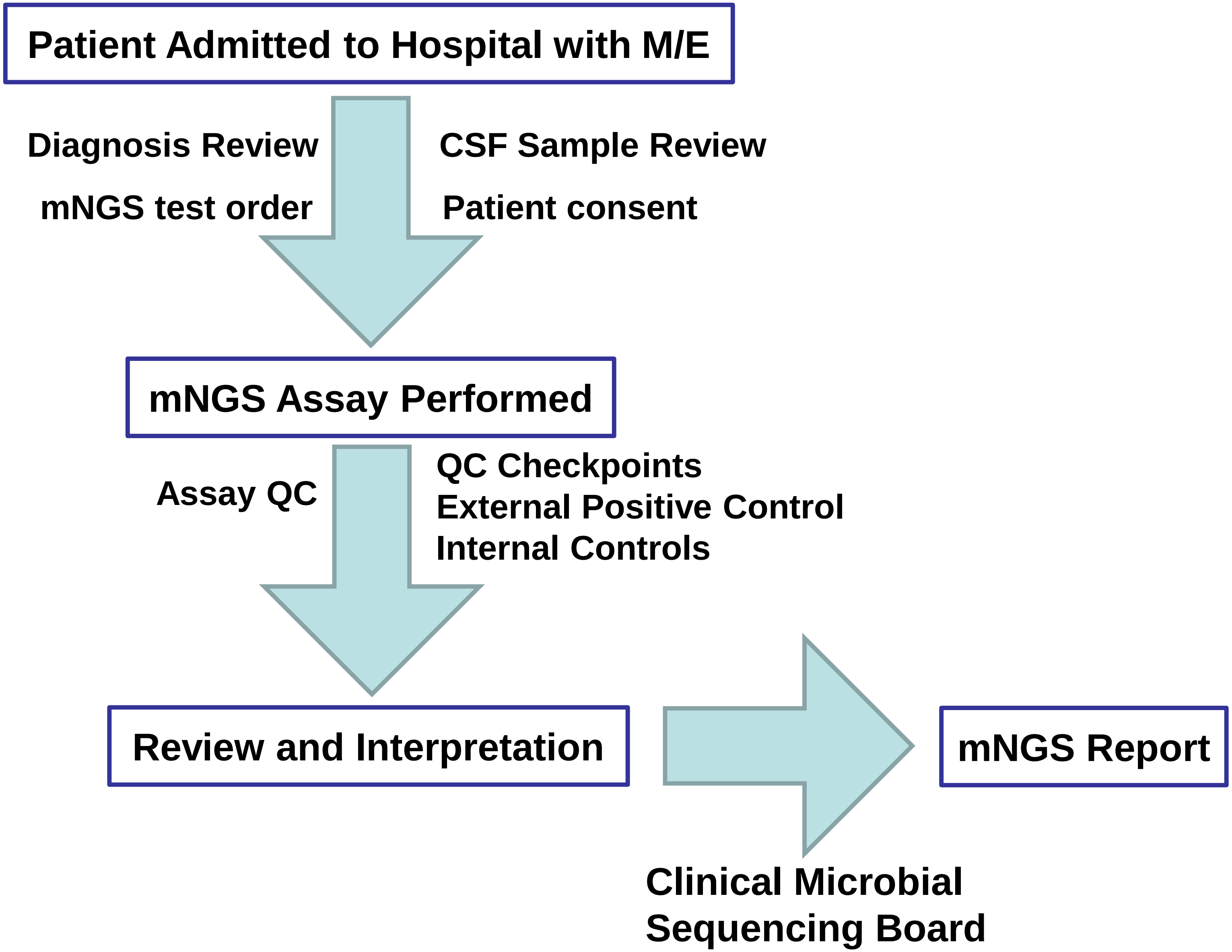
- Double barcoded adaptors

- Need caution interpreting significance of a few sequence reads when high positive sample is on same or recent run

- Bacterial background reads complicate detection of low levels of bacteria in clinical samples

- Use ratio of bacteria, fungal and parasite reads to NTC
  - Threshold of 10x reads in sample compared to NTC

Precision Diagnosis of Acute Infectious Disease



Patient Admitted to Hospital with M/E

Diagnosis Review

mNGS test order

CSF Sample Review

Patient consent

mNGS Assay Performed

Assay QC

QC Checkpoints
External Positive Control
Internal Controls

Review and Interpretation

mNGS Report

Clinical Microbial
Sequencing Board

mNGS Clinical Cases

An 11-year-old female with HA and Back pain

- From Mexico
- 1 month PTA, she was admitted to a local hospital in Mexico due to HA, nausea, abnormal jerking movements of fingers, hands, and legs.
- CSF showed 183 WBC (100% mono), glucose 25, protein 270
  EBV and HHV-7 detected in CSF
- She was treated with acyclovir, then ganciclovir.
- 2 weeks after discharge, she developed back pain and worsening HA.

An 11-year-old female with HA and Back pain

- Repeat admission for worsening symptoms
- Brain MRI w/ + w/o contrast: normal
- CSF showed WBC 137 (L91%), glu 25, prot 200
  Negative Gram-stain, bacterial culture
  Negative HSV PCR, EBV PCR, enterovirus PCR
- Multiple ID tests (blood and CSF) were sent
- INH, rifampin, PZA, ETB + pyridoxine initiated for empiric coverage of tuberculosis

An 11-year-old female with HA and Back pain

MRI spine
- Leptomeningeal enhancement along the conus medullaris involving the cauda equina without underlying signal abnormality of the spinal cord.
-”...these findings represent an infectious or inflammatory process...”

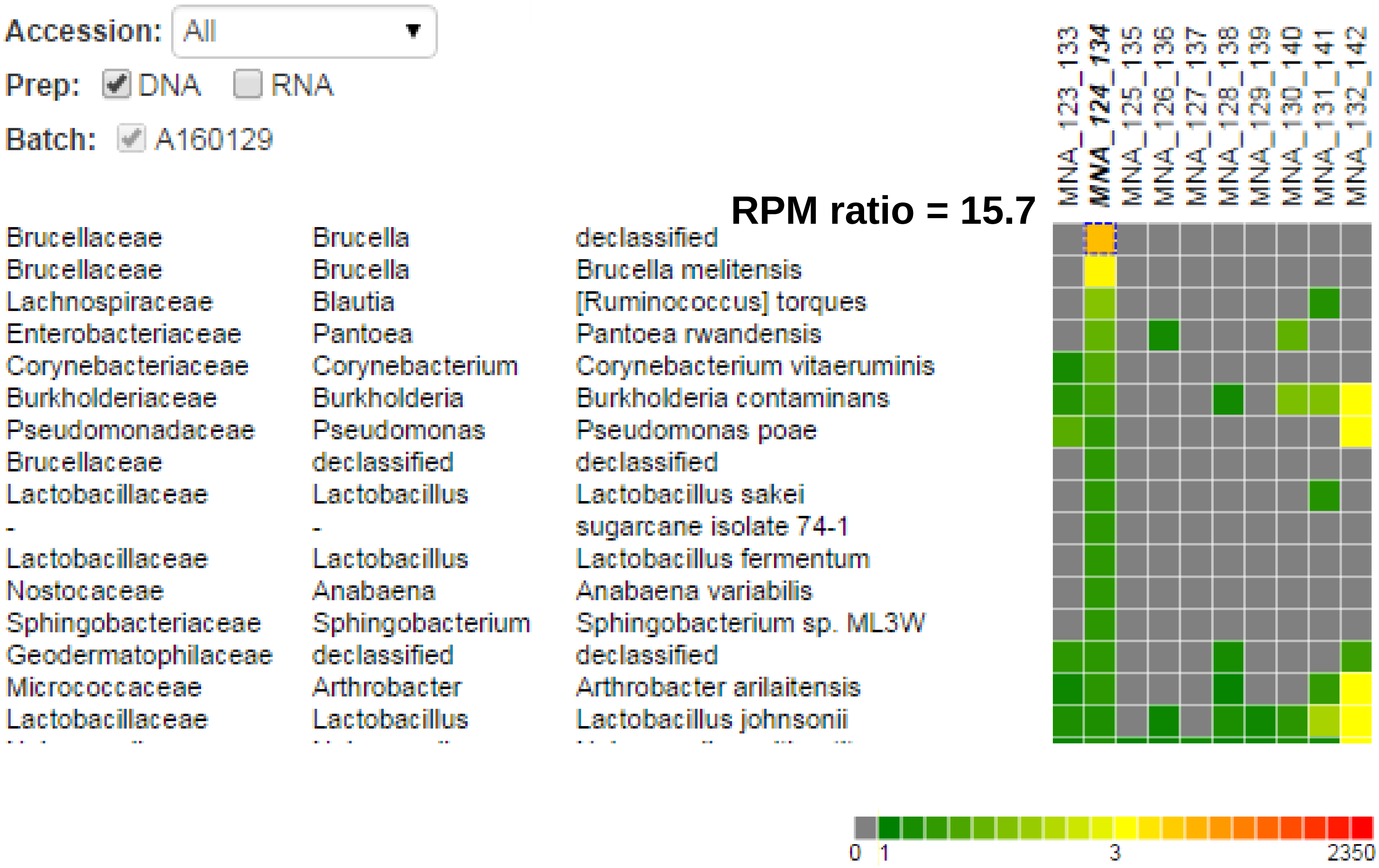
An 11-year-old female with HA and Back pain

- Clonazepam added for polyminimyoclonus
- Negative CSF *M. tb* PCR
- No improvement after 8 days of therapy – repeat LP
- CSF (#2): WBC 176 (N53%, L38%, M9%),
  glucose 25, protein 131.
  CSF#2 was positive for EBV PCR (Ct=36.44) and HHV-7 PCR
  - EBV serology: c/w remote infection
  - Blood for EBV PCR was not detected
- Concern for drug-resistant TB:
  - ETB was changed to ethionamide
  - Levofloxacin added to RIPE

An 11-year-old female with HA and Back pain

- TB PCR CSF #2 negative
- she improved substantially and was discharged home with <u>5 anti-TB medications</u>
- At 1 week and 1 month follow-up after discharge, she had near resolution of symptoms, but still reported several episodes of shaking of her extremities
- Because of clinical improvement, she returned to Mexico and continued her anti-TB therapy.
- CSF #2 submitted for mNGS analysis

Reads per Million Normalized to NTC



Accession: All ▼

Prep: ☑ DNA ☐ RNA

Batch: ☑ A160129

RPM ratio = 15.7

Brucellaceae	Brucella	declassified
Brucellaceae	Brucella	Brucella melitensis
Lachnospiraceae	Blautia	[Ruminococcus] torques
Enterobacteriaceae	Pantoea	Pantoea rwandensis
Corynebacteriaceae	Corynebacterium	Corynebacterium vitaeruminis
Burkholderiaceae	Burkholderia	Burkholderia contaminans
Pseudomonadaceae	Pseudomonas	Pseudomonas poae
Brucellaceae	declassified	declassified
Lactobacillaceae	Lactobacillus	Lactobacillus sakei
-	-	sugarcane isolate 74-1
Lactobacillaceae	Lactobacillus	Lactobacillus fermentum
Nostocaceae	Anabaena	Anabaena variabilis
Sphingobacteriaceae	Sphingobacterium	Sphingobacterium sp. ML3W
Geodermatophilaceae	declassified	declassified
Micrococcaceae	Arthrobacter	Arthrobacter arilaitensis
Lactobacillaceae	Lactobacillus	Lactobacillus johnsonii

https://surpiviz.ucsf.edu/heatmap/surpi_clin_HVald004_EP_ES_160201/

Followup

- Patient started on targeted *Brucella* therapy
  - Doxycycline + Rifampin
- Symptoms fully resolved in 2 weeks
- Serum antibody agglutination positive 1:80 titer
- PCR performed confirming mNGS findings of *Brucella* DNA

58 y/o woman with fever, headache, and nausea/vomiting

- History of idiopathic pulmonary fibrosis s/p bilateral lung transplant, migraines, hypercoagulability with DVT, and multiple sclerosis (MS) on chronic immunosuppression
- Admitted 8 days of fever, headache, refractory nausea/vomiting, neck stiffness, and photophobia
- Had been hospitalized prior complaining of “worst headache of her life” – treated with abortive migraine medications with only partial relief

58 y/o woman with fever, headache, and nausea/vomiting

- Resident of southern California
- Denied sick contacts, pets, insect bites, or eating shellfish or game meats
- Reported travel to mountains in Utah in Aug 2016, the Caribbean in 2010, and throughout Europe decades prior to admission
- Outpatient medications notable for immunosuppressive agents (tacrolimus, mycophenolate mofetil, prednisone, teriflunomide) and antimicrobial prophylaxis with trimethoprim/sulfamethoxazole and acyclovir; also on an intrathecal morphine pump

58 y/o woman with fever, headache, and nausea/vomiting

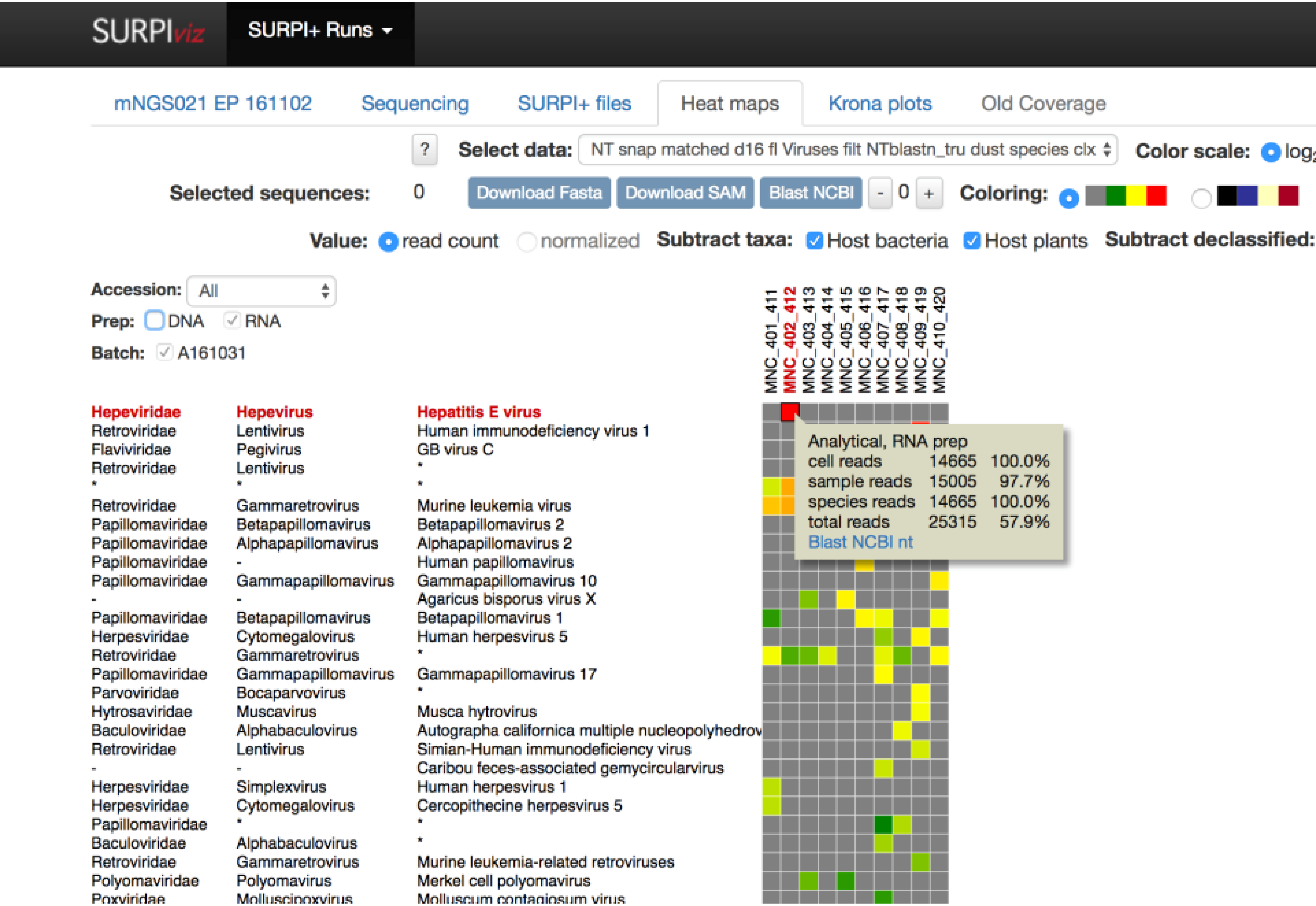
- On admission
  - Fever to 38.3° C, otherwise vital signs normal
  - Physical exam remarkable only for R leg tenderness attributed to known DVT
  - Initial laboratories remarkable for stable pancytopenia (WBC 9, HgB 10.9, platelets 124,000), mildly elevated LFTs (AST 130 U/L, ALT 83 U/L, total bili 1.1 mg/dL, alkaline phosphatase 144 U/L) and borderline elevated prothrombin time of 14.3 (11-14 seconds)
  - MRI – baseline periventricular and subcortical T2/FLAIR white matter intensities associated with her MS, no acute changes
  - Started on empiric vancomycin, ceftazidime, acyclovir, voriconazole
- Lumbar puncture performed on day 3
  - CSF pleocytosis (10 WBC/mm63; 88% lymphocytes; 12% monocytes; protein 29; glucose 48)

Microbiological Studies Negative for Etiology

Hospitalization at UCLA (Oct 2016)	Hospitalization at UCLA (Nov 2016)
Blood CMV DNA quantitative PCR Cryptococcal Antigen EBV PCR detected <10 HSV-1 and 2 PCR Fungal Culture Bacterial Culture Toxoplasma gondii DNA PCR MTB-Quantiferon-Gold Adenovirus DNA PCR Parvovirus B19 DNA PCR West Nile IgG and IgM Rickettsia RMSF and typhus IgG and IgM Varicella zoster DNA PCR Coccidioides IgG and IgM EIA Hepatitis A Ab total Hepatitis A Ab IgM CSF Cryptococcal Antigen Enterovirus PCR Fungal Culture Bacterial gram stain and culture Varicella Zoster PCR HSV 1 and 2 PCR CMV DNA quantitative PCR	Blood Hepatitis A IgM Hepatitis B core IgM Hepatitis B surface antigen Hepatitis C Antibody HBV DNA PCR Cryptococcal antigen HIV 4th generation Ag/Ab JCV Ab 2.31 (>0.40 positive) RPR Aspergillus antigen EIA Coccidioides IgG/IgM EIA Toxoplasma gondii DNA PCR Peritoneal fluid Bacterial gram stain and Culture Nasal swab Respiratory Virus Panel PCR

58 y/o woman with fever, headache, and nausea/vomiting

- By day 6, patient clinically improved with resolution of all symptoms and fever, except for mild persistent headache
- Discharge diagnosis was meningoencephalitis due to viral infection or tacrolimus toxicity
- mNGS performed on CSF sample obtained on day 3



Select data: NT snap matched d16 fl Viruses filt NTblastn_tru dust species clx

Color scale: ● log

Selected sequences: 0

0

Coloring: ● ○

Value: ● read count ○ normalized **Subtract taxa:** ☑ Host bacteria ☑ Host plants **Subtract declassified:**

Accession: All

Prep: ☐ DNA ☑ RNA

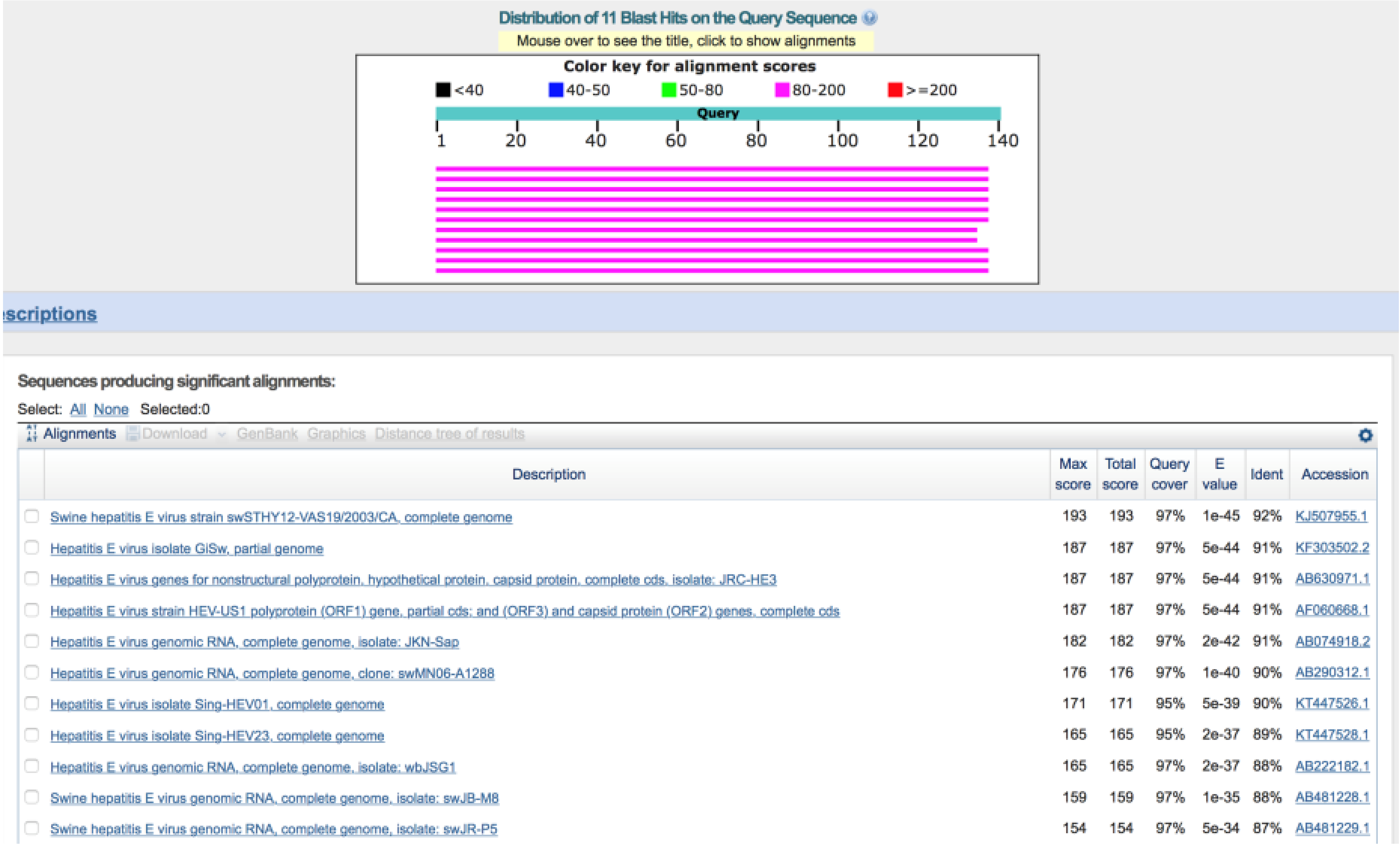
Batch: ☑ A161031

Hepeviridae	**Hepevirus**	**Hepatitis E virus**
Retroviridae	Lentivirus	Human immunodeficiency virus 1
Flaviviridae	Pegivirus	GB virus C
Retroviridae	Lentivirus	*
*	*	*
Retroviridae	Gammaretrovirus	Murine leukemia virus
Papillomaviridae	Betapapillomavirus	Betapapillomavirus 2
Papillomaviridae	Alphapapillomavirus	Alphapapillomavirus 2
Papillomaviridae	-	Human papillomavirus
Papillomaviridae	Gammapapillomavirus	Gammapapillomavirus 10
-	-	Agaricus bisporus virus X
Papillomaviridae	Betapapillomavirus	Betapapillomavirus 1
Herpesviridae	Cytomegalovirus	Human herpesvirus 5
Retroviridae	Gammaretrovirus	*
Papillomaviridae	Gammapapillomavirus	Gammapapillomavirus 17
Parvoviridae	Bocaparvovirus	*
Hytrosaviridae	Muscavirus	Musca hytrovirus
Baculoviridae	Alphabaculovirus	Autographa californica multiple nucleopolyhedrov
Retroviridae	Lentivirus	Simian-Human immunodeficiency virus
-	-	Caribou feces-associated gemycircularvirus
Herpesviridae	Simplexvirus	Human herpesvirus 1
Herpesviridae	Cytomegalovirus	Cercopithecine herpesvirus 5
Papillomaviridae	*	*
Baculoviridae	Alphabaculovirus	*
Retroviridae	Gammaretrovirus	Murine leukemia-related retroviruses
Polyomaviridae	Polyomavirus	Merkel cell polyomavirus
Poxviridae	Molluscipoxvirus	Molluscum contagiosum virus

Analytical, RNA prep

cell reads	14665	100.0%
sample reads	15005	97.7%
species reads	14665	100.0%
total reads	25315	57.9%

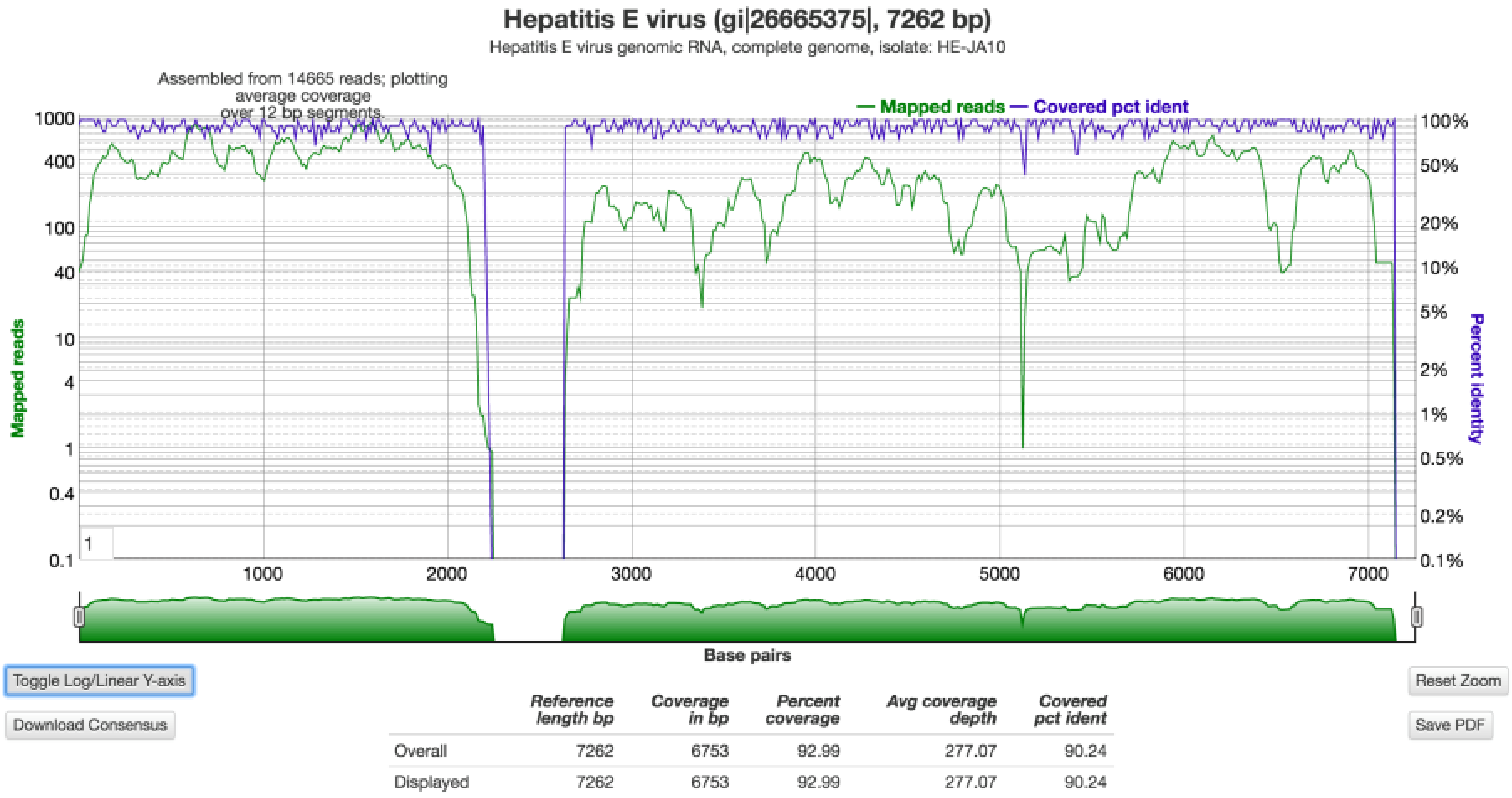
Blast NCBI nt



Sequences producing significant alignments:

Select: All None Selected:0

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Swine hepatitis E virus strain swSTHY12-VAS19/2003/CA, complete genome	193	193	97%	1e-45	92%	KJ507955.1
☐	Hepatitis E virus isolate GiSw, partial genome	187	187	97%	5e-44	91%	KF303502.2
☐	Hepatitis E virus genes for nonstructural polyprotein, hypothetical protein, capsid protein, complete cds, isolate: JRC-HE3	187	187	97%	5e-44	91%	AB630971.1
☐	Hepatitis E virus strain HEV-US1 polyprotein (ORF1) gene, partial cds; and (ORF3) and capsid protein (ORF2) genes, complete cds	187	187	97%	5e-44	91%	AF060668.1
☐	Hepatitis E virus genomic RNA, complete genome, isolate: JKN-Sap	182	182	97%	2e-42	91%	AB074918.2
☐	Hepatitis E virus genomic RNA, complete genome, clone: swMN06-A1288	176	176	97%	1e-40	90%	AB290312.1
☐	Hepatitis E virus isolate Sing-HEV01, complete genome	171	171	95%	5e-39	90%	KT447526.1
☐	Hepatitis E virus isolate Sing-HEV23, complete genome	165	165	95%	2e-37	89%	KT447528.1
☐	Hepatitis E virus genomic RNA, complete genome, isolate: wbJSG1	165	165	97%	2e-37	88%	AB222182.1
☐	Swine hepatitis E virus genomic RNA, complete genome, isolate: swJB-M8	159	159	97%	1e-35	88%	AB481228.1
☐	Swine hepatitis E virus genomic RNA, complete genome, isolate: swJR-P5	154	154	97%	5e-34	87%	AB481229.1



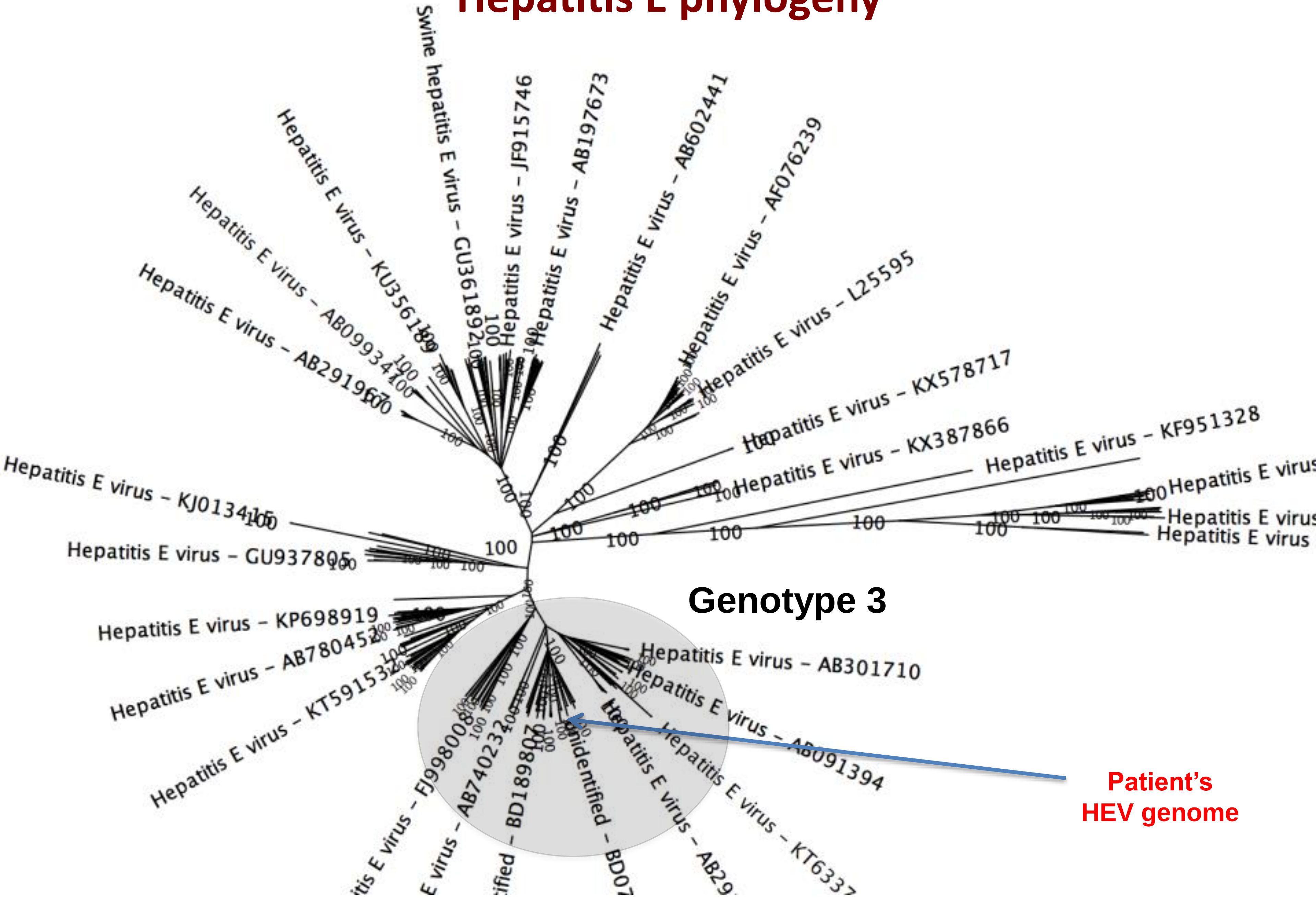
Hepatitis E virus (gi|26665375|, 7262 bp)

Hepatitis E virus genomic RNA, complete genome, isolate: HE-JA10

Assembled from 14665 reads; plotting average coverage over 12 bp segments.

	Reference length bp	*Coverage in bp*	*Percent coverage*	*Avg coverage depth*	*Covered pct ident*
Overall	7262	6753	92.99	277.07	90.24
Displayed	7262	6753	92.99	277.07	90.24

Hepatitis E phylogeny



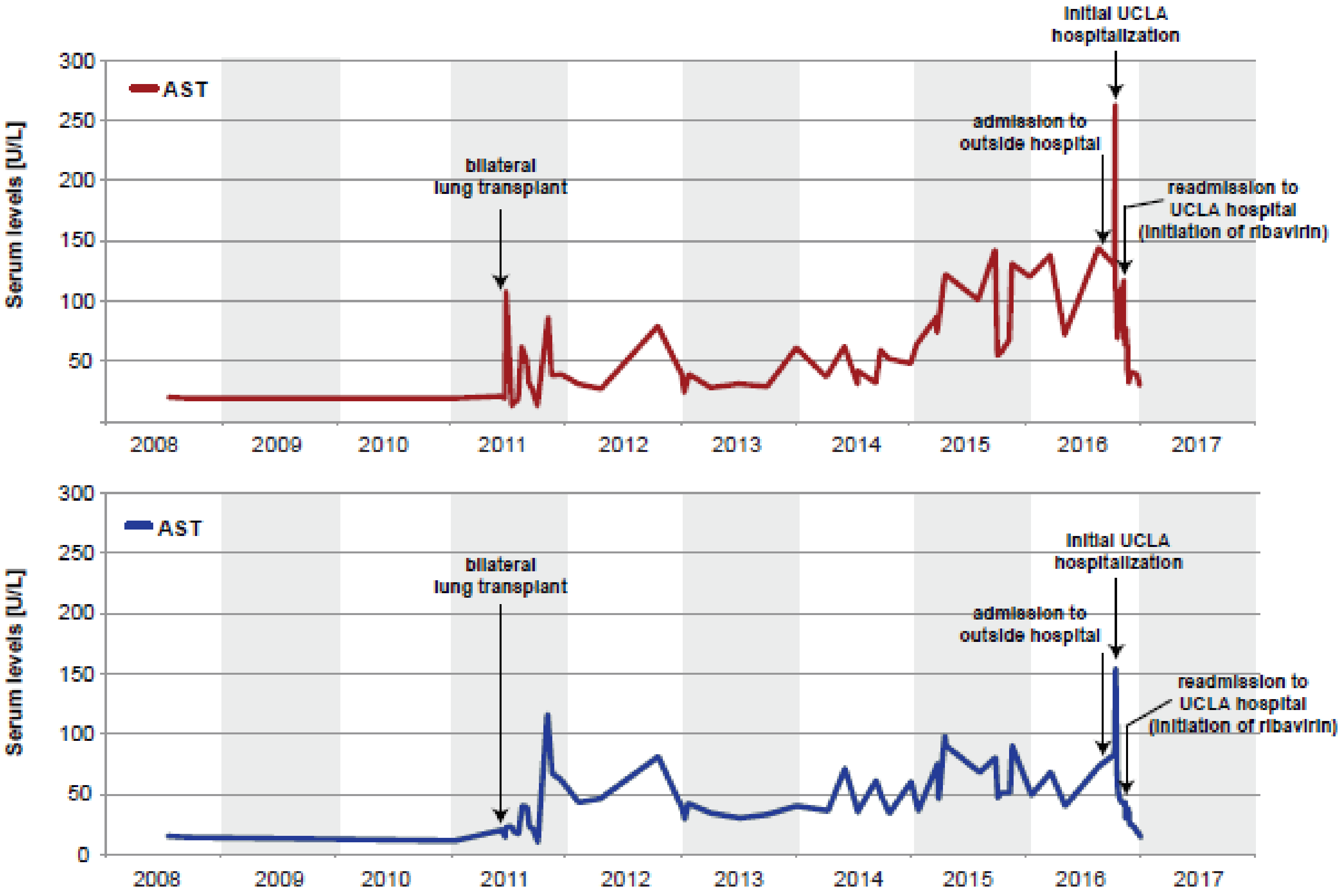
Genotype 3

Patient's HEV genome

58 y/o woman with fever, headache, and nausea/vomiting

- Diagnosis of HEV-associated meningoencephalitis communicated to patient
- Retrospective review of EMR showed normal transaminase levels prior to lung transplantation, with persistent low-level transaminase elevations following transplantation in 2011
- Immediate follow up HEV testing showed serum IgM positivity, negative IgG, and plasma HEV viremia (5,960,000 IU/mL)
- Ribavirin monotherapy was started for this patient
- Case reported to United Network for Organ Sharing donor safety net
- HEV IgG/IgM donor serum was positive, but HEV RNA not detected
  - Potential donor-derived infection

Patient LFTs



Hepatitis E – Neurologic Manifestations

- Commonest cause of Acute hepatitis worldwide
- Neurological disorders associated with HEV
    - First case- 2000: GBS
    - 2011: 5.5% of well characterized patients with acute and chronic Hepatitis E cases from Europe
    - 100 cases reported to date

Box 1

Neurological manifestations of hepatitis E

Conditions in which there is probably a causal relationship (*n* = cases reported)

- Guillain–Barré syndrome (*n* = 36)
- Brachial neuritis (*n* = 30)
- Meningoencephalitis (*n* = 12)

Conditions in which causality remains to be established (*n* = cases reported)

- Mononeuritis multiplex (*n* = –6)
- Bell’s palsy (*n* = 3)
- Myositis (*n* = 2)
- Vestibular neuritis (*n* = 1)
- Peripheral neuropathy (*n* = 1)

Case Discussion

- First report of potential HEV transmission via lung transplantation
- Evidence in support: positive anti-HEV IgG/IgM testing of donor serum
- Persistent low-level transaminase elevations post-transplant
- Prior HEV donor transmission reports include liver transplant recipient and 2 renal transplant recipients (from same donor)
  - (Sclosser, et al., J hepatol 2012; Pourbaix, et al., Transplant Infect Dis 2016)

68 year old male with fever and AMS

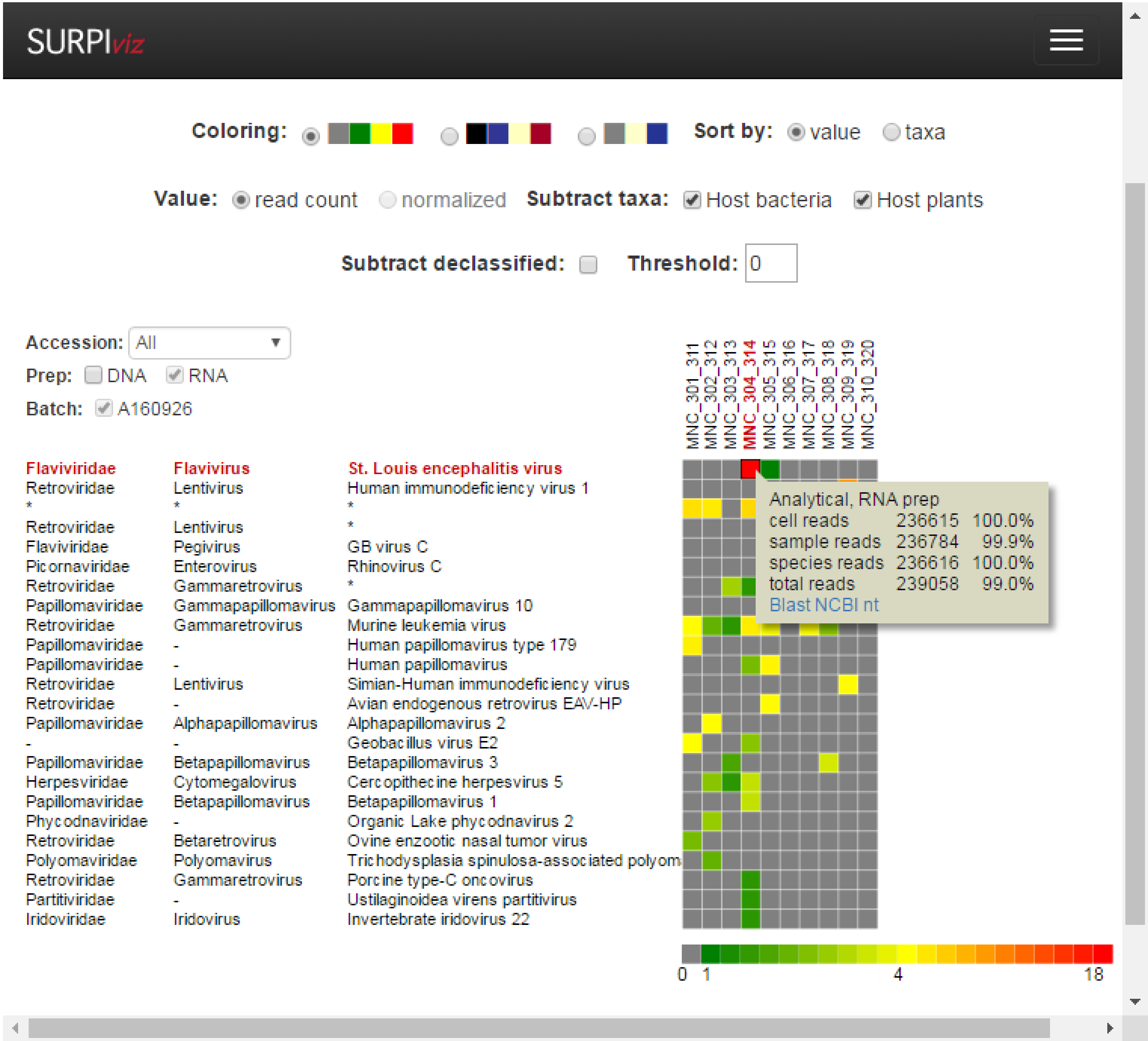
- History of CAD, HTN, mantle cell lymphoma
- Developed fever, chills lethargy, fatigue, confusion
- Admitted and started on empirical vancomycin, meropenem and levofloxacin
- 3 days later became hypoxic with worsening AMS
  - LP showed 18 WBC (35% monocytes, 33% lymphocytes, 32% neutrophils), normal glucose and protein
- Added acylovir for viral meningitis

68 year old male with fever and AMS

- Patient was retired oil field worker living in Kern County, CA
- Dog owner
- Recent travel to mountains of Arizona

68 year old male with fever and AMS

- Patient continued to spike fevers
  - Antifungal therapy added
- Repeat LP showed persistent pleocytosis
  - Extensive infectious workup non-revealing
- CSF analyzed by mNGS



SURPI*viz*

Coloring: ● ○ ○ **Sort by:** ● value ○ taxa

Value: ● read count ○ normalized **Subtract taxa:** ☑ Host bacteria ☑ Host plants

Subtract declassified: ☐ **Threshold:** 0

Accession: All ▾

Prep: ☐ DNA ☑ RNA

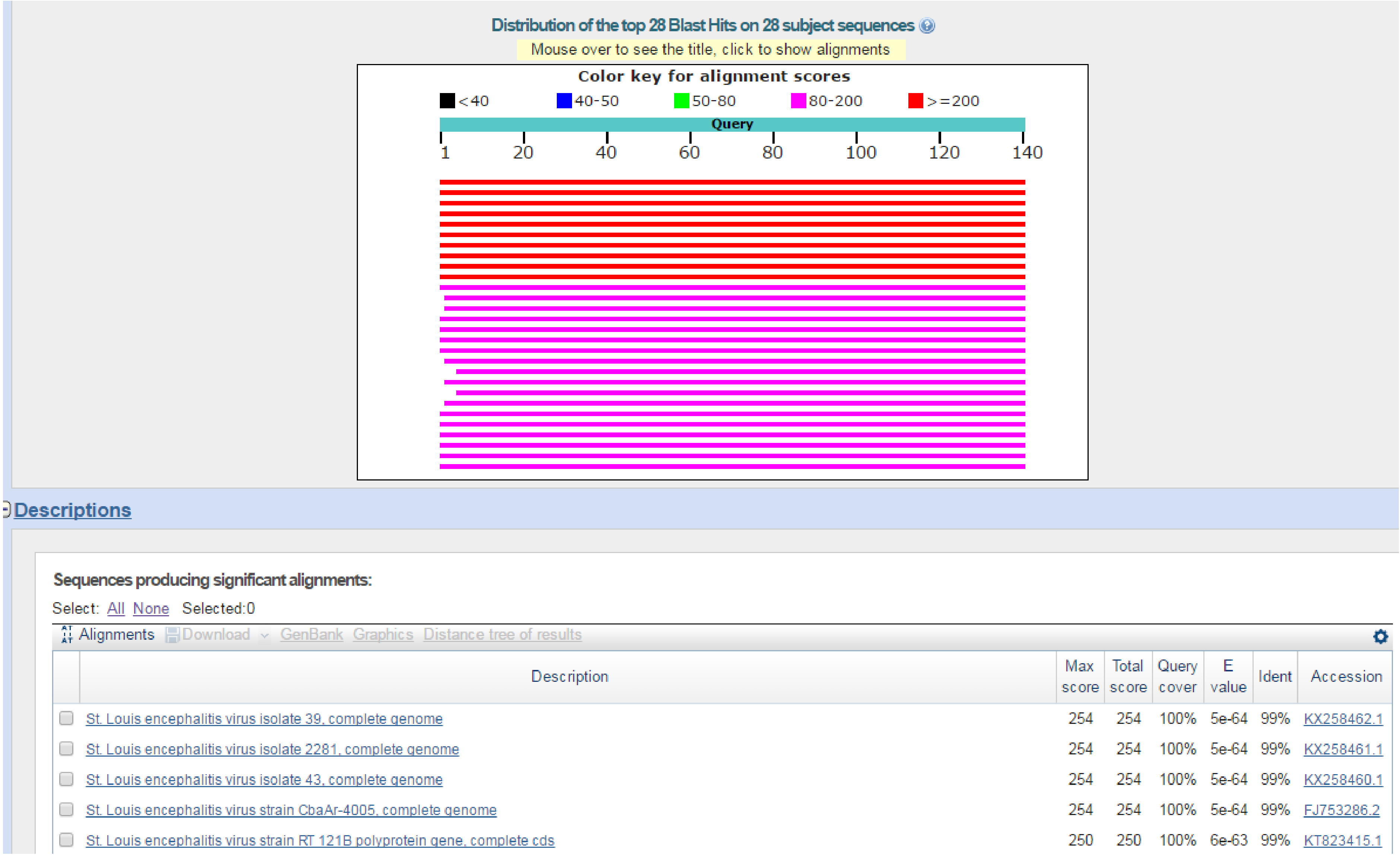
Batch: ☑ A160926

Flaviviridae	**Flavivirus**	**St. Louis encephalitis virus**
Retroviridae	Lentivirus	Human immunodeficiency virus 1
*	*	*
Retroviridae	Lentivirus	*
Flaviviridae	Pegivirus	GB virus C
Picornaviridae	Enterovirus	Rhinovirus C
Retroviridae	Gammaretrovirus	*
Papillomaviridae	Gammapapillomavirus	Gammapapillomavirus 10
Retroviridae	Gammaretrovirus	Murine leukemia virus
Papillomaviridae	-	Human papillomavirus type 179
Papillomaviridae	-	Human papillomavirus
Retroviridae	Lentivirus	Simian-Human immunodeficiency virus
Retroviridae	-	Avian endogenous retrovirus EAV-HP
Papillomaviridae	Alphapapillomavirus	Alphapapillomavirus 2
-	-	Geobacillus virus E2
Papillomaviridae	Betapapillomavirus	Betapapillomavirus 3
Herpesviridae	Cytomegalovirus	Cercopithecine herpesvirus 5
Papillomaviridae	Betapapillomavirus	Betapapillomavirus 1
Phycodnaviridae	-	Organic Lake phycodnavirus 2
Retroviridae	Betaretrovirus	Ovine enzootic nasal tumor virus
Polyomaviridae	Polyomavirus	Trichodysplasia spinulosa-associated polyom
Retroviridae	Gammaretrovirus	Porcine type-C oncovirus
Partitiviridae	-	Ustilaginoidea virens partitivirus
Iridoviridae	Iridovirus	Invertebrate iridovirus 22

Analytical, RNA prep

cell reads	236615	100.0%
sample reads	236784	99.9%
species reads	236616	100.0%
total reads	239058	99.0%

Blast NCBI nt



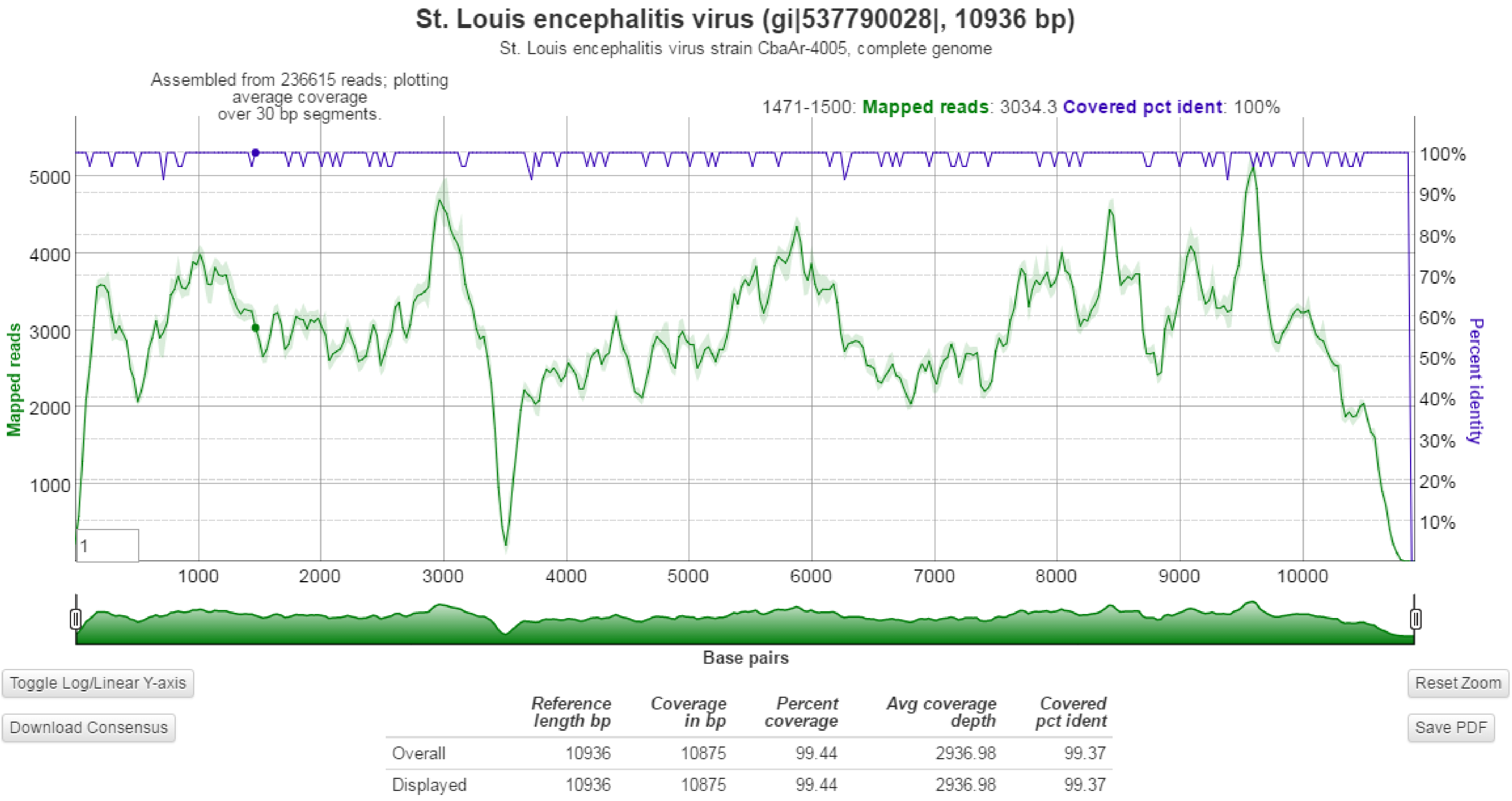
Descriptions

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	St. Louis encephalitis virus isolate 39, complete genome	254	254	100%	5e-64	99%	KX258462.1
☐	St. Louis encephalitis virus isolate 2281, complete genome	254	254	100%	5e-64	99%	KX258461.1
☐	St. Louis encephalitis virus isolate 43, complete genome	254	254	100%	5e-64	99%	KX258460.1
☐	St. Louis encephalitis virus strain CbaAr-4005, complete genome	254	254	100%	5e-64	99%	FJ753286.2
☐	St. Louis encephalitis virus strain RT 121B polyprotein gene, complete cds	250	250	100%	6e-63	99%	KT823415.1



St. Louis encephalitis virus (gi|537790028|, 10936 bp)

St. Louis encephalitis virus strain CbaAr-4005, complete genome

Assembled from 236615 reads; plotting average coverage over 30 bp segments.

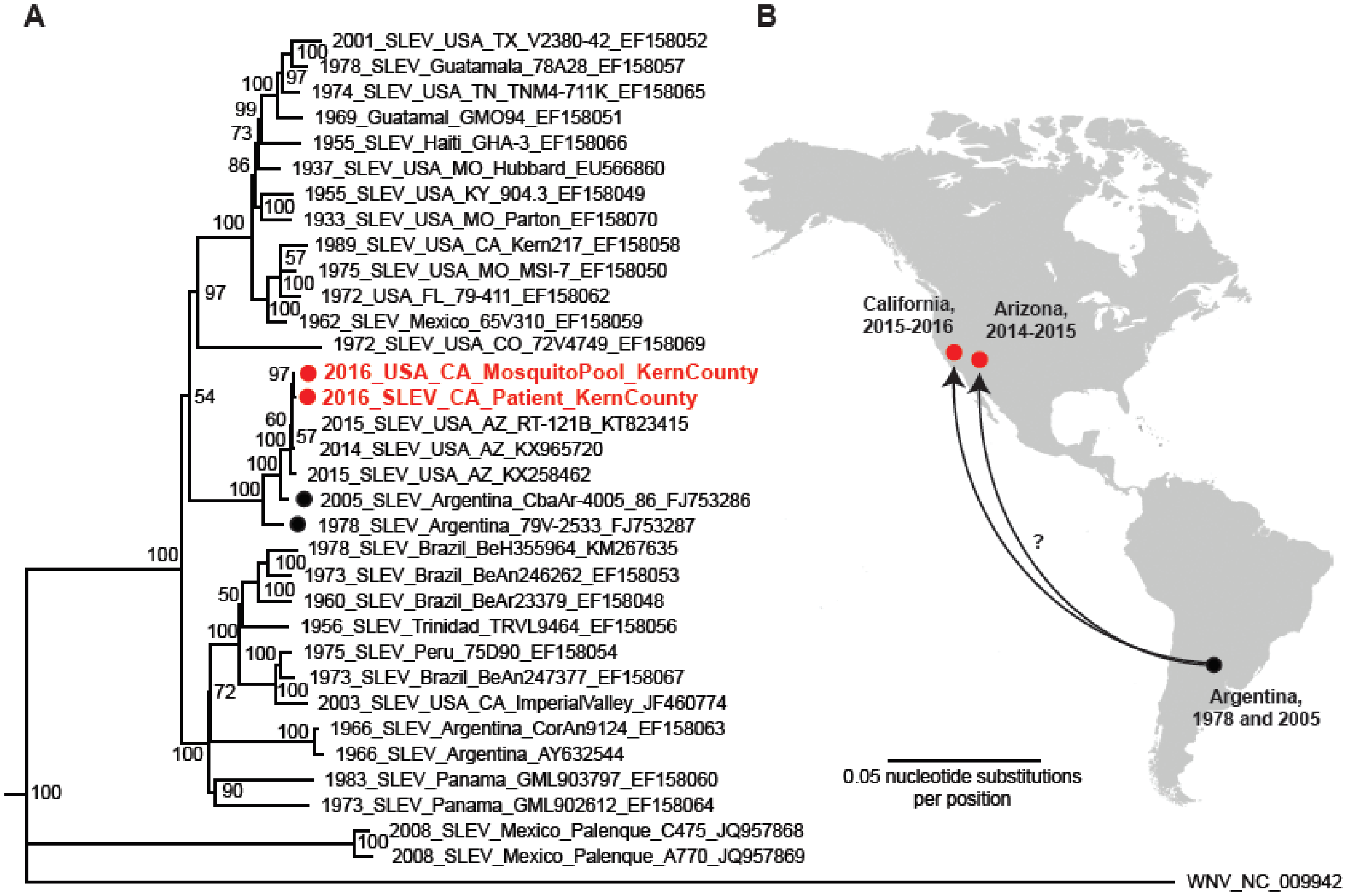
1471-1500: **Mapped reads**: 3034.3 **Covered pct ident**: 100%

	Reference length bp	*Coverage in bp*	*Percent coverage*	*Avg coverage depth*	*Covered pct ident*
Overall	10936	10875	99.44	2936.98	99.37
Displayed	10936	10875	99.44	2936.98	99.37

St. Louis Encephalitis Virus

- Normally self-limiting
- Can be prolonged / severe in immunocompromised patient
- Patient continued to deteriorate with supportive care
  - Placed on comfort care and passed away
- Autopsy revealed mantle cell lymphoma and aspiration pneumonia

SLEV Reintroduced into North America from Argentina



Case Discussion

- SLEV re-introduced into Arizona in 2014 from Argentina
- Sequence analysis shows most similar to mosquito pool from Kern County, CA
    - Patient likely acquired locally rather than from trip to Arizona
- Patient immunocompromised due to underlying disease leading to fatal infection

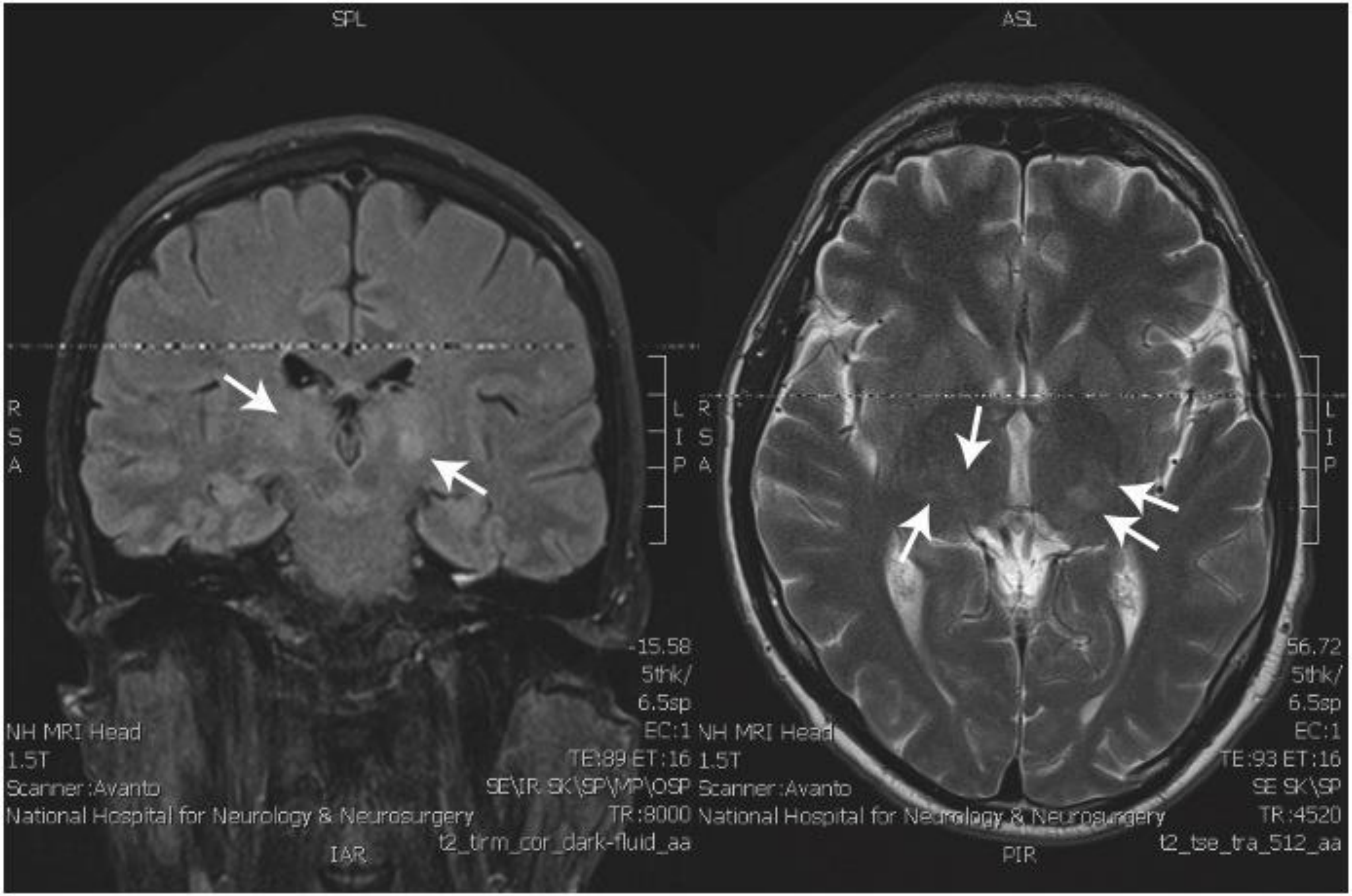
55 y/o male with deafness and behavioral change

- **55 y/o male with bone marrow transplant for CLL. Prior immunosuppressive therapy. In mid-October, developed rapidly progressive hearing loss over 2-3 weeks.**

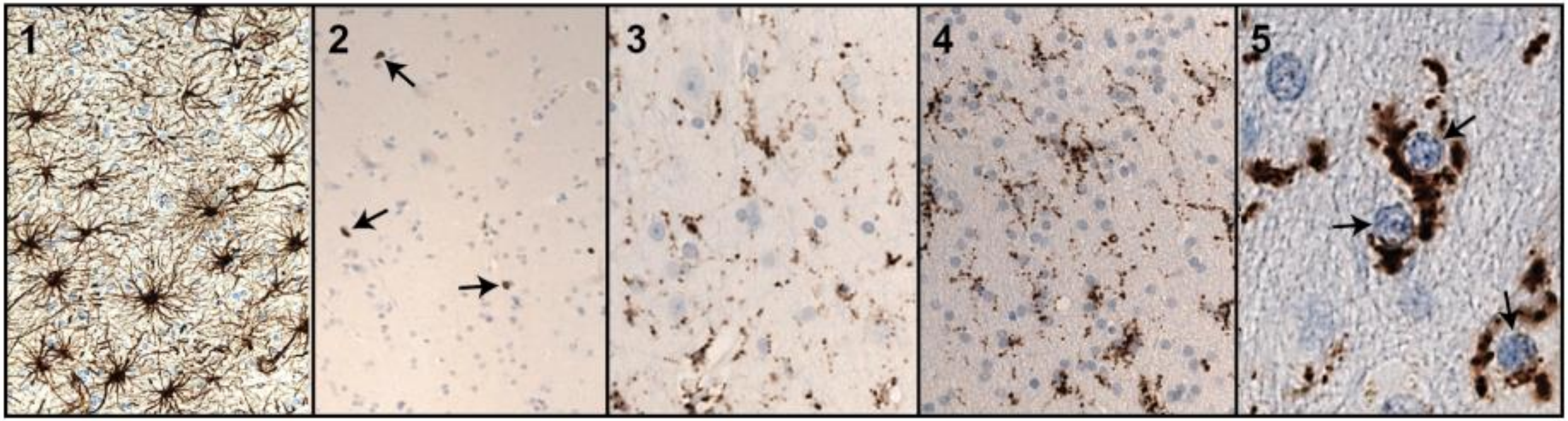
- **CSF unremarkable; MRI negative; PCR for HSV and enterovirus negative; treated empirically with high-dose valacyclovir, antibiotics, IVIg and steroids daily to no effect**

- **Over next few weeks, developed nausea, fatigue, ataxia, persistent hearing loss, then depressed, irritable mood (unusual for patient per his wife).**

- **Repeat MRI late December → abnormal signal in thalamus and midbrain bilaterally; frontal lobe biopsy performed (only accessible region)**


SPL
R
S
A
L
I
P
-15.58
5thk/
6.5sp
EC:1
NH MRI Head
1.5T
Scanner:Avanto
National Hospital for Neurology & Neurosurgery
TE:89 ET:16
SE\IR SK\SP\MP\OSP
TR:8000
t2_tirm_cor_dark-fluid_aa
IAR
ASL
R
S
A
L
I
P
56.72
5thk/
6.5sp
EC:1
NH MRI Head
1.5T
Scanner:Avanto
National Hospital for Neurology & Neurosurgery
TE:93 ET:16
SE SK\SP
TR:4520
t2_tse_tra_512_aa
PIR

Right frontal lobe **Cortex** **White matter**



GFAP stain

Diffuse reactive gliosis

CD3 stain

T lymphocytes

CD8 stain

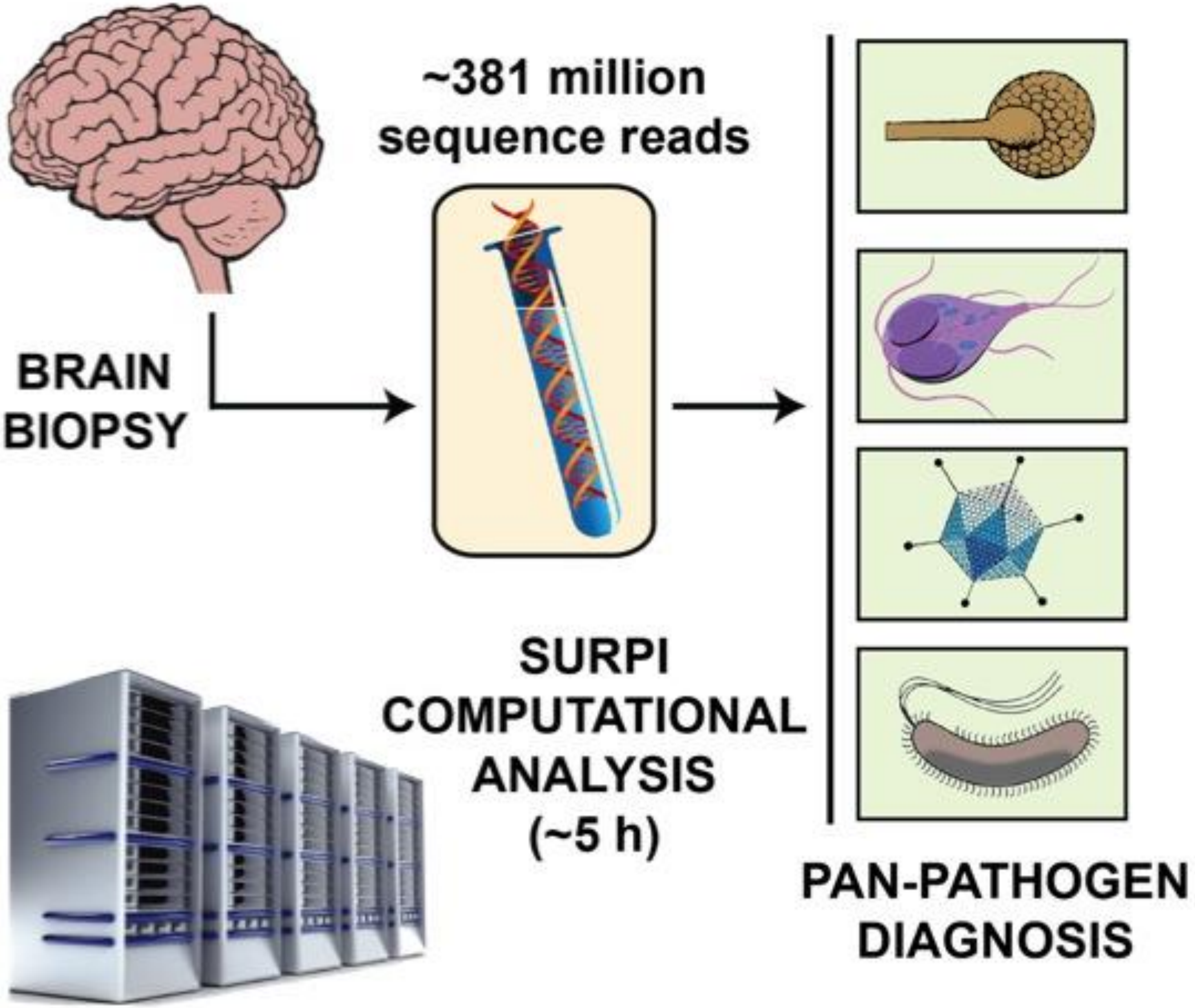
Microglial activation

CD8 stain

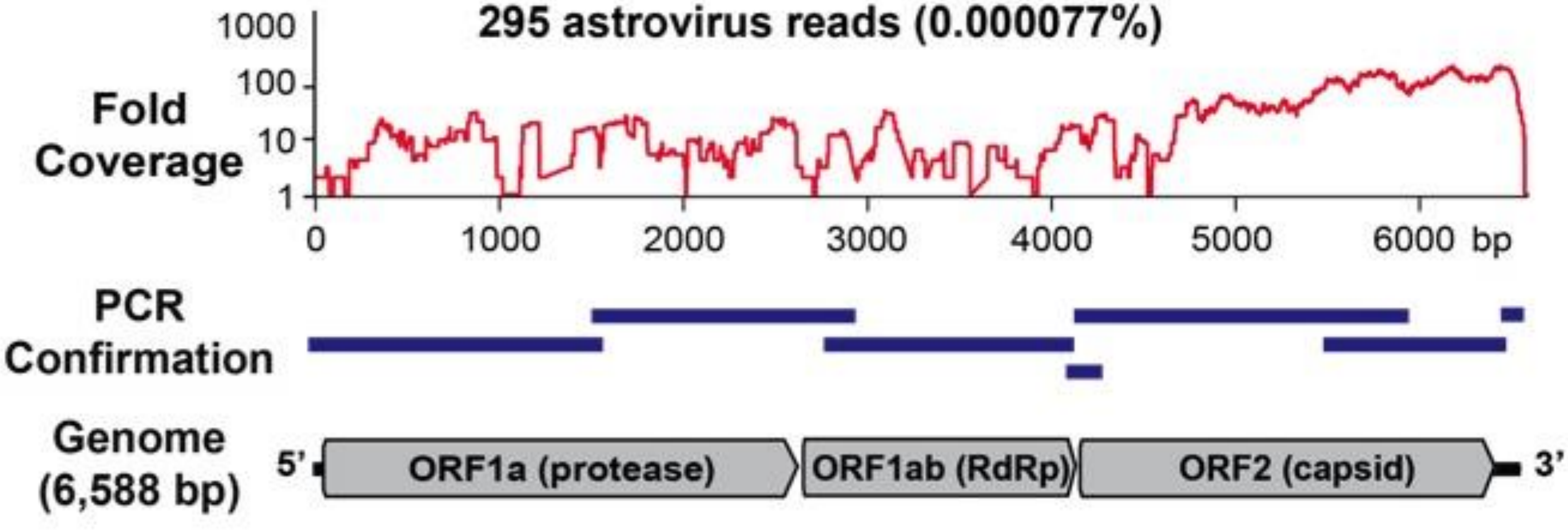
Microglial activation

CD8 stain

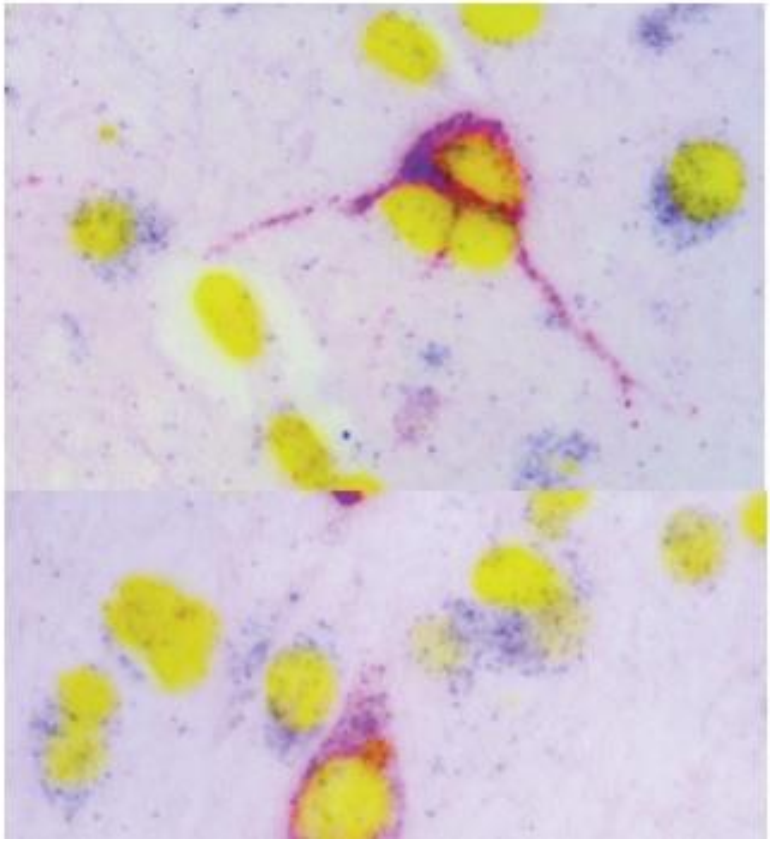
Microglial cell processes in proximity to neurons


~381 million
sequence reads
BRAIN
BIOPSY
SURPI
COMPUTATIONAL
ANALYSIS
(~5 h)
PAN-PATHOGEN
DIAGNOSIS

Genome Assembly of a Novel Astrovirus



DNA *In situ* Hybridization of Brain Tissue For Astrovirus



Neuronal cytoplasmic staining

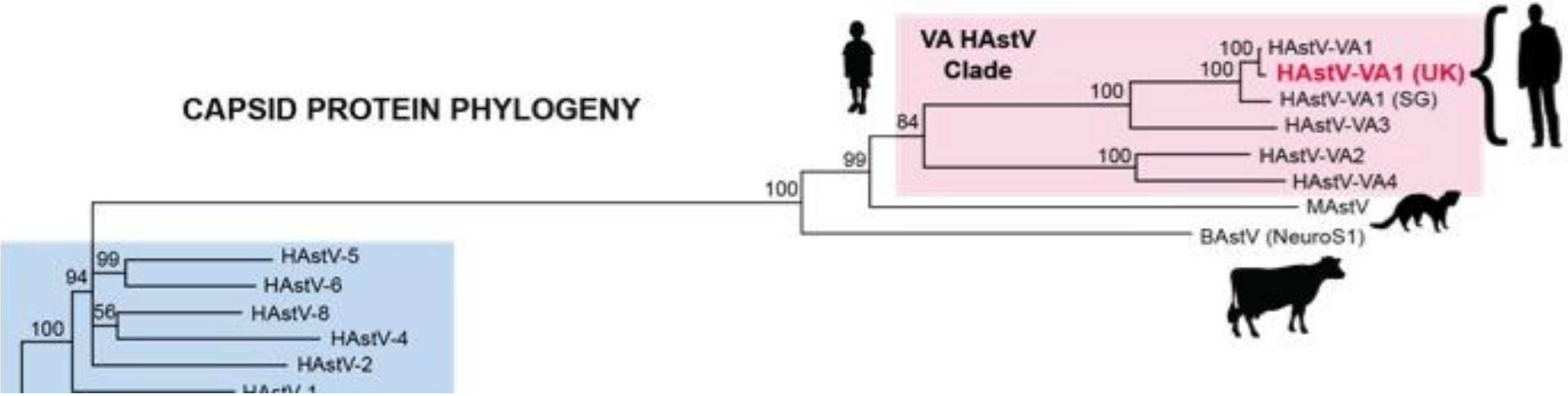
“Encephalitic” Astrovirus Clade in Humans

Volume 16, Number 6—June 2010

Research

Astrovirus Encephalitis in Boy with X-linked Agammaglobulinemia

Phenix-Lan Quan, Thor A. Wagner, Thomas Briese, Troy R. Torgerson, Mady Hornig, Alla Tashmukhamedova, Cadhla Firth, Gustavo Palacios, Ada Baisre-De-Leon, Christopher D. Paddock, Stephen K. Hutchison, Michael Egholm, Sherif R. Zaki, James E. Goldman, Hans D. Ochs, and W. Ian Lipkin ✉



Case Discussion

- Patient started on ribavirin and IVIg
- Origin of virus unknown, but presumably community-acquired
- Despite treatment, he continued to deterioriate, and passed away

Summary

- NGS assay shows acceptable performance for clinical testing
  - Current study investigating utility of CSF testing
  - Evaluation of other sample types (plasma for sepsis, BAL for pneumonia)
- Allows for diagnosis of severe infections of unknown etiology after extensive conventional testing
  - Sensitive and specific but some limitations
  - Pathogen DNA may be transient / serology used for diagnosis
  - Contamination control important
- Pathogen detection can guide optimal therapy

Acknowledgements

UCSF Chiu Lab and VDDC

Charles Chiu, MD/PhD
Erik Samayoa, CLS
Shaun Arevalo, CLS
Becky Fung, CLS
Hannah Sample, BS
Samia Naccache, PhD
Scot Federman, BS
Doug Stryke, BS
Joseph DeRisi, PhD
Michael Wilson, MD
Jeffrey Gelfand, MD
Michael Geschwind, MD

UCLA

Jeffrey Klausner, MD/MPH
Romney Humphries, PhD (ABMM)

University of California, Berkeley

Brent Fulton, PhD/MBA

UCDavis

Chris Polage, MD
Stuart Cohen, MD

Children’s Hospital Los Angeles

Jeffrey Bender, MD
Jennifer Dien-Bard, MD

Children’s National Medical Center

Brittany Goldberg, MD
Joseph Campos, MD

St. Jude’s Medical Center

Randall Hayden, MD/PhD