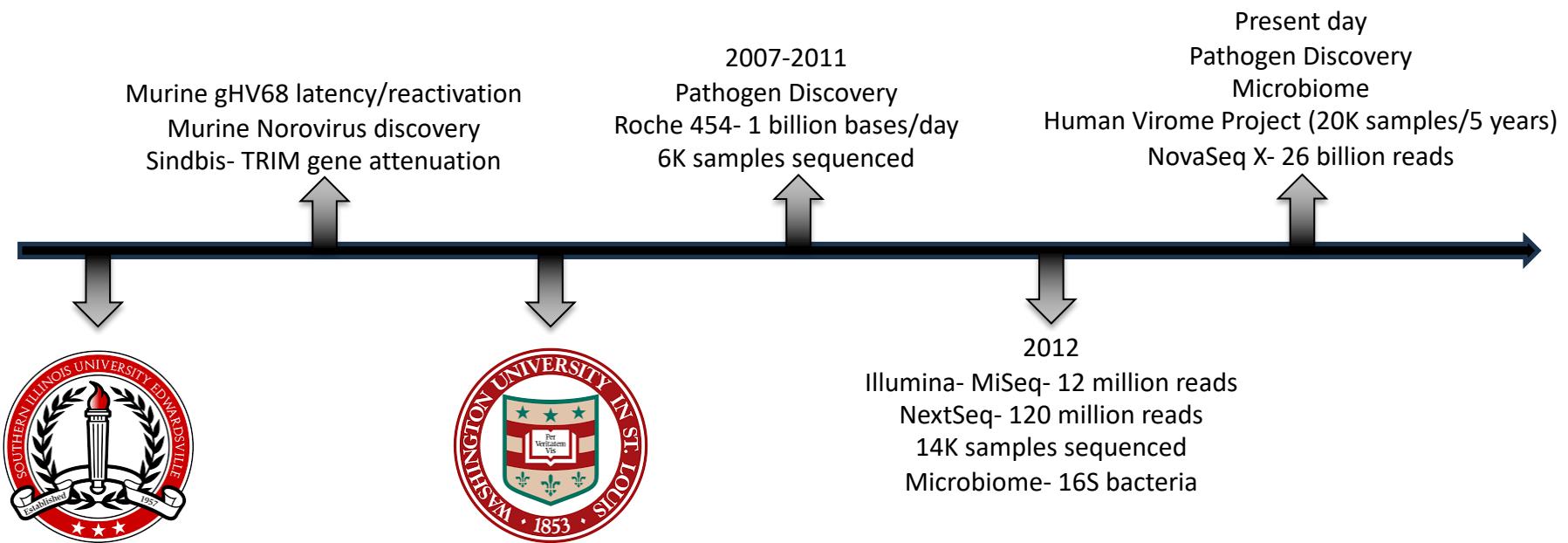


From Sample to Seq

Lindsay Droit

Washington University School of Medicine
St. Louis, Missouri

Career Path



Research Interests

Women's health in developing countries



Pathogen Discovery



Marsabit, Kenya

Capacity Building and Training/Teaching



Durban, South Africa



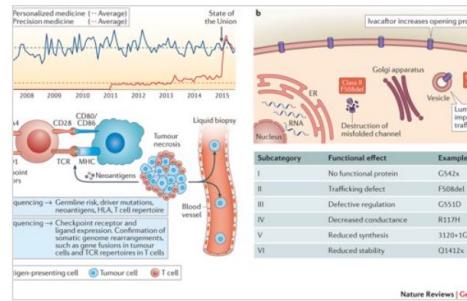
Kathmandu, Nepal



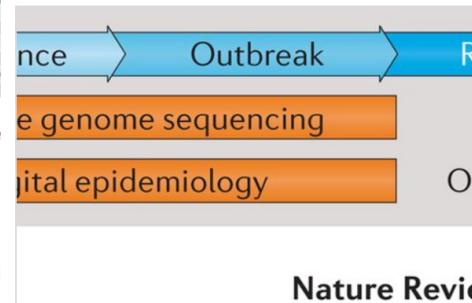
Addis Ababa, Ethiopia

Next Gen Sequencing Applications

Nature Reviews Genetics | Review Article



Nature Reviews Genetics | Review Article



Nature Review

Towards precision medicine

Precision medicine is a strategy for tailoring clinical decision making to the underlying genetic causes of disease. This Review describes how, despite the straightforward overall principles of precision medicine, adopting it responsibly into clinical practice will require many technical and conceptual hurdles to be overcome. Such challenges include optimized sequencing strategies, clinically focused bioinformatics pipelines and reliable metrics for the disease causality of genetic variants.

[show less](#)

Euan A. Ashley

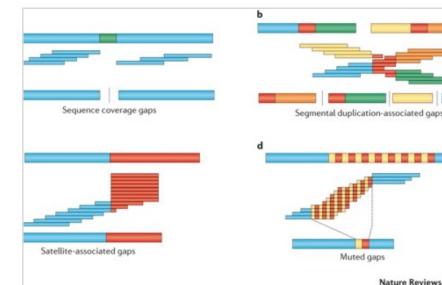
Towards a genomics-informed, real-time, global pathogen surveillance system

Next-generation sequencing has the potential to support public health surveillance systems to improve the early detection of emerging infectious diseases. This Review delineates the role of genomics in rapid outbreak response and the challenges that need to be tackled for genomics-informed pathogen surveillance to become a global reality.

[show less](#)

Jennifer L. Gardy & Nicholas J. Loman

Nature Reviews Genetics | Review Article



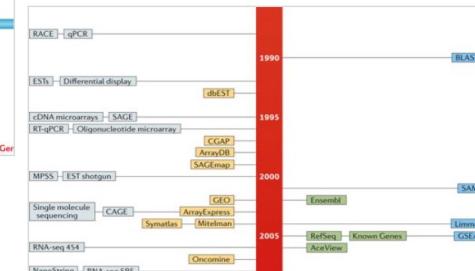
Genetic variation and the de novo assembly of human genomes

The wealth of existing and emerging DNA-sequencing data provides an opportunity for a comprehensive understanding of human genetic variation, including the discovery of disease-causing variants. This Review describes how the limitations of current reference-genome assemblies confound the characterization of genetic variation and how this can be mitigated by important advances in algorithms and sequencing technology that facilitate the *de novo* assembly of genomes.

[show less](#)

Mark J. P. Chaisson, Richard K. Wilson & Evan E. Eichler

Nature Reviews Genetics | Review Article



Cancer transcriptome profiling at the juncture of clinical translation

Although cancer genome sequencing is becoming routine in cancer research, cancer transcriptome profiling through methods such as RNA sequencing (RNA-seq) provides information not only on mutations but also on their functional cellular consequences. This Review discusses how technical and analytical advances in cancer transcriptomics have provided various clinically valuable insights into gene expression signatures, driver gene prioritization, cancer microenvironments, immuno-oncology and prognostic biomarkers.

[show less](#)

Marcin Cieślik & Arul M. Chinnaiyan

Original Sample



Chip/Aliquot



Lyse



Extract Nucleic Acid



Amplify (if necessary)



Construct Library



Pool

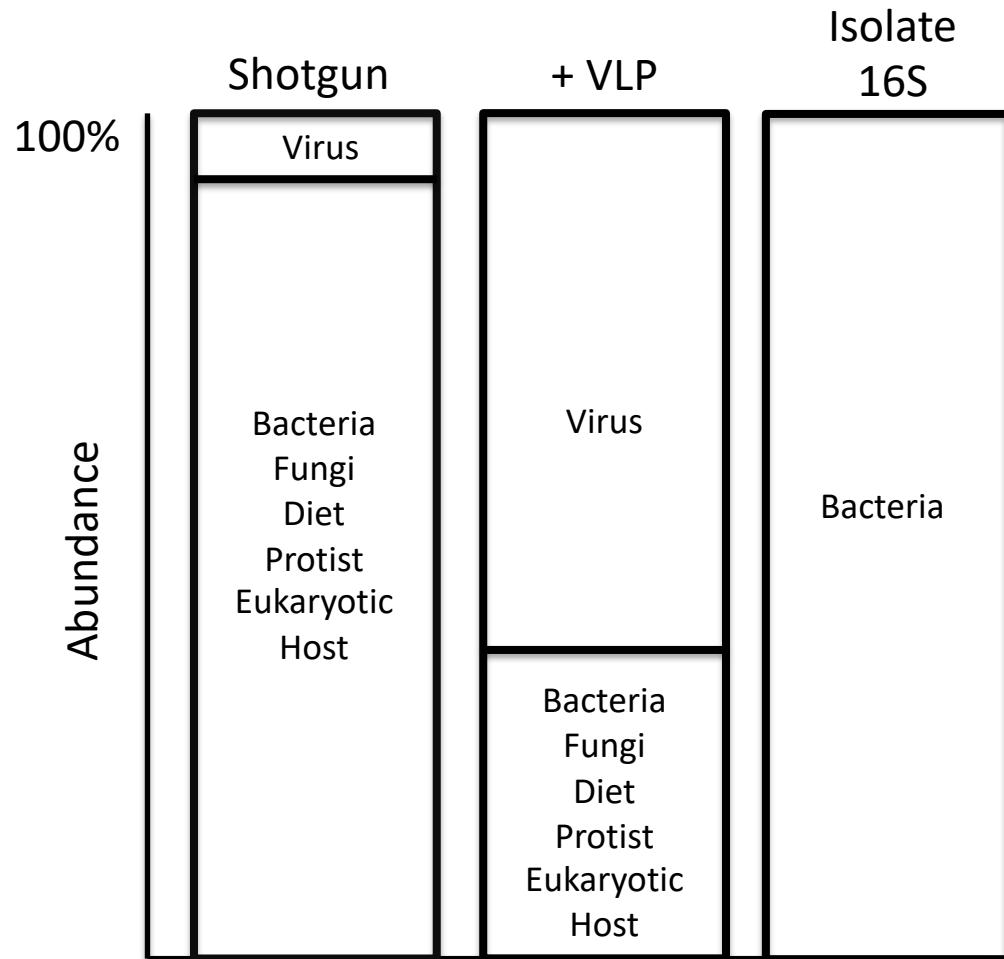


Sequence

Using Next Generation Sequencing to Define the Microbiome

- 1.) Amplicon Surveys- targeted amplicons
 - 16S (bacteria)
 - 18S (eukaryotic microbes)
 - ITS (fungi)

- 2.) Shotgun Metagenomics-
 - entire metagenome: sacrifice resolution
 - enriched metagenome- Virome



extraction, enrichment, sequencing depth

How to participate?



 [Copy participation link](#)



- 1
- 2

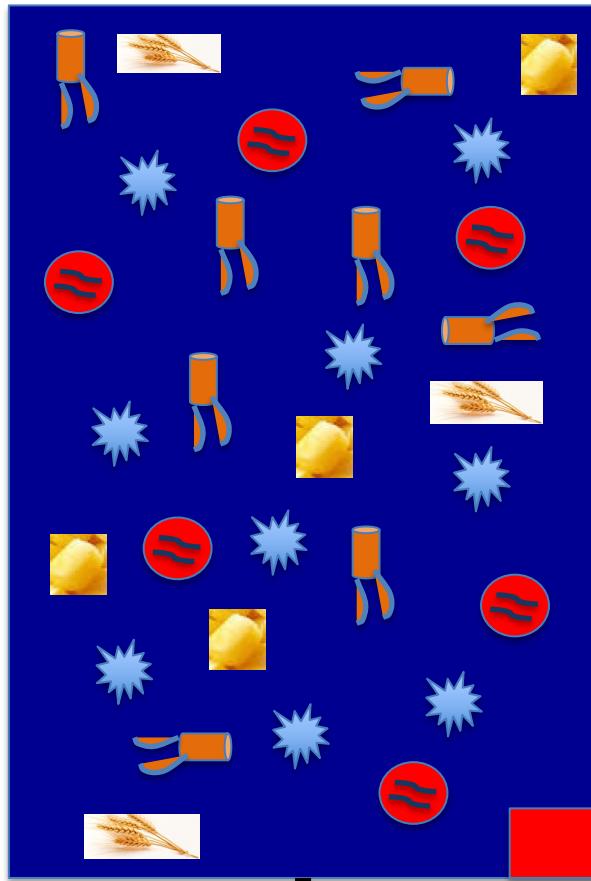
Go to wooclap.com

Enter the event code
in the top banner

Event code
DURBAN

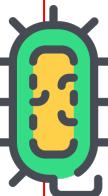
 [Enable answers by SMS](#)

Stool Sample



Amplicon Sequencing

16S/18S/ITS
-DNA
-targeted amplicons



Enriched Metagenome- Virome

Virus Like Particle (VLP)

-DNA, RNA

-remove bacteria

-remove host

Shotgun Sequencing



Sample Types

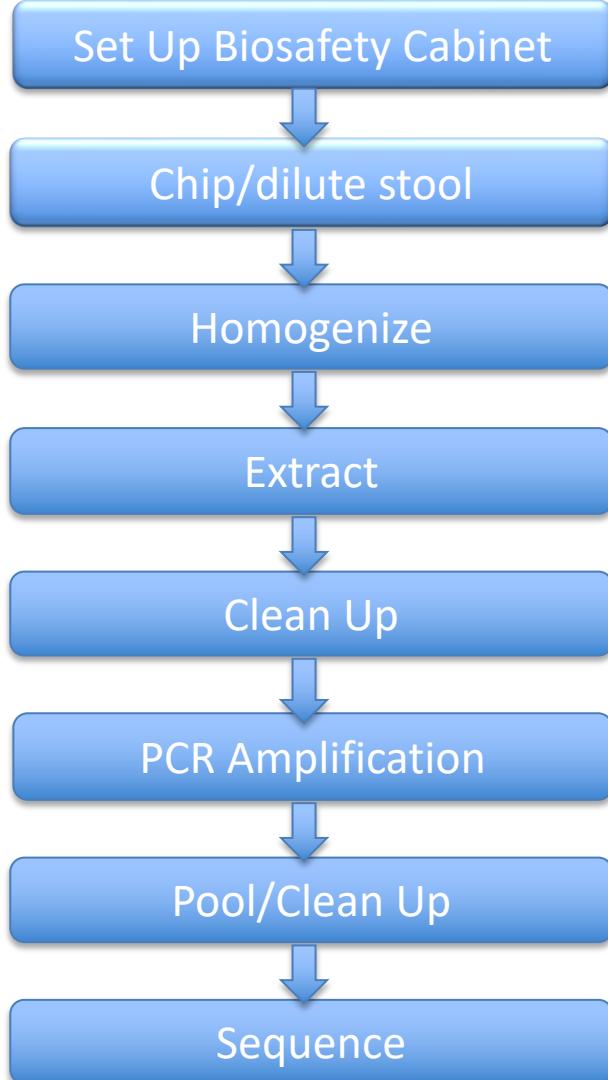
Stool
Urine
Tissue
Cerebral Spinal Fluid
Cervicovaginal Swab
Nasopharyngeal Swab
Bronchoalveolar Lavage
Sputum
Serum
Synovial Fluid
Whole Blood
Cerebral Spinal Fluid
Allantoic Fluid
Amniotic Fluid
Breastmilk
Oral Wash
Saliva
Pericardial Fluid
Semen
Sewage

How much sample do you need?

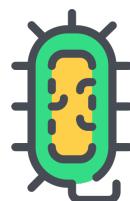
- Stool
 - VLP- at least 200mg
 - 16S- ~20mg 
- Fluid type samples eg; serum, sputum, etc: ~1mL
- May want to consider requesting additional specimen for follow up experiments

Storage Conditions

- Frozen at -80 degrees as quickly as possible
- No medium eg; RNAlater etc



Preparing Samples for 16S/ITS/18S Amplicon Sequencing



Set Up Biosafety Cabinet

Chip/dilute stool

Homogenize

Extract

Clean Up

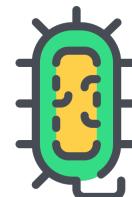
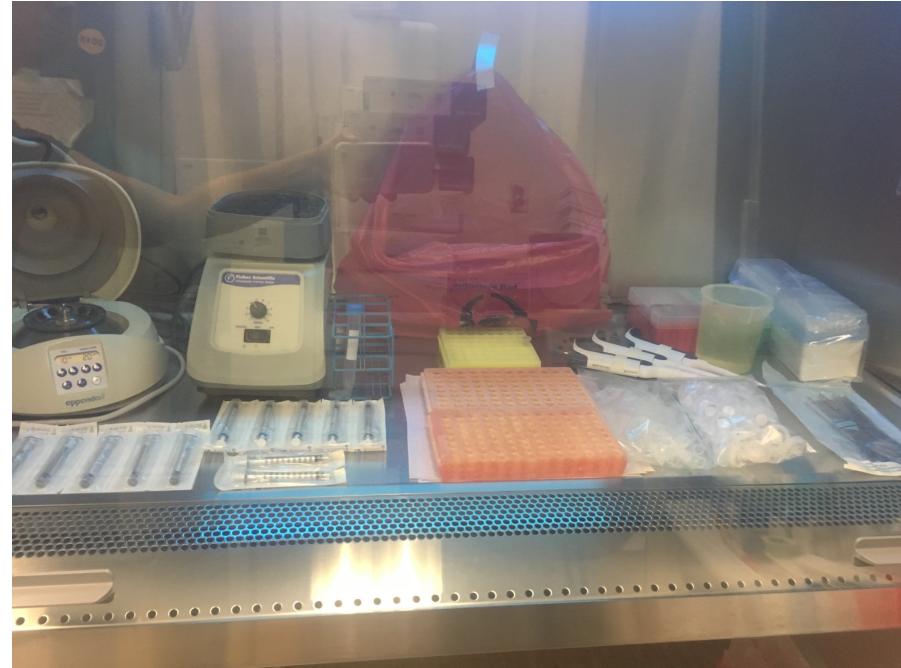
PCR Amplification

Pool/Clean Up

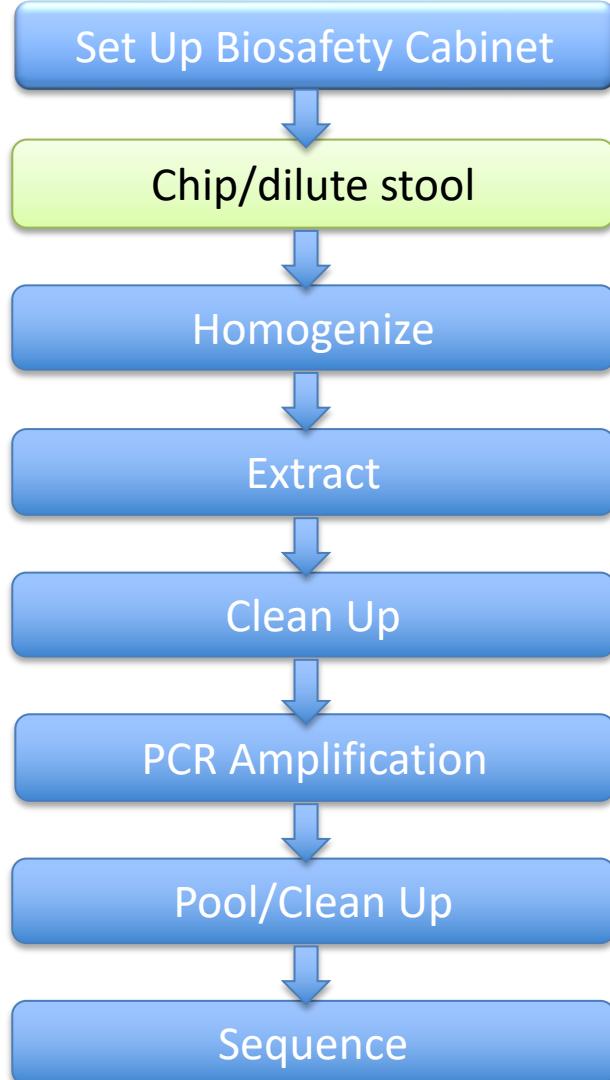
Sequence

Biosafety Cabinet Set Up

- Samples are handled at BSL2+- gown and double glove
- Decon with 10% bleach solution, 70% ethanol, and UV for 30 minutes
- Waste collected in biohazard bags and autoclaved

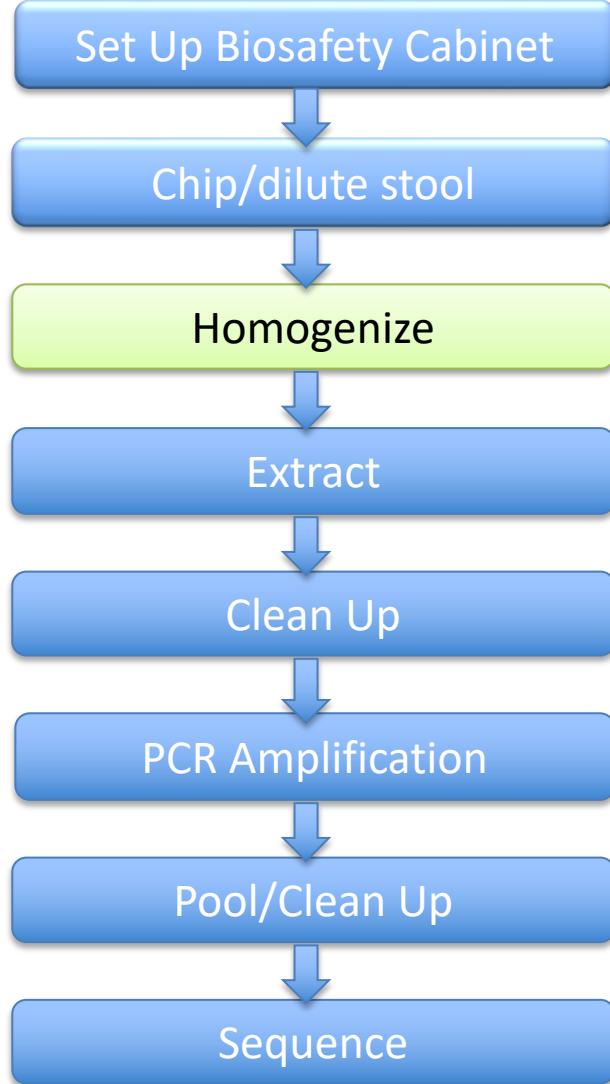


Chip Stool Sample



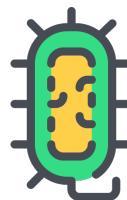
- Keep sample frozen
- Chip ~20mg of stool
- 200ul of autoclaved 1.0mm silica beads
- Add 20% SDS, Buffer A (NaCl, Tris, EDTA), PCI



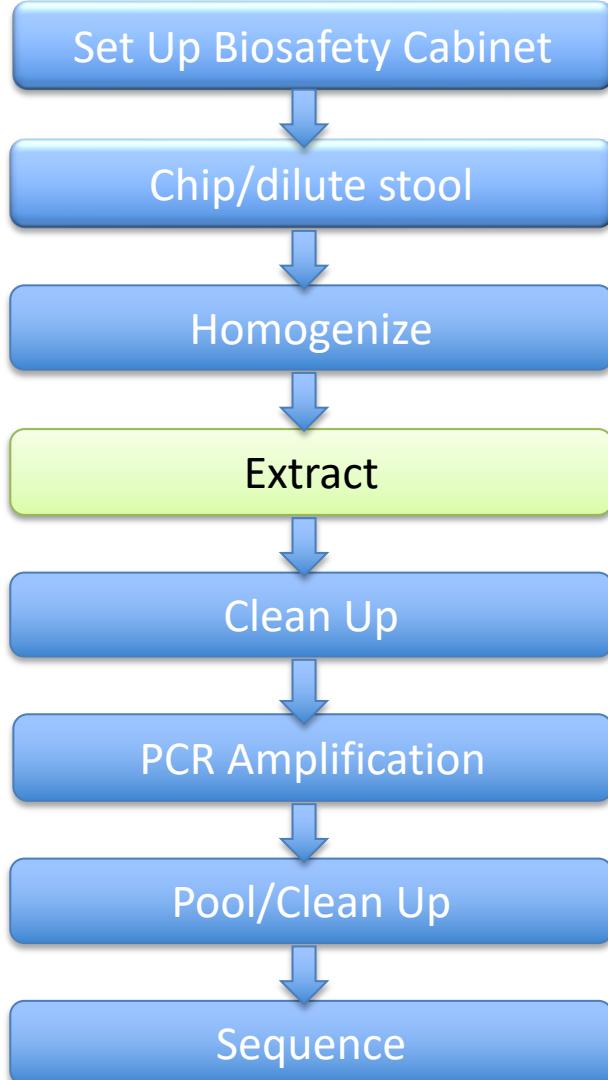


Homogenize Samples

- Bead beat for 1 minute
- Lyse cells to release bacterial DNA



Crude DNA Extraction



Aqueous solution of homogenized stool



Equal volume phenol:chloroform:IAA



Vortex



Centrifuge



Pipette Aqueous Layer



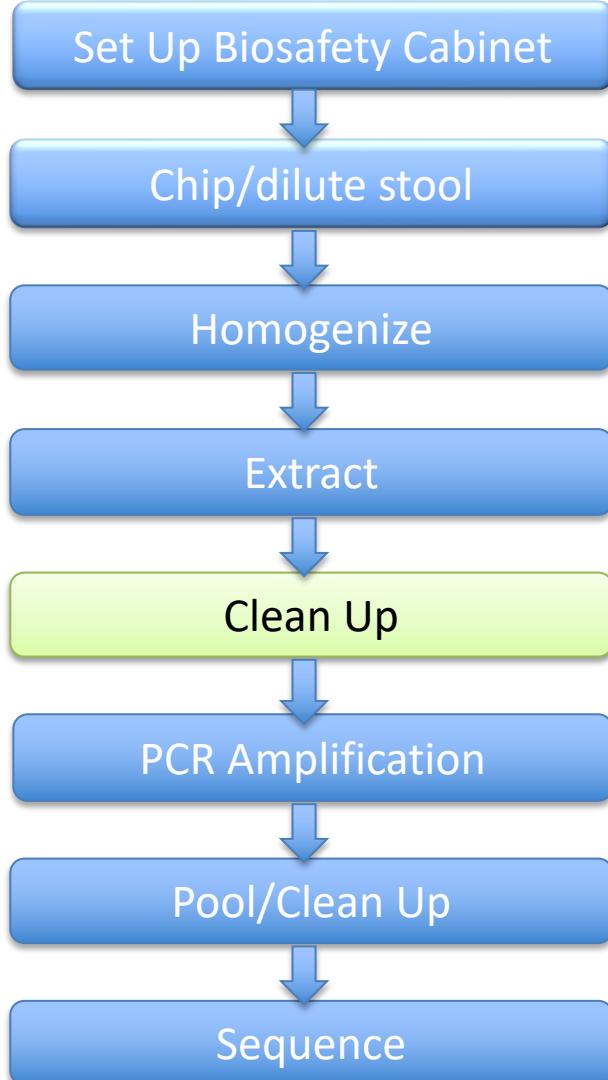
Isopropanol Precipitation



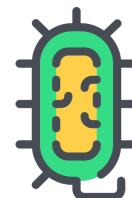
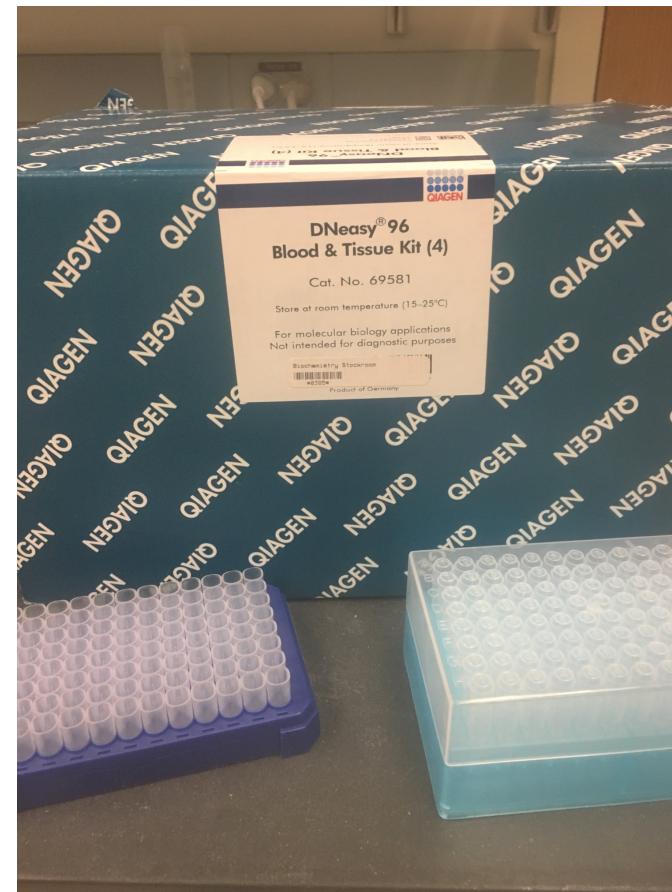
Resuspend in 50ul of H₂O



Qiagen DNeasy

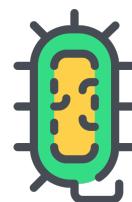
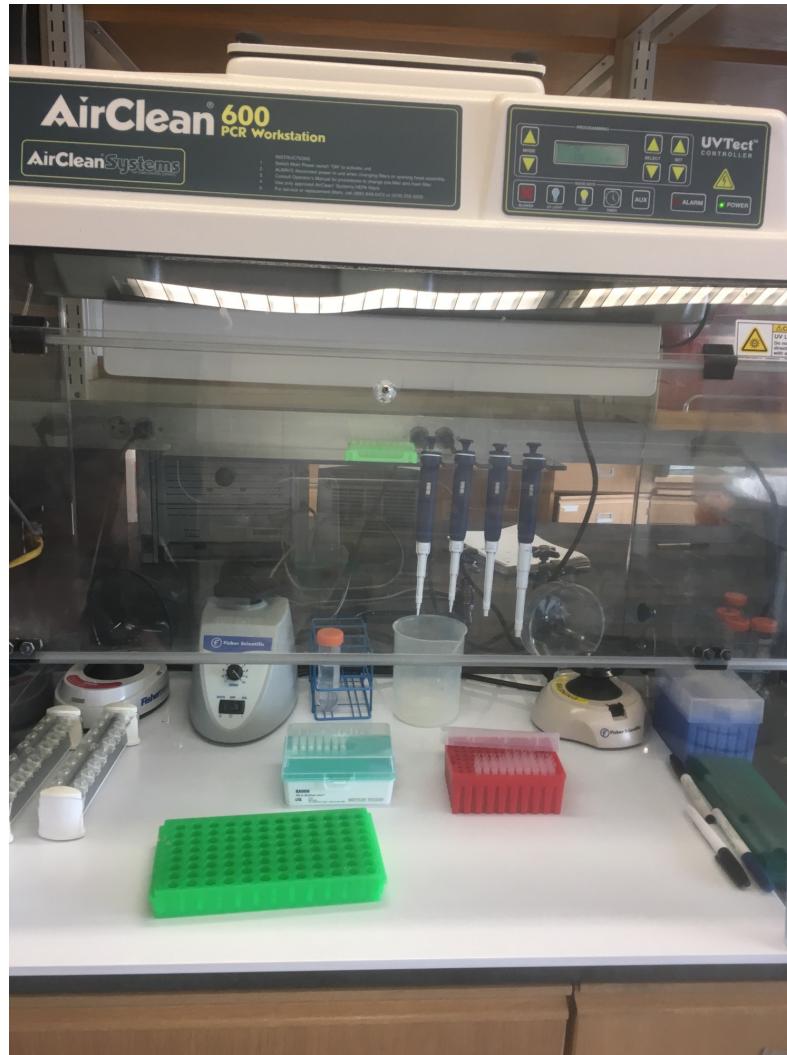


- P:C:I extracted nucleic acid is not pure enough for PCR
- Samples arranged in 96 well format for remainder of protocol



PCR Workstation

- Vertical Laminar air flow
- Hepa filtration system
- Built in UV



PCR Amplification Plate Set Up

Set Up Biosafety Cabinet

Chip/dilute stool

Homogenize

Extract

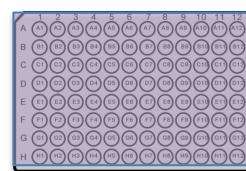
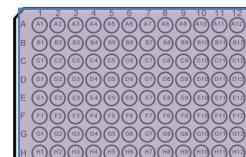
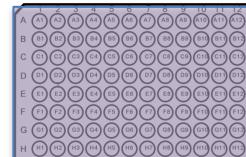
Clean Up

PCR Amplification

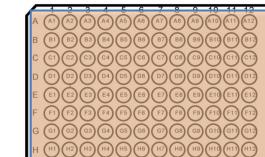
Pool/Clean Up

Sequence

Triplicate Sample Plates



Negative Control Plate



Sample Plate

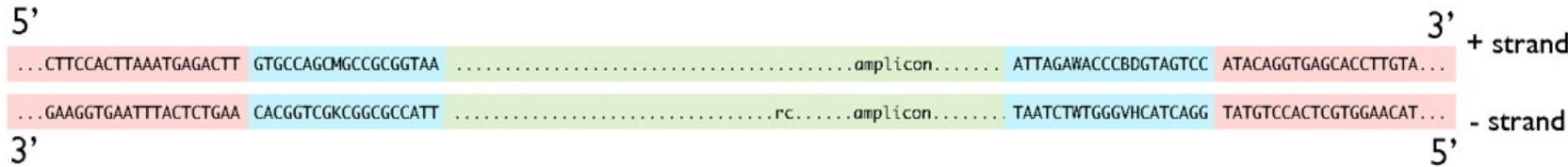
	1	2	3	4	5	6
A		1322	1346	1348	1434	1332
B	1329		1334	1352	1393	1316
C	1330	1331		1333	1350	1351
D	1382	1383	1384		1385	1388

Primer Plate

	1	2	3	4	5	6
A	806rcbc0	806rcbc1	806rcbc2	806rcbc3	806rcbc4	806rcbc5
B	806rcbc12	806rcbc13	806rcbc14	806rcbc15	806rcbc16	806rcbc17
C	806rcbc24	806rcbc25	806rcbc26	806rcbc27	806rcbc28	806rcbc29
D	806rcbc36	806rcbc37	806rcbc38	806rcbc39	806rcbc40	806rcbc41



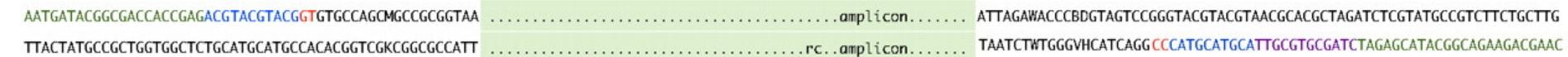
Target gene:



Amplification primers with annealing sites:



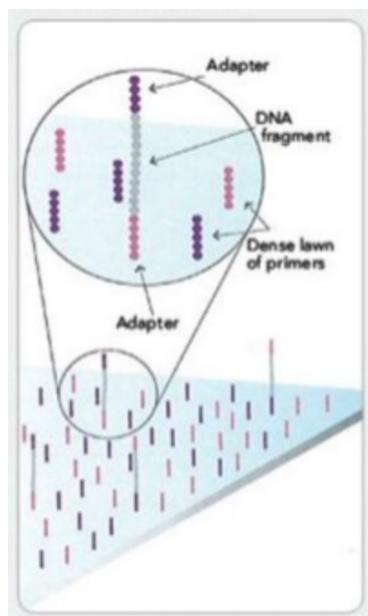
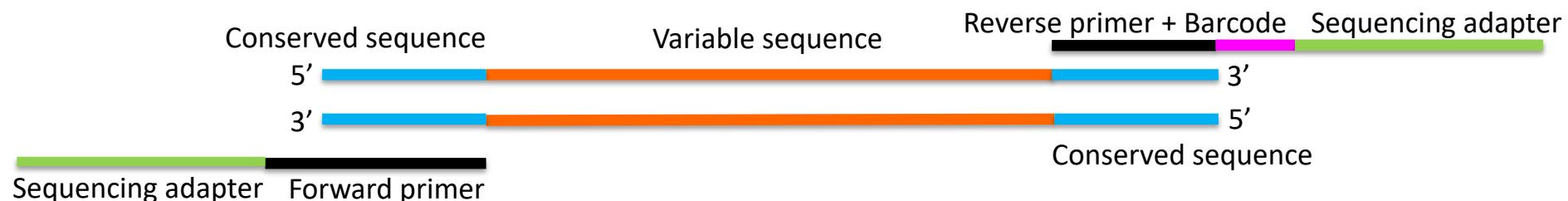
Amplification products:



Sequencing primers with annealing sites:



Primer Design



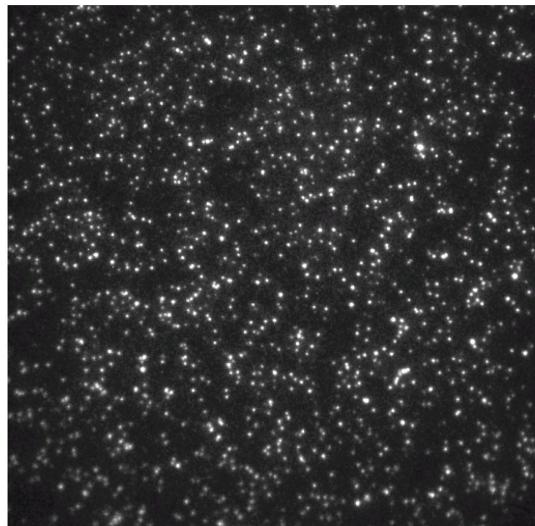
- Variable sequence
- Conserved sequence
- Forward/Reverse Primer
- Barcode
- Illumina sequencing adapter



Illumina Sequencing Technology

- Sample preparation
- Cluster generation
- Sequencing
- Data analysis

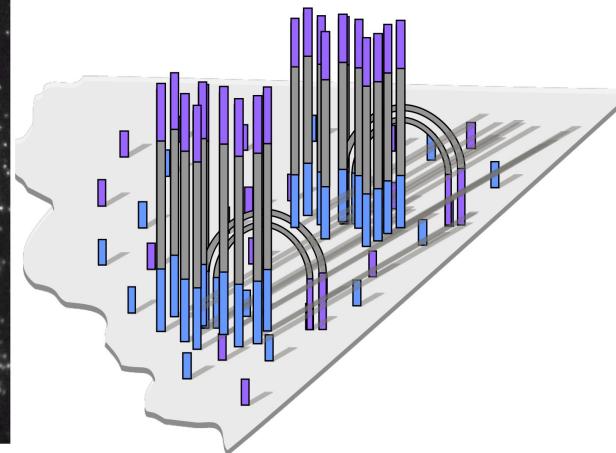
Step 2: Clonal Single Molecule Arrays



100um

Random array of clusters

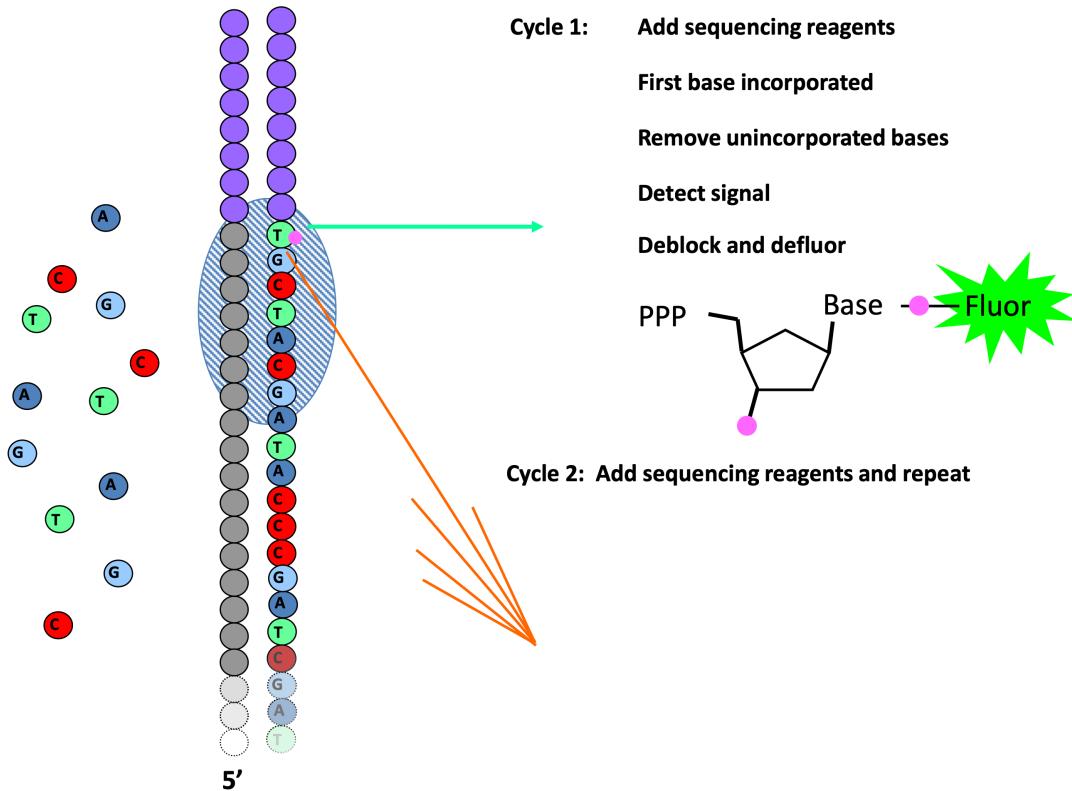
Attach single molecules to surface
Amplify to form clusters



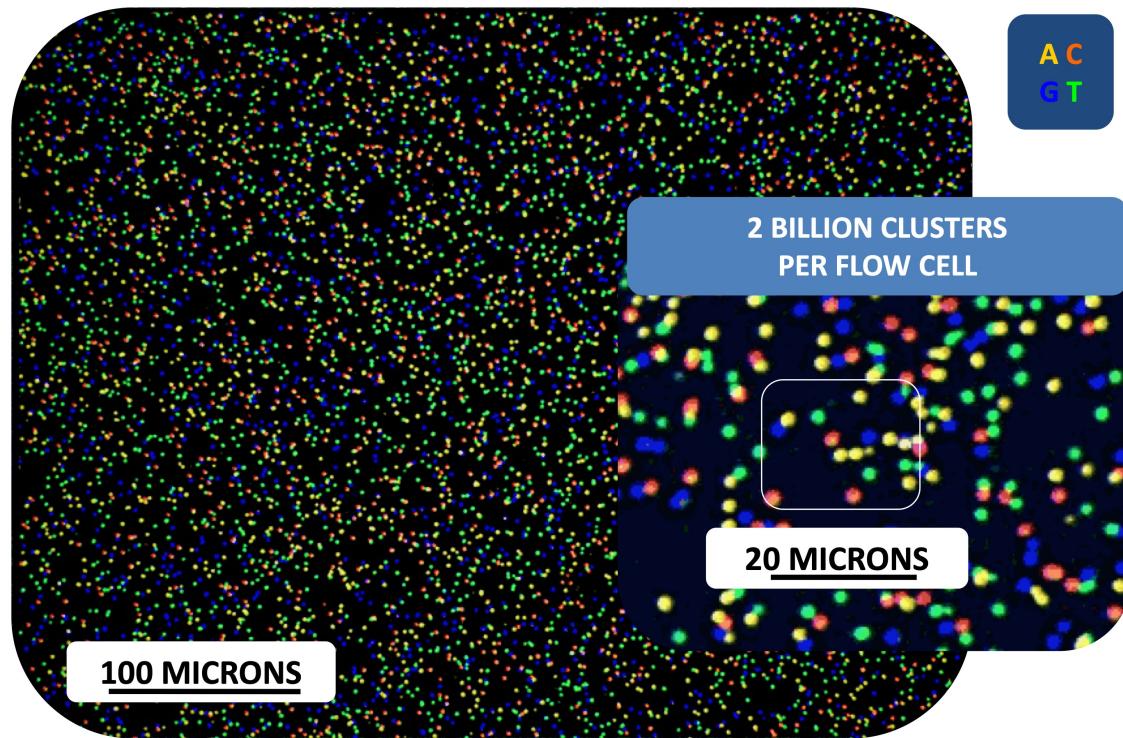
~1000 molecules per ~ 1 um cluster
~2 billion clusters per flowcell

1 cluster = 1 sequence

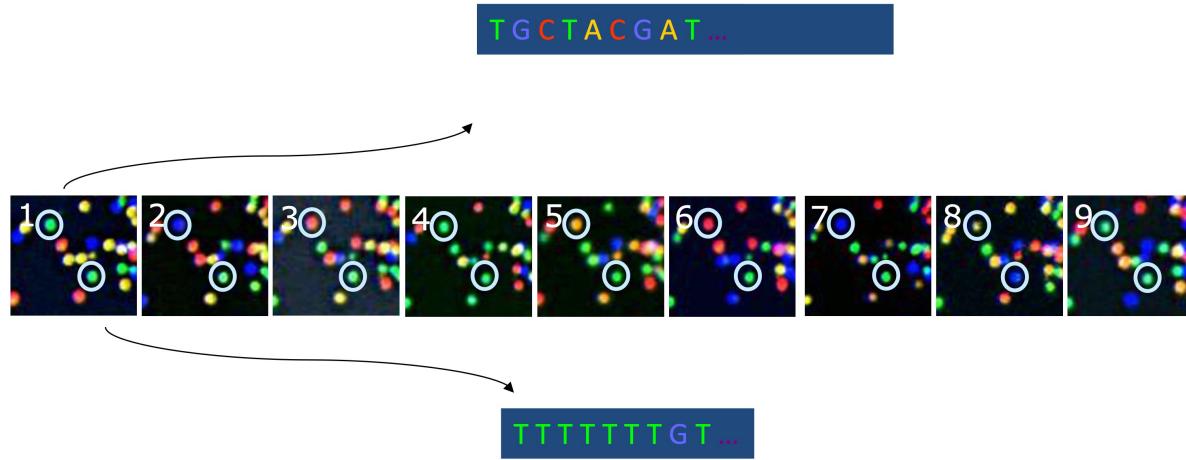
Step 3: Sequencing By Synthesis (SBS)



Illumina Sequencing : How it looks

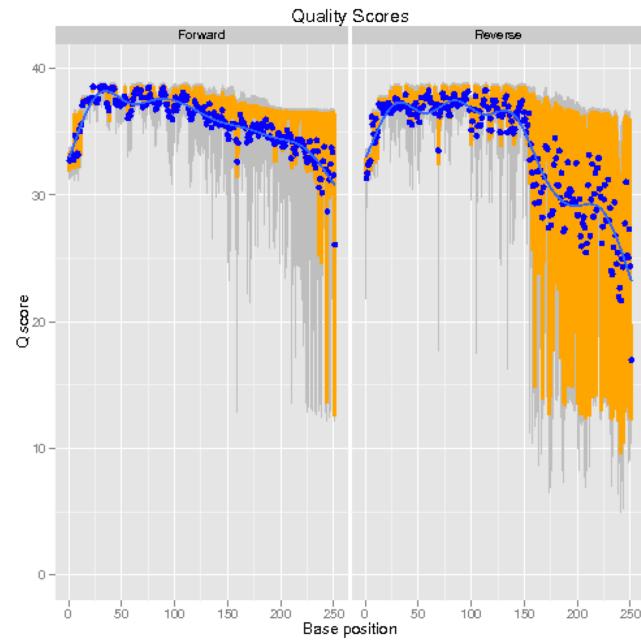


Base calling from raw data



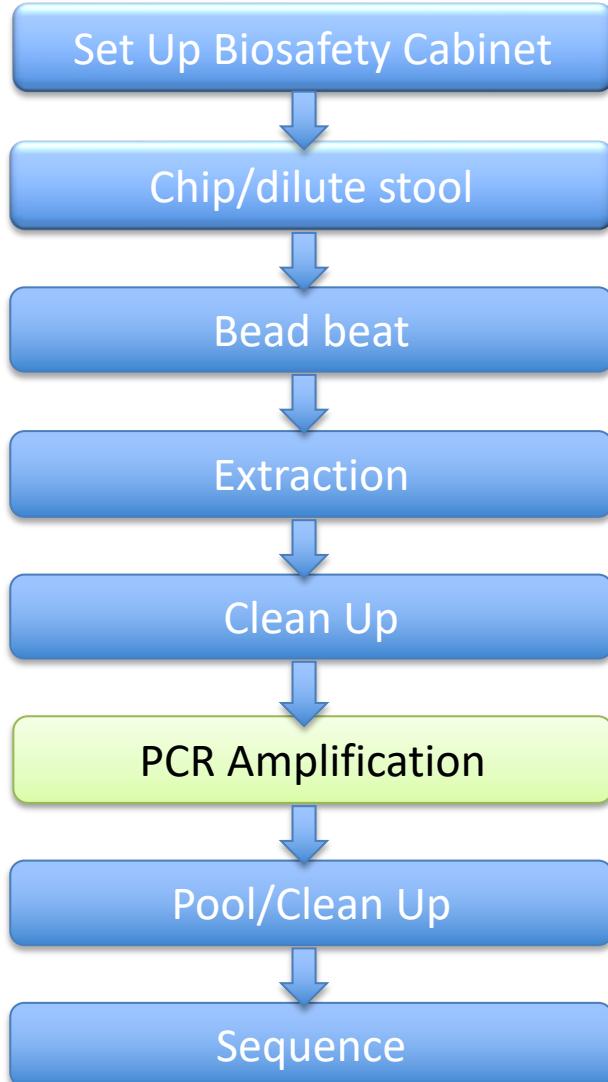
The identity of each base of a cluster is read off from sequential images.

Why do quality scores drop towards the end of a read?

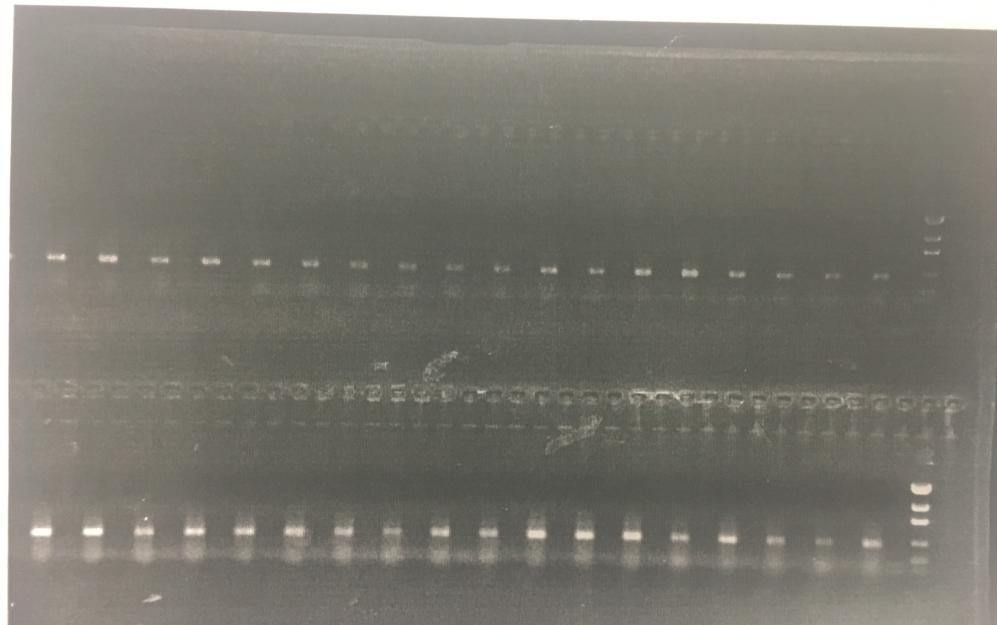


<https://www.youtube.com/watch?v=fCd6B5HRaz8>

Post PCR Amplification

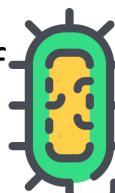


- Triplicate reactions are pooled into single plate
- 1% Agarose gel- 5ul of amplified DNA
- Expected band 16S=390bp, 18S=260bp +/- 50bp, ITS= ~230bp

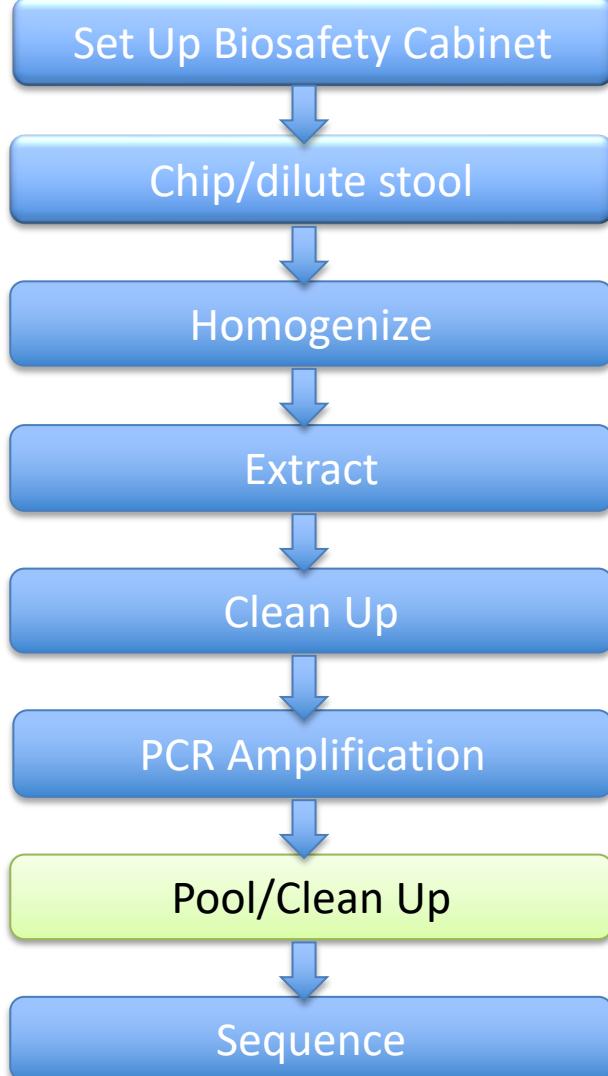


Failure Rate: up to 40% for some projects
Troubleshooting No Band:

- Antibiotics treated samples often don't show a band
- PCR inhibitors- sometimes a 1:10 or 1:100 dilution of the sample will work

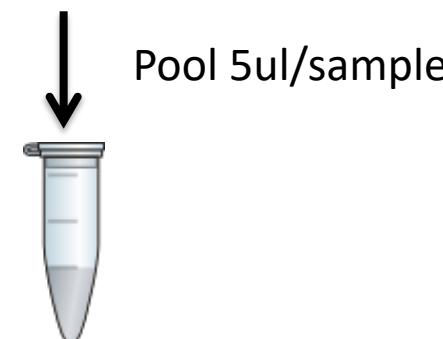


Sample Pooling and Clean Up



- Pool 5ul per sample. Including H₂O negative controls
- Clean Up
- Quantify- Qubit

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12



Set Up Biosafety Cabinet

Chip/dilute stool

Homogenize

Extract

Clean Up

PCR Amplification

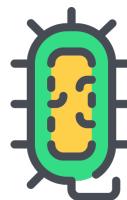
Pool/Clean Up

Sequence

MiSeq V2 2X250



(pick your favorite)





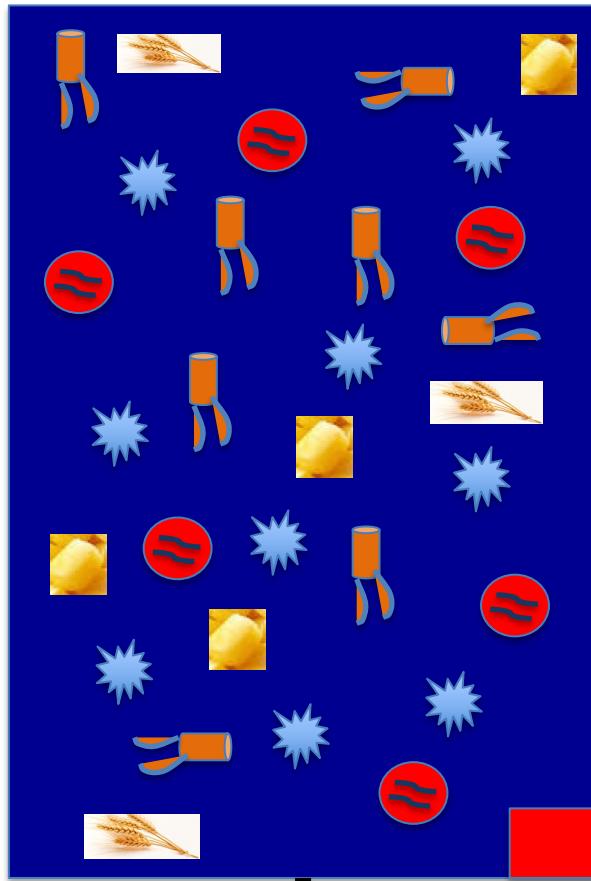
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<http://www.earthmicrobiome.org>



Stool Sample

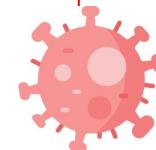


Amplicon Sequencing

16S/18S/ITS
-DNA
-targeted amplicons

Enriched Metagenome- Virome

Virus Like Particle (VLP)
-DNA, RNA
-remove bacteria
-remove encapsulated DNA



Shotgun Sequencing

Preparing Samples for Virome Sequencing: 3 Steps

- 1.) Virus Like Particle (VLP)
Enrichment and Total Nucleic Acid Extraction
- 2.) Reverse Transcription, Second Strand Synthesis and PCR Amplification
- 3.) Library Construction



Preparing Samples for Virome Sequencing: 3 Steps

- 1.) Virus Like Particle (VLP)
Enrichment and Total Nucleic Acid Extraction
- 2.) Reverse Transcription, Second Strand Synthesis and PCR Amplification
- 3.) Library Construction



Biosafety Cabinet Set Up

Set Up Biosafety Cabinet



Chip/Dilute



Vortex



Centrifuge



Filter

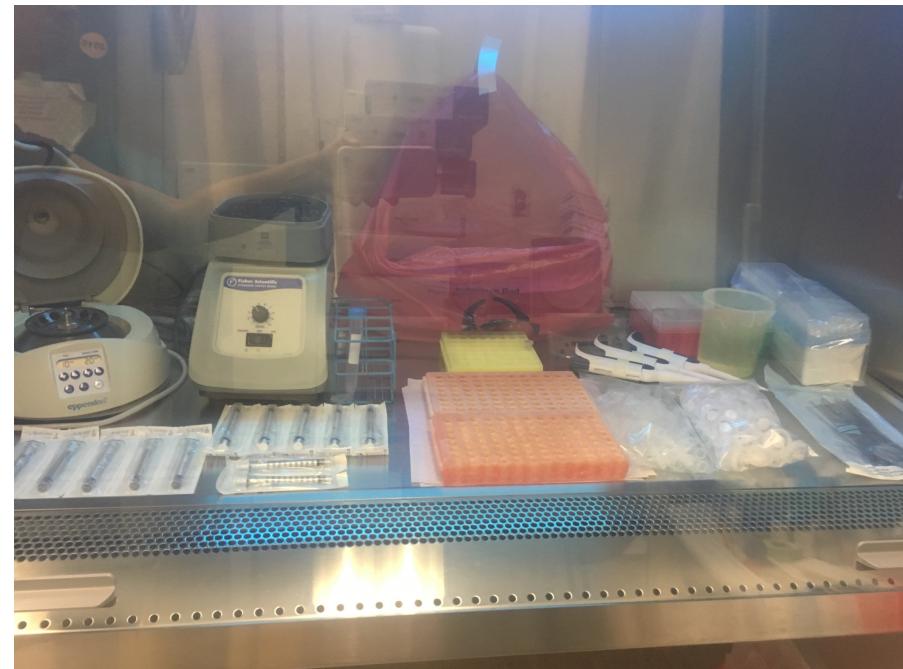


DNase/Lysozyme



TNA Extraction

- Samples are handled at BSL2+- gown and double glove
- Decon with 10% bleach solution, 70% ethanol, and UV for 30 minutes
- Waste collected in biohazard bags and autoclaved



Chip Stool Sample

Set Up Biosafety Cabinet

Chip/Dilute

Vortex

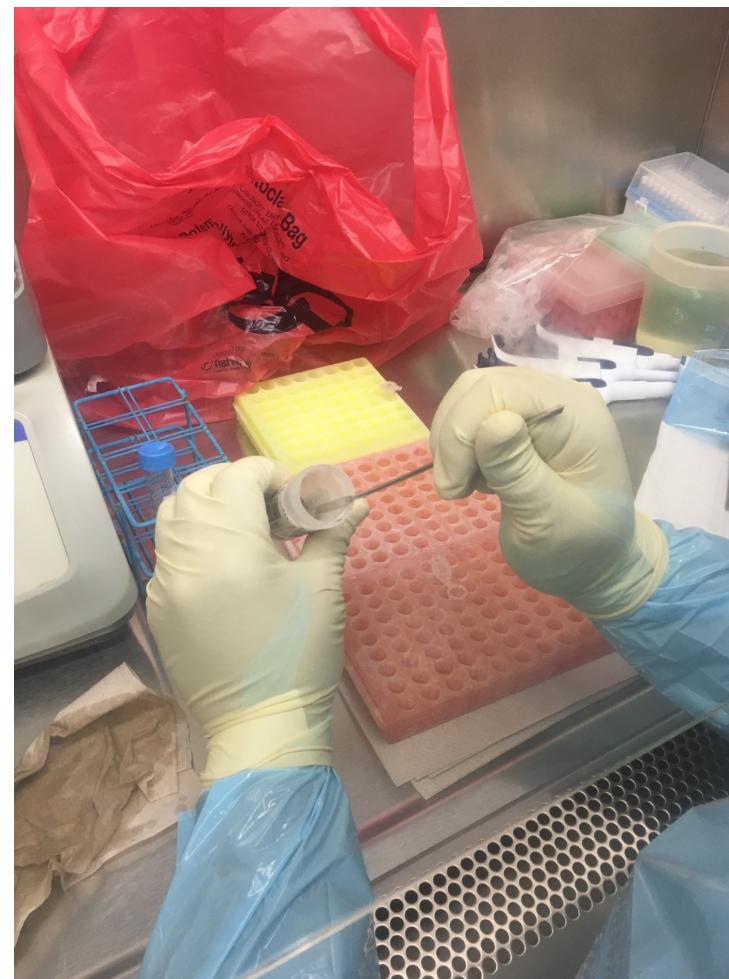
Centrifuge

Filter

DNase/Lysozyme

TNA Extraction

- Keep sample frozen
- Chip ~200mg of stool
- Add SM Buffer (NaCl, Tris, MgSO₄)



Homogenize Samples

Set Up Biosafety Cabinet



Chip/Dilute



Vortex



Centrifuge



Filter



DNase/Lysozyme



TNA Extraction



Centrifuge

Set Up Biosafety Cabinet



Chip/Dilute



Vortex



Centrifuge



Filter

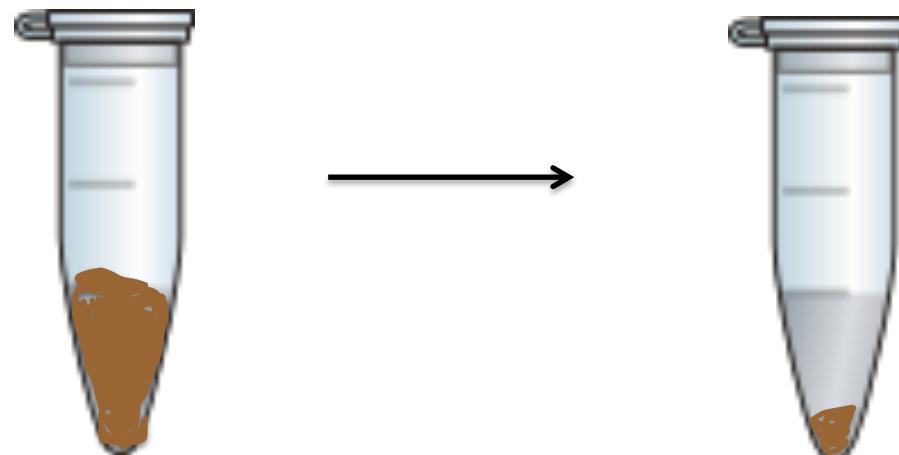


DNase/Lysozyme



TNA Extraction

- 7,000G for 10 minutes
- Pellet stool particles



Filter to Remove Bacteria

Set Up Biosafety Cabinet

Chip/Dilute

Vortex

Centrifuge

Filter

DNase/Lysozyme

TNA Extraction

- .45 μ filter



Non Encapsulated DNA Removal

Set Up Biosafety Cabinet



Chip/Dilute



Vortex



Centrifuge



Filter



DNase/Lysozyme



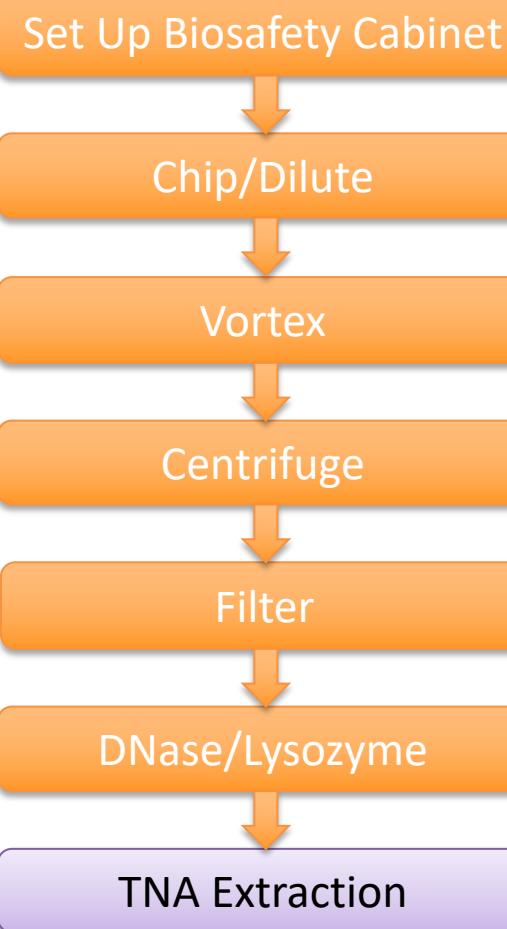
TNA Extraction

- Lysozyme DNase Enzyme cocktail
- Degrade non-encapsulated DNA

	Per 800ul sample	$12 +1 =13$ samples
Turbo DNase buffer	108 <u>ul</u>	1,404
TurboDNaseI (2U/ <u>ul</u>)	20 <u>ul</u>	260
Baseline zero (1U/ <u>ul</u>)	4 <u>ul</u>	52
Lysozyme (10mg/ml)	80 <u>ul</u>	1,040
H2O	68 <u>ul</u>	884
	280ul	



Total Nucleic Acid Extraction



- Extract both DNA and RNA
- Automated systems
- Manual Kits- Qiagen DNeasy



Preparing Samples for Virome Sequencing: 3 Steps

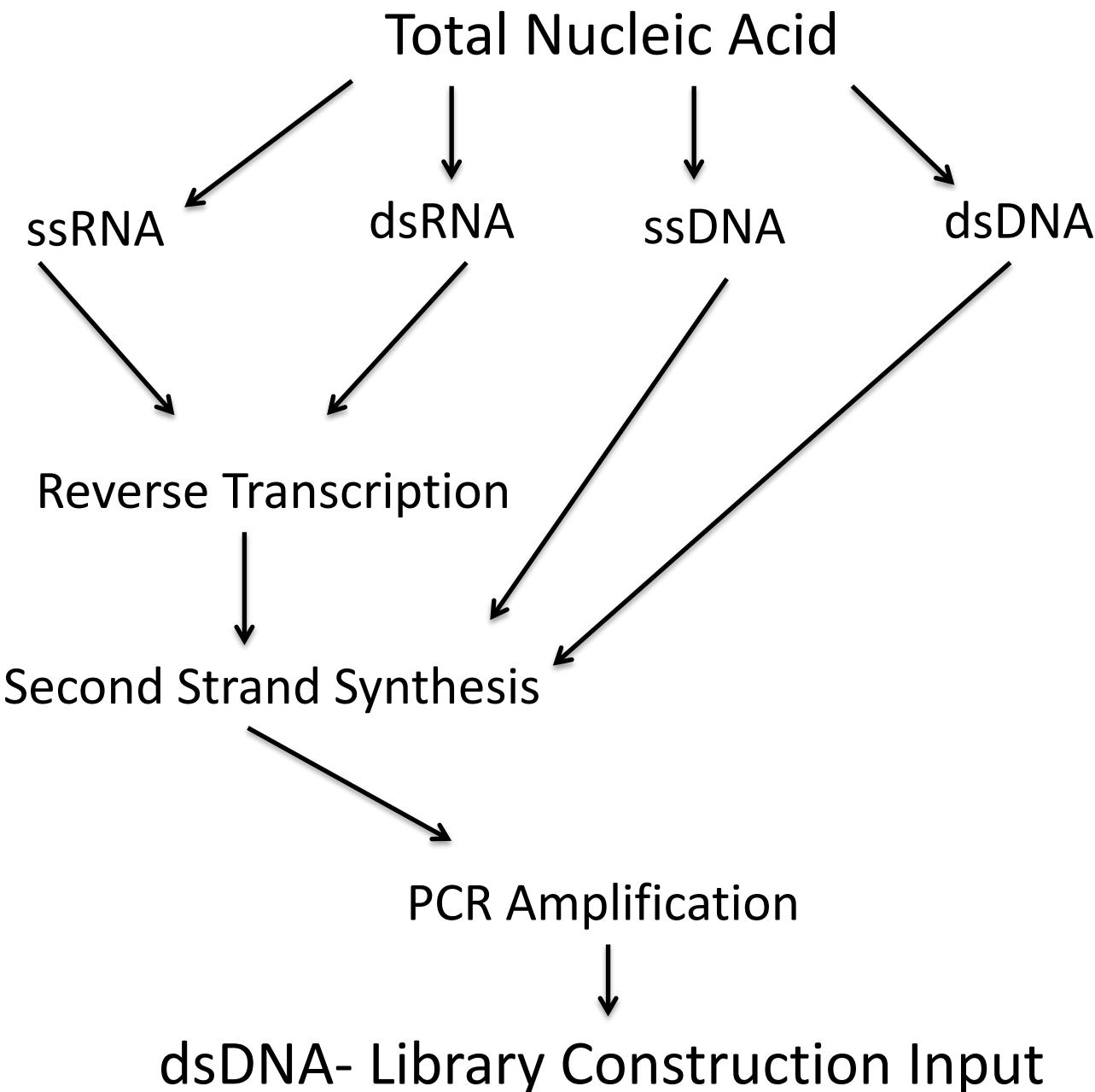
1.) Virus Like Particle (VLP)

Enrichment and Total Nucleic Acid Extraction

2.) Reverse Transcription, Second Strand Synthesis and PCR Amplification

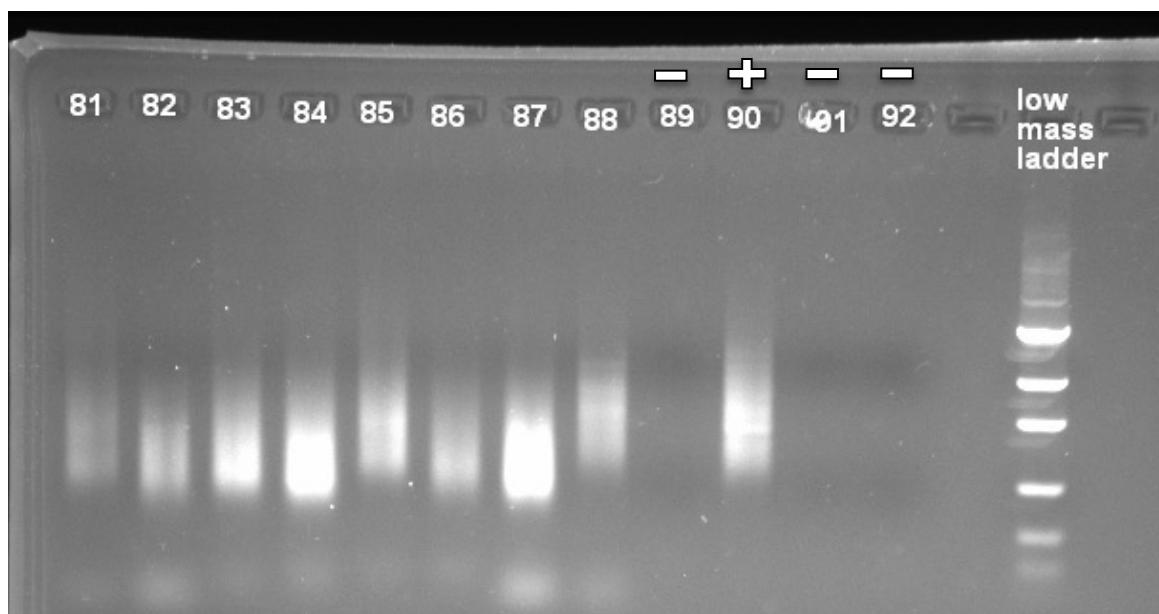
3.) Library Construction



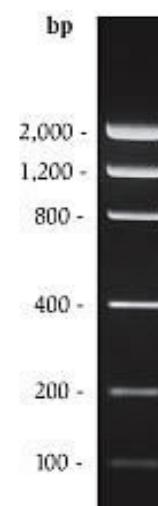


Post PCR Amplification

- 1% Agarose gel- 10ul of amplified DNA
- Expected smear 200bp-1kb
- Failure rate averages 8-10%



Invitrogen Low Mass Ladder



Preparing Samples for Virome Sequencing: 3 Steps

1.) Virus Like Particle (VLP)

Enrichment and Total Nucleic Acid Extraction

2.) Reverse Transcription, Second

Strand Synthesis, and PCR Amplification

3.) Library Construction





Clean Up/Size Selection

End Repair

Adapter Ligation

Clean Up/Size Selection

PCR Amplification

PCR Clean Up

Quality Control

Pool

Sequence

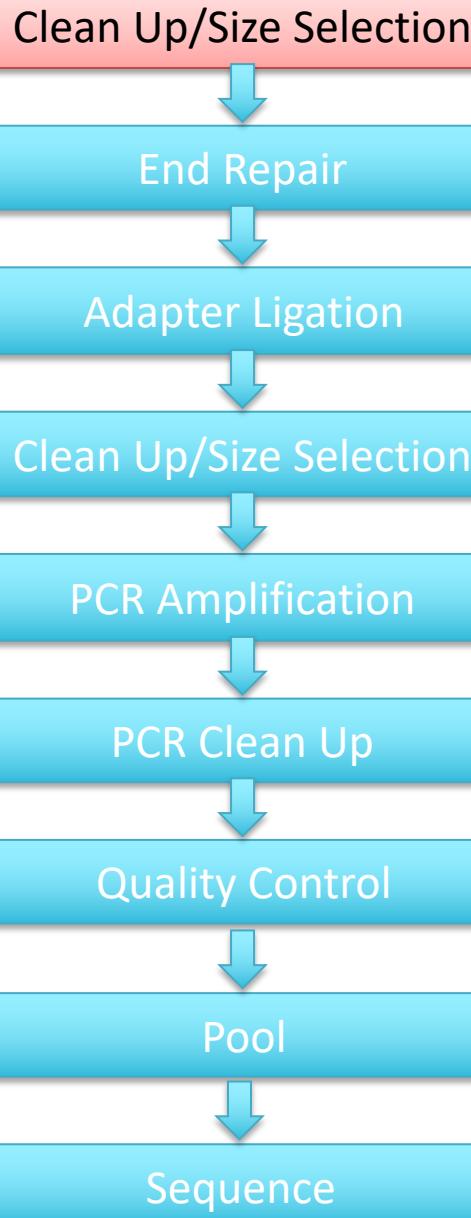
New England Biolabs NEB Next DNA Library Construction



PCR Workstation

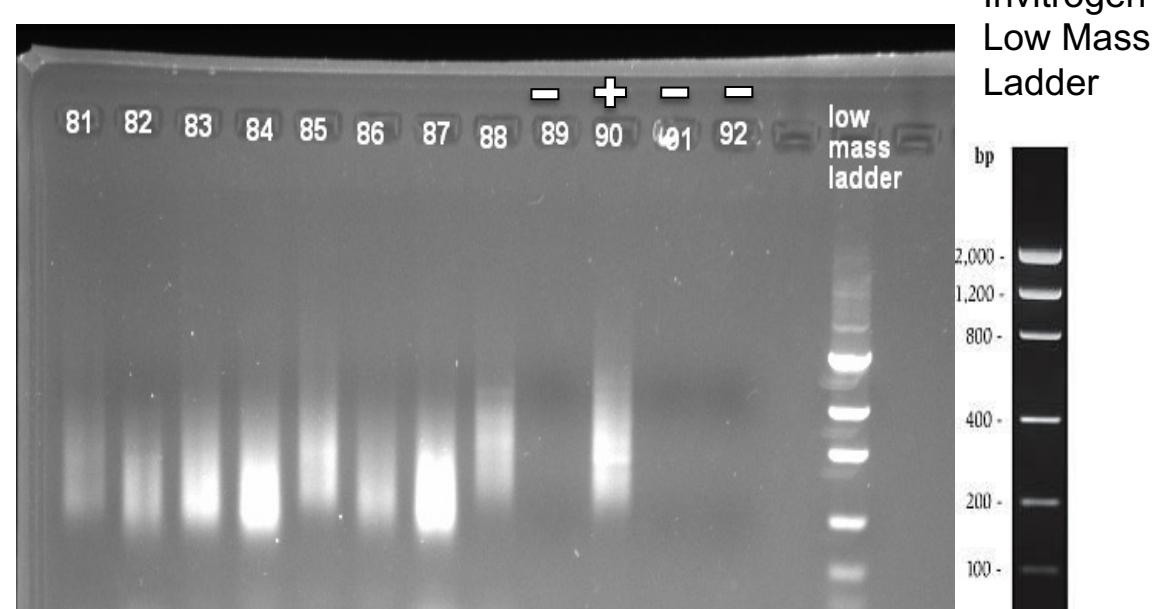
- Vertical Laminar air flow
- Hepa filtration system
- Built in UV





Post PCR Amplification

- 1% Agarose gel- 10ul of amplified DNA
- Expected smear 200bp-1kb
- Target 400-600bp for library construction



Beckman Coulter- AmPure Bead

Clean Up/Size Selection



End Repair



Adapter Ligation



Clean Up/Size Selection



PCR Amplification



PCR Clean Up



Quality Control

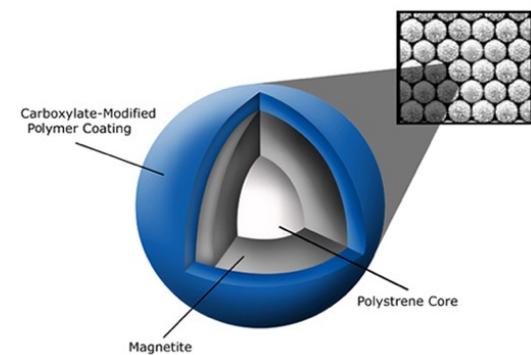
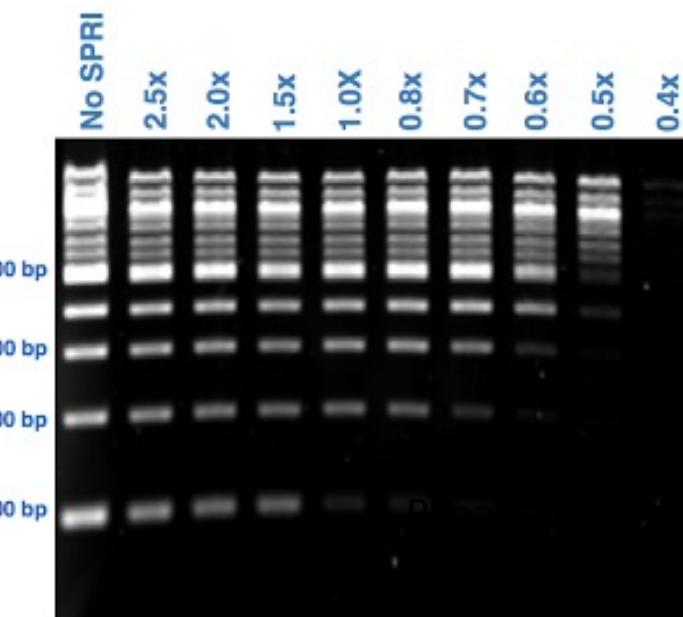


Pool

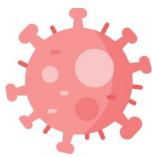


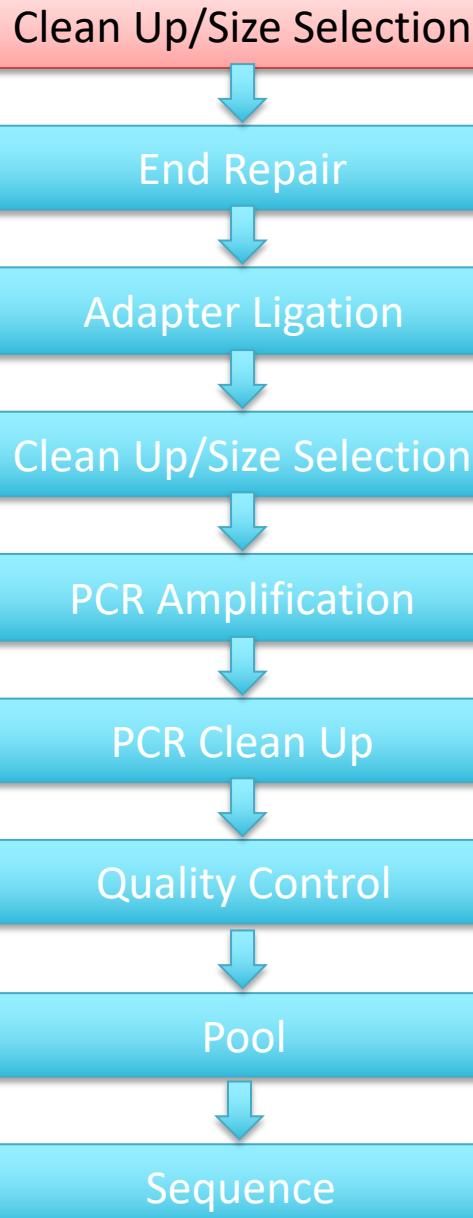
Sequence

- SPRI Bead (Solid Phase Reversible Immobilization)
- Uses Paramagnetic beads to selectively bind nucleic acid by size
- PEG (polyethylene glycol) causes the negatively charged DNA to bind to the carboxyl molecules on bead surface
- Lower the ratio of SPRI:DNA= larger final fragments at elution



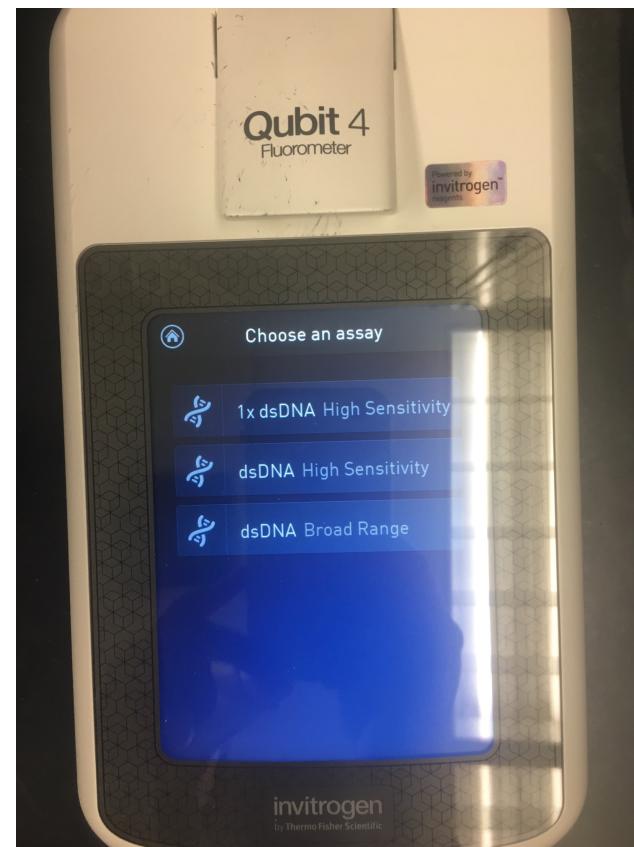
<https://youtu.be/zGV0SjCe0CU>





Sample Quantification

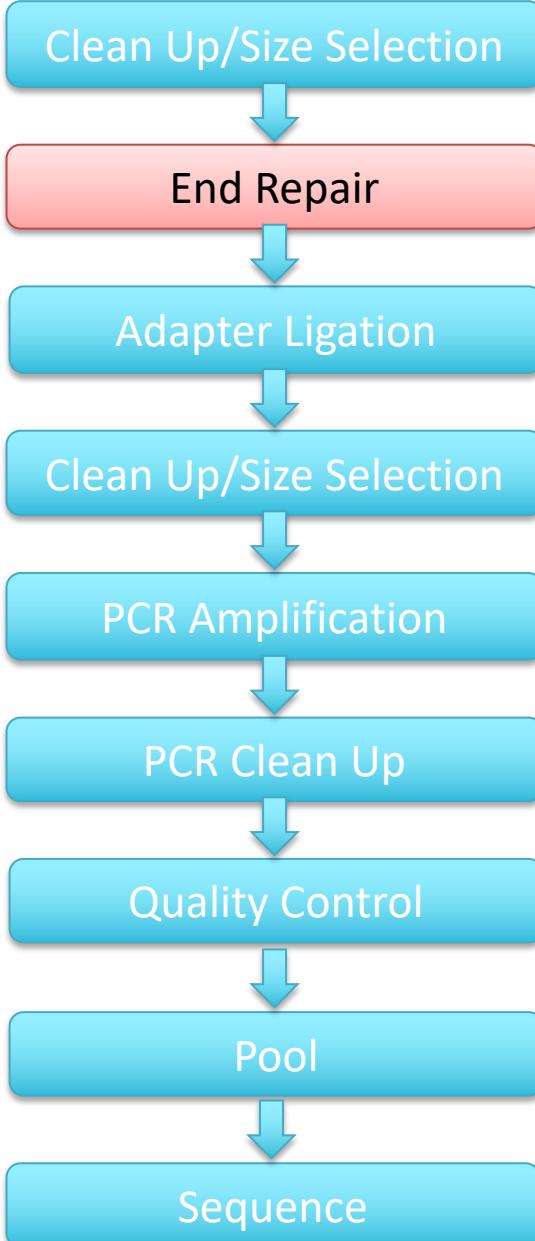
- Library input DNA 20-100ng
- Can go as low as 5ng
- Knowing input is critical for downstream steps- adapter concentration and PCR amplification cycle number



End Repair

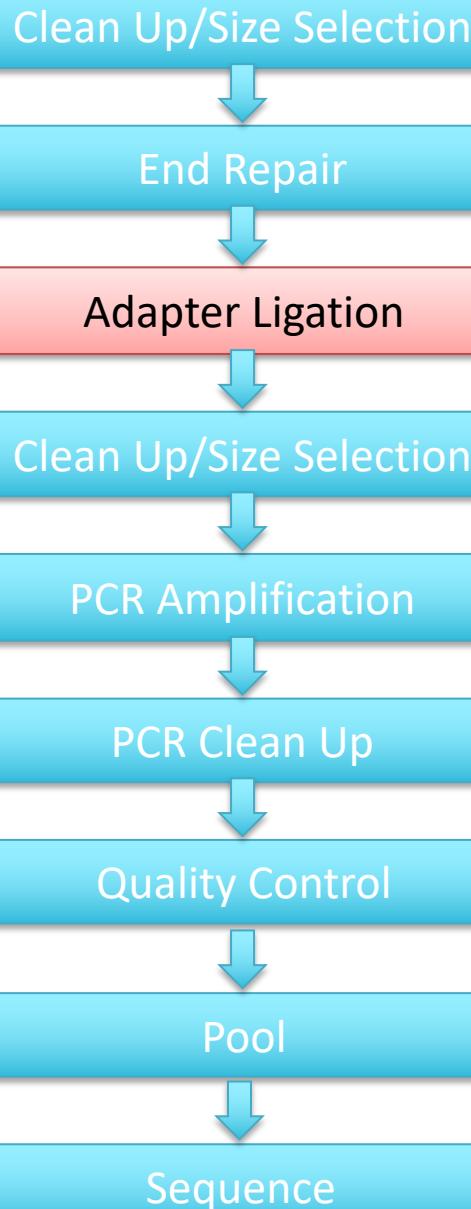
5' Phosphorylation and dA-Tailing

- Strands are blunted and phosphorylated
- Adding an A to 3' ends



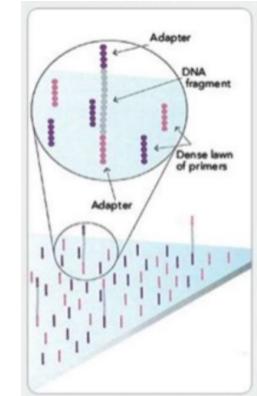
End Repair, 5' Phosphorylation and dA-Tailing





Adapter Ligation

- Adapters with single T overhang ligated on the end repair dA fragment
- Amount of adapter is critical
- User enzyme used to cleave hairpin loop

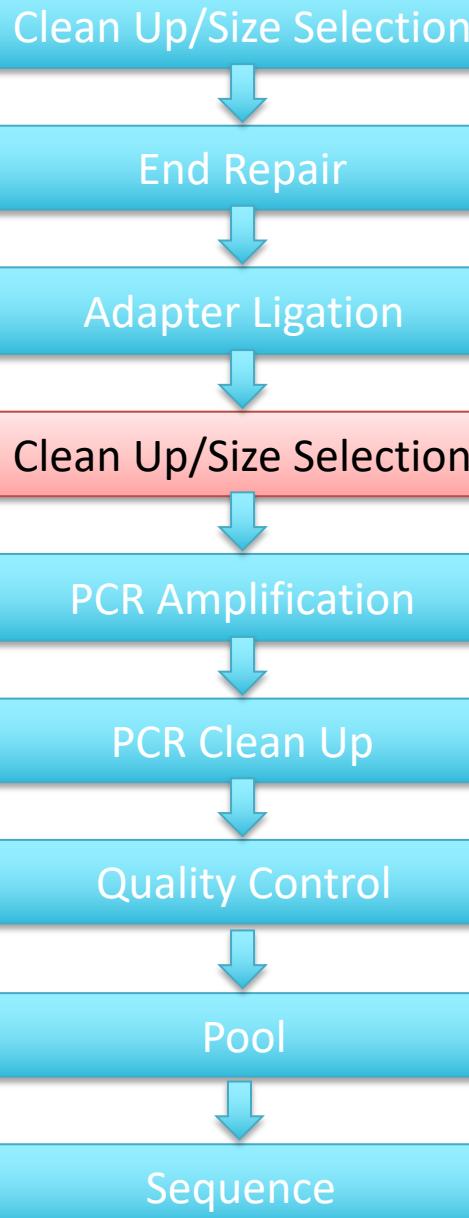


Adaptor Ligation with optional NEBNext Adaptor



U Excision





Clean Up- Post Adapter Ligation

- Size Selection 400-600bp
- Remove unused ligation reaction components, adapter dimers, and concatemers



Clean Up/Size Selection

End Repair

Adapter Ligation

Clean Up/Size Selection

PCR Amplification

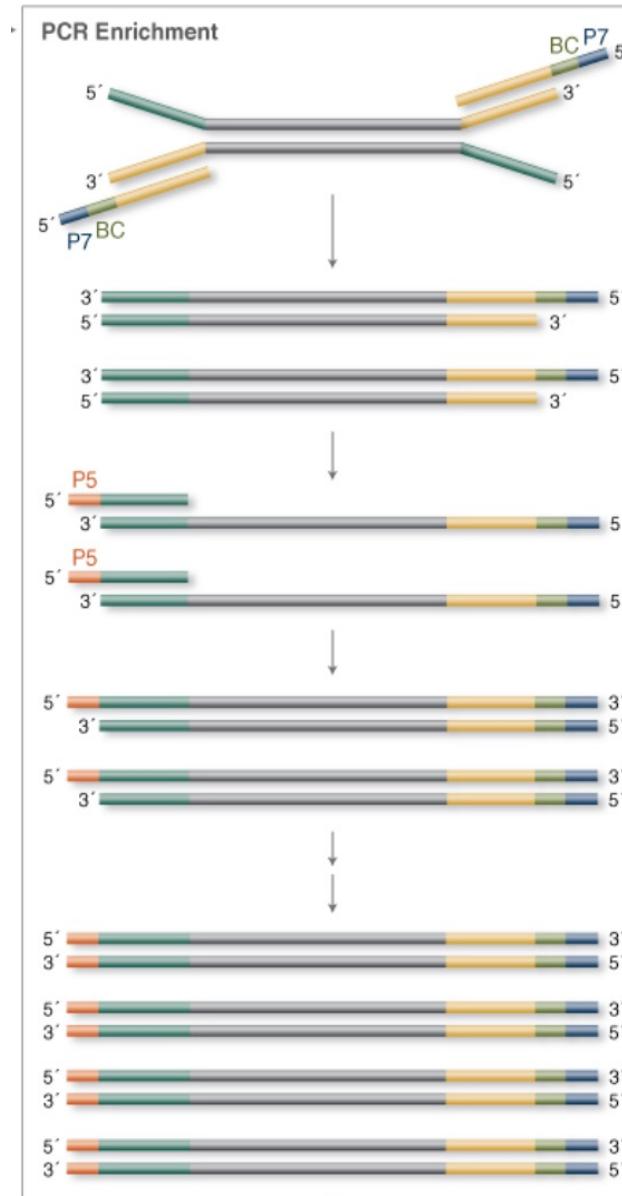
PCR Clean Up

Quality Control

Pool

Sequence

Library Amplification by PCR



- Increase the amount of library
- Select for libraries with adapters on each end
- Indexes can be added for multiplexing- 24 unique indexes



Clean Up/Size Selection



End Repair



Adapter Ligation



Clean Up/Size Selection



PCR Amplification



PCR Clean Up



Quality Control



Pool



Sequence

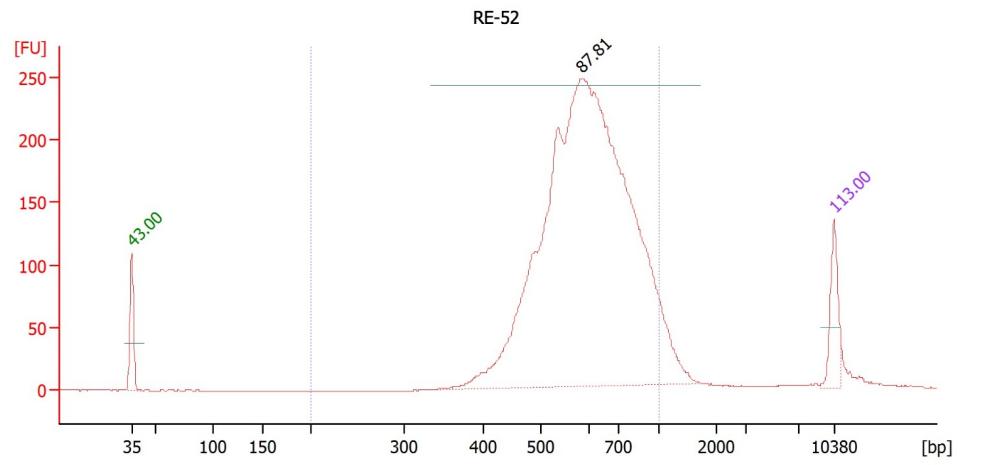
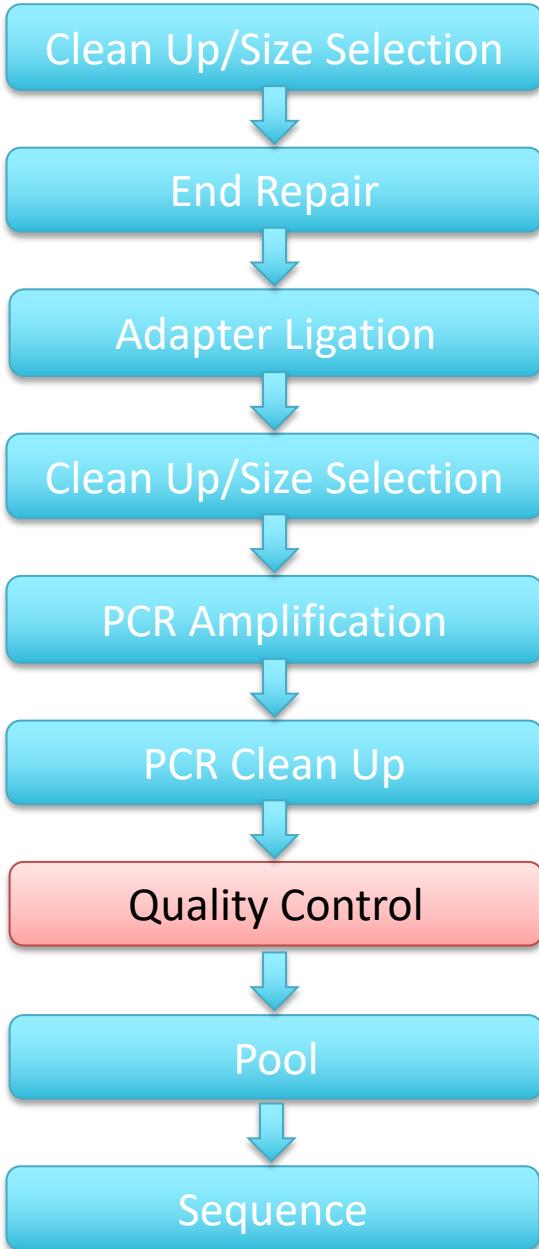
Clean Up- Post PCR Amplification

- Remove free barcodes, nucleotides
- Remove adapter dimers



Quality Control

- Agilent Bioanalyzer 2100
- Microfluidics platform for sizing and quantification



Overall Results for sample 9 : RE-52

Number of peaks found: 1 Corr. Area 1: 2,893.5
Noise: 0.3

Peak table for sample 9 : RE-52

Peak	Size [bp]	Conc. [pg/μl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	585	2,754.89	7,140.6	
3	10,380	75.00	10.9	Upper Marker



Pool Final Libraries

Clean Up/Size Selection



End Repair



Adapter Ligation



Clean Up/Size Selection



PCR Amplification



PCR Clean Up



Quality Control

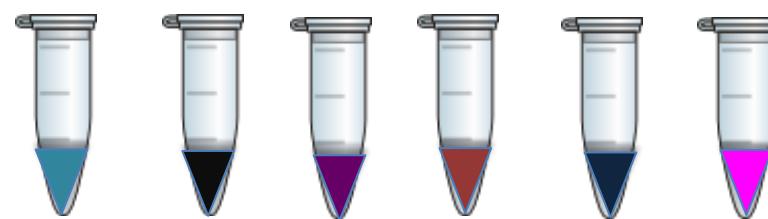
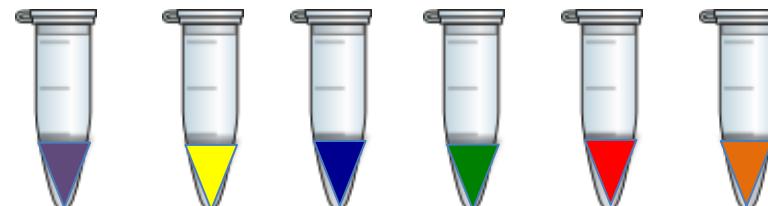


Pool



Sequence

- Individual Barcode for multiplexing
- Pool equal molar concentration
- Sequencing Core requires 20ul at 2-10nM



Clean Up/Size Selection



End Repair



Adapter Ligation



Clean Up/Size Selection



PCR Amplification



PCR Clean Up



Quality Control



Pool



Sequence

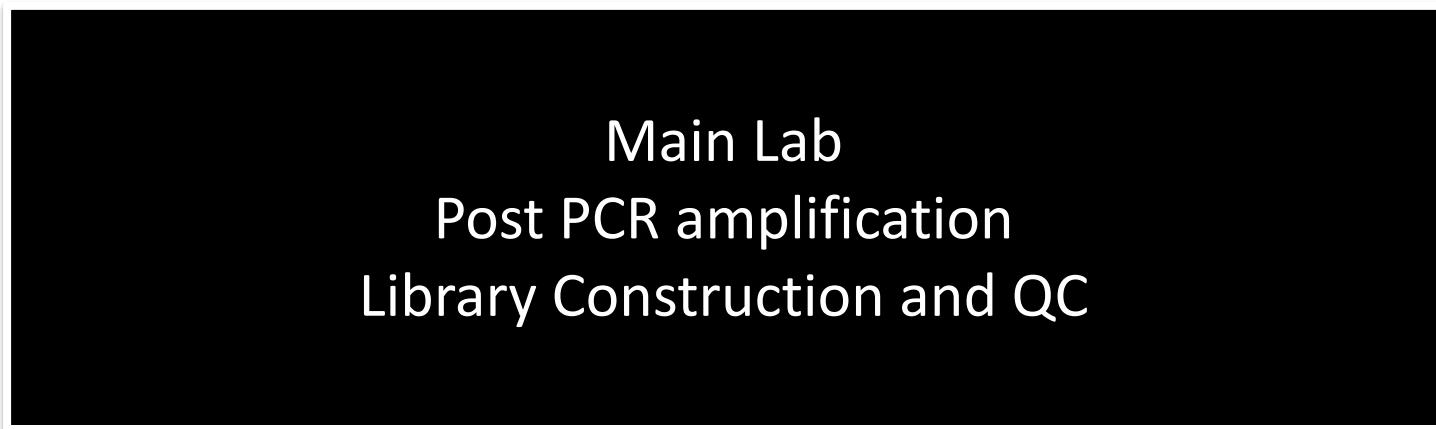
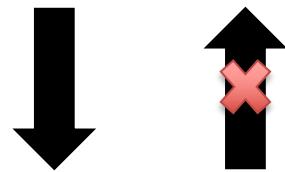
MiSeq V2 2X250



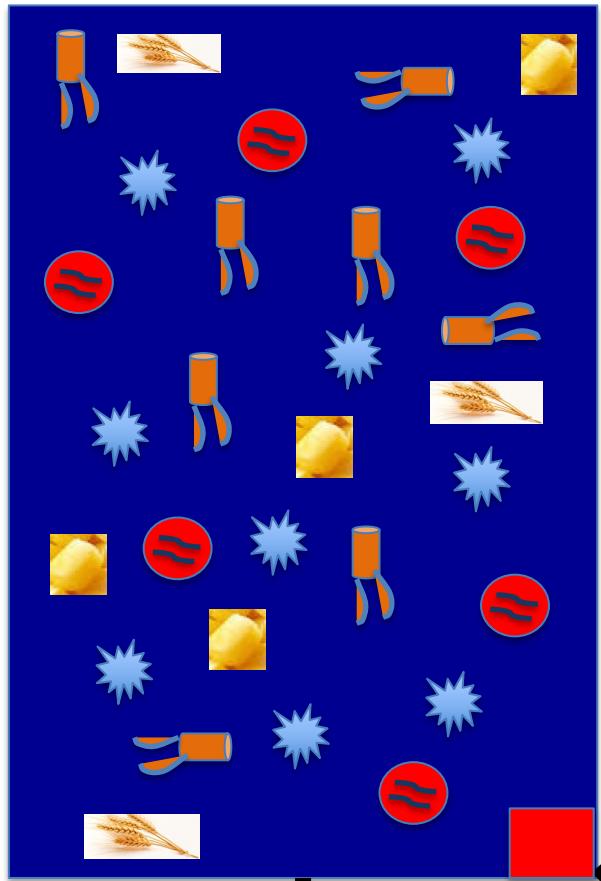
(pick your favorite)



Laboratory Layout



Stool Sample



Amplicon Sequencing

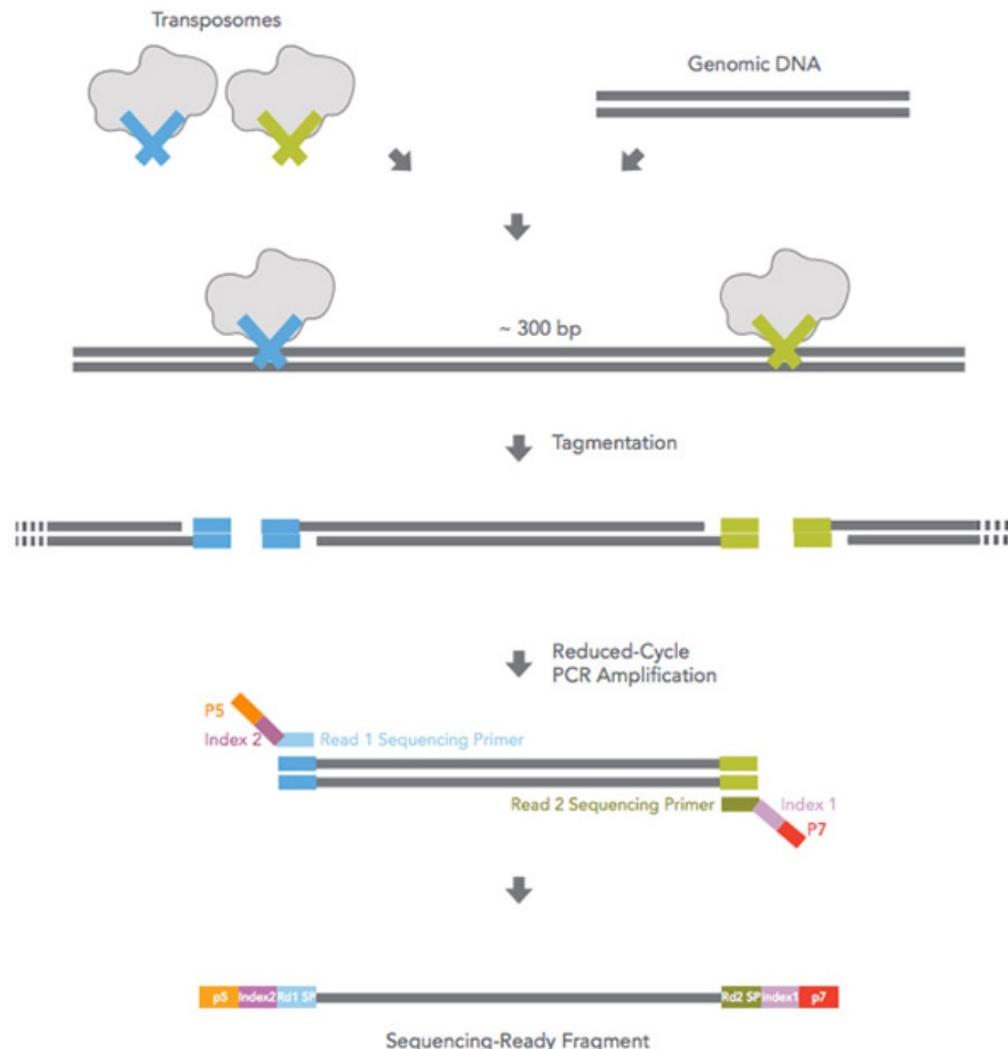
16S/18S/ITS
-DNA
-targeted Amplicons

Enriched Metagenome- Virome
Virus Like Particle (VLP)
-DNA, RNA
-remove bacteria
-remove host



Shotgun Sequencing

Nextera Library Preparation Biochemistry



Nextera chemistry simultaneously fragments and tags DNA in a single step. A simple PCR amplification then appends sequencing adapters and sample indexes to each fragment.

16S/ITS vs Virome

16S/18S/ITS- DNA only

- Chip-20mg/Buffer A
- Bead Beat- Lyse
- Extraction
- PCR Amplification/
Library Construction
- QC
- Sequence

Virome- DNA + RNA

- Chip-200mg/SM Buffer
- Vortex- Homogenize
- VLP enrichment
- Extraction
- Reverse Transcription,
Second Strand Synthesis,
PCR Amplification
- Library Construction
- QC
- Sequence

Shotgun

- Chip
- Bead Beat- Lyse
- Extraction
- Library Construction
- QC
- Sequence

Acknowledgements



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