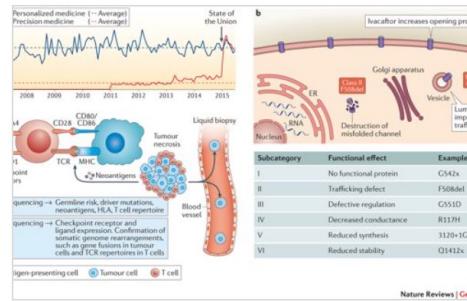


# From Sample to Seq

Lindsay Droit  
Washington University School of  
Medicine  
St. Louis, Missouri

# Next Gen Sequencing Applications

Nature Reviews Genetics | Review Article



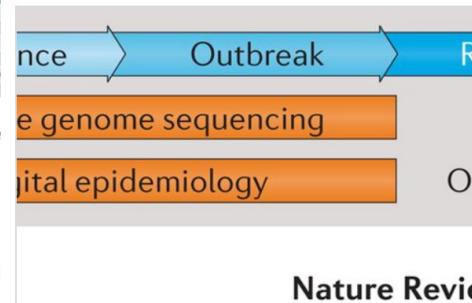
## Towards precision medicine

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

Nature Reviews Genetics | Review Article

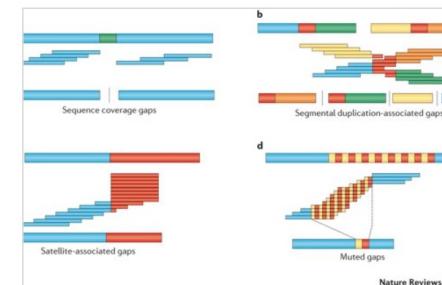


## Towards a genomics-informed, real-time, global pathogen surveillance system

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

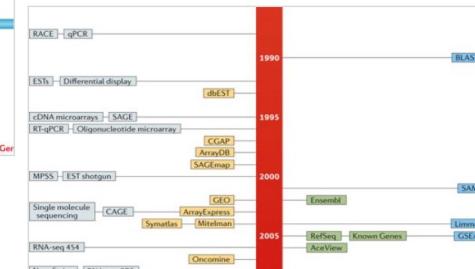


## Genetic variation and the *de novo* assembly of human genomes

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

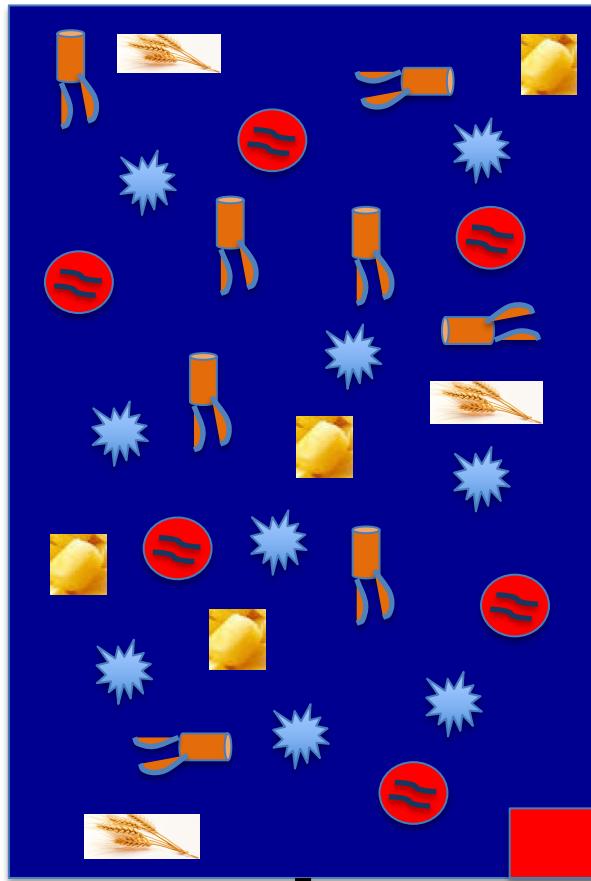
Although cancer genome sequencing is becoming routine in cancer research, cancer transcriptome profiling through methods such as RNA sequencing (RNA-seq) provides information not only on mutations but also on their functional cellular consequences. This Review discusses how technical and analytical advances 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

# Using Next Generation Sequencing to Define the Microbiome

- 1.) Amplicon Surveys- targeted amplicons
  - 16S (bacteria)
  - 18S (bacteria)
  - ITS (fungi)
  
- 2.) Shotgun Metagenomics-
  - entire metagenome: sacrifice resolution
  - enriched metagenome- Virome

# 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 at least 200mg, <1500mg 
- Fluid type samples eg; serum, sputum, etc: 1mL

## Storage Conditions

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

Original Sample



Chip/Aliquot



Lyse



Extract DNA



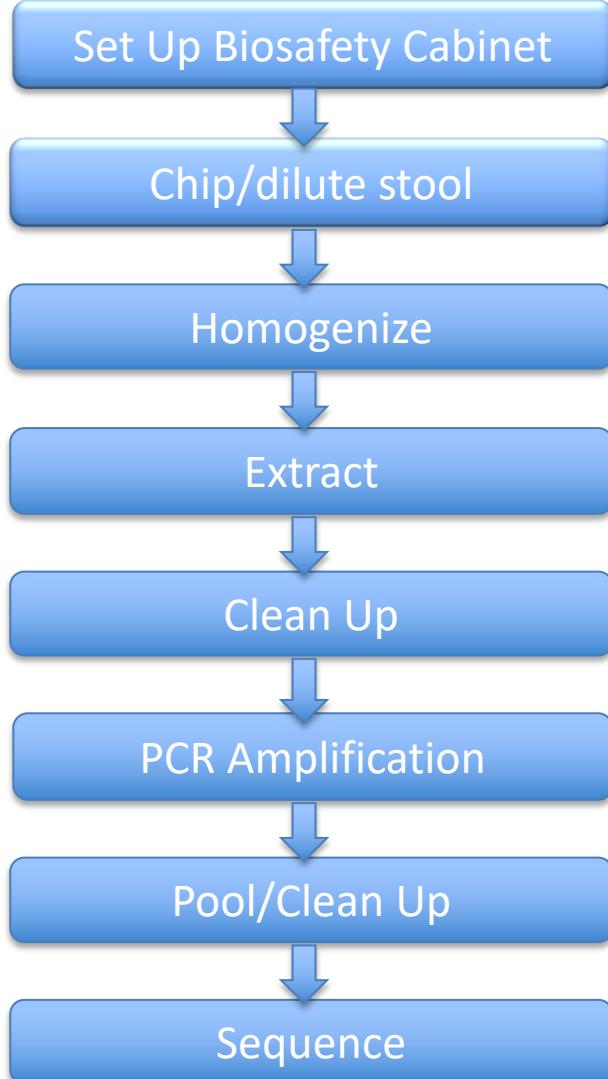
Amplification



Pool



Final Library



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

Set Up Biosafety Cabinet

Chip/dilute stool

Homogenize

Extract

Clean Up

PCR Amplification

Pool/Clean Up

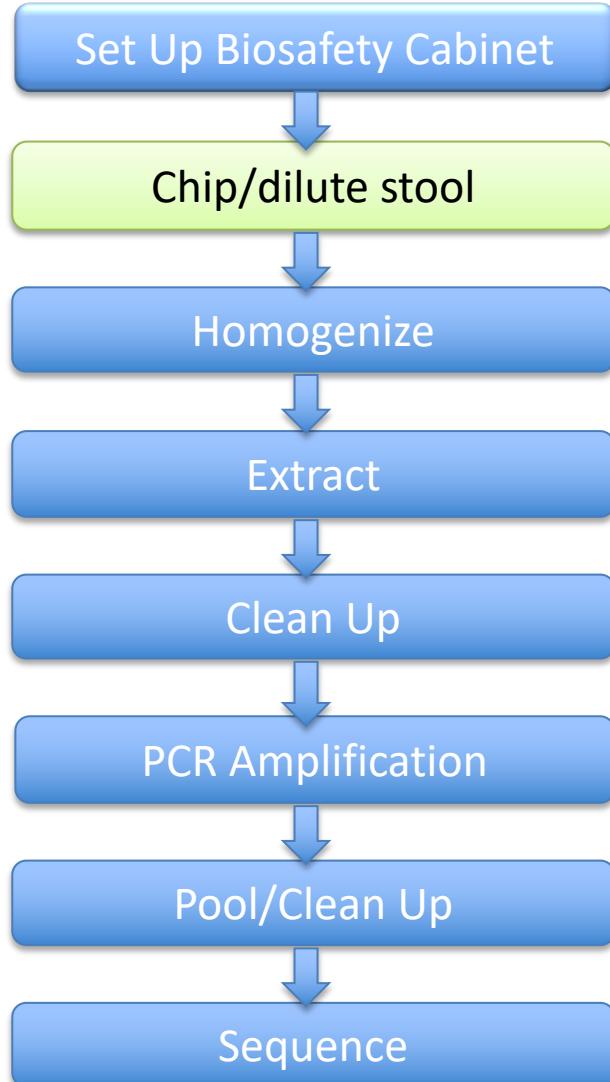
Sequence

# Biosafety Cabinet Set Up

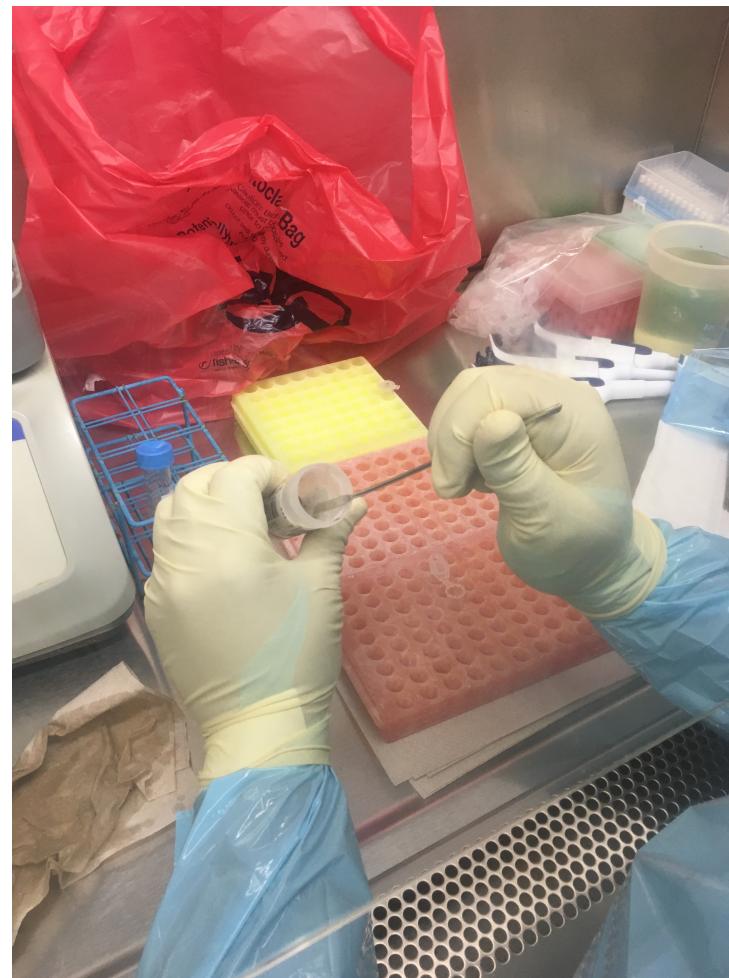
- Samples are handled at BSL2+- gown and double glove
- Decon with 10% bleach solution, 70% ethanol, and UV for 30 minutes
- Waste collected in biohazard bags and autoclaved

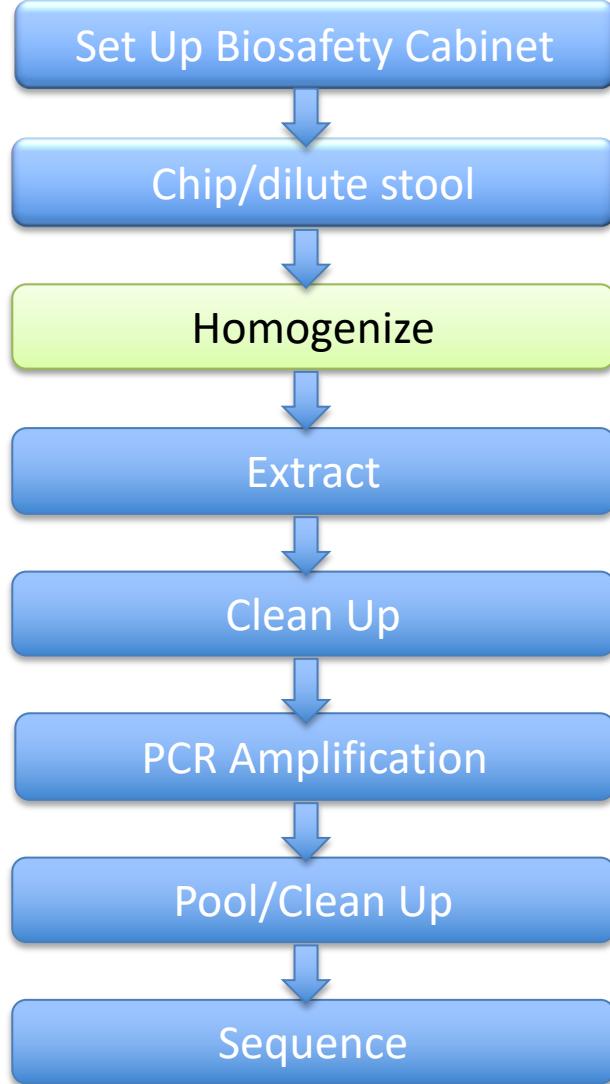


# Chip Stool Sample



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



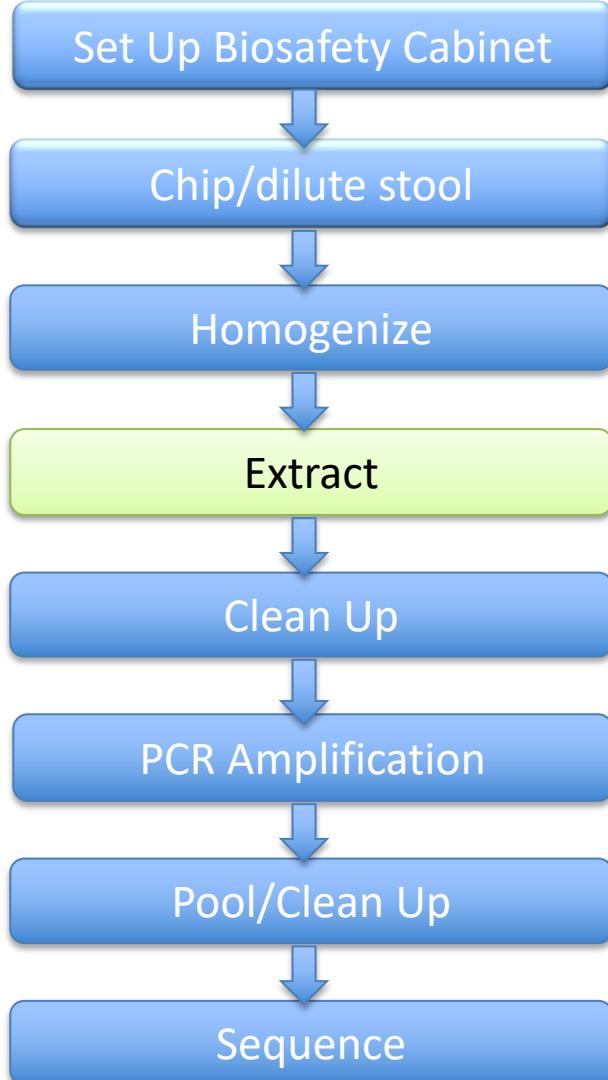


# Homogenize Samples

- Bead beat for 1 minute
- Lyse cells to release bacterial nucleic acid



# Crude DNA Extraction



Aqueous solution of homogenized stool



Equal volume phenol:chloroform:IAA



Vortex



Centrifuge



Pipette Aqueous Layer

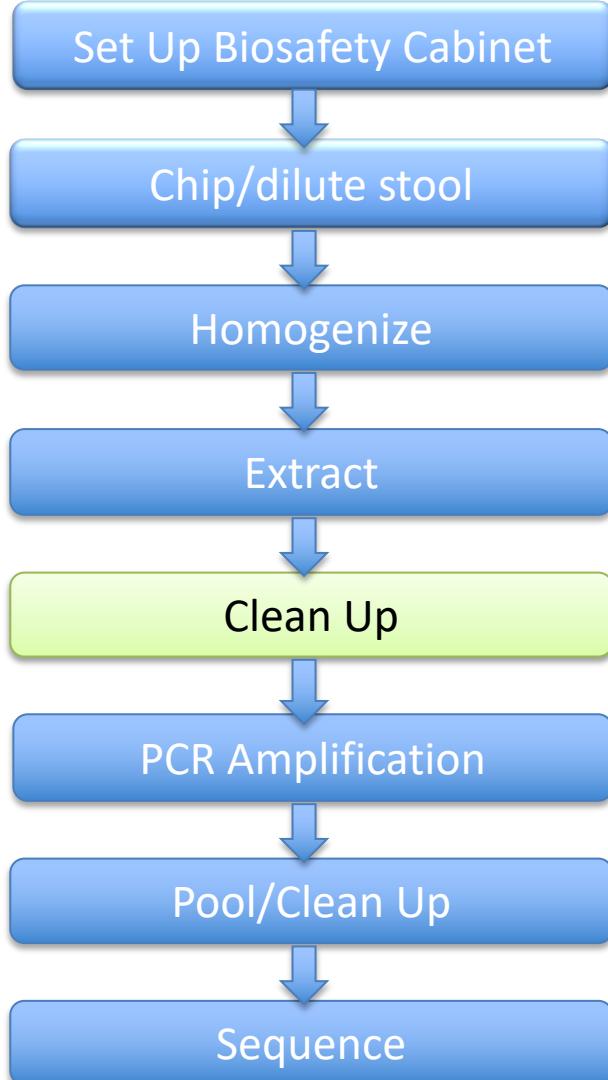


Isopropanol Precipitation

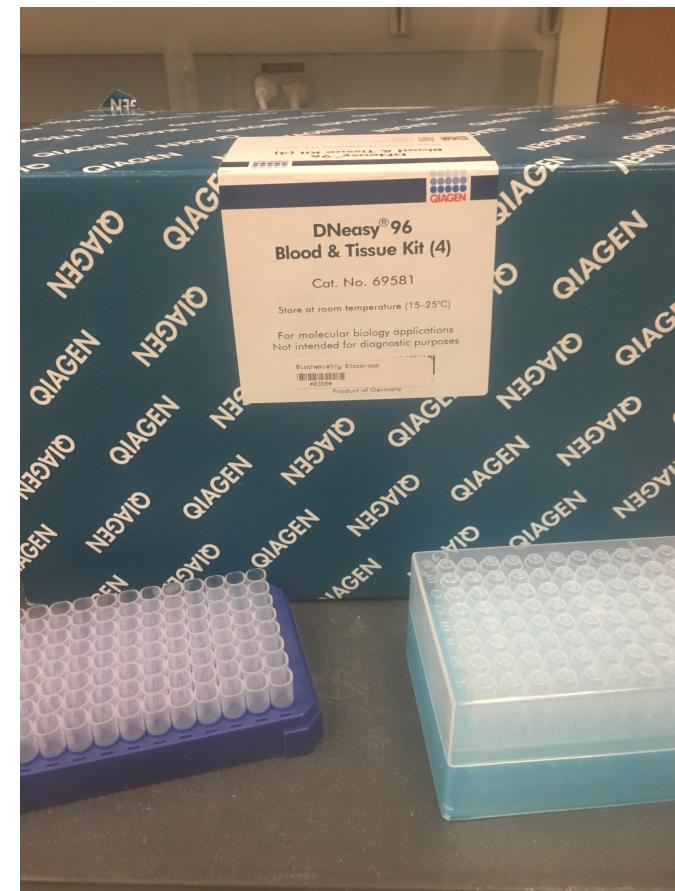


Resuspend in 50ul of H<sub>2</sub>O

# Qiagen DNeasy

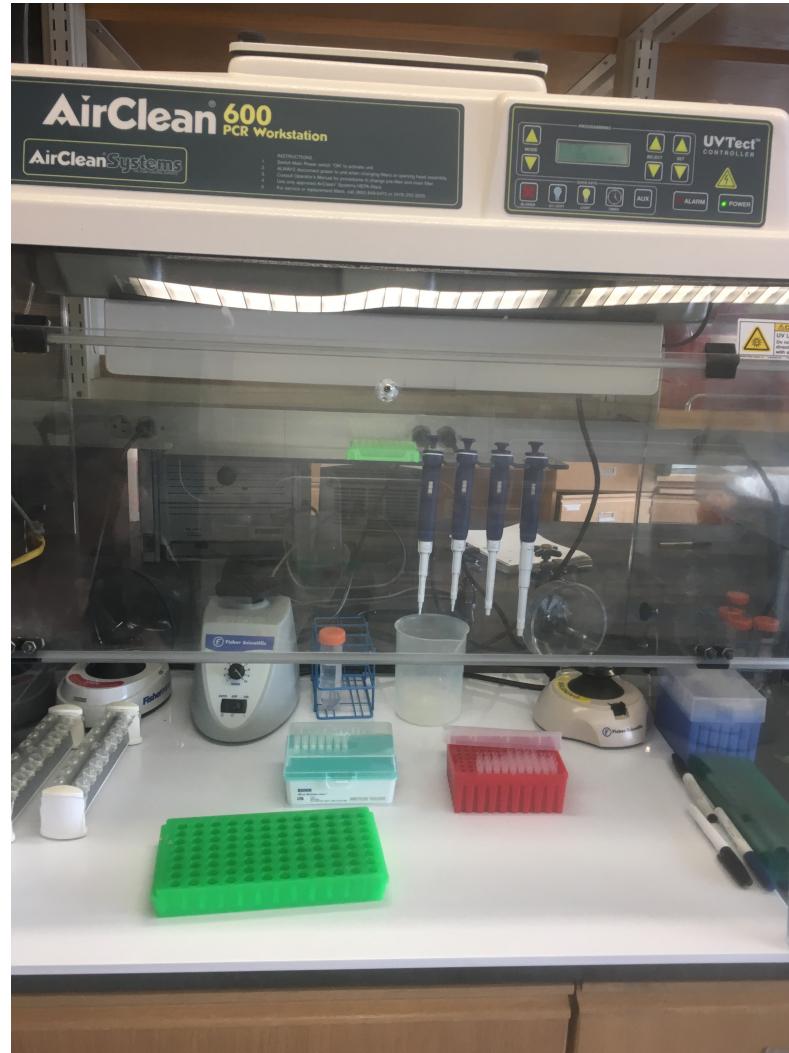


- P:C:I extracted nucleic acid is not pure enough for PCR
- Samples arranged in 96 well format for remainder of protocol



# 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

A	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96
B	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96
C	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96
D	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96

Negative Control Plate

A	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96
B	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96
C	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96
D	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96

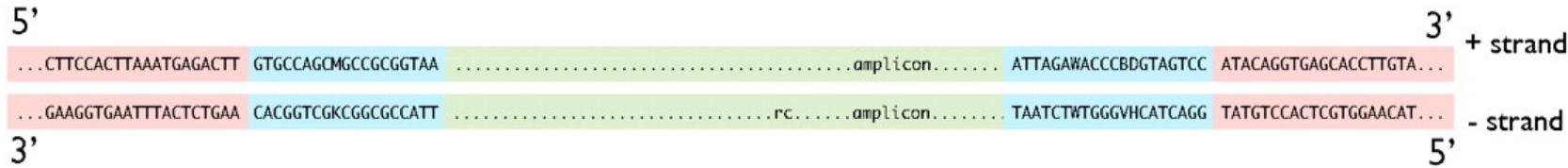
Sample Plate

	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	.....

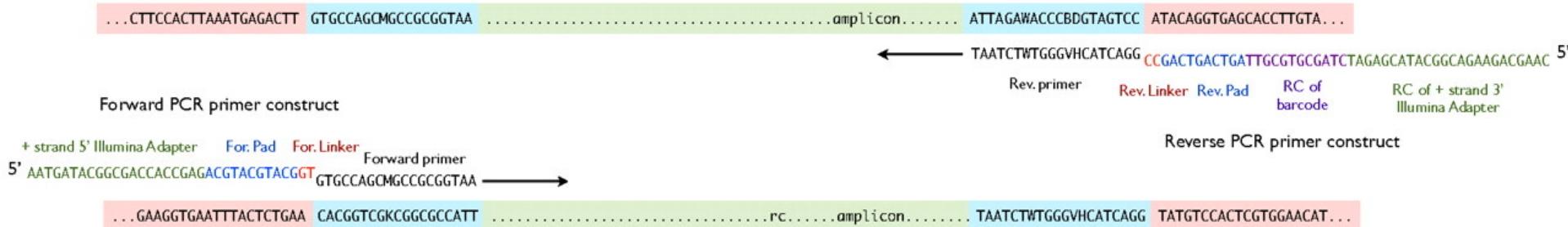
Primer Plate

	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:



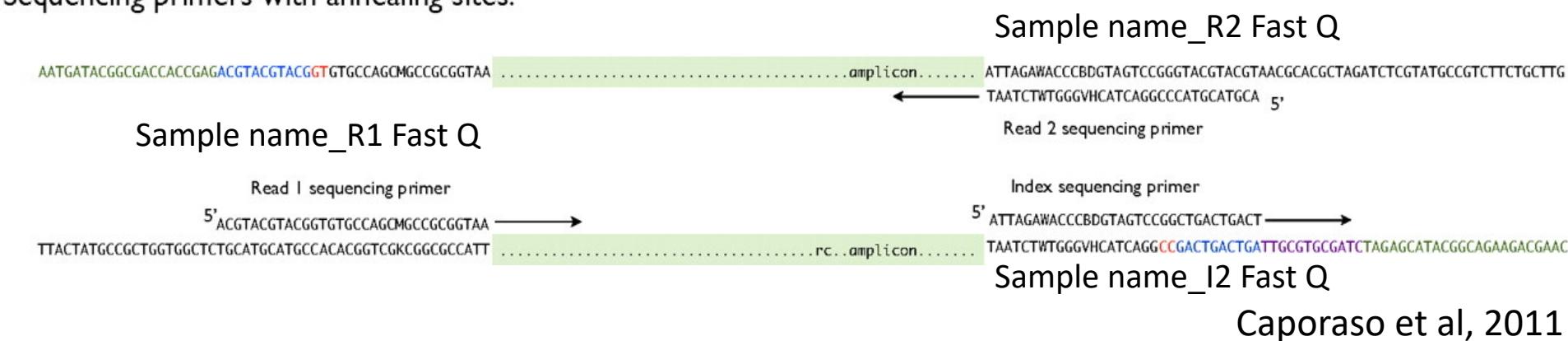
Amplification primers with annealing sites:



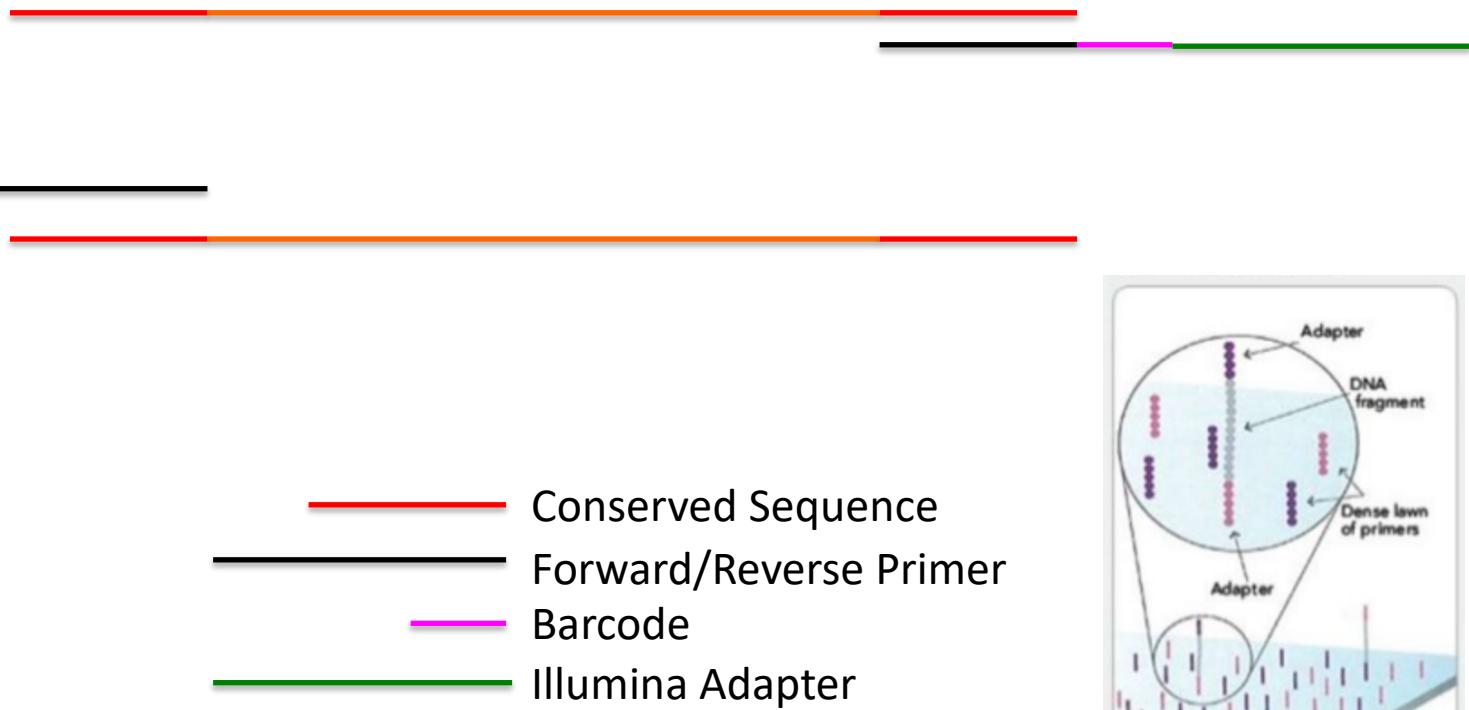
Amplification products:



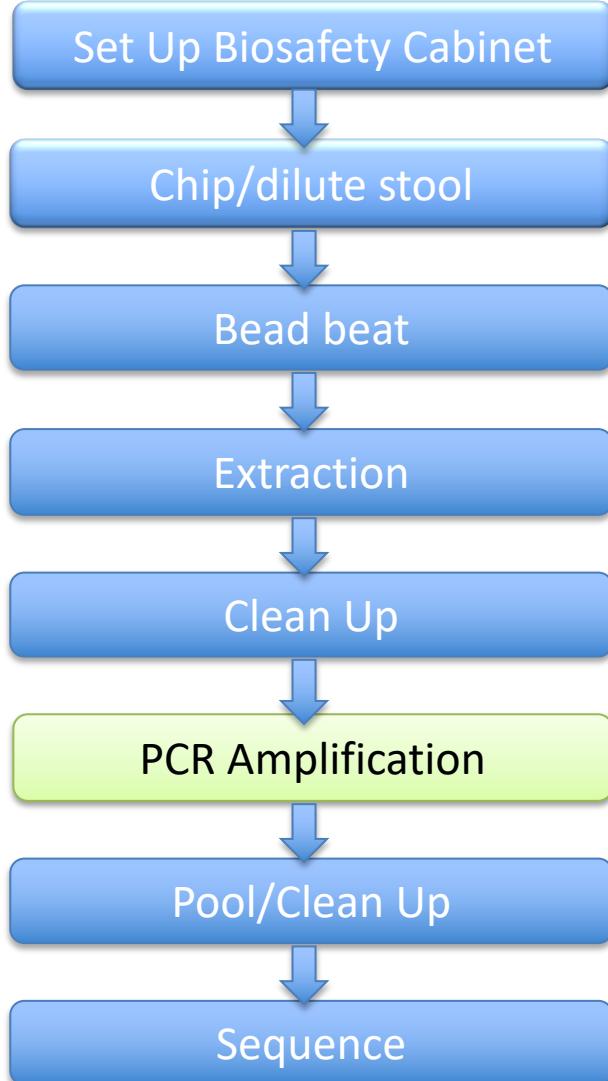
Sequencing primers with annealing sites:



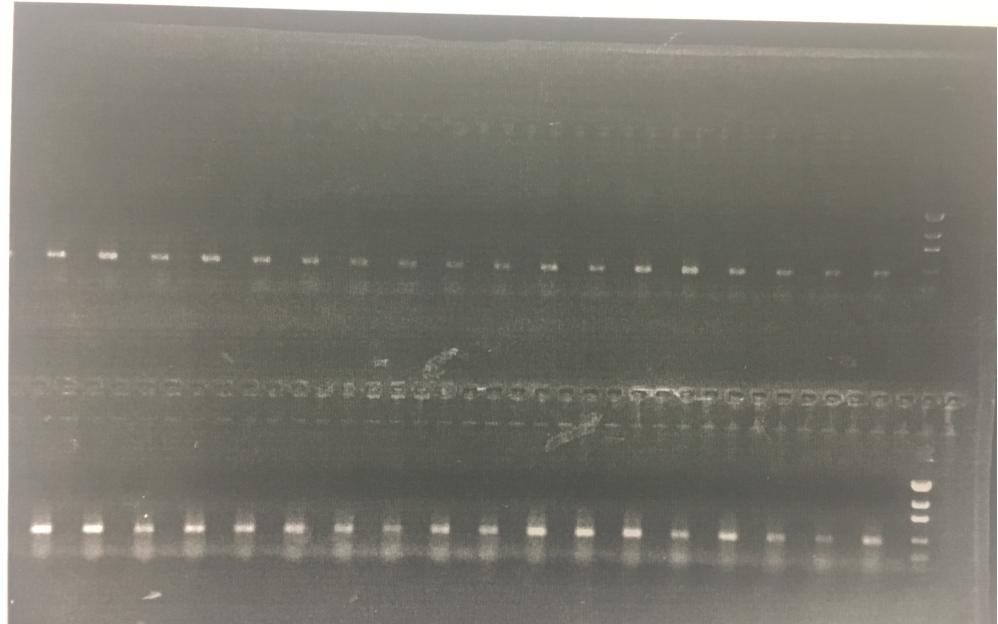
# Primer Design



# Post PCR Amplification



- Triplicate reactions are pooled into single plate
- 1% Agarose gel- 5ul of amplified DNA
- Expected band 16S=390bp, 18S=260bp, ITS= 250-600bp

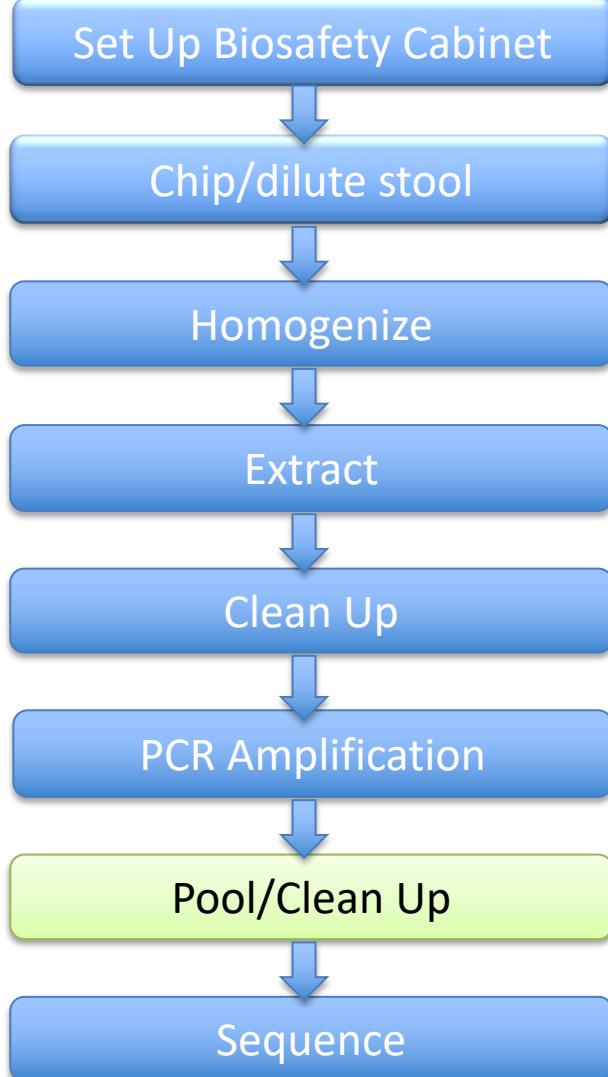


Failure Rate: ~5%

Troubleshooting No Band:

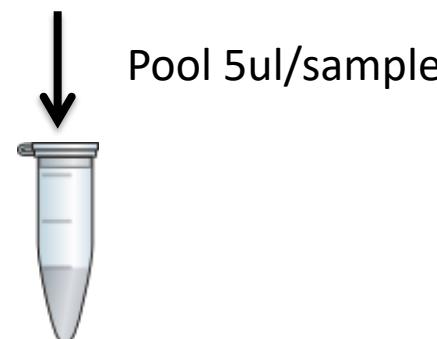
- Antibiotics treated samples often don't show a band
- PCR inhibitors- sometimes a 1:10 or 1:100 dilution of the sample will work

# Sample Pooling and Clean Up

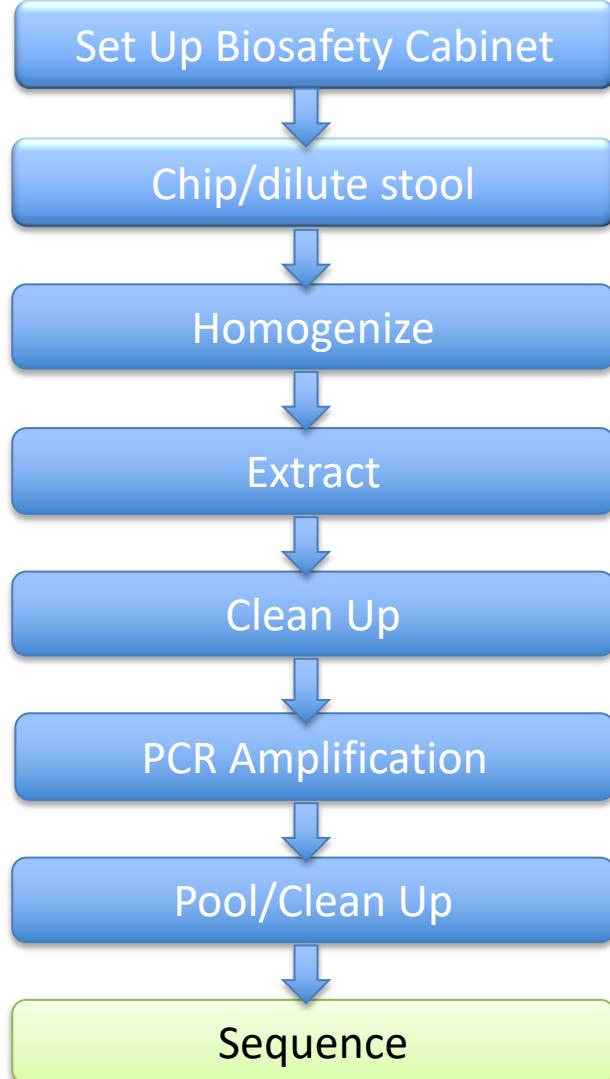


- Pool 5ul per sample. Including H2O negative controls
- Clean Up
- Quantify Using Nanodrop

	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



Pool 5ul/sample



MiSeq V2 2X250



(pick your favorite)

# 16S/18S/ITS Summary

16S/18S/ITS are all set up the same way in 96 well plate format.

Differences in protocol:

- Primers
- Thermocycler conditions
- Amplicon size
  - 16S amplicon size is 390bp
  - 18S amplicon size is 260bp
  - ITS amplicon size is variable ~250-600bp

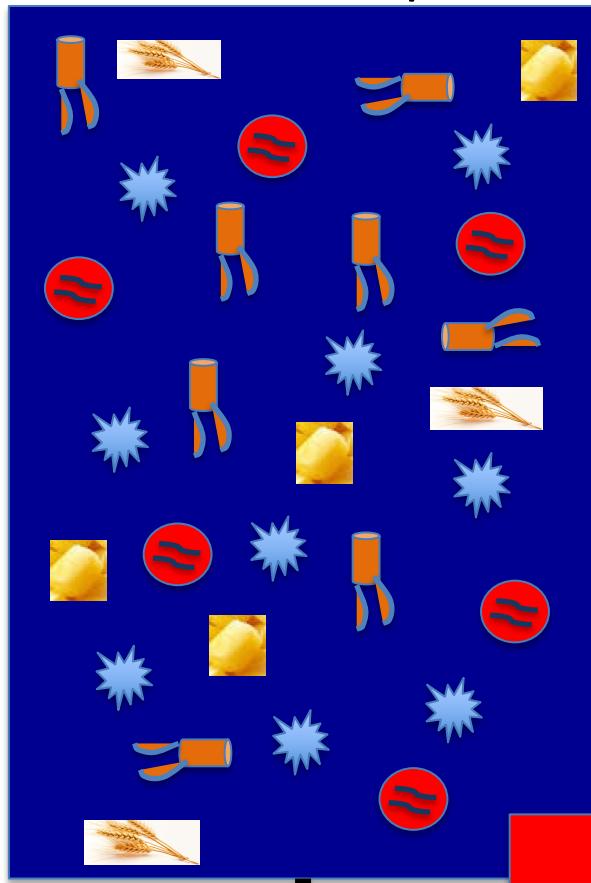


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<http://www.earthmicrobiome.org>

# Stool Sample



## Amplicon Sequencing

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

## Enriched Metagenome- Virome

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



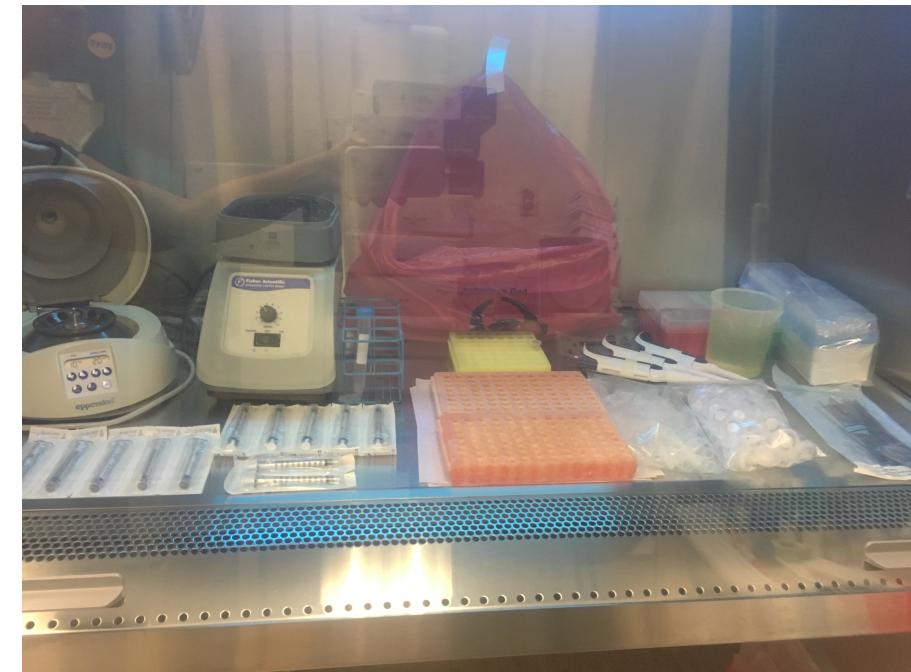
Filter



DNase/Lysozyme



TNA Extraction



- Samples are handled at BS<sub>L</sub>2+- 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

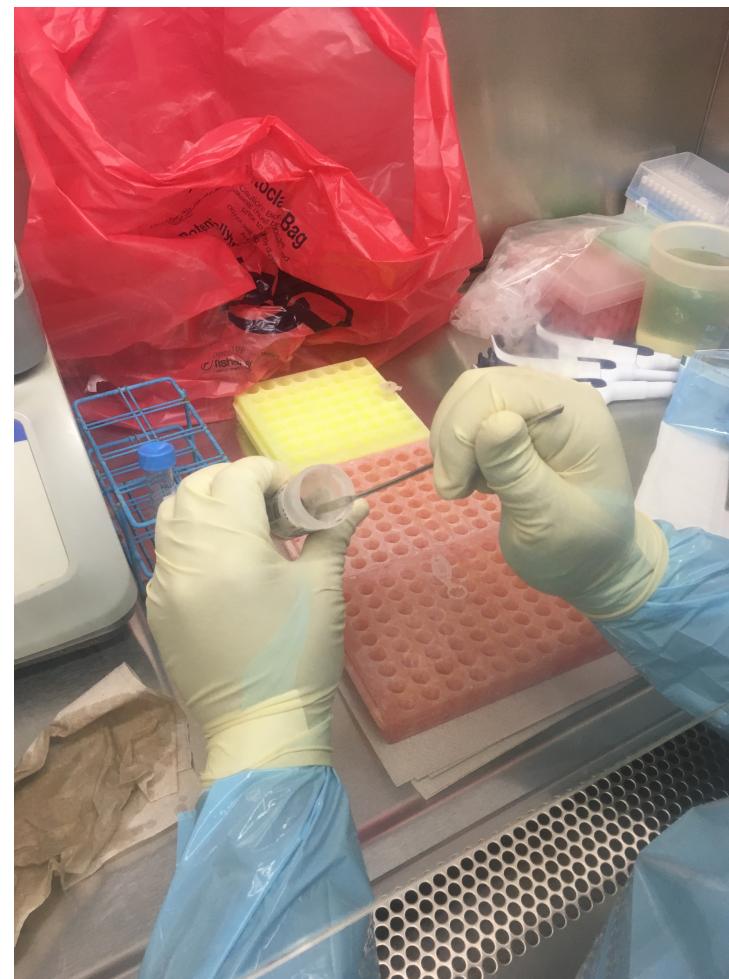
Centrifuge

Filter

DNase/Lysozyme

TNA Extraction

- Keep sample frozen
- Chip ~200mg of stool
- Add SM Buffer (NaCl, Tris, MgSO<sub>4</sub>)



# Homogenize Samples

Set Up Biosafety Cabinet



Chip/Dilute



Vortex



Centrifuge



Filter



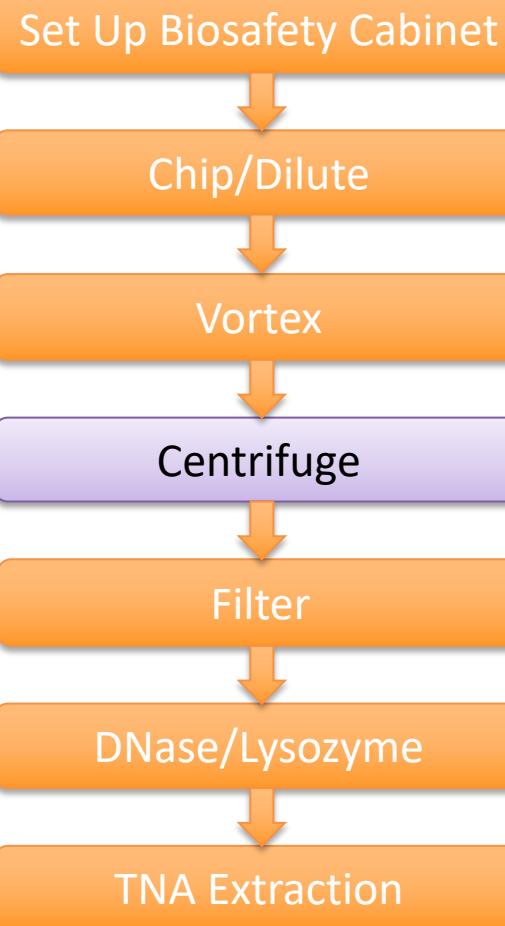
DNase/Lysozyme



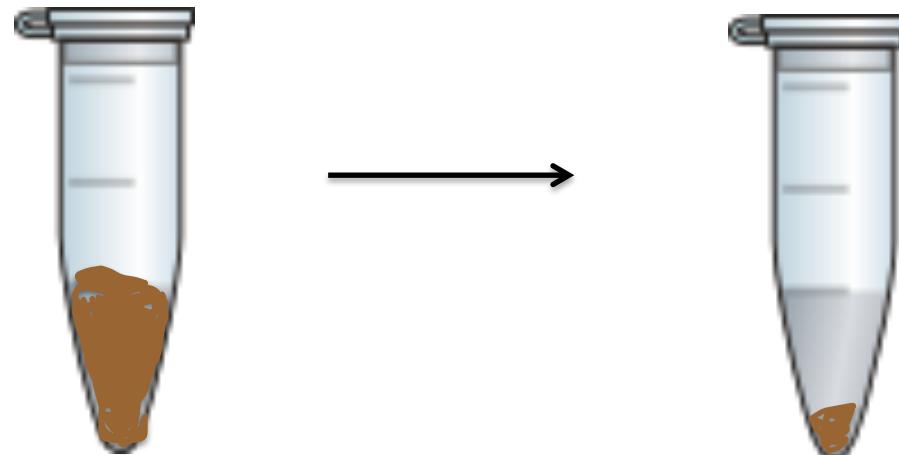
TNA Extraction



# Centrifuge



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



# Filter to Remove Bacteria

Set Up Biosafety Cabinet



Chip/Dilute



Vortex



Centrifuge



Filter



DNase/Lysozyme

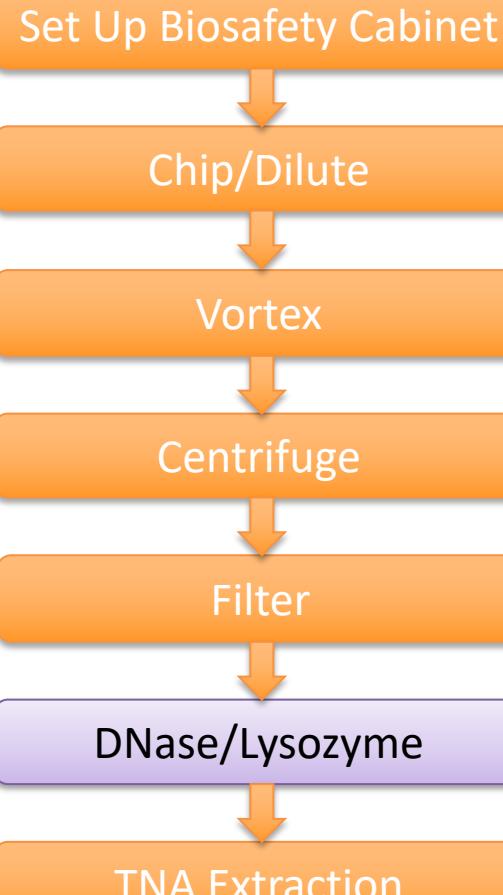


TNA Extraction

- .45 $\mu$  filter



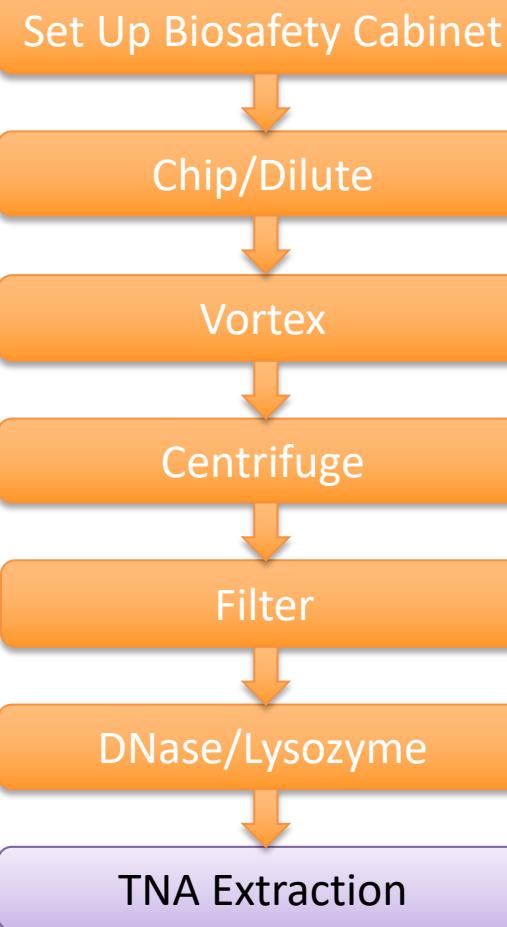
# Non Encapsulated DNA Removal



- Lysozyme DNase Enzyme cocktail
- Degrade non-encapsulated DNA

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

# Total Nucleic Acid Extraction



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



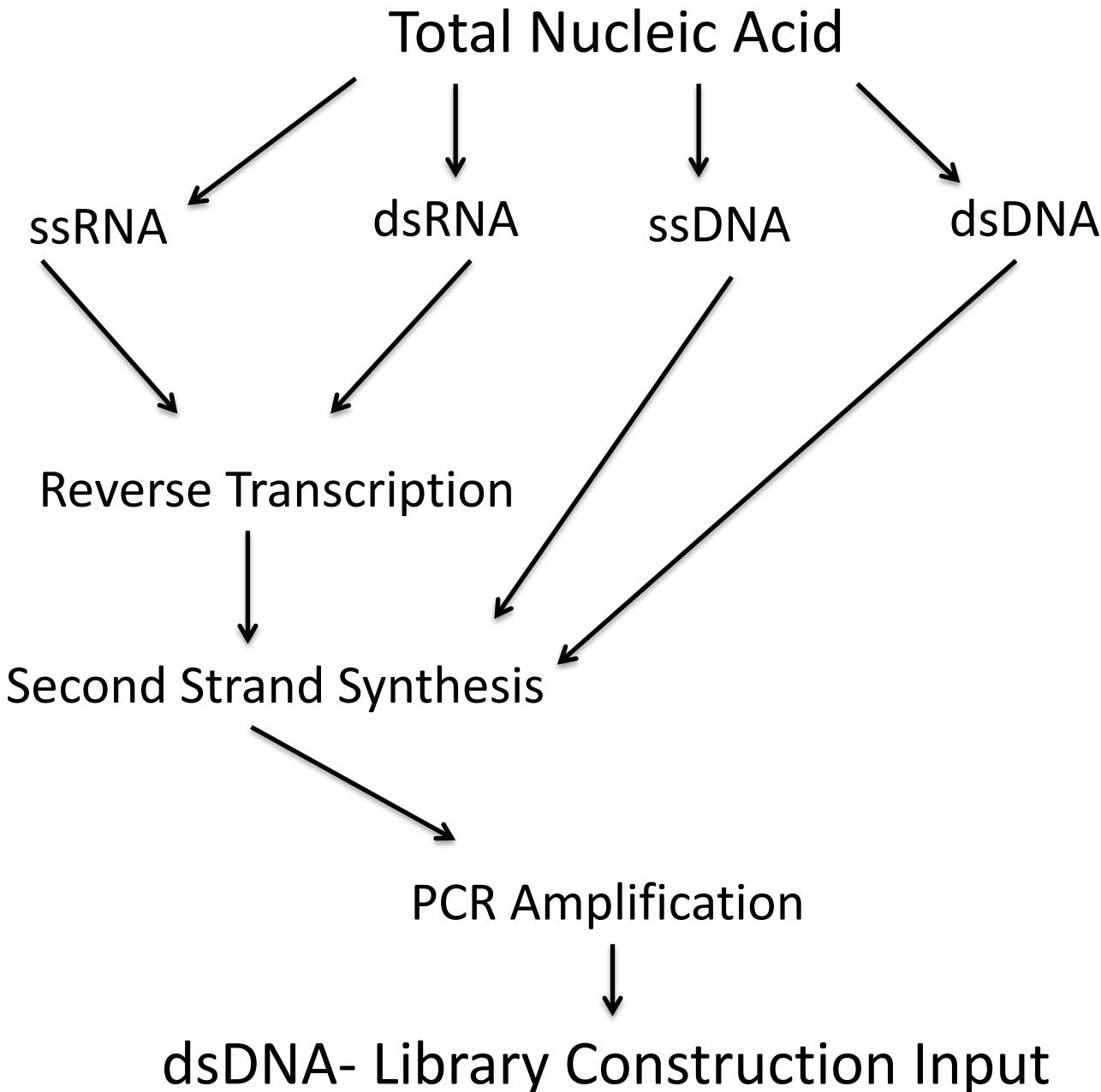
# Preparing Samples for Virome Sequencing: 3 Steps

1.) Virus Like Particle (VLP)

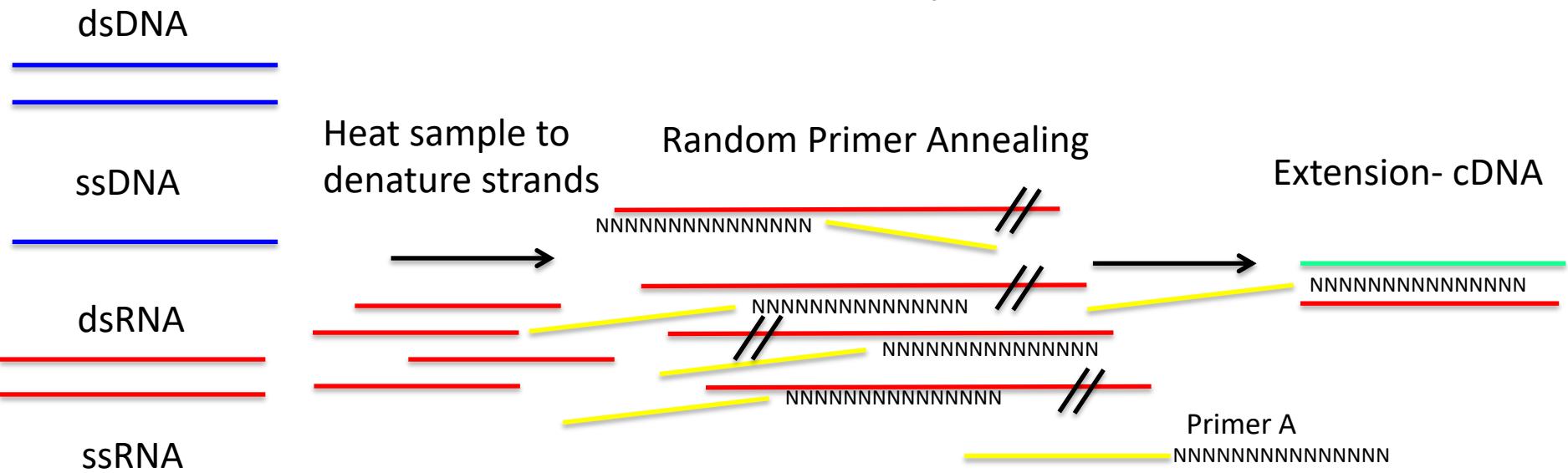
Enrichment and Total Nucleic Acid Extraction

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

3.) Library Construction



# Reverse Transcription



## Second Strand Synthesis

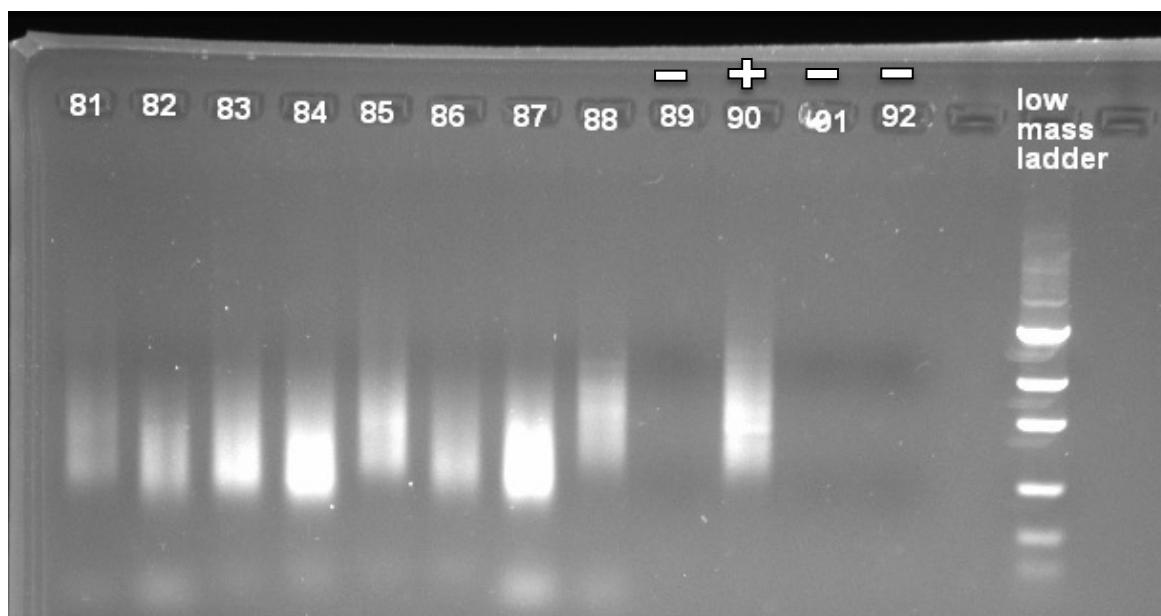


## PCR Amplification

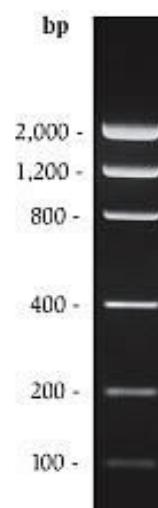


# Post PCR Amplification

- 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

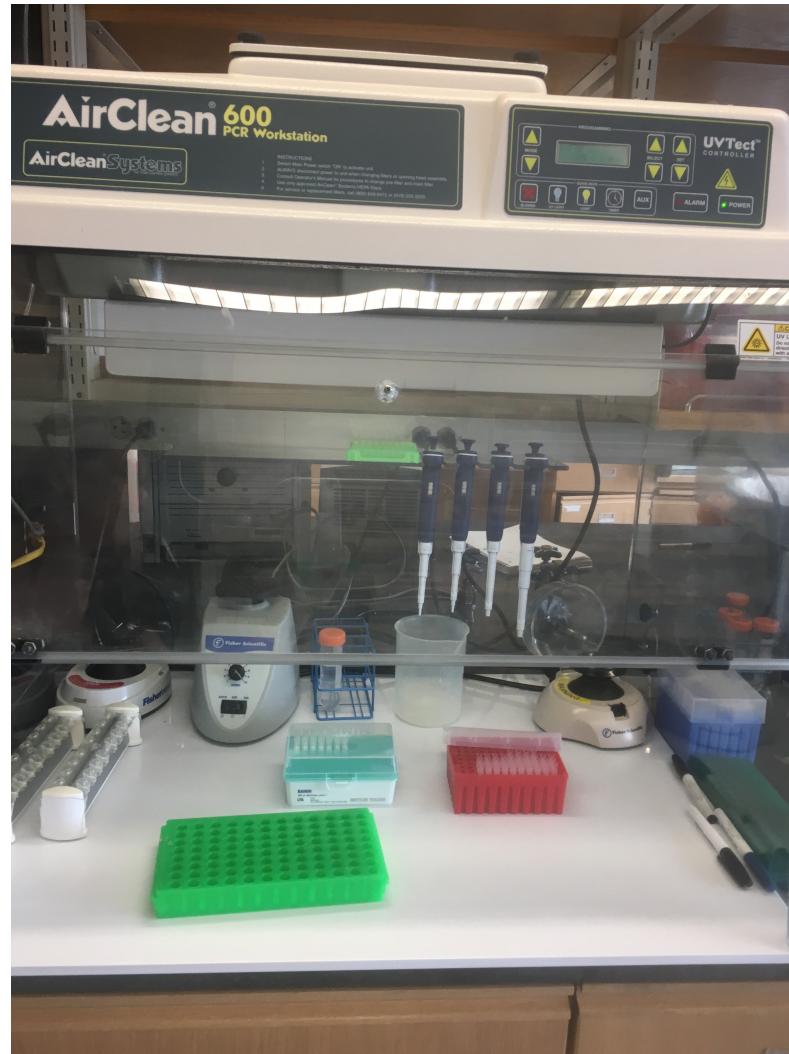
Pool

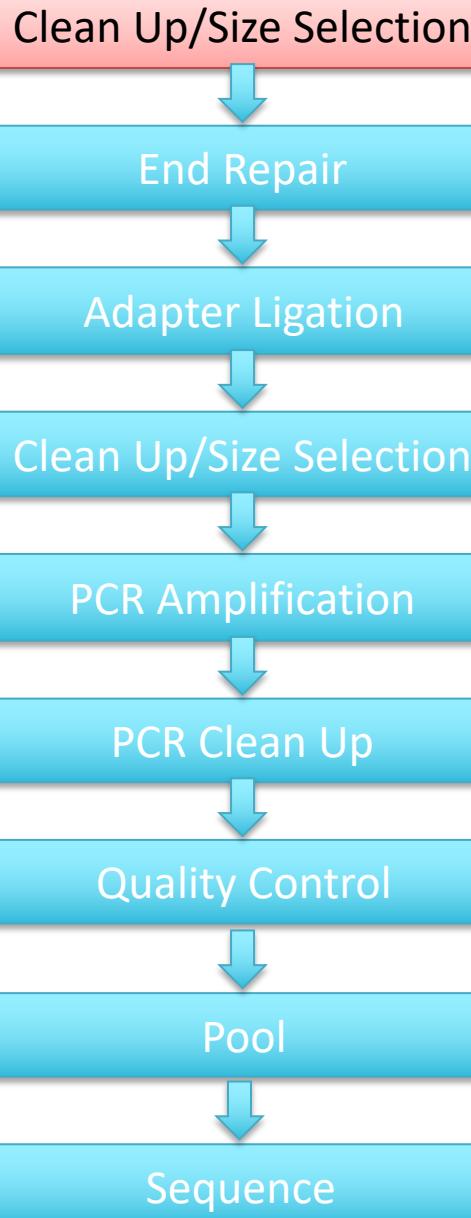
Sequence

# New England Biolabs NEB Next DNA Library Construction

# PCR Workstation

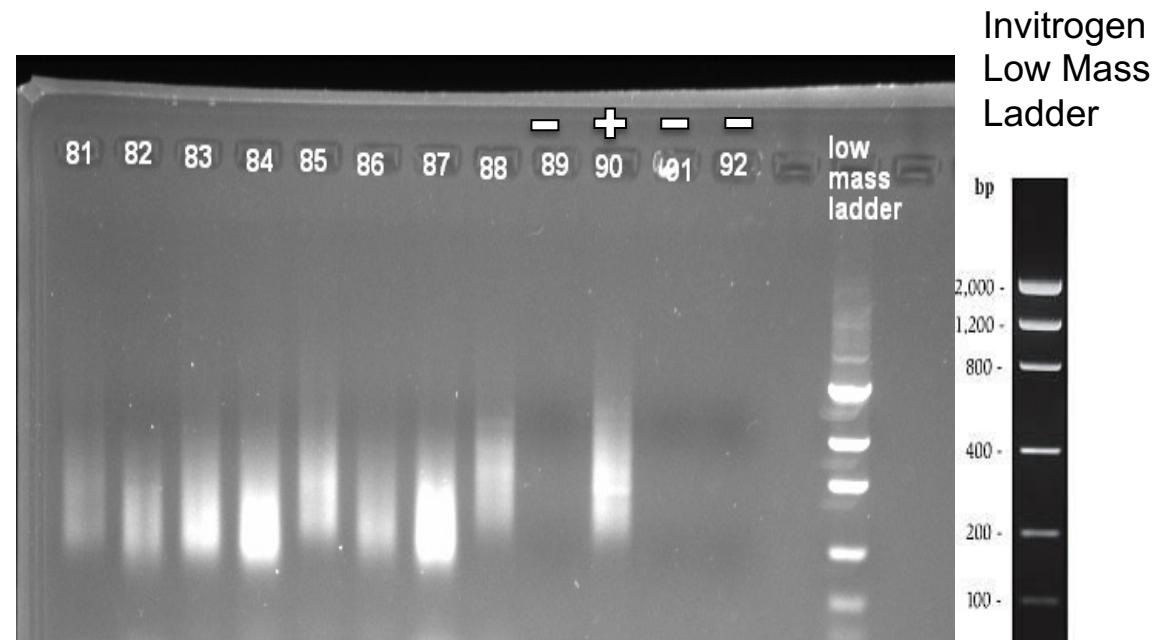
- Vertical Laminar air flow
- Hepa filtration system
- Built in UV





## 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

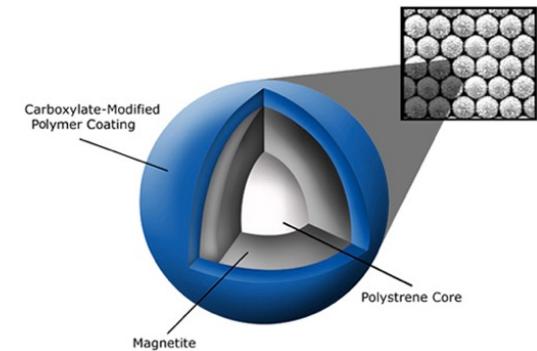
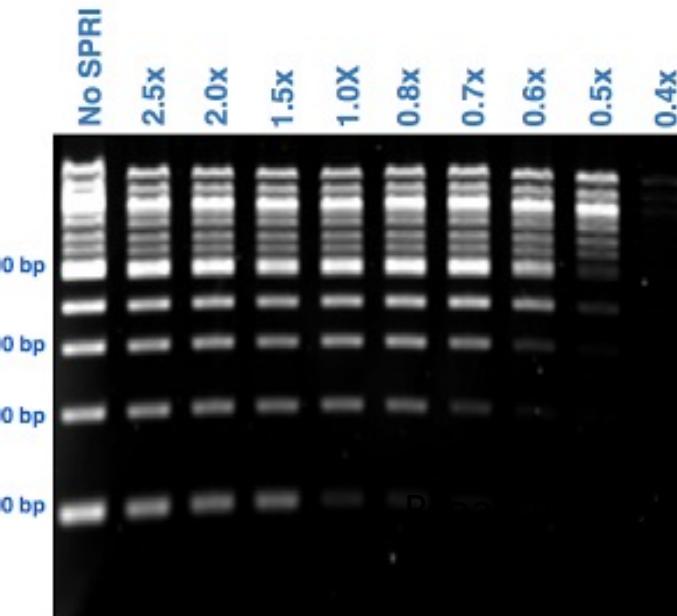


Pool

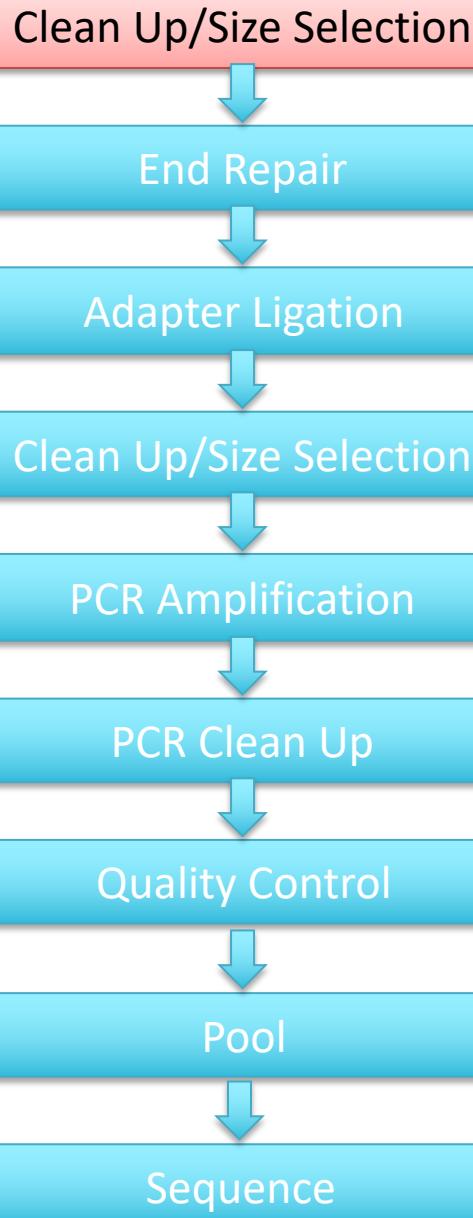


Sequence

- 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

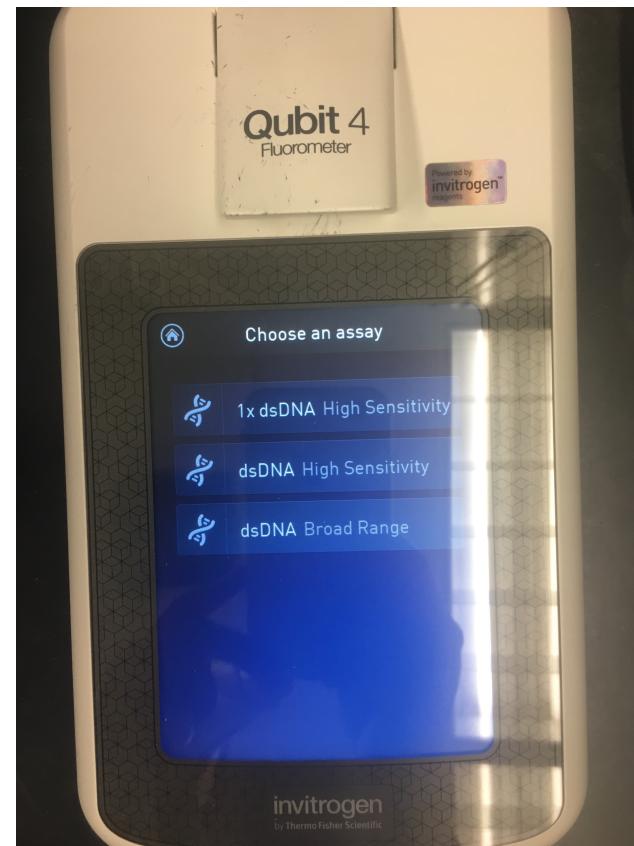


<https://youtu.be/zGV0SjCe0CU>



## Sample Quantification

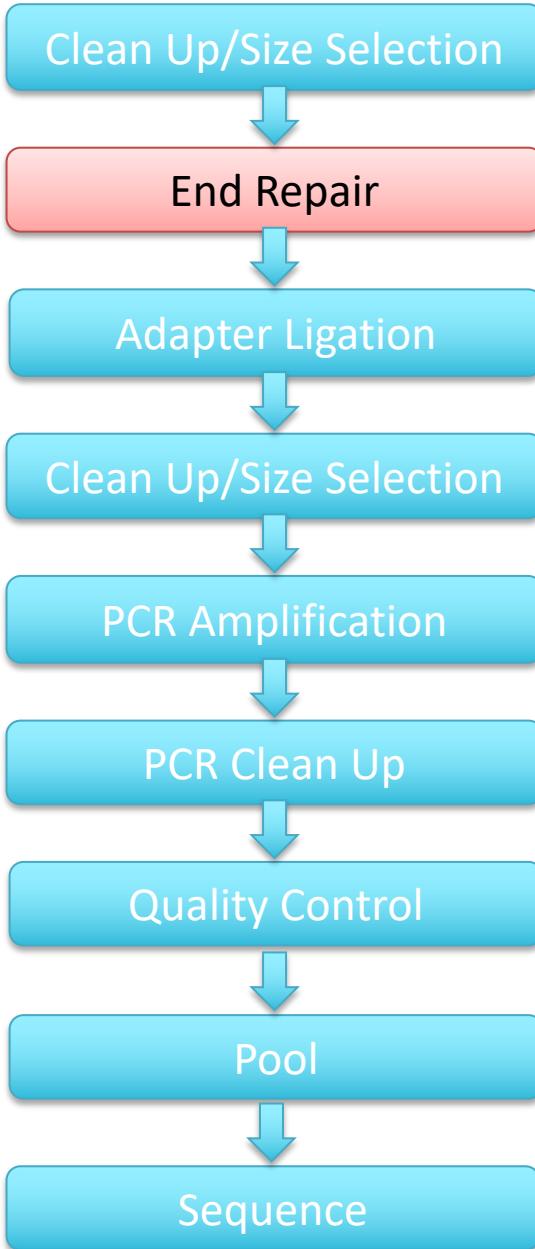
- Library input DNA 20-100ng
- Can go as low as 5ng
- Knowing input is critical for downstream steps- adapter concentration and PCR amplification cycle number



# End Repair

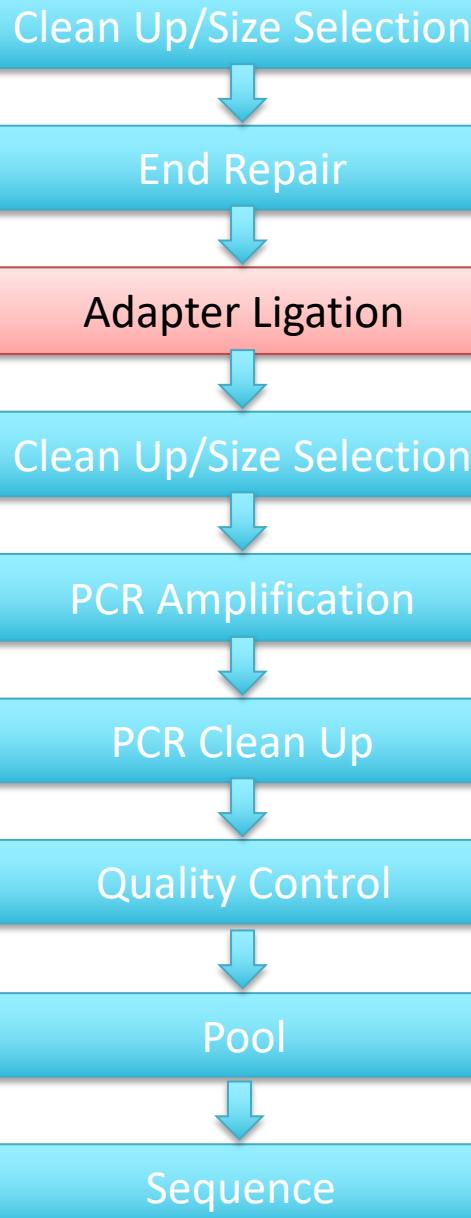
## 5' Phosphorylation and dA-Tailing

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



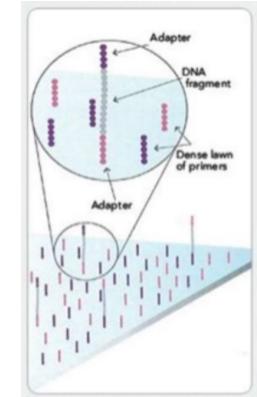
### End Repair, 5' Phosphorylation and dA-Tailing





# 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

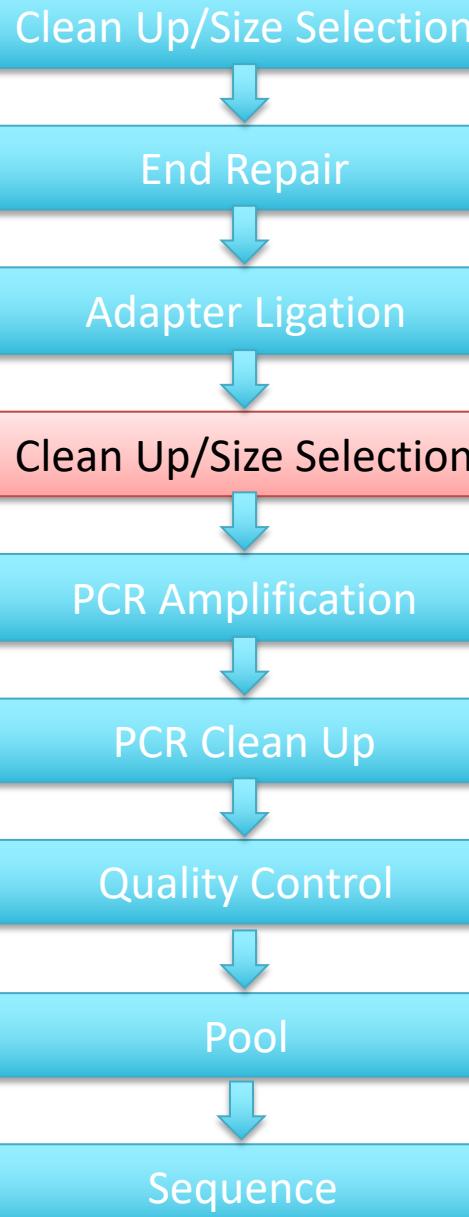


## Adaptor Ligation with optional NEBNext Adaptor



## 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

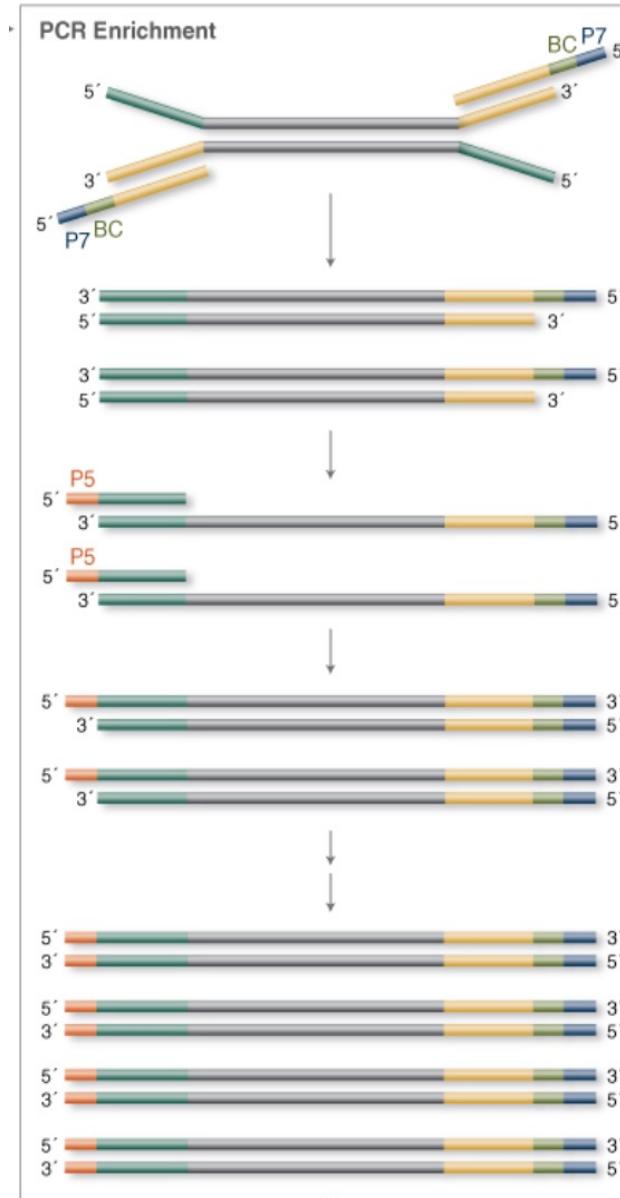
PCR Clean Up

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



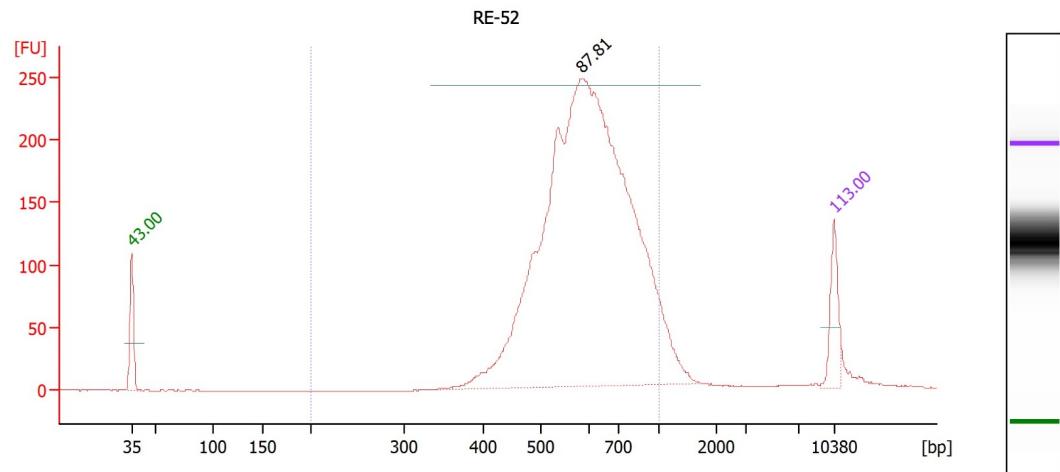
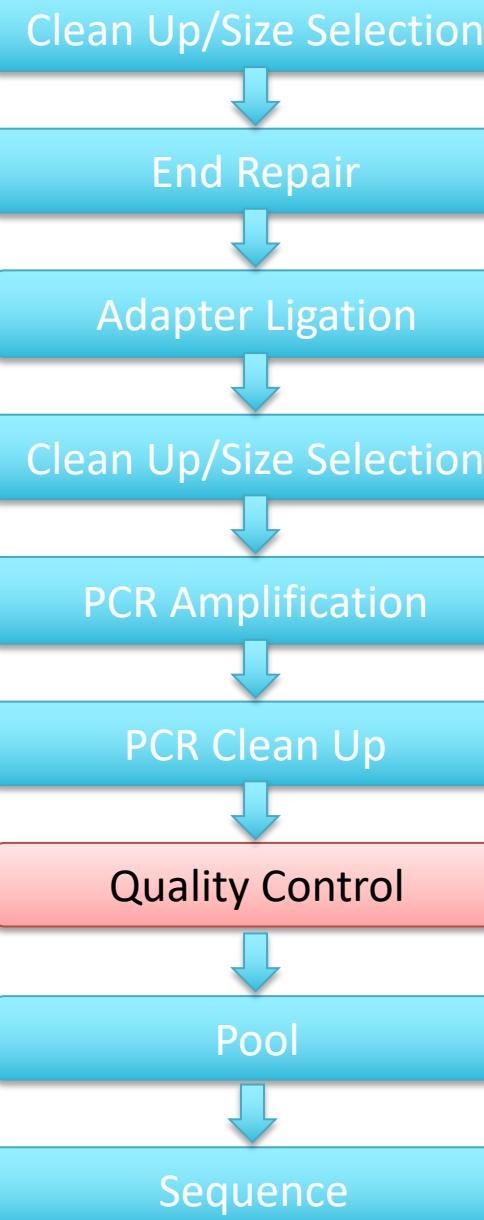
Sequence

## Clean Up- Post PCR Amplification

- Remove free barcodes, nucleotides
- Remove adapter dimers

# Quality Control

- Agilent Bioanalyzer 2100
- Microfluidics platform for sizing and quantification



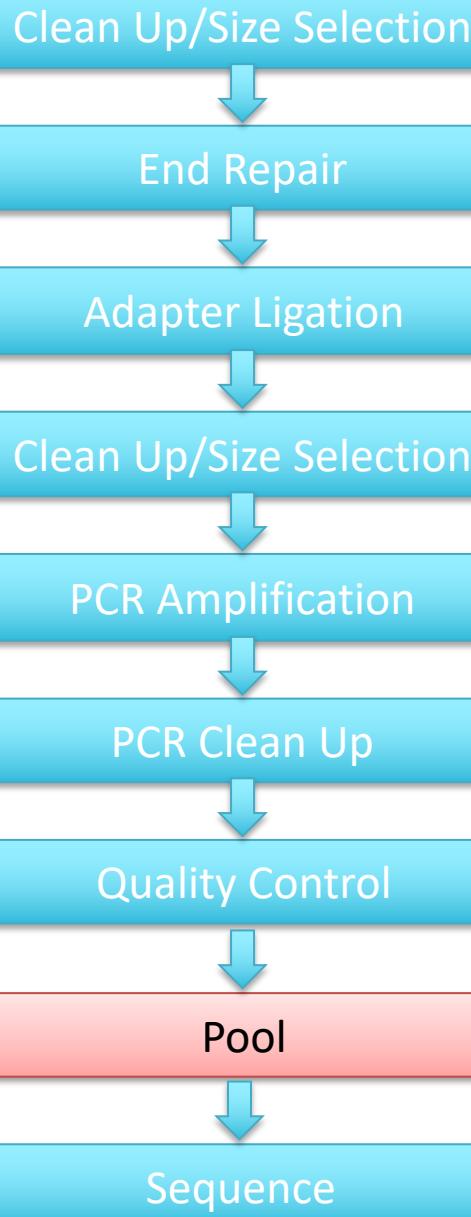
Overall Results for sample 9 : RE-52

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

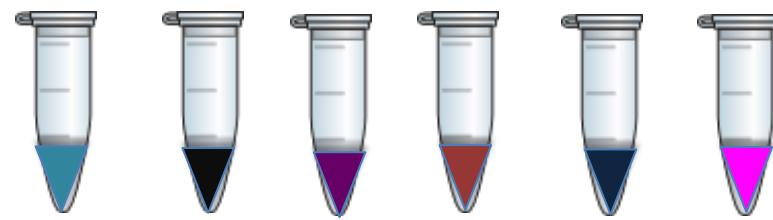
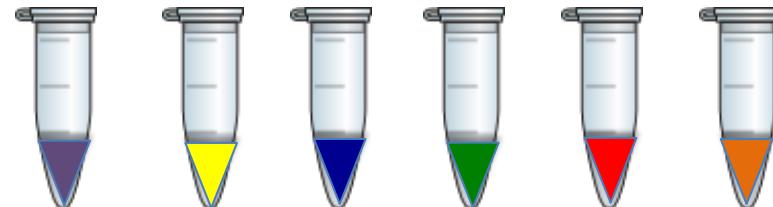
Peak table for sample 9 : RE-52

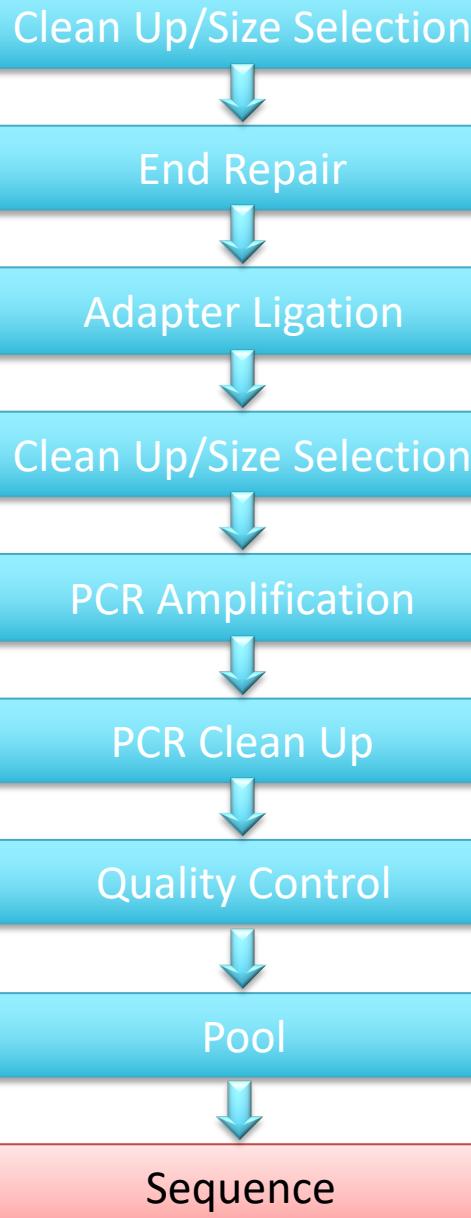
Peak	Size [bp]	Conc. [pg/μl]	Molarity [pmol/l]	Observations
1	35	125.00	5,411.3	Lower Marker
2	585	2,754.89	7,140.6	
3	10,380	75.00	10.9	Upper Marker

# Pool Final Libraries



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



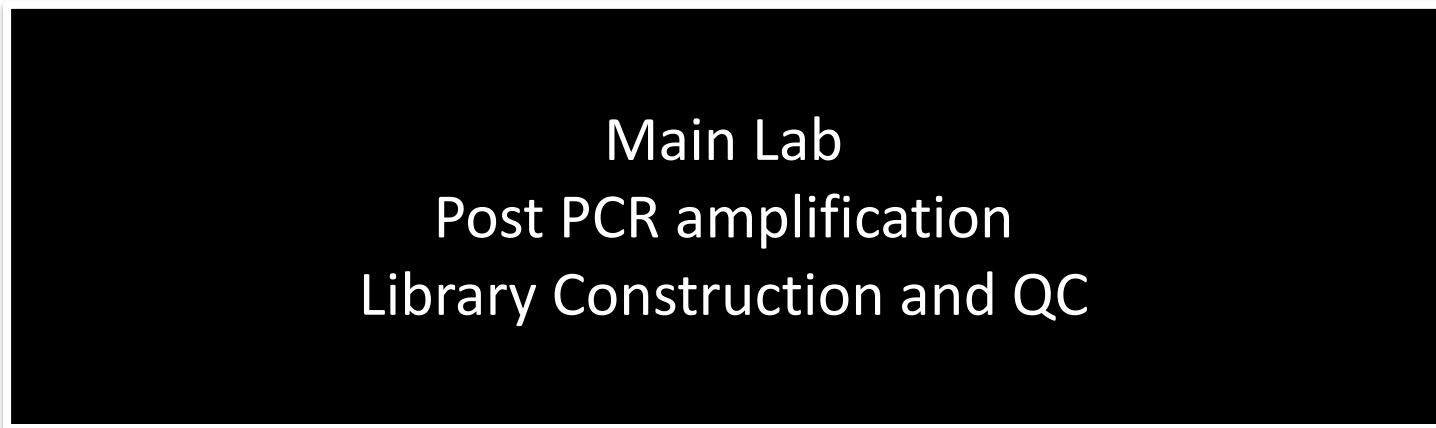
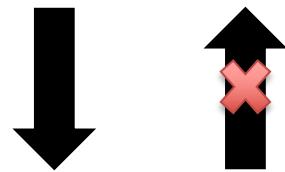
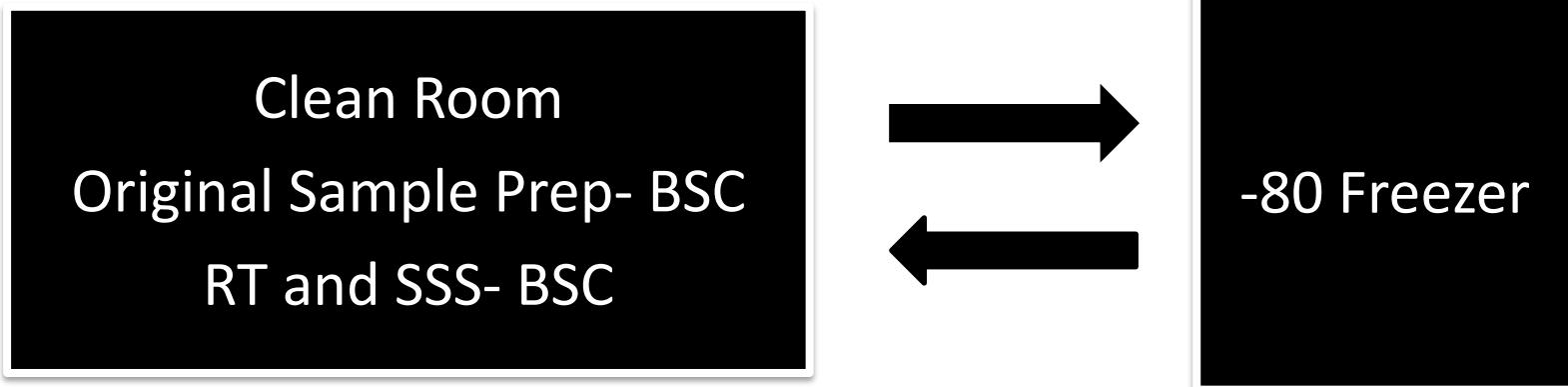


MiSeq V2 2X250

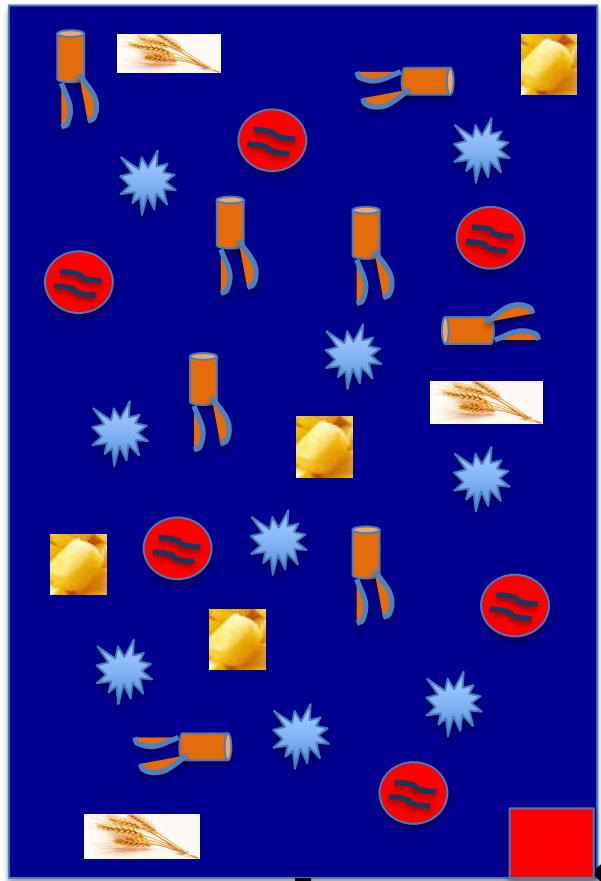


(pick your favorite)

# Laboratory Layout



# Stool Sample



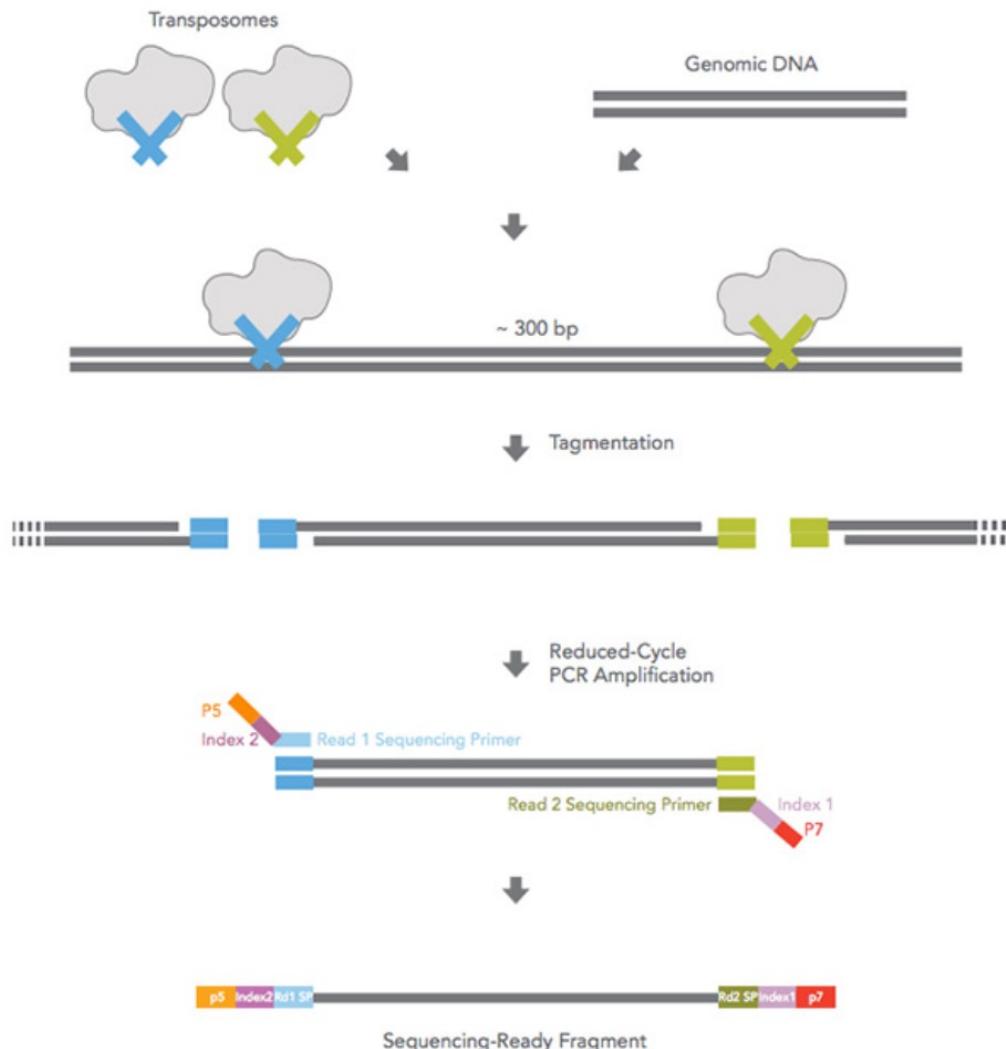
## Amplicon Sequencing

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/18S/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

# RNAseq for Expression Analysis

# A Plethora of Biological Sequence Analyses Enabled by RNA-Seq

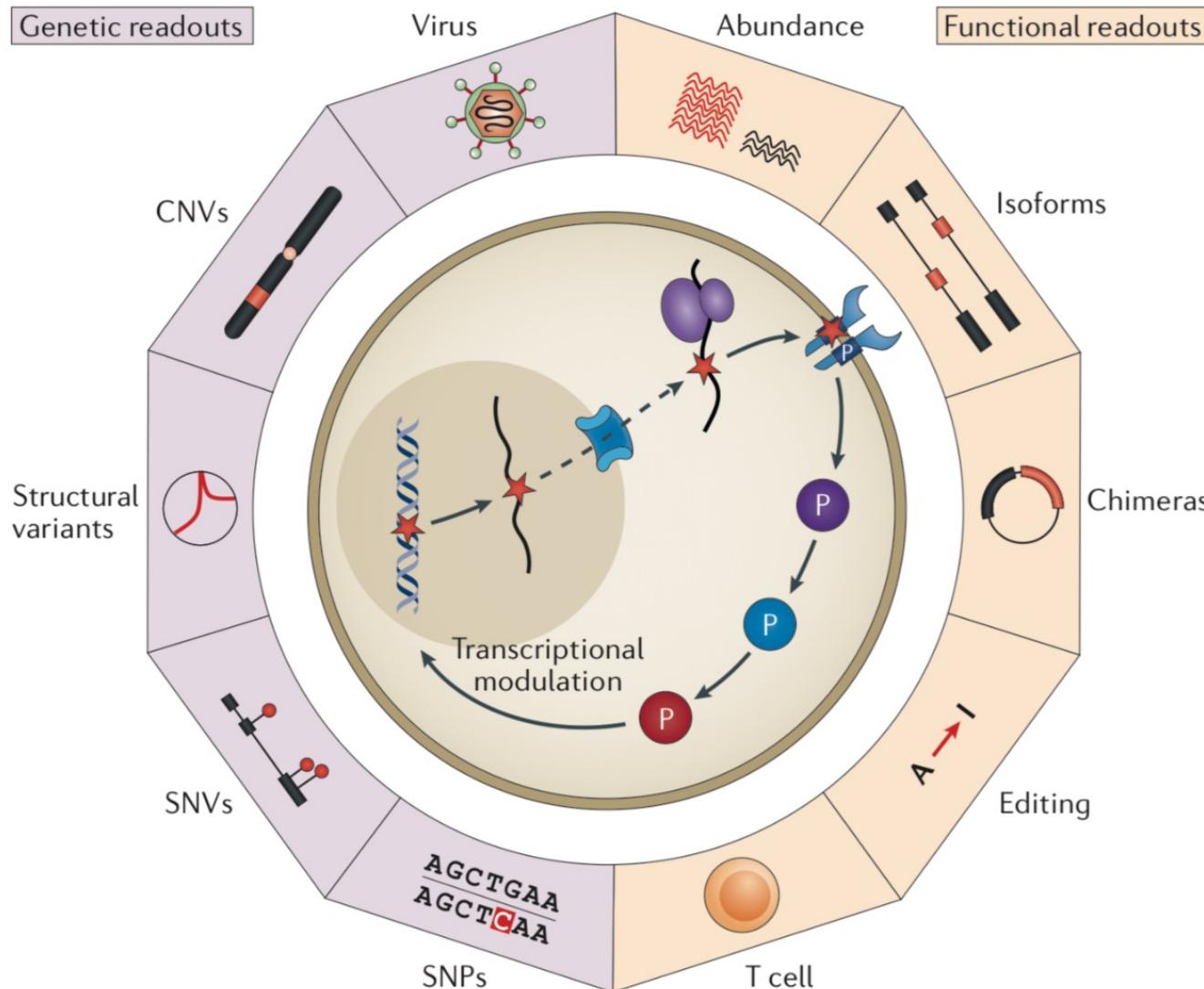
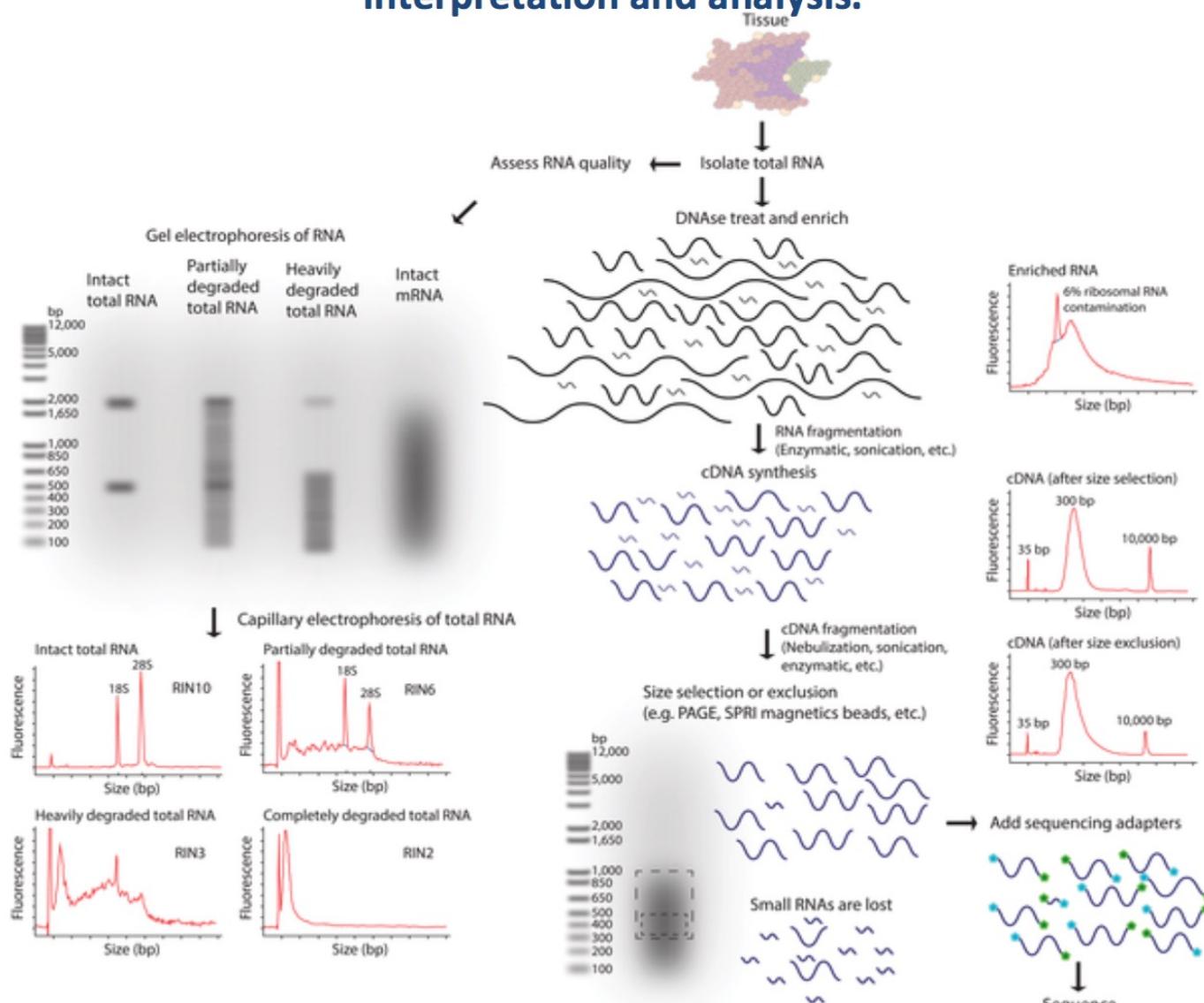


Figure 2 | Transcriptome profiling for genetic causes and functional phenotypic readouts.

From Cieslik and Chinnaiyan, NRG, 2017

# RNA-seq library fragmentation and size selection strategies that influence interpretation and analysis.



rRNA Depletion/Fragment

Clean Up Size Selection

First Strand cDNA

Second Strand cDNA

Clean Up/Size Selection

Adenylate 3' Ends

Ligate Adapters

Clean Up/Size Selection

PCR Amplification

Clean Up/Size Selection

Final Library

Quality Control

Pool

Sequence

# Preparing Sample for Transcriptomics

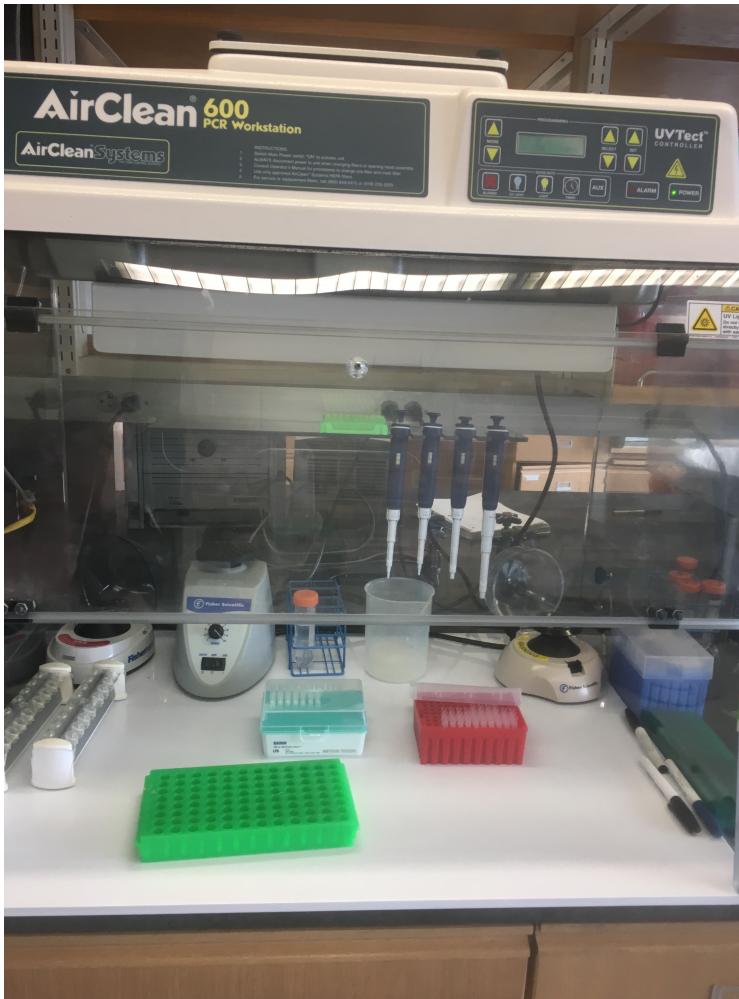
## Sequencing (RNaseq): 3 steps

- 1.) Extraction
- 2.) ribosomal RNA depletion
- 3.) Library Construction



# PCR Workstation

- Vertical Laminar air flow
- Hepa filtration system
- Build in UV
- Reduce Exposure to RNases



rRNA Depletion/Fragment



Clean Up Size Selection



First Strand cDNA



Second Strand cDNA



Clean Up/Size Selection



Adenylate 3' Ends



Ligate Adapters



Clean Up/Size Selection



PCR Amplification



Clean Up/Size Selection



Final Library



Quality Control



Pool



Sequence

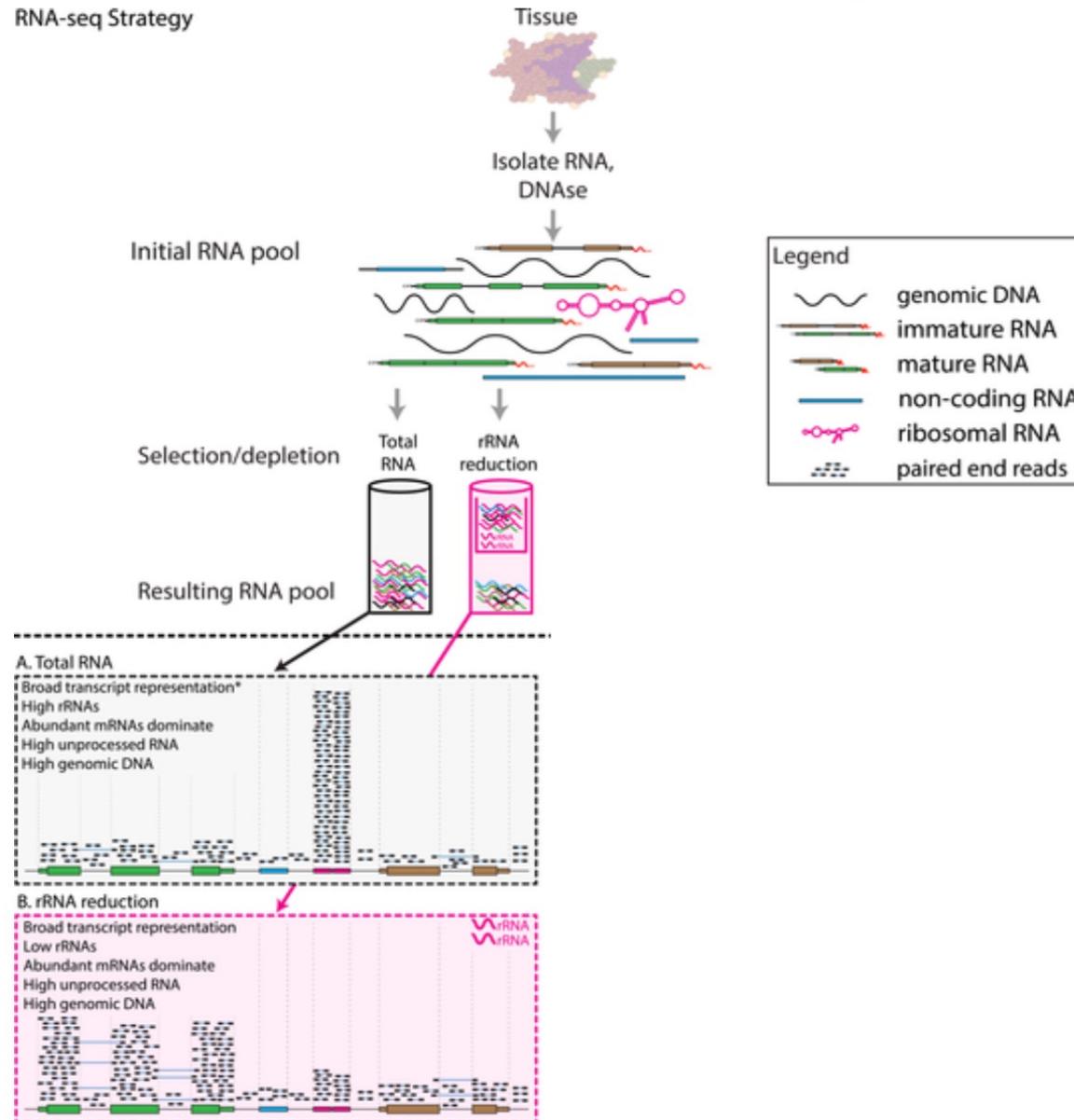
# Ribosomal RNA Depletion and Fragmenting

- Ribo Zero: iron core bead with rDNA conserved sequence hybridized to rRNA in sample
- Fragmented 150-300bp and primed for cDNA synthesis



# RNA-seq library enrichment strategies that influence interpretation and analysis.

## RNA-seq Strategy





## Clean Up-Post Fragmentation

- Select for fragments 150-300bp
- Remove left over components from rRNA depletion

rRNA Depletion/Fragment



Clean Up Size Selection

First Strand cDNA

Second Strand cDNA

Clean Up/Size Selection

Adenylate 3' Ends

Ligate Adapters

Clean Up/Size Selection

PCR Amplification

Clean Up/Size Selection

Final Library

Quality Control

Pool

Sequence

## First Strand Synthesis

- Cleaved RNA fragments are copied into cDNA using RT and random primers



First strand cDNA



## Second Strand Synthesis

- Double stranded cDNA
- Incorporation of dUTP in SSS quenches the second strand during amplification



## Strand Specificity

- Important for looking at overlapping genes
- Identification of antisense expression



## Clean Up- Post ds cDNA synthesis

- Select for fragments 150-300bp
- Remove left over components from cDNA synthesis

rRNA Depletion/Fragment

Clean Up Size Selection

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Second Strand cDNA

Clean Up/Size Selection

Adenylate 3' Ends

Ligate Adapters

Clean Up/Size Selection

PCR Amplification

Clean Up/Size Selection

Final Library

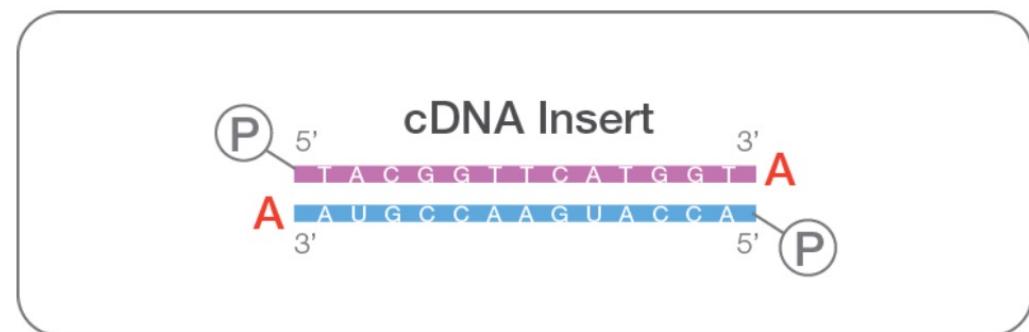
Quality Control

Pool

Sequence

## A-tailing

- Single A nucleotide is added to 3' ends of blunt fragments
- Single T nucleotide on the adapter provides complementary overhang for ligating the adapter



rRNA Depletion/Fragment

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Ligate Adapters

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PCR Amplification

Clean Up/Size Selection

Final Library

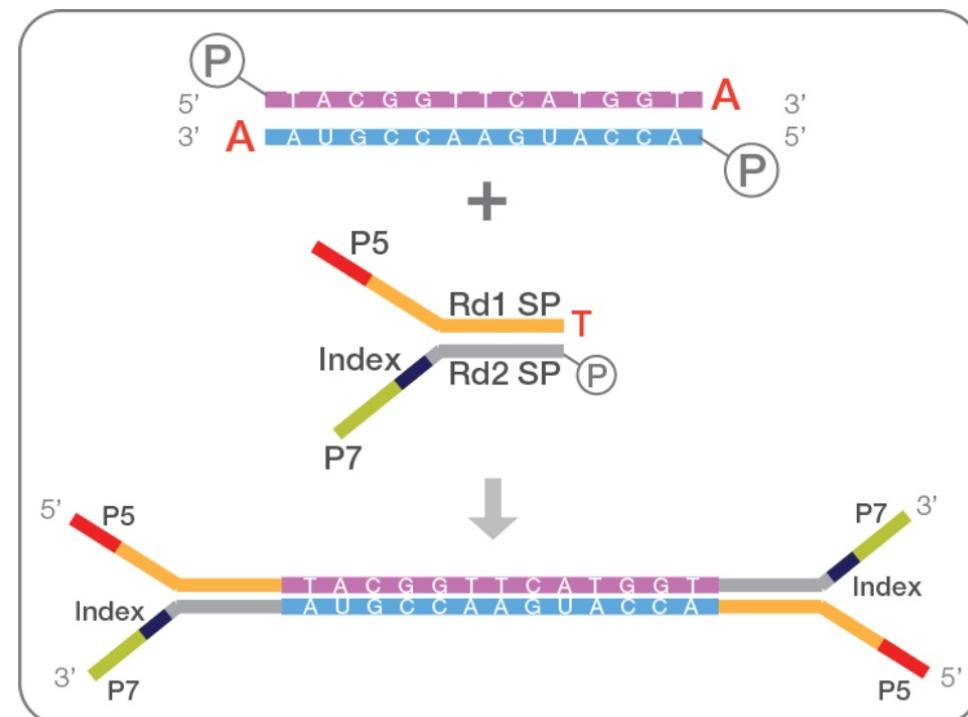
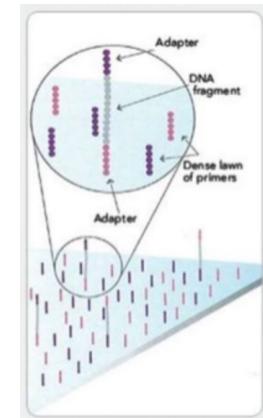
Quality Control

Pool

Sequence

## Adapter Ligation

- P5 and P7 are used for amplification
- Barcodes are part of adapter





## Clean Up- Post Adapter Ligation

- Size Selection 150-300bp
- Remove unused ligation reaction components, adapter dimers, and concatemers

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Clean Up/Size Selection

PCR Amplification

Clean Up/Size Selection

Final Library

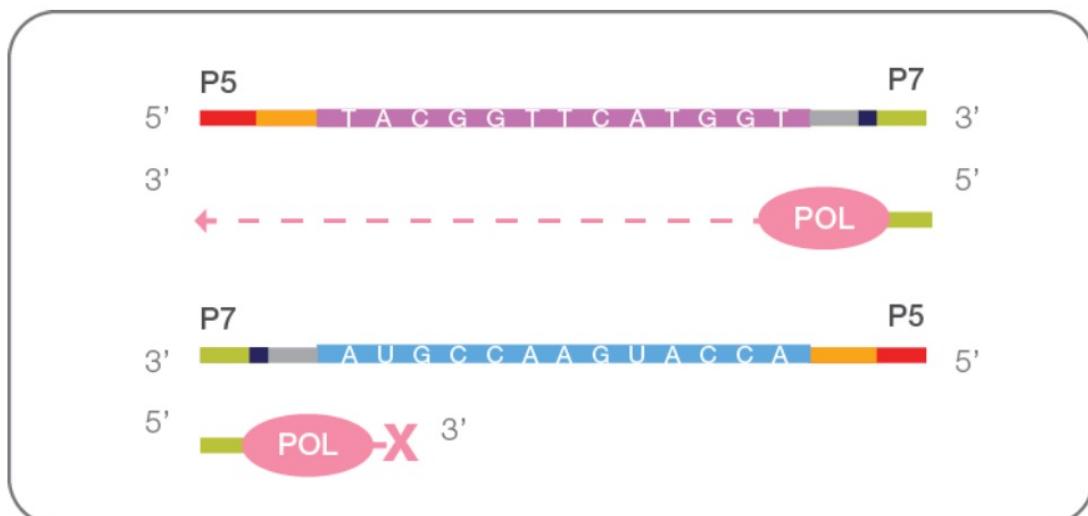
Quality Control

Pool

Sequence

## PCR Amplification

- Polymerase does not incorporate past dUTP. Second strand is quenched during amplification.
- Products enriched with PCR amplification.





## Clean Up- Post PCR Amplification

- Remove free barcodes, nucleotides
- Remove adapter dimers

rRNA Depletion/Fragment

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Second Strand cDNA

Clean Up/Size Selection

Adenylate 3' Ends

Ligate Adapters

Clean Up/Size Selection

PCR Amplification

Clean Up/Size Selection

Final Library

Quality Control

Pool

Sequence

Final Library



rRNA Depletion/Fragment

Clean Up Size Selection

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Ligate Adapters

Clean Up/Size Selection

PCR Amplification

Clean Up/Size Selection

Final Library

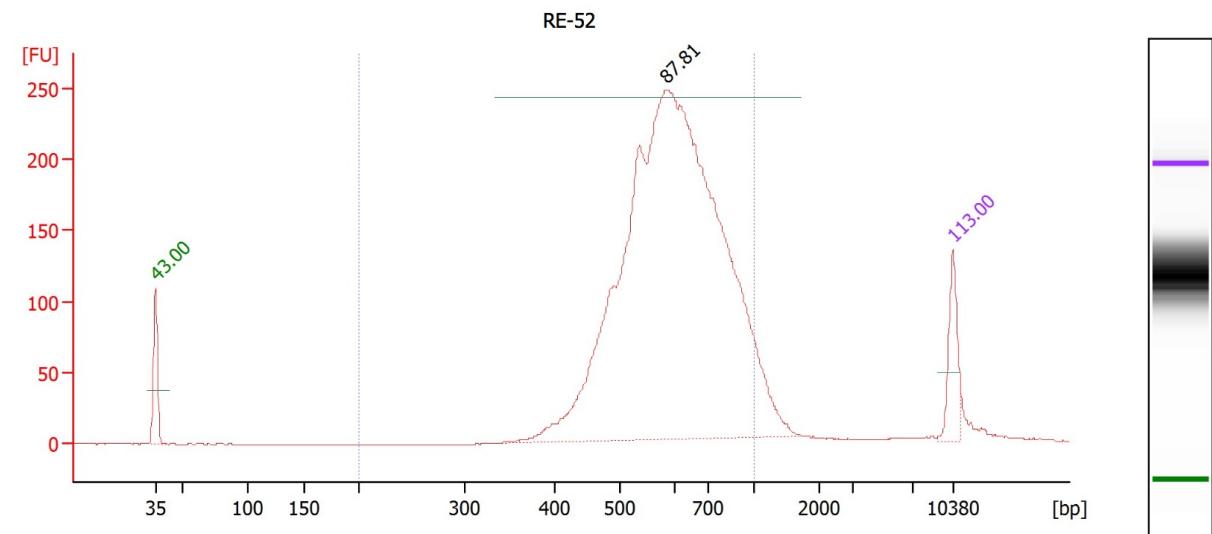
Quality Control

Pool

Sequence

# 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

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Clean Up/Size Selection

PCR Amplification

Clean Up/Size Selection

Final Library

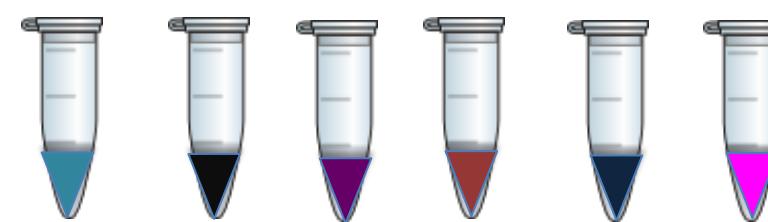
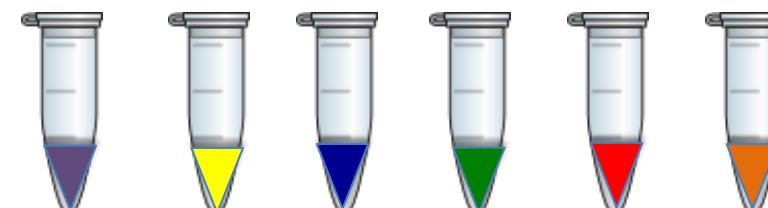
Quality Control

Pool

Sequence

## Pool Final Libraries

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



rRNA Depletion/Fragment

Clean Up Size Selection

First Strand cDNA

Second Strand cDNA

Clean Up/Size Selection

Adenylate 3' Ends

Ligate Adapters

Clean Up/Size Selection

PCR Amplification

Clean Up/Size Selection

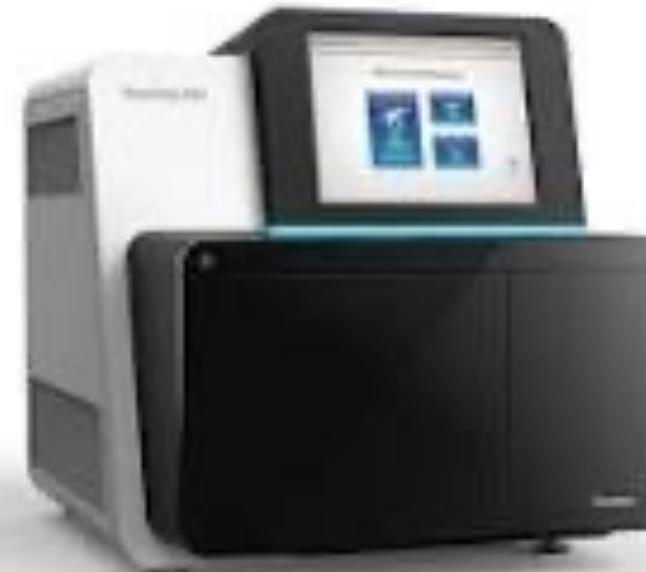
Final Library

Quality Control

Pool

Sequence

## Illumina NextSeq



# How much will my experiment cost?

## Generating RNA-Seq: *How to Choose?*

Platform	Project Firefly 2018	MiniSeq	HiSeq 2500 RR	HiSeq 2500 V3	HiSeq 2500 V4	HiSeq 4000	HiSeq X	Nova Seq S1 2018	Nova Seq S2	Nova Seq S4	5500 XL	318 HiQ 520	Ion Proton P1	PGM HiQ 540	RS P6-C4	Sequel	R&D end 2018	Smidg ION RnD	Mini ION R9.5	Grid ION X5	Prome thION RnD	Prome thION theor etical	QiaGen Gene Reader	BGI SEQ 500	BGI SEQ 50	#			
Reads: (M)	4	25	25	400	600	3000	4000	5000	6000	3300	6600	20000	1400	3-5	15-20	165	60-80	5.5	38.5	--	--	--	--	--	400	1600	1600	--	
Read length: (paired-end*)	150*	150*	300*	150*	100*	100*	125*	150*	150*	150*	150*	150*	60	200	200	200	15K	12K	32K	--	--	--	--	--	--	100*	50	--	
Run time: (d)	0.54	1	2	1.2	1.125	11	6	3.5	3	1.66	1.66	1.66	7	0.37	0.16	--	0.16	4.3	--	--	2	2	2	--	--	1	0.4	--	
Yield: (Gb)	1	7.5	15	120	120	600	1000	1500	1800	1000	2000	6000	180	1.5	7	10	12	12	5	150	4	8	40	2400	11000	80	200	8	
Rate: (Gb/d)	1.85	7.5	7.5	100	106.6	55	166	400	600	600	1200	3600	30	5.5	50	--	93.75	2.8	--	--	--	4	20	1200	5500	--	200	20	--
Reagents: (\$K)	0.1	1.75	1	5	6.145	23.47	29.9	--	--	--	--	--	10.5	0.6	--	1	1.2	2.4	--	1	--	0.5	1.5	--	--	0.5	--	--	
per-Gb: (\$)	100	233	66	50	51.2	39.1	31.7	20.5	7.08	18	15	5.8	58.33	--	--	100	--	200	80	6.6	--	62.5	37.5	20	4.3	--	--	--	
hg-30x: (\$)	12000	28000	8000	5000	6144	4692	3804	2460	849.6	1800	1564	700	7000	--	--	12000	--	24000	9600	1000	--	7500	4500	2400	500	--	600	--	
Machine: (\$)	30K	49.5K	99K	250K	740K	690K	690K	900K	1M	999K	999K	999K	595K	50K	65K	243K	242K	695K	350K	350K	--	--	125K	75K	75K	--	200K	--	

#Page maintained by <http://twitter.com/albertvilella> <http://tinyurl.com/ngslytics> #Editable version: <http://tinyurl.com/ngsspecshared>  
#curl "https://docs.google.com/spreadsheets/d/1GMGmfhyLk0-q8Xklo3YxiWaZA5vVMuhU1kg41g4xLkXc/export?gid=4&format=csv" | grep -v '^#' | grep -v '^"' | column -t -s\| | less -S



\*Not all shown at scale

Brian Haas, Broad

16S/ITS- \$18/sample

Virome- \$250/sample

Transcriptomics RNAseq- \$390/sample

# Thank You!



Scott Handley

Dave Wang

Leah Heath

Jessica Hoisington-Lopez



Doug Kwon

Joseph Elsherbini

Blythe Gulley