5- Overview of Laboratory Protocols for Pathogen Sequencing (non SARS-CoV-2)

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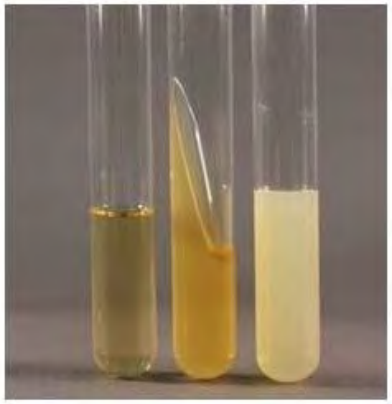
Library Prep is Dependent on Sample Type & Desired Sequencing Method

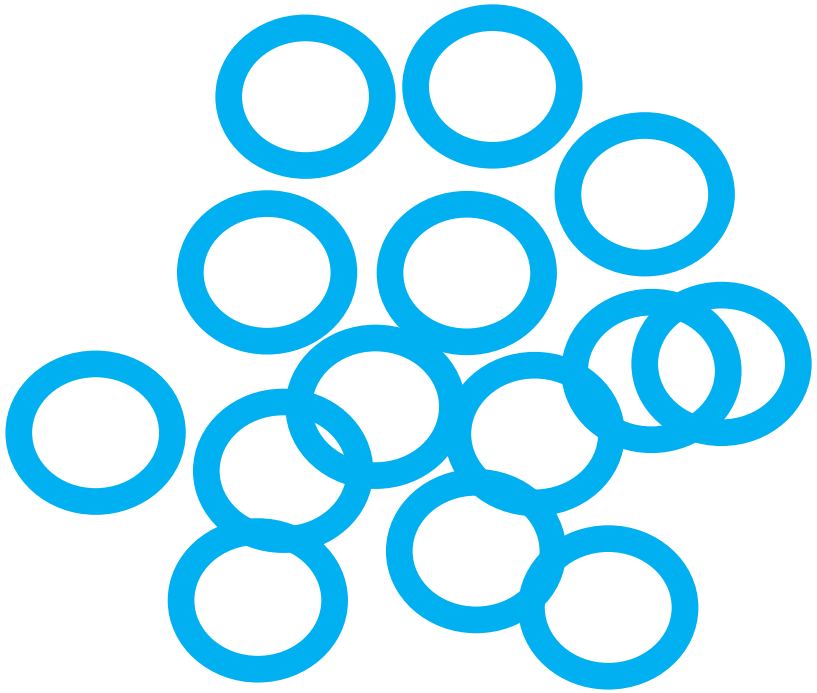
Sample Type- Pure Culture/Isolate

- High Concentration of DNA
  - Suitable for protocols that require high input amounts (>μg)
- High Quality DNA
  - Suitable for long sequencing read protocols (ONT & PacBio)
- Isolation Protocols May Need to Consider Hard to Lyse Bacteria
  - Bead-bashing/stringent lysis buffers



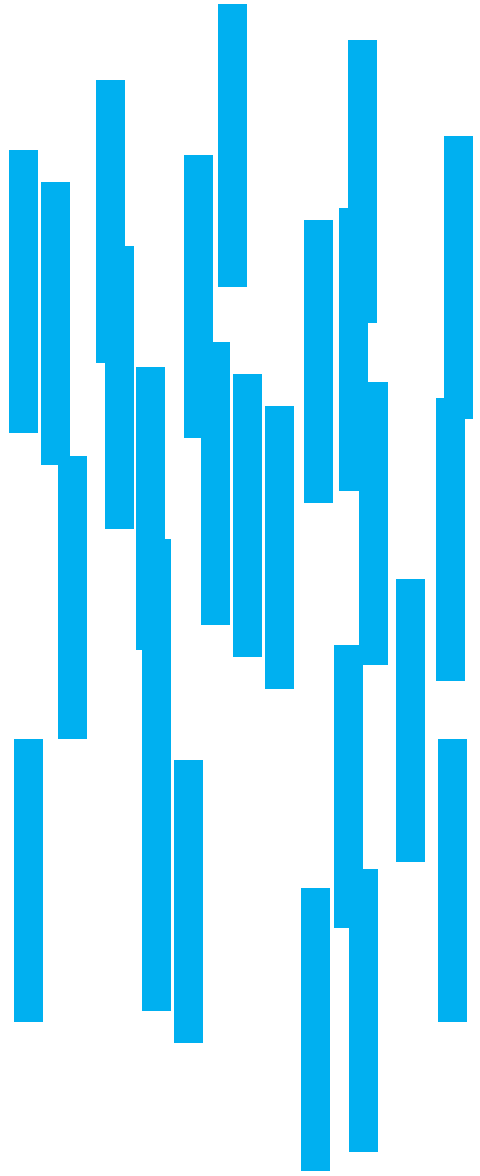
Shotgun Sequencing

Pure Population of Bacteria

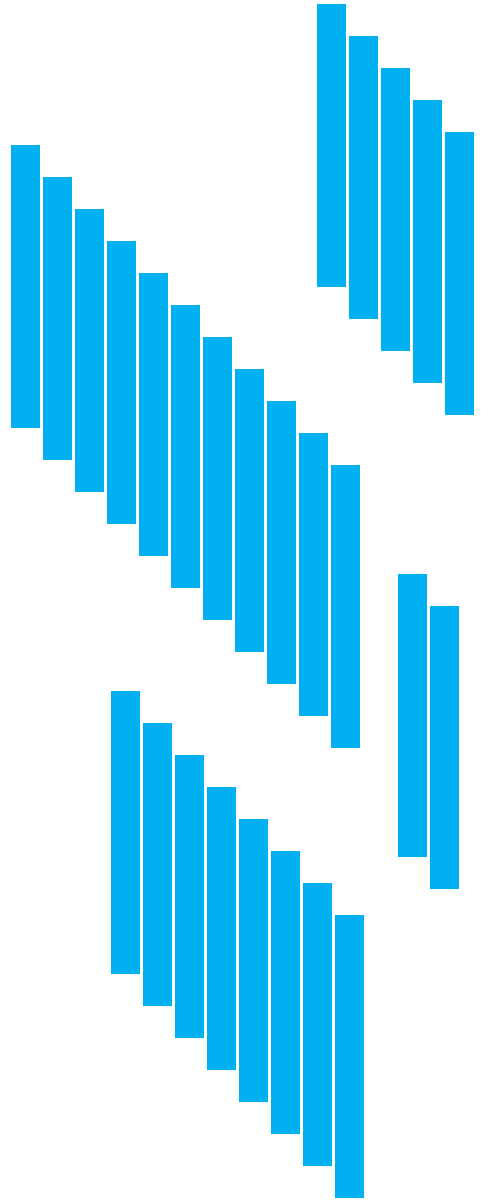


Cultured Isolate
Genome of ~4.9 Mbp

Shotgun Whole Genome Sequencing



Bioinformatic Assembly of Bacteria



Illumina- DNA Prep

Nextera DNA Flex products are now called Illumina DNA Prep

1. **Tagment Genomic DNA**
   Hands-on: 20 minutes
   Total: 40 minutes
   Reagents: BLT, TB1
2. **Post Tagmentation Clean Up**
   Hands-on: 20 minutes
   Total: 45 minutes
   Reagents: TSB, TWB
3. **Amplify Tagmented DNA**
   Hands-on: 15 minutes
   Total: 45 minutes
   Reagents: EPM, Index Adapters

Safe Stopping Point

4. **Clean Up Libraries**
   Hands-on: 10 minutes
   Total: 50 minutes
   Reagents: RSB, IPB, EtOH

Safe Stopping Point

5. **Pool Libraries**

Pre-PCR Post-PCR

Illumina- DNA Prep

Can sequence up to 384 samples together.
Mix Set A, B, C, D

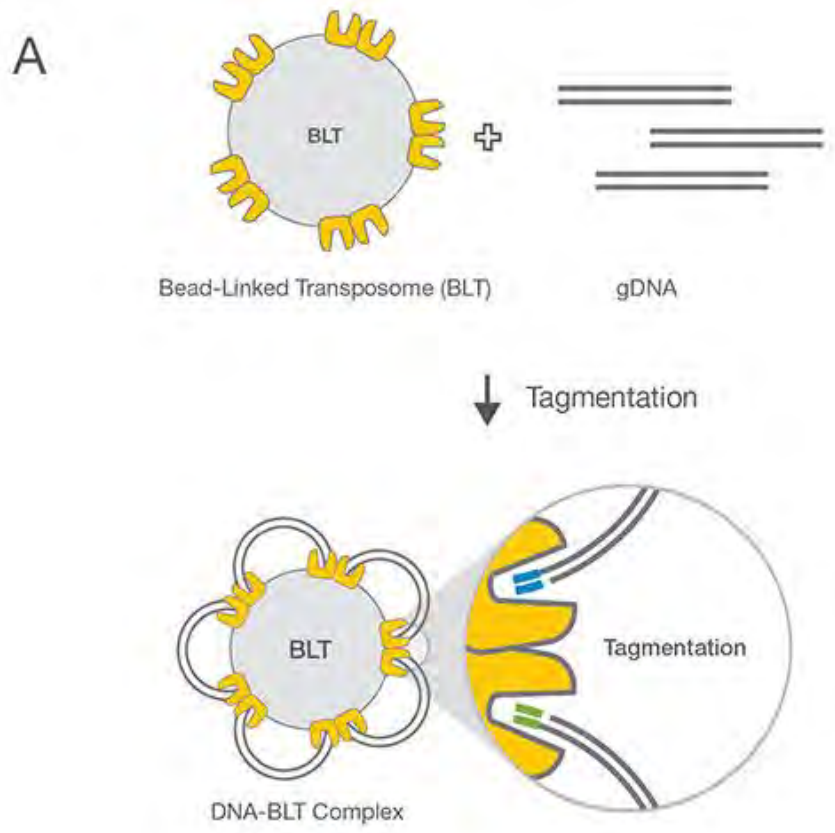
Library Prep

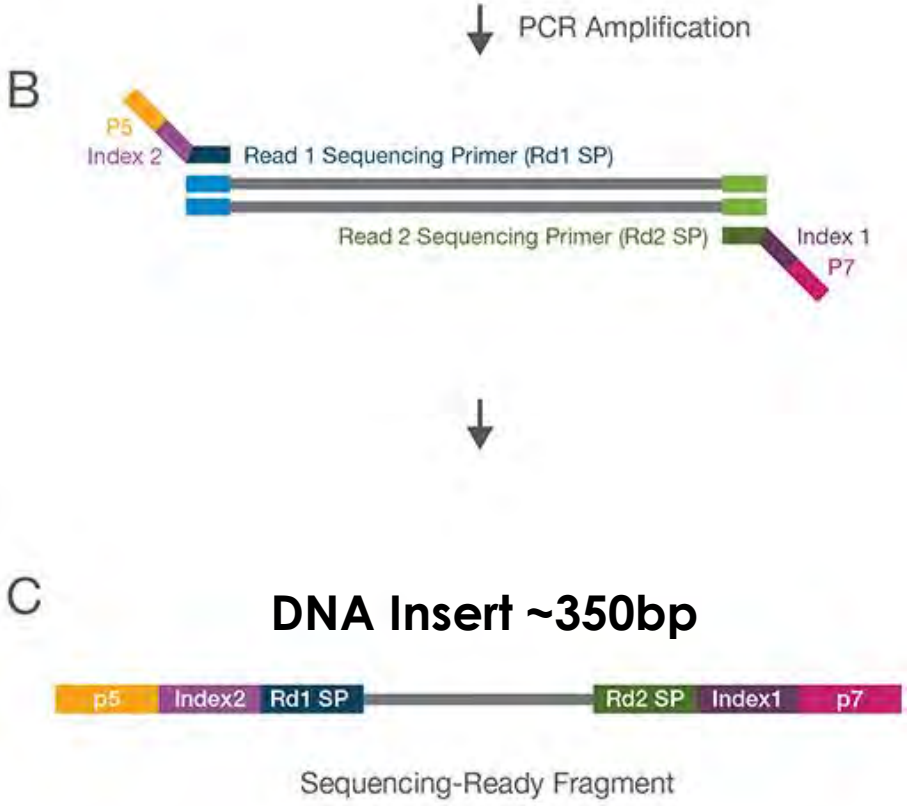
- 0 — Illumina® DNA Prep, (M) Tagmentation (24 Samples, IPB) 20060060
- 0 — Illumina® DNA Prep, (M) Tagmentation (96 Samples, IPB) 20060059
- 0 — Illumina® DNA Prep, (M) Tagmentation (24 Samples) 20018704
- 0 — Illumina® DNA Prep, (M) Tagmentation (96 Samples) 20018705

Index Adapters

- 0 — IDT® for Illumina® DNA/RNA UD Indexes Set A, Tagmentation (96 Indexes, 96 Samples) 20027213
- 0 — IDT® for Illumina® DNA/RNA UD Indexes Set B, Tagmentation (96 Indexes, 96 Samples) 20027214
- 0 — IDT® for Illumina Nextera DNA Unique Dual Indexes Set C (96 Indexes, 96 Samples) 20027215
- 0 — IDT® for Illumina Nextera DNA Unique Dual Indexes Set D (96 Indexes, 96 Samples) 20027216
- 0 — Nextera™ DNA CD Indexes (24 Indexes, 24 Samples) 20018707
- 0 — Nextera™ DNA CD Indexes (96 Indexes, 96 Samples) 20018708

Illumina- DNA Prep





Illumina- DNA Prep

Illumina Library Prep Checklists are Often Easier to Follow

illumina®

Illumina DNA Prep Checklist

For Research Use Only. Not for use in diagnostic procedures.

Tagment Genomic DNA

- ☐ 1 Add 2–30 µl DNA to a 96-well PCR plate.
- ☐ 2 If DNA volume < 30 µl, add nuclease-free water to bring the volume to 30 µl.
- ☐ 3 Vortex BLT vigorously to resuspend.
- ☐ 4 For each sample, combine the following volumes.
  - BLT (11 µl)
  - TB1 (11 µl)
- ☐ 5 Vortex the master mix to resuspend.
- ☐ 6 Divide the master mix volume into an 8-tube strip.
- ☐ 7 Transfer 20 µl to each well containing a sample.
- ☐ 8 Discard the 8-tube strip.
- ☐ 9 Pipette 10 times to resuspend.
- ☐ 10 Seal the plate, place on the thermal cycler, and run the TAG program.

Post Tagmentation Cleanup

- ☐ 1 Add 10 µl TSB.
- ☐ 2 Slowly pipette 10 times to resuspend the beads.
- ☐ 3 Seal the plate, place on the thermal cycler, and run the PTC program.
- ☐ 4 Place the plate on the magnetic stand until liquid is clear.
- ☐ 5 Remove and discard supernatant.
- ☐ 6 Remove from the magnetic stand and 100 µl TWB.
- ☐ 7 Pipette to resuspend or shake at 1600 rpm for 1 minute
- ☐ 8 Repeat steps 4–7 two times for a total of 3 washes.
- ☐ 9 Seal the plate and place on the magnetic stand until the liquid is clear

Amplify Tagmented DNA

- ☐ 1 For each sample, combine the following volumes.
  - EPM (22 µl)
  - Nuclease-free water (22 µl)
- ☐ 2 Vortex, and then centrifuge at 280 × g for 10 seconds.
- ☐ 3 Remove and discard supernatant.
- ☐ 4 Remove from the magnet.
- ☐ 5 Add 40 µl master mix in each sample well.
- ☐ 6 Pipette 10 times or shake at 1600 rpm for 1 minute.
- ☐ 7 Seal the plate and centrifuge at 280 × g for 3 seconds.
- ☐ 8 Add the appropriate index adapters to each sample.
- ☐ 9 Pipette 10 times or shake at 1600 rpm for 1 minute.
- ☐ 10 Seal the plate, and then centrifuge at 280 × g for 30 seconds.
- ☐ 11 Place on the thermal cycler and run the BLT PCR program.

SAFE STOPPING POINT

If you are stopping, store at 2°C to 8°C for up to 3 days.

illumina®

Illumina DNA Prep Checklist

Clean Up Libraries

- ☐ 1 Centrifuge at 280 × g for 1 minute.
- ☐ 2 Place on the magnetic stand until the liquid is clear.
- ☐ 3 Transfer 45 µl supernatant to a new midi plate.
- ☐ 4 Vortex and invert SPB to resuspend.
- ☐ 5 For standard DNA input, perform the following steps.
  - ☐ a Add 40 µl nuclease-free water.
  - ☐ b Add 45 µl SPB.
  - ☐ c Pipette 10 times or shake at 1600 rpm for 1 minute.
  - ☐ d Seal the plate and incubate for 5 minutes.
  - ☐ e Place on the magnetic stand until the liquid is clear.
  - ☐ f Vortex SPB (*undiluted* stock tube), and then add 15 µl to a *new* midi plate.
  - ☐ g Transfer 125 µl supernatant to the new plate.
  - ☐ h Pipette 10 times or shake at 1600 rpm for 1 minute.
  - ☐ i Discard the first plate.
- ☐ 6 For small PCR amplicon input, perform the following steps.
  - ☐ a Add 81 µl SPB.
  - ☐ b Pipette 10 times or shake at 1600 rpm for 1 minute.
- ☐ 7 Incubate at room temperature for 5 minutes.
- ☐ 8 Place on the magnetic stand until the liquid is clear.
- ☐ 9 Remove and discard supernatant.
- ☐ 10 Wash two times with 200 µl fresh 80% EtOH.
- ☐ 11 Remove and discard residual EtOH.
- ☐ 12 Air-dry for 5 minutes.
- ☐ 13 Remove from the magnetic stand.
- ☐ 14 Add 32 µl RSB.
- ☐ 15 Pipette to resuspend.
- ☐ 16 Incubate at room temperature for 2 minutes.
- ☐ 17 Place the plate on the magnetic stand until the liquid is clear.
- ☐ 18 Transfer 30 µl supernatant to a new plate.

SAFE STOPPING POINT

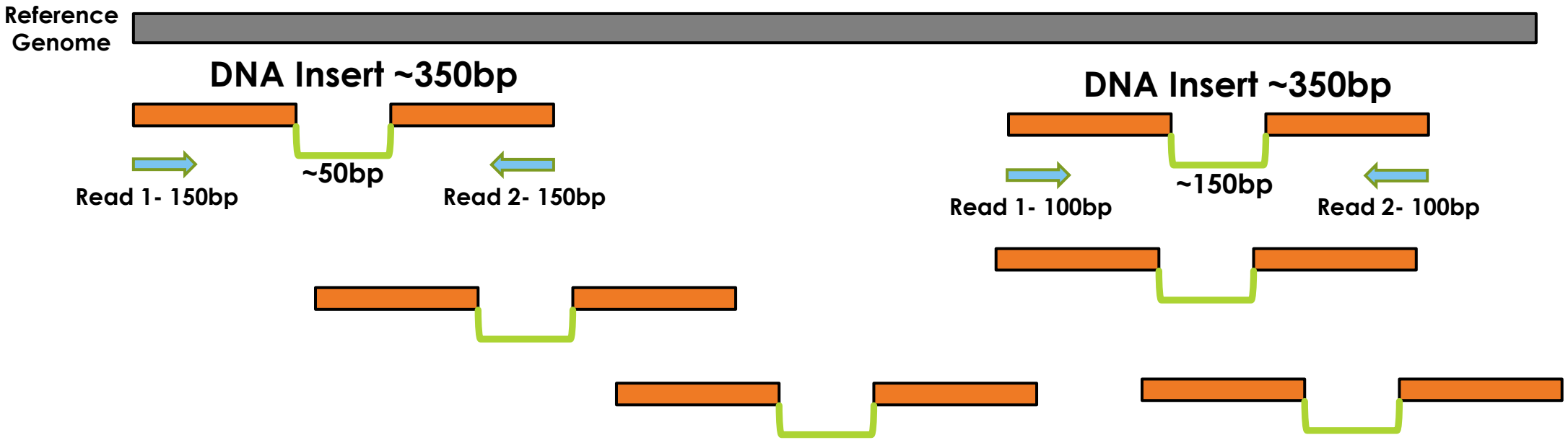
If you are stopping, seal the plate with Microseal 'B' adhesive or Microseal 'F' foil seal, and store at -25°C to -15°C for up to 30 days.

Check Library Quality (Optional)

- ☐ 1 Run 1 µl library or pooled libraries on one of the following instruments:
  - Add 1 µl RSB to the library to achieve the 2 µl volume required for Fragment Analyzer.

Illumina- Paired End Sequencing

Read Length Impacts Portion of DNA Insert not Sequenced



ONT- Ligation Sequencing Kit

Can sequence up to 96 samples together.

	Ligation Sequencing Kit
Preparation time	60 min
Input requirement	1,000 ng HMW gDNA
Fragmentation	Optional
Read length	Equal to fragment length
Multiplexing options	Yes

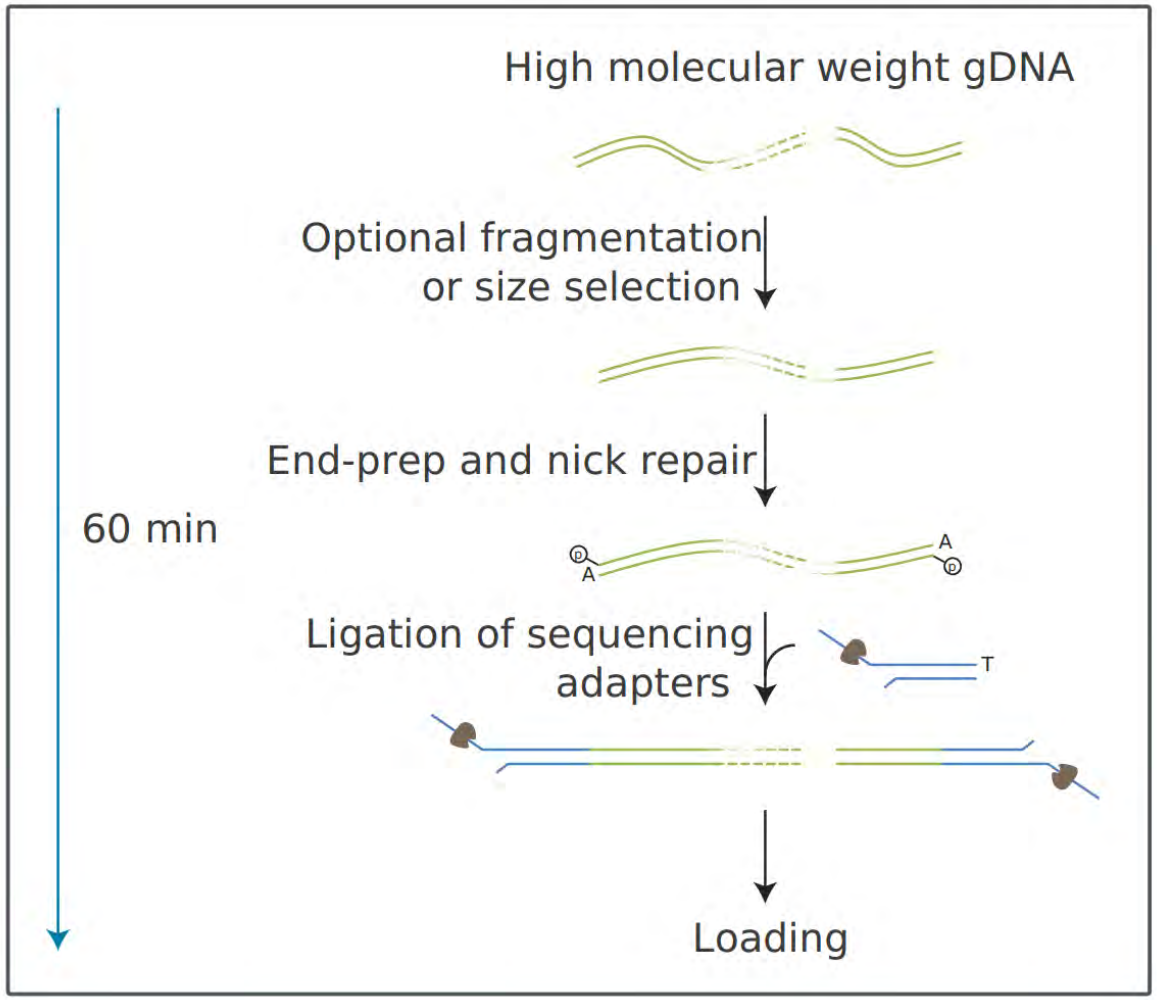
Native Barcoding Kit 24 (SQK-NBD112.24)
Native Barcoding Kit 96 (SQK-NBD112.96)

https://nanoporetech.com/products/kits

ONT- Ligation Sequencing Kit

DCS : DNA control strand
AMX-F : Adapter mix F
LNB : Ligation buffer
LFB : L fragment buffer
SFB : S fragment buffer
SBII : Sequencing buffer II
EB : Elution buffer
LBII : Loading beads II
LS : Loading solution
FB : Flush buffer
FLT : Flush tether

ONT- Ligation Sequencing Kit



ONT- Ligation Sequencing Kit

Name	Acronym	Cap colour	No. of vials	Fill volume per vial (µl)
DNA CS	DCS	Yellow	1	35
Adapter Mix F	AMX-F	Green	1	40
Ligation Buffer	LNB	Clear	1	200
L Fragment Buffer	LFB	White cap, orange stripe on label	2	1,800
S Fragment Buffer	SFB	Grey	2	1,800
Sequencing Buffer II	SBII	Red	1	500
Elution Buffer	EB	Black	1	200
Loading Beads II	LBII	Pink	1	360
Loading Solution	LS	White cap, pink sticker on label	1	360
Flush Buffer	FB	Blue	6	1,170
Flush Tether	FLT	Purple	1	200

ONT- Ligation Sequencing Recommendations

18 **Remove and retain 15 µl of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.**

Dispose of the pelleted beads

Quantify 1 µl of eluted sample using a Qubit fluorometer.

IMPORTANT

We recommend loading the final prepared library onto a flow cell following one of our recommendations depending on the flow cell type:

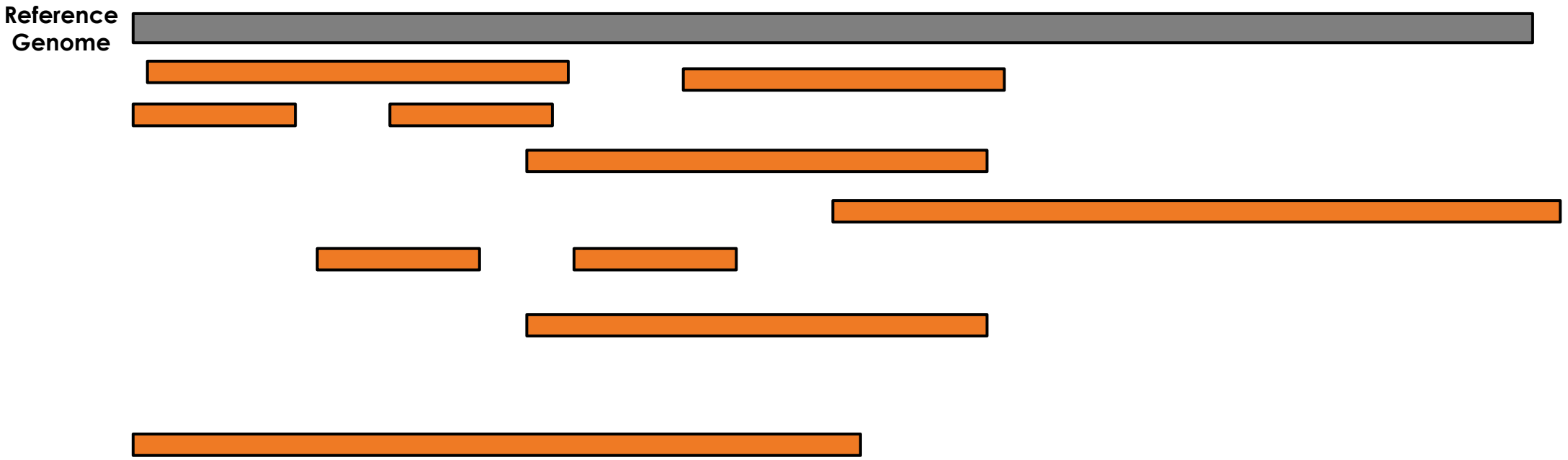
- R9.4.1 flow cells, load 5-50 fmol
- R10.3 flow cells, load 25-75 fmol

Loading more than the maximal recommended amount of DNA can have a detrimental effect on output as higher quantities of DNA results in a larger number of ligated DNA ends with loaded motor protein. This depletes fuel in the Sequencing Buffer, regardless of whether or not the DNA fragments are being sequenced. This leads to fuel depletion and speed drop-off early in the sequencing run. Dilute the libraries in Elution Buffer if required.

If you are using the Flongle for sample prep development, we recommend loading 3-20 fmol instead.

Mass	No. of moles if fragment length = 2 kb	No. of moles if fragment length = 8 kb	No. of moles if fragment length = 50 kb
10 µg	7.7 pmol	1.9 pmol	308 fmol
5 µg	3.9 pmol	963 fmol	154 fmol
3.5 µg	2.7 pmol	674 fmol	108 fmol
2 µg	1.5 pmol	385 fmol	62 fmol
1.5 µg	1.2 pmol	289 fmol	46 fmol
1 µg	770 fmol	193 fmol	31 fmol
500 ng	385 fmol	96 fmol	15 fmol
400 ng	308 fmol	77 fmol	12 fmol
200 ng	154 fmol	39 fmol	6.2 fmol
100 ng	77 fmol	19 fmol	3.1 fmol
30 ng	23 fmol	5.8 fmol	0.9 fmol
10 ng	7.7 fmol	1.9 fmol	0.3 fmol
10 pg	0.0077 fmol	0.009 fmol	0.0003 fmol

ONT- Single End Sequencing

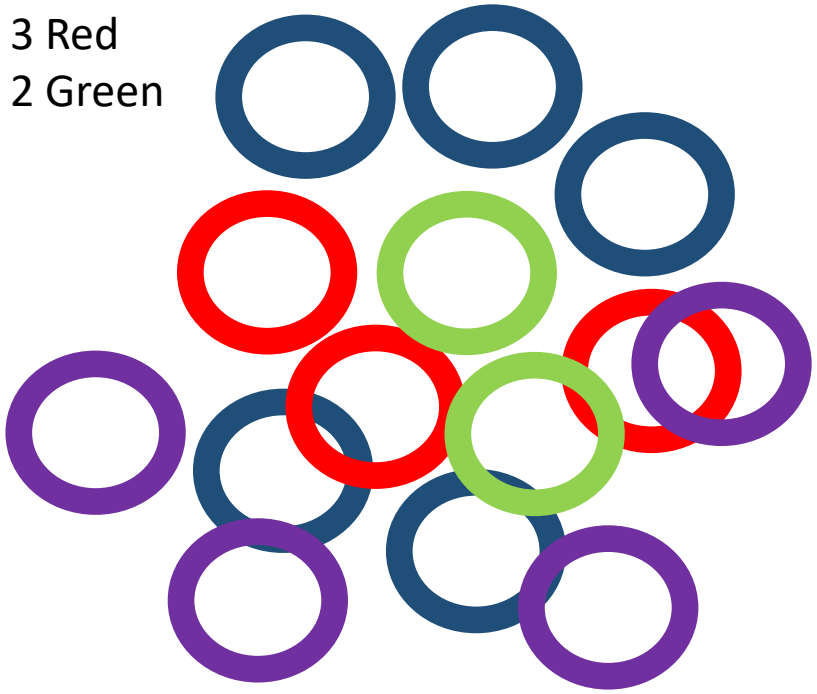


Sample Type- Mixed Samples

- Mixed Sample
  - Includes human cells (DNA/RNA), host microbial flora, pathogen, environmental organisms
- Clinical
  - Nasal/Oral Swab, Stool, Blood, Puss, CSF, Urine, Sputum
- Environmental
  - Swabs, Soil or water
  - Food
  - Wastewater
- Variable Amounts of Pathogen

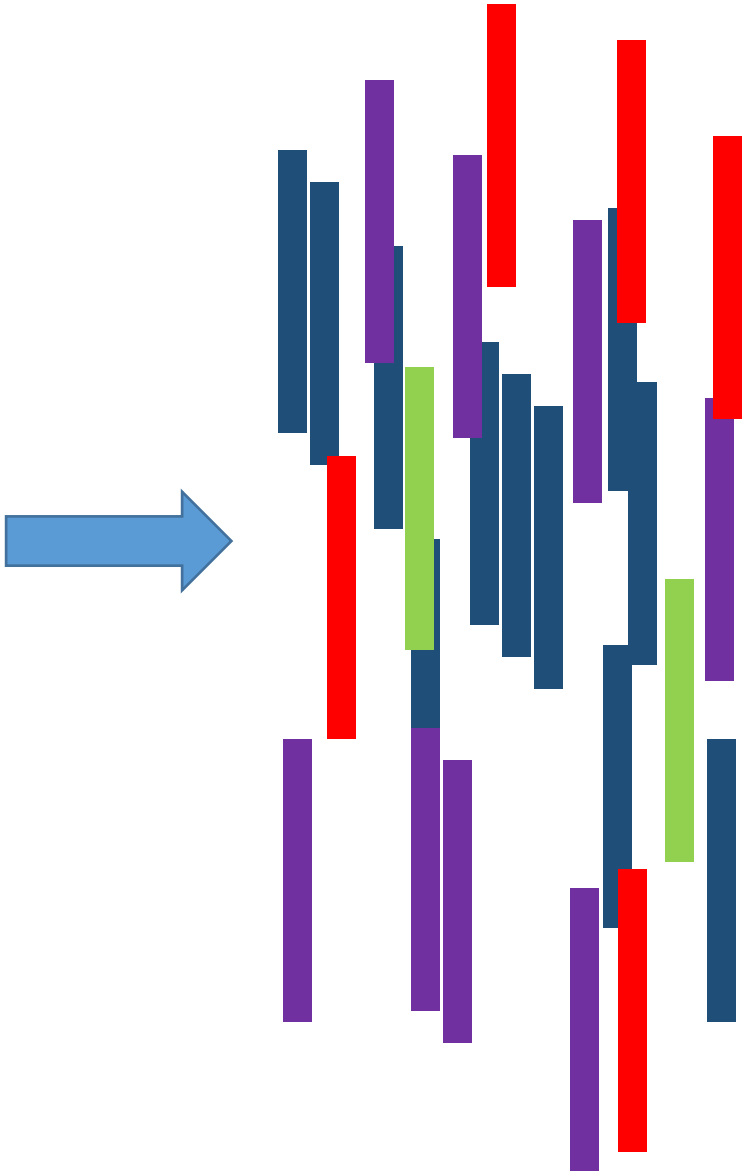
Mixed Population of Bacteria

5 Dark Blue
4 Purple
3 Red
2 Green

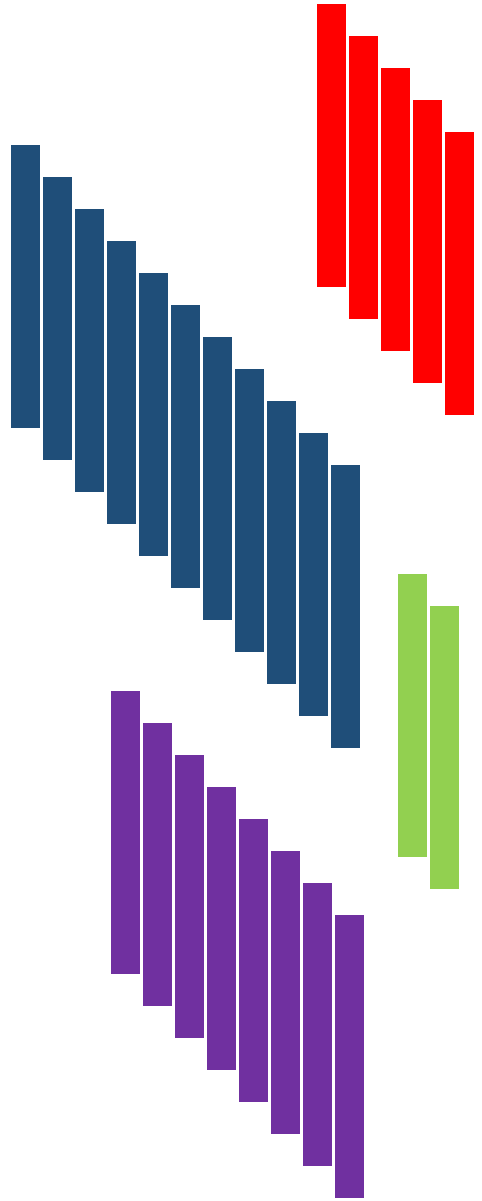


Environmental Swab
Clinical Swab

Shotgun Metagenomic Sequencing



Bioinformatic Assembly of Each Bacteria

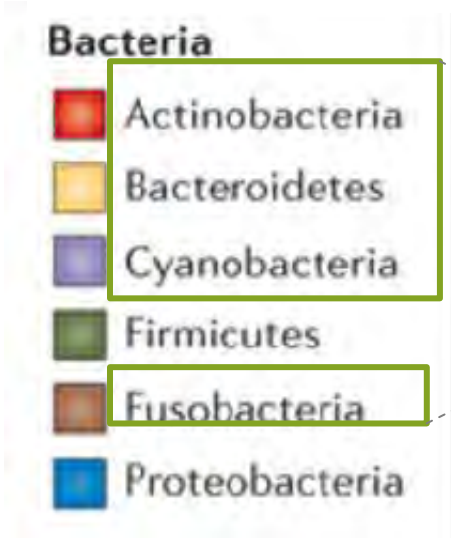


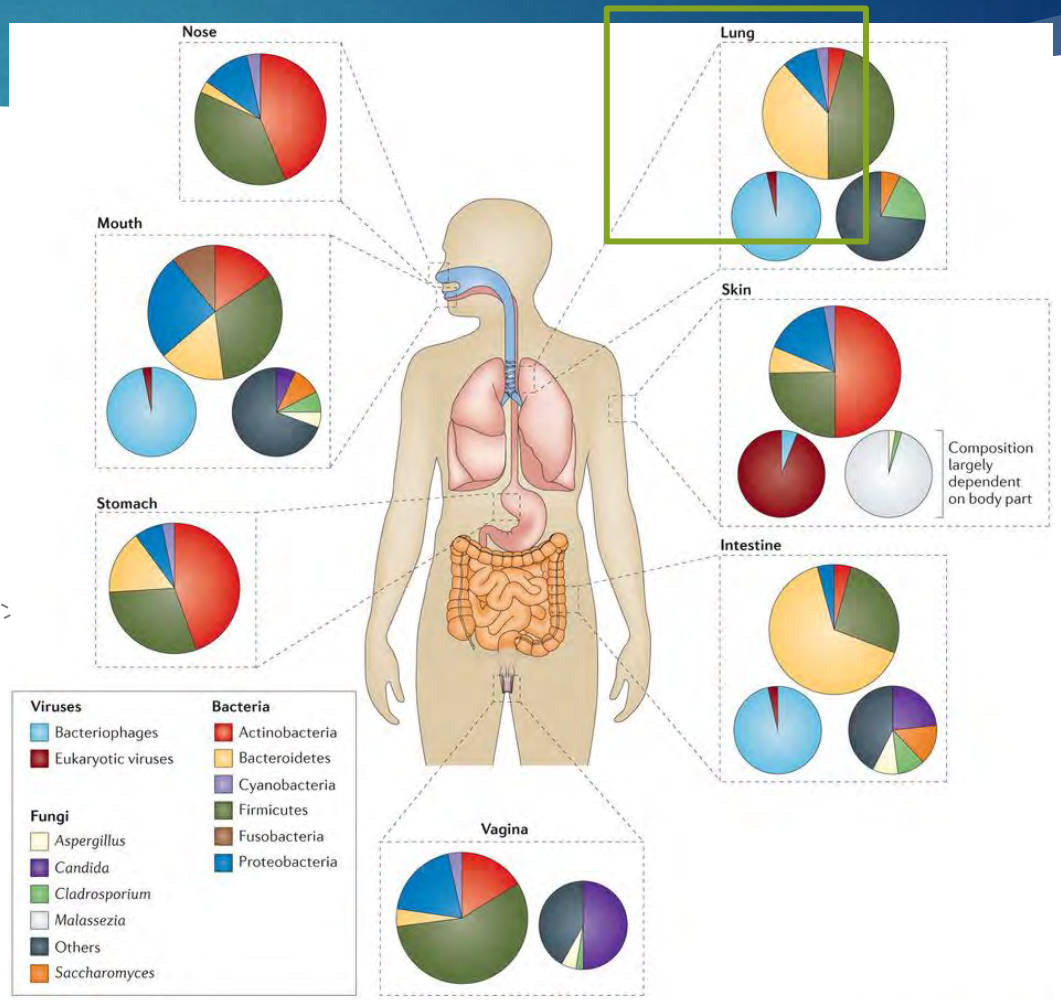
Mixed Samples- Metagenomic

- Same DNA library prep methods as purified genomic DNA can be used
- Will require significantly more sequence depth
- May require longer read lengths to differentiate between organisms or determine strains
- 16S rRNA sequencing can be used to identify bacterial communities present

16S rRNA (Microbiome) Sequencing

Viral, bacterial and fungal microbiota





Nature Reviews Immunology, Marsland and Gollwitzer 2014

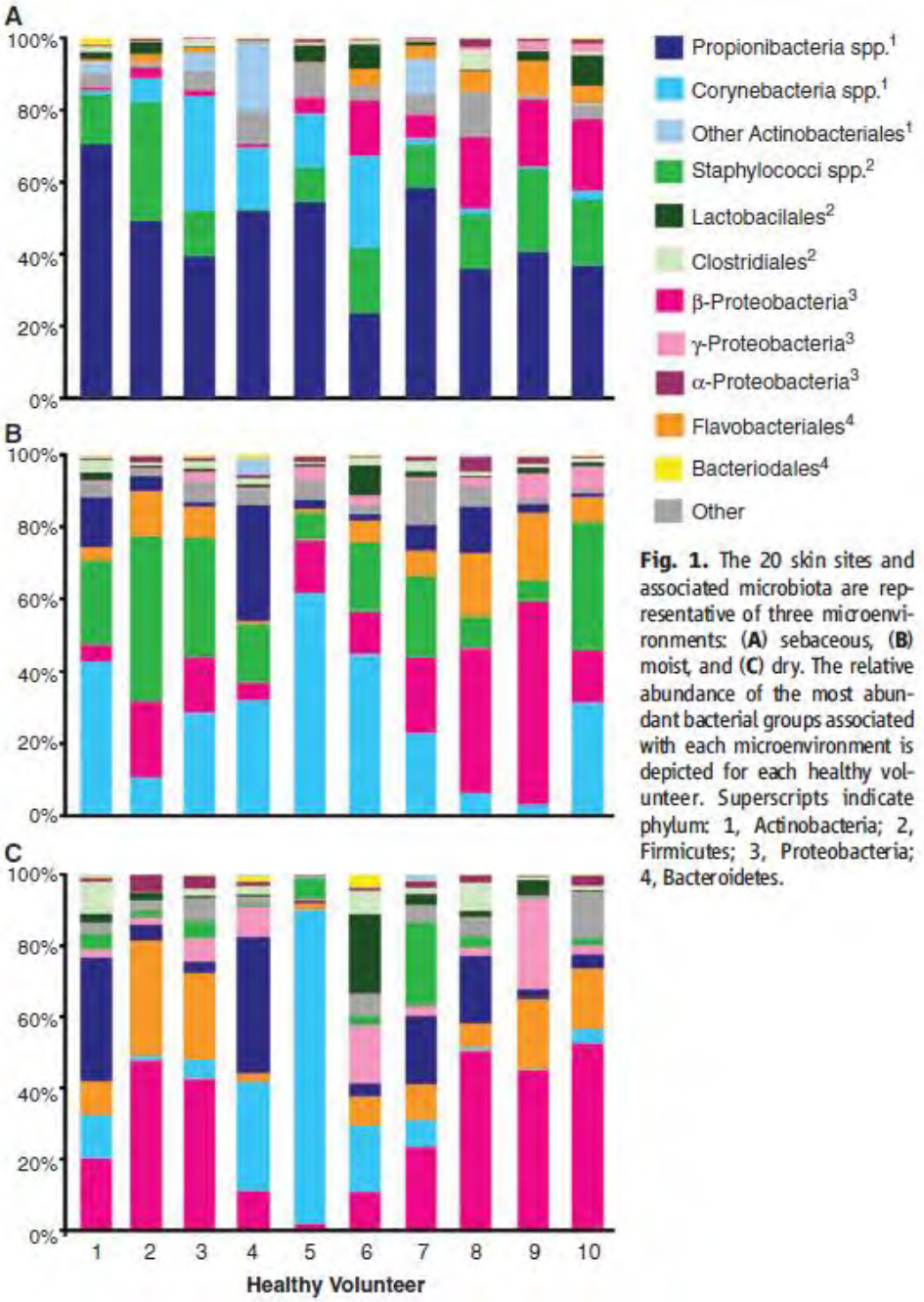
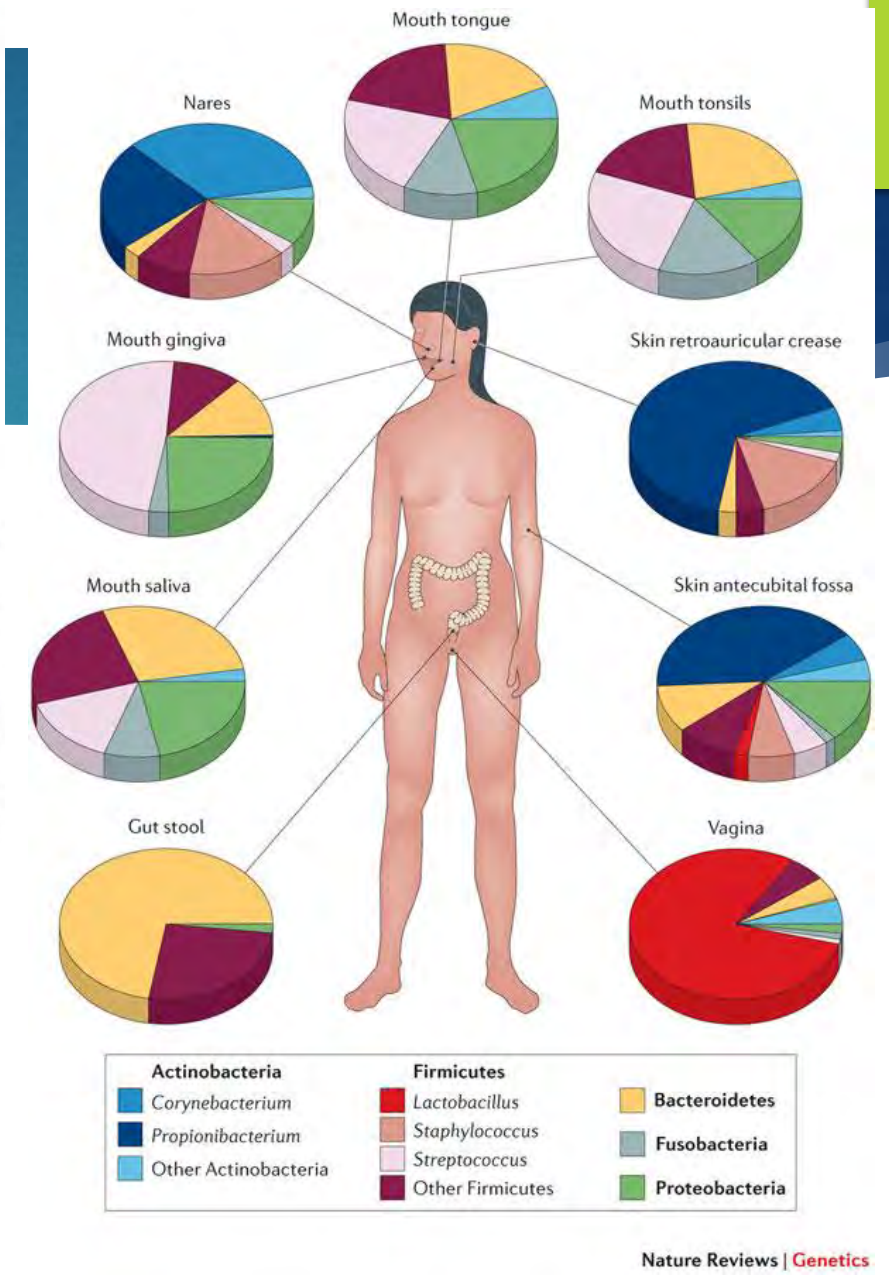


Fig. 1. The 20 skin sites and associated microbiota are representative of three microenvironments: **(A)** sebaceous, **(B)** moist, and **(C)** dry. The relative abundance of the most abundant bacterial groups associated with each microenvironment is depicted for each healthy volunteer. Superscripts indicate phylum: 1, Actinobacteria; 2, Firmicutes; 3, Proteobacteria; 4, Bacteroidetes.

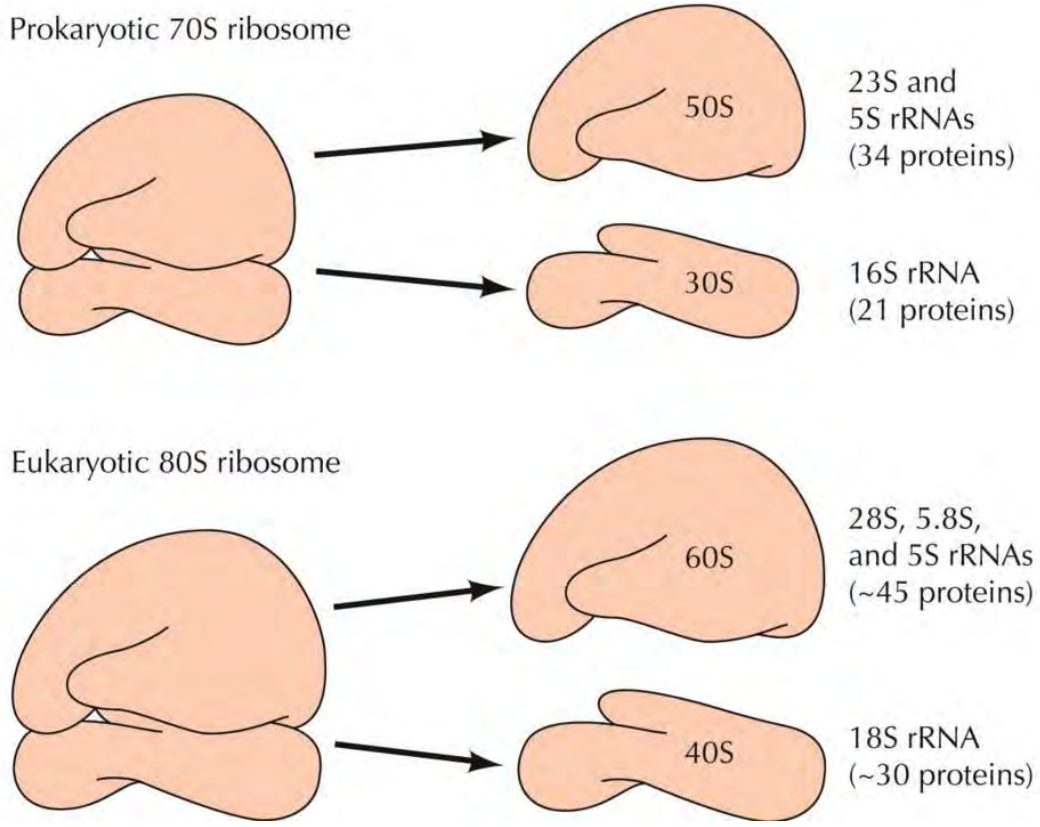
Science 29 May 2009: Vol. 324 no. 5931 pp. 1190-1192.



Nature Reviews Genetics 15, 577–584, 2014.

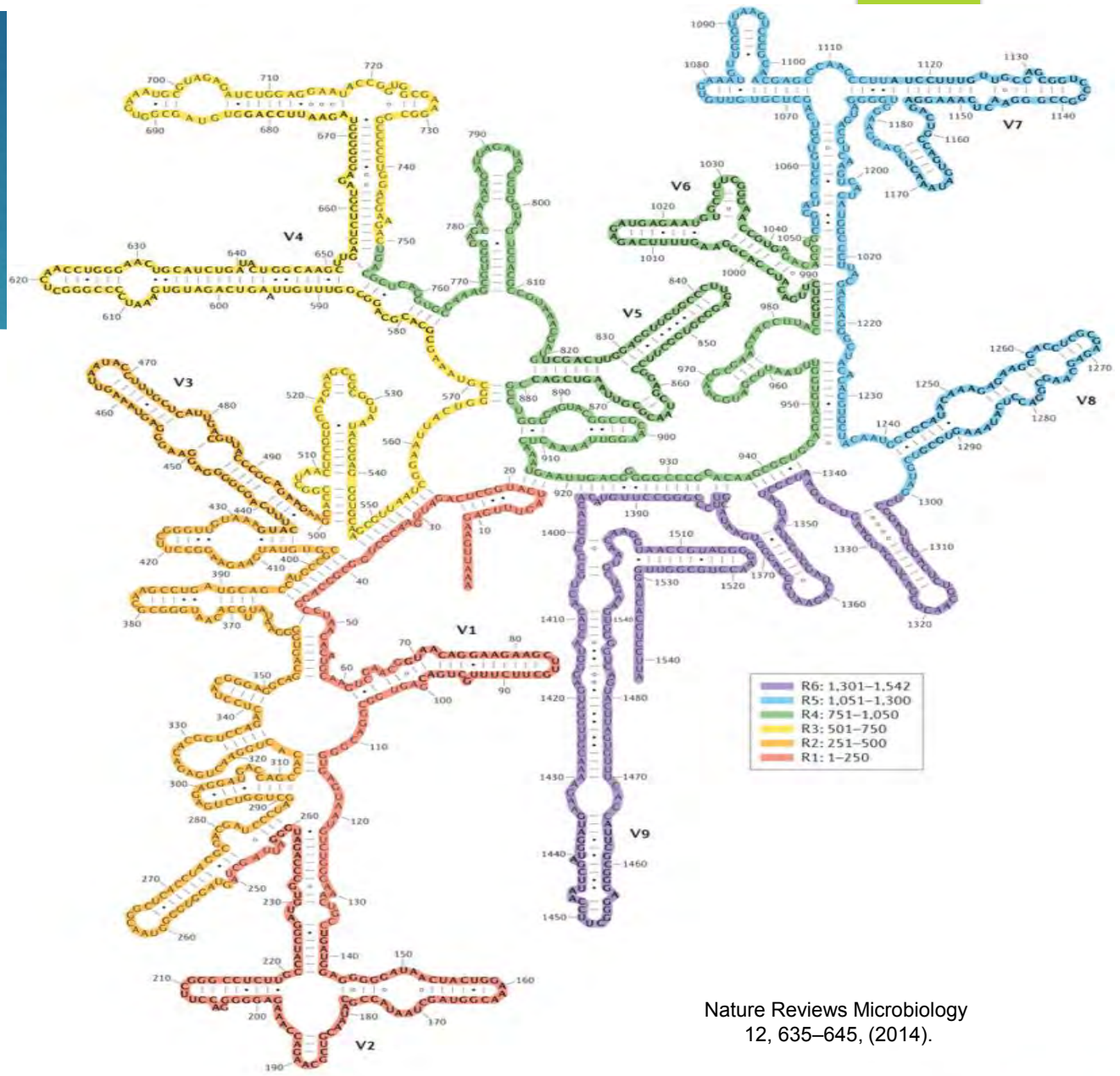
16S rRNA Gene

- Small subunit of prokaryotic ribosomes
- Sequencing DNA gene sequence of a functional rRNA
- Compare 16S sequence to database of known sequences



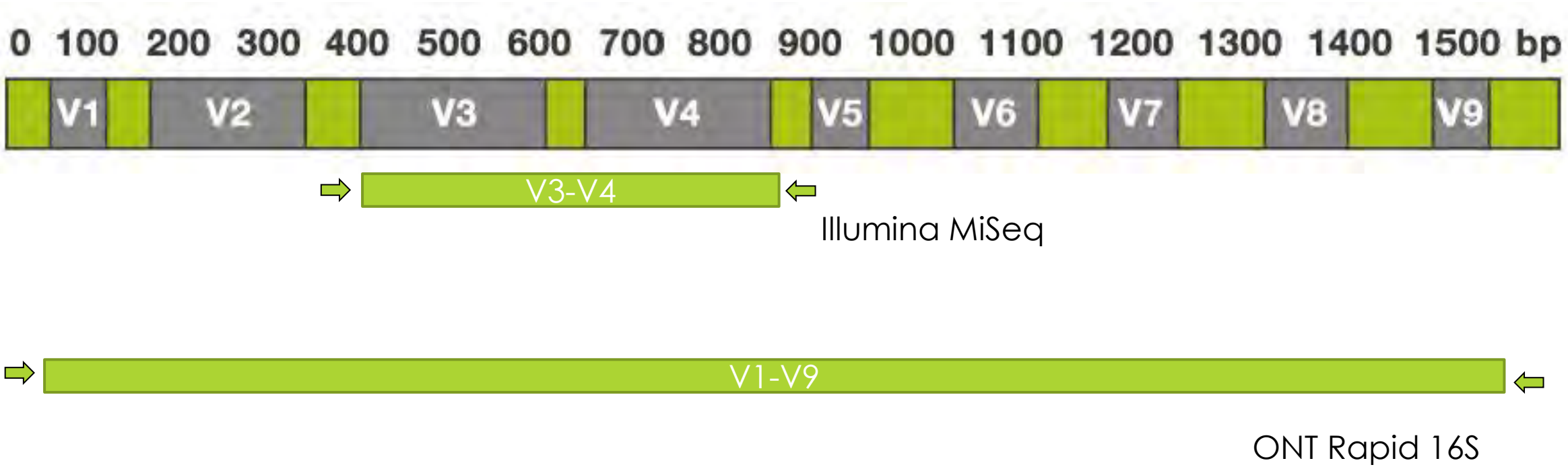
16S rRNA

- Used for reconstructing phylogenies, due to slow rate of evolution
- PCR with primers in conserved regions to amplify variable regions (V)
- Sequence 16S rRNA PCR amplicon



Nature Reviews Microbiology
12, 635–645, (2014).

16S rRNA Gene



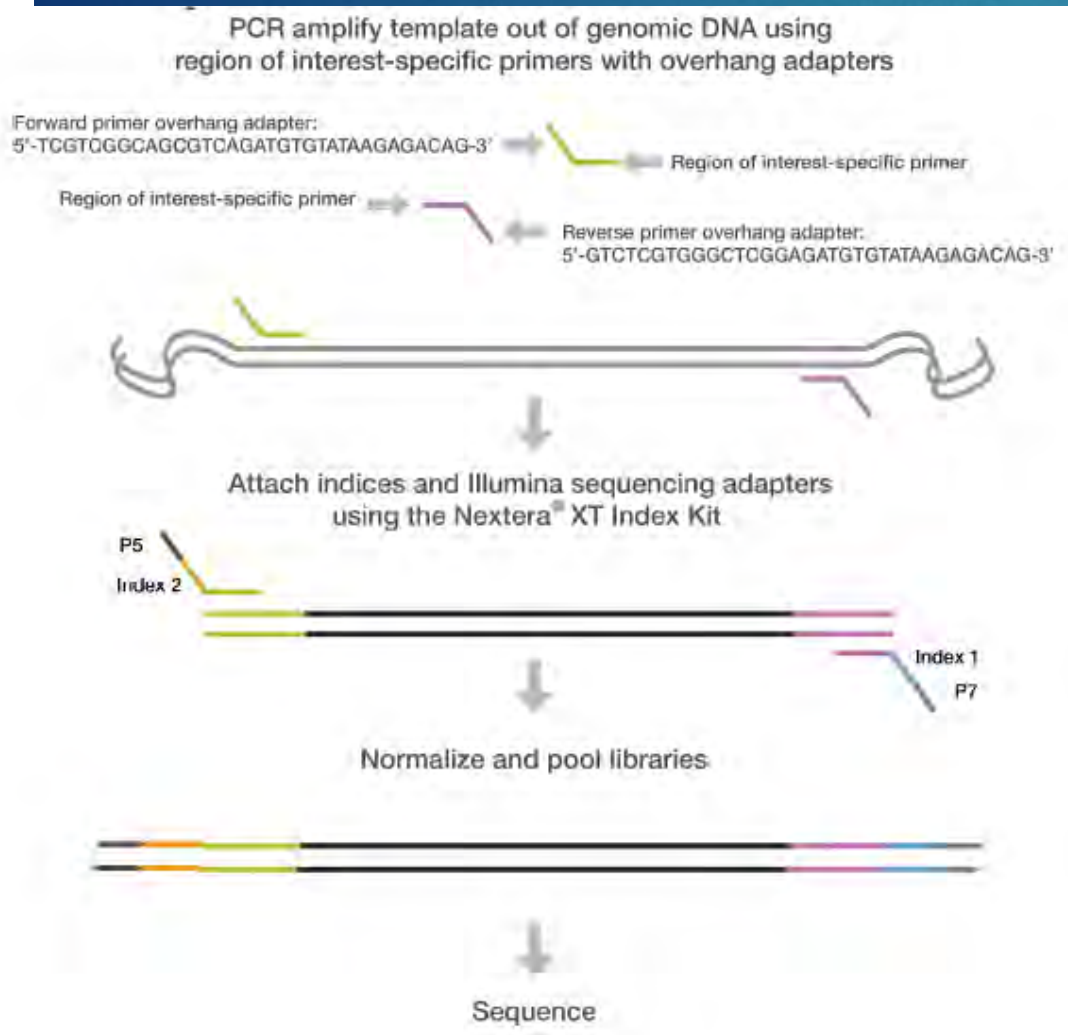
Illumina 16S rRNA Sequencing

16S Metagenomic Sequencing Library Preparation

Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System

https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf

Illumina 16S rRNA



Isolate DNA

V3-V4
PCR

V3-V4-Nextera Primers **PCR**
30 cycles

Clean up

Illumina Nextera **PCR**
Set A,B,C,D Index Primers
PCR- 8 cycles

Clean up

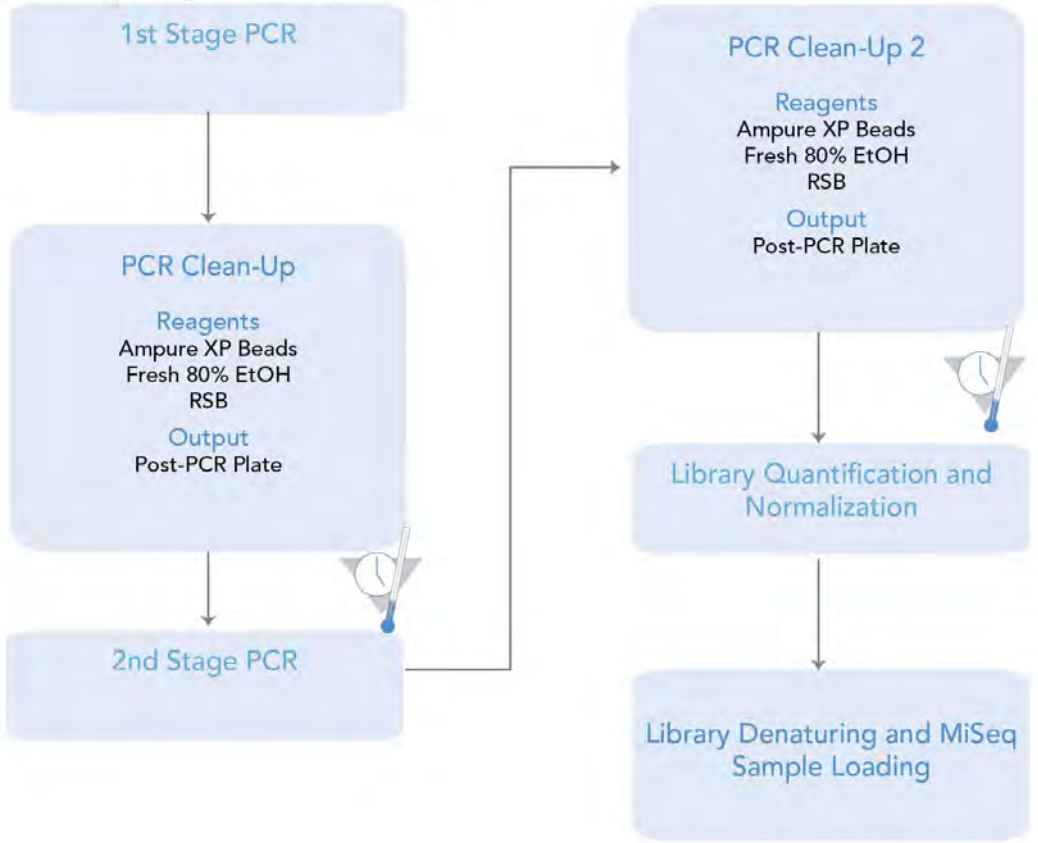
QC- Qubit
Bioanalyzer

Can sequence up to 384 samples together

Pool 96-384 samples → Sequence MiSeq 2x300bp

Illumina 16S rRNA Sequencing

Figure 2 16S Library Preparation Workflow



Illumina 16S rRNA Primers

- 16S Amplicon PCR Forward Primer = 5'
  TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

- 16S Amplicon PCR Reverse Primer = 5'
  GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

- Illumina Adapter Overhang- 16S rRNA Primer

ONT 16S Barcoding Kit 1-24

Can sequence up to 24 samples together

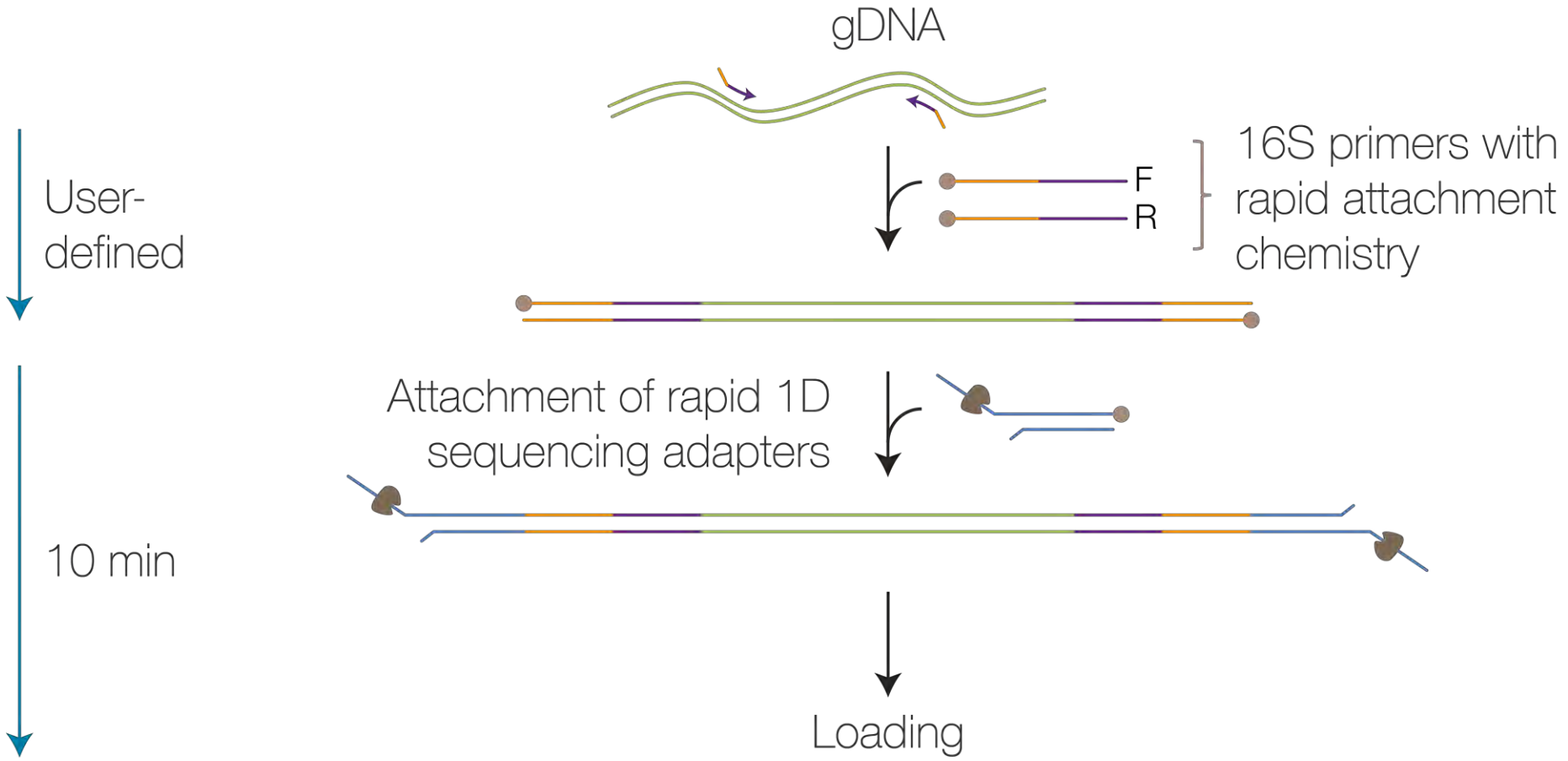
16S Barcoding Kit 1-24 SQK-16S024

Genus-level bacterial identification with barcoding for up to 24 samples.

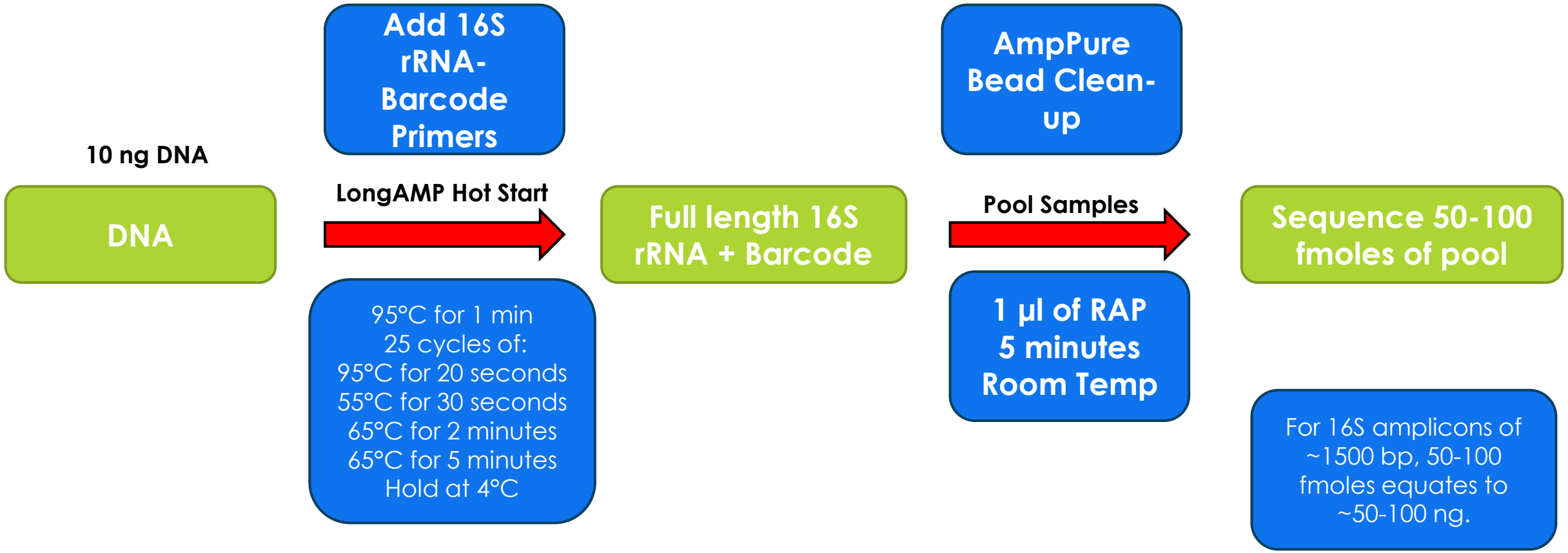
Includes a Flow Cell Priming Kit

- Preparation time: PCR + 10
- Read length: = full-length 16S gene (~1.5kb)
- Input amount: 10 ng gDNA

ONT 16S Barcoding Kit 1-24



ONT 16S Barcoding

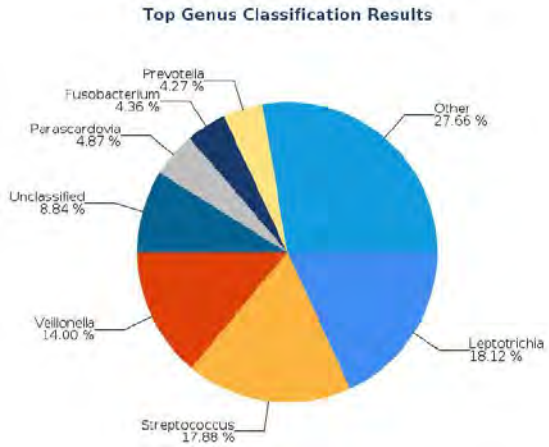


Example 16S rRNA Pipeline

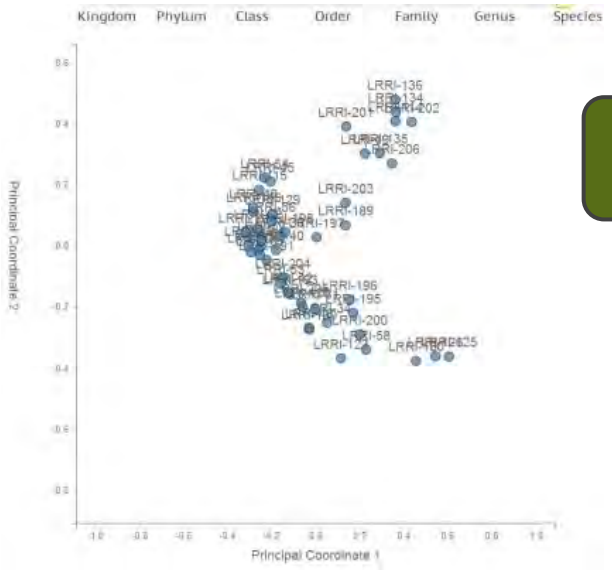
Fastq sequencing reads → 16S Metagenomic Pipeline → Identify Bacteria and Archaea Present (OTU)

16S Metagenomics App
Kraken Metagenomics App

GREENGENES
The 16S rRNA Gene Database and Tools

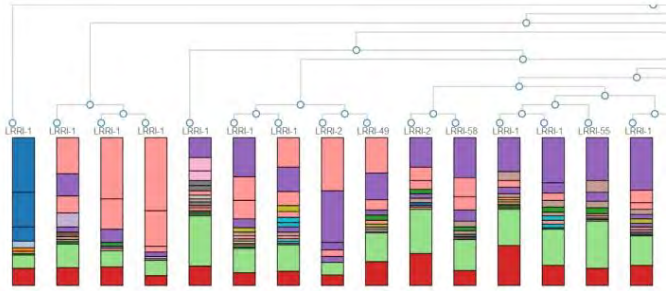


Assess Diversity of Microbiome

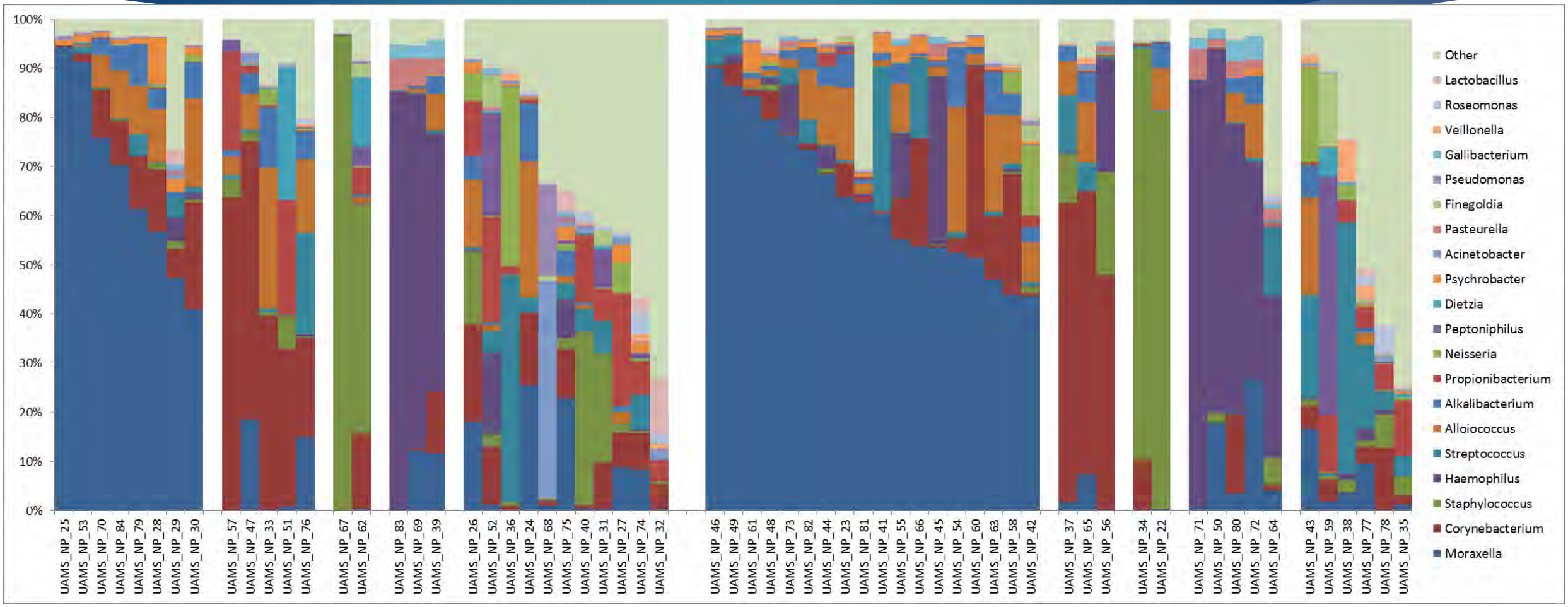


Assess # and % of Genera/Species Identified

Compare Within and Across Individuals



Nasopharyngeal Microbiome in Asthmatics

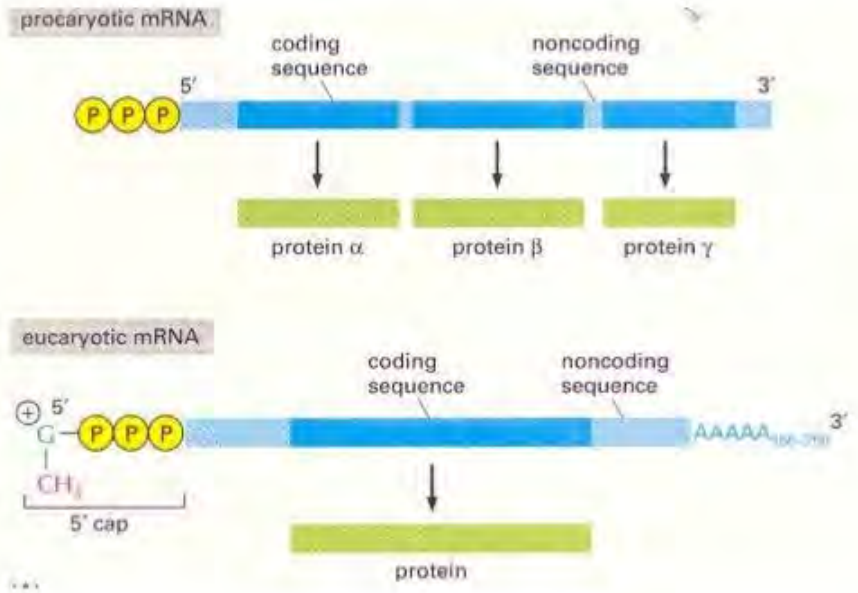


RNA Sequencing (metatranscriptomic)

RNA Library Prep

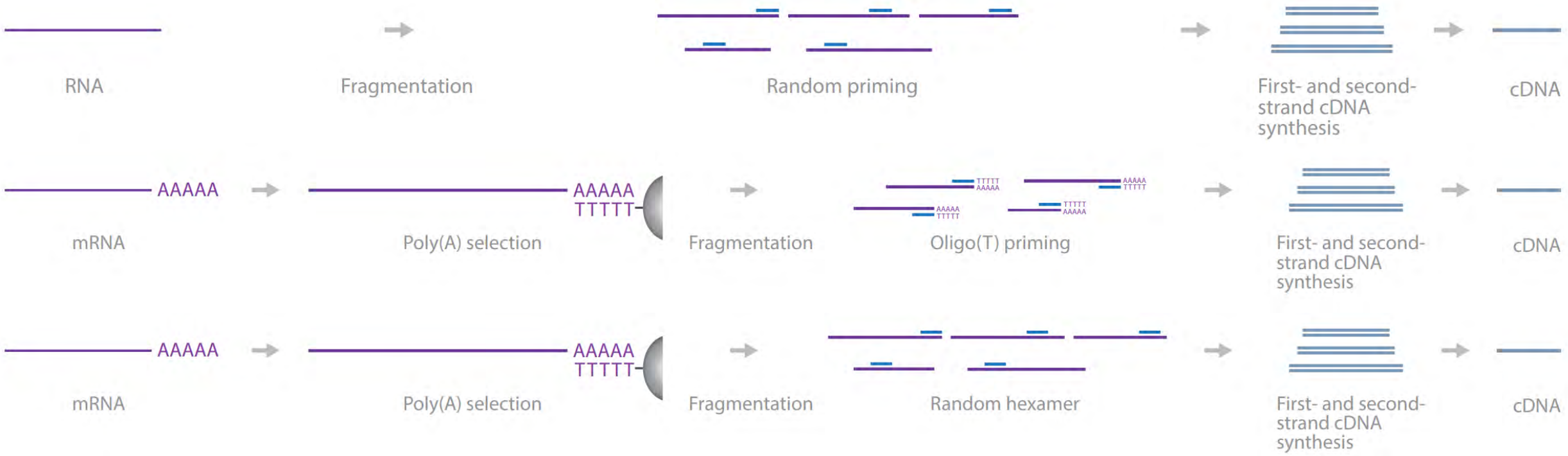
- Total RNA
  - Ribosomal Depletion
- Stranded RNA
  - Differentiate between - & + sense RNA strand (-ssRNA virus: genomic vs mRNA)
- mRNA/Poly-A Selected RNA
  - Eukaryotic organisms only;

Comparison of prokaryotic and Eukaryotic mRNA molecules:

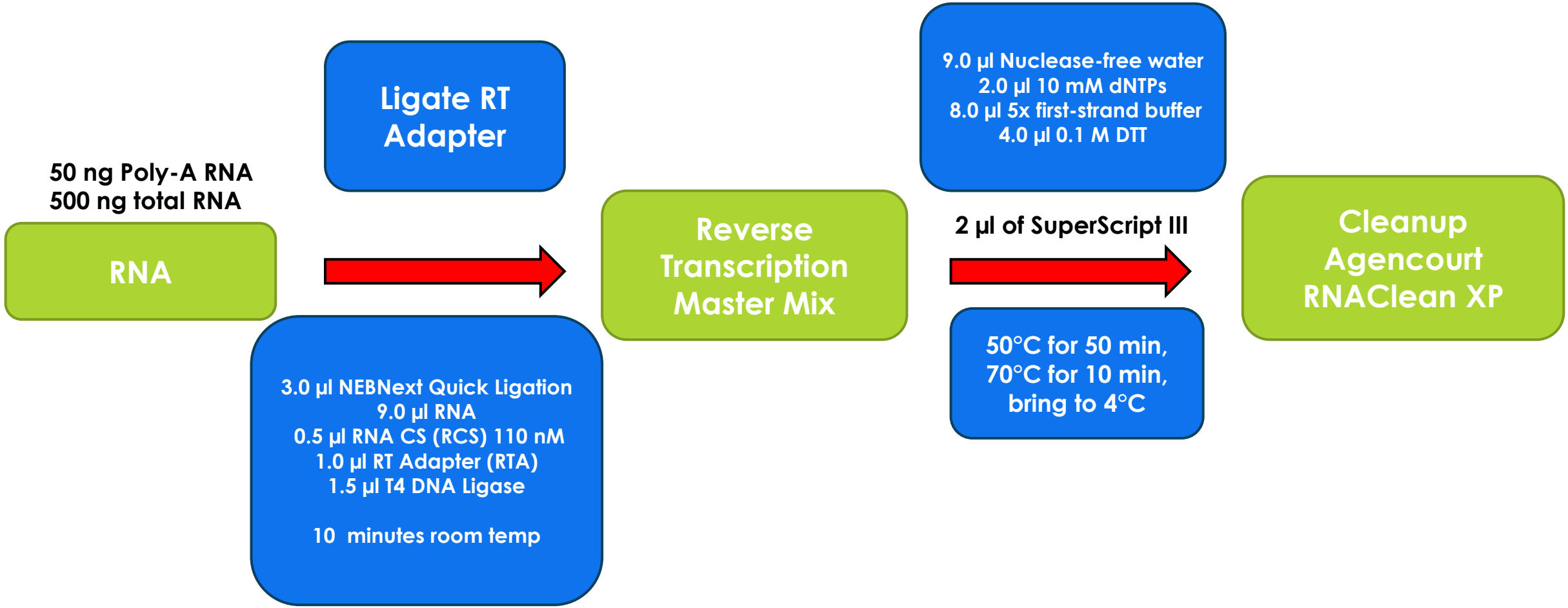


Mol. Biol. Of the Cell, Alberts et al., 2002.

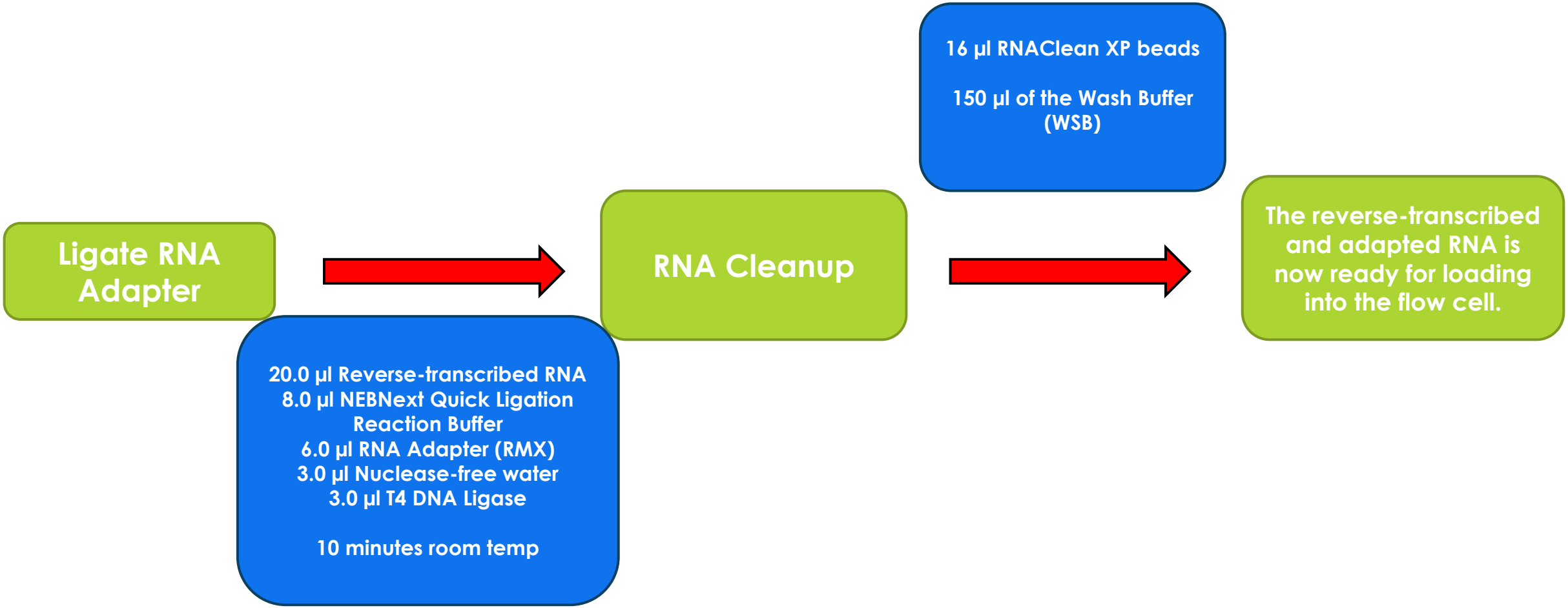
Overview of RNA Sequencing



ONT Direct RNA Sequencing



ONT Direct RNA Sequencing



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Correction: Author Correction

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Direct RNA Sequencing of the Coding Complete Influenza A Virus Genome

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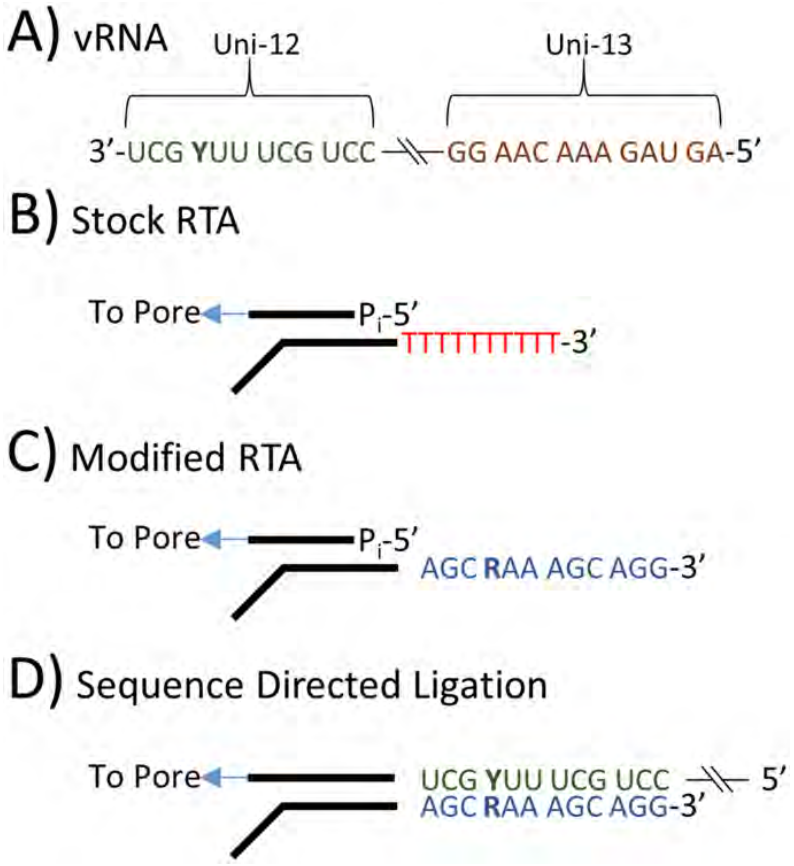
Influenza Virus ONT Direct RNA Sequencing

- (**A**) Influenza A viruses contain highly conserved 12 and 13 nt sequences at the 3′ and 5′ termini.
- (**B**) The key component of Oxford Nanopore direct RNA sequencing is a Reverse Transcriptase Adapter (RTA) which targets poly(**A**) mRNA and is ligated to the 3′ end of the mRNA. A sequencing adapter is then ligated to the RTA which directs the RNA strand into the pore for sequencing.
- (**C**) The RTA was modified to target the 3′ conserved 12 nt of the influenza A virus genome.
- (**D**) The modified RTA hybridizes and is ligated to vRNA in the first step of direct RNA sequencing.



Questions?