Better lives through livestock

Library Preparation – Illumina

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Africa CDC Genomics Workshop

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ASLM AFRICAN SOCIETY FOR LABORATORY MEDICINE

African Union

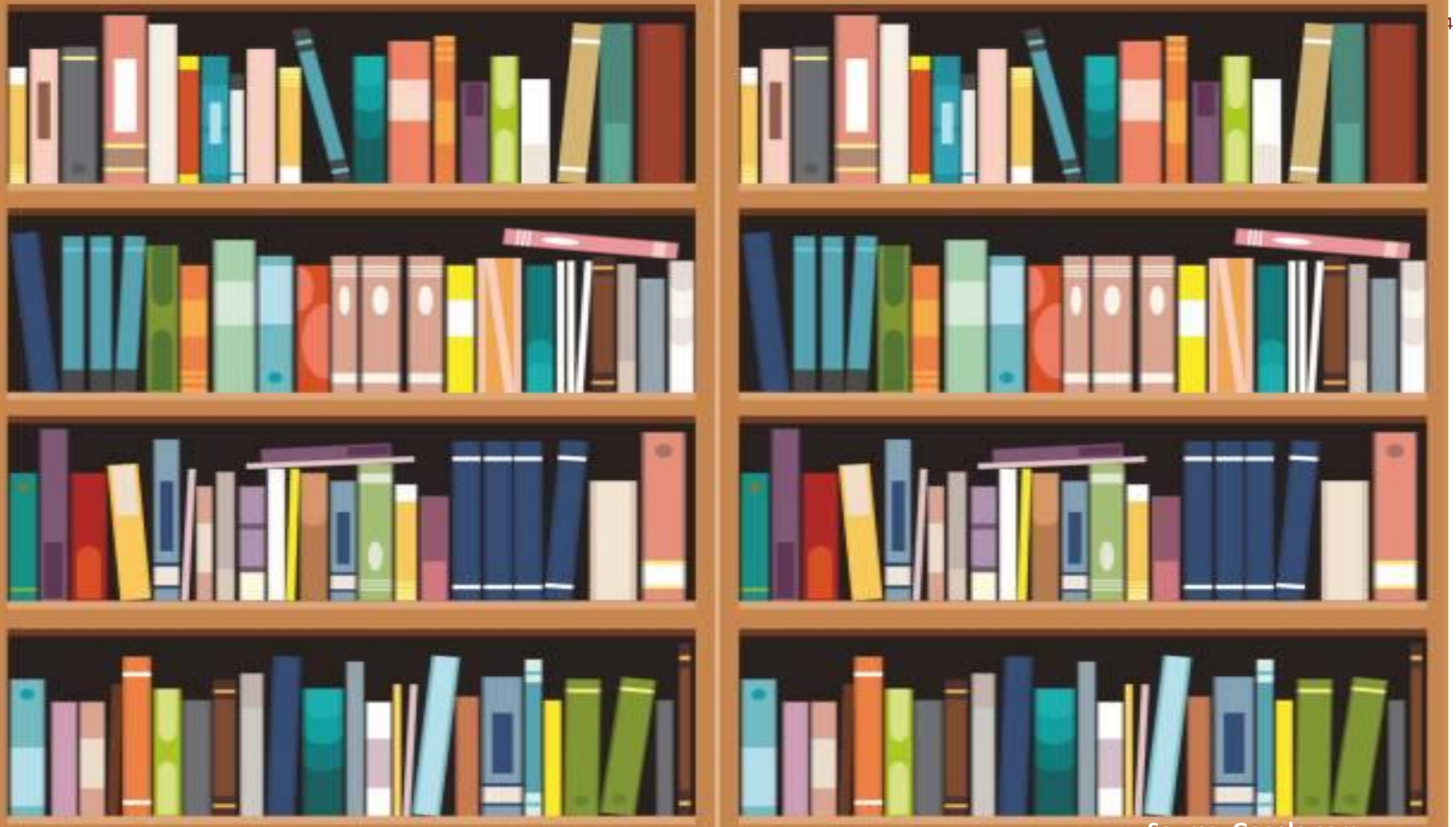
AFRICA CDC Centres for Disease Control and Prevention Safeguarding Africa's Health


Source: Google.com

What is a library?

- A **collection of books** used for reading or study, or the **building** or room in which such a collection is kept (Britannica).

- A **collection of DNA** or **RNA fragments** that have been **prepared for sequencing**.

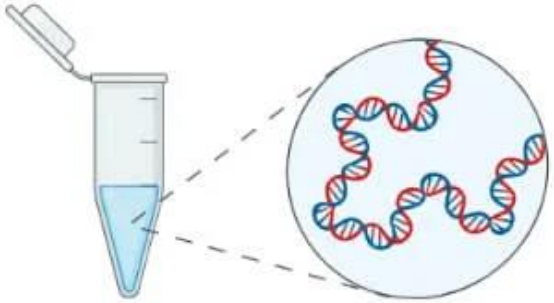


Source: Google.com

NGS Workflow - Illumina

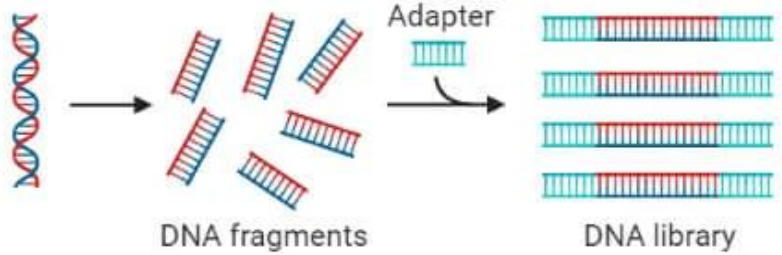
Step 1:

Nucleic acid extraction



Step 2:

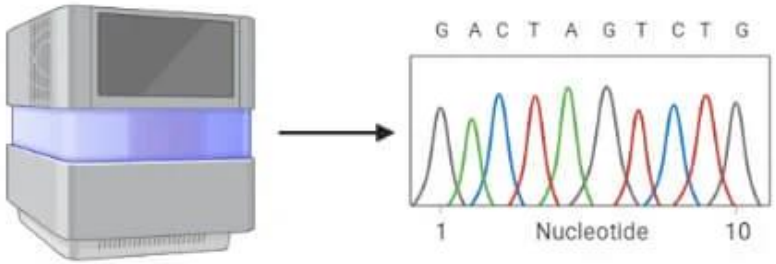
Library preparation



Next Generation Sequencing Workflow

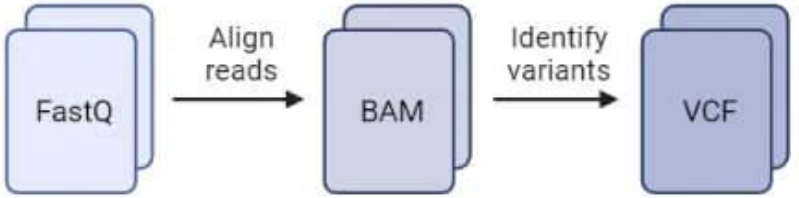
Step 3:

Sequencing



Step 4:

Analysis



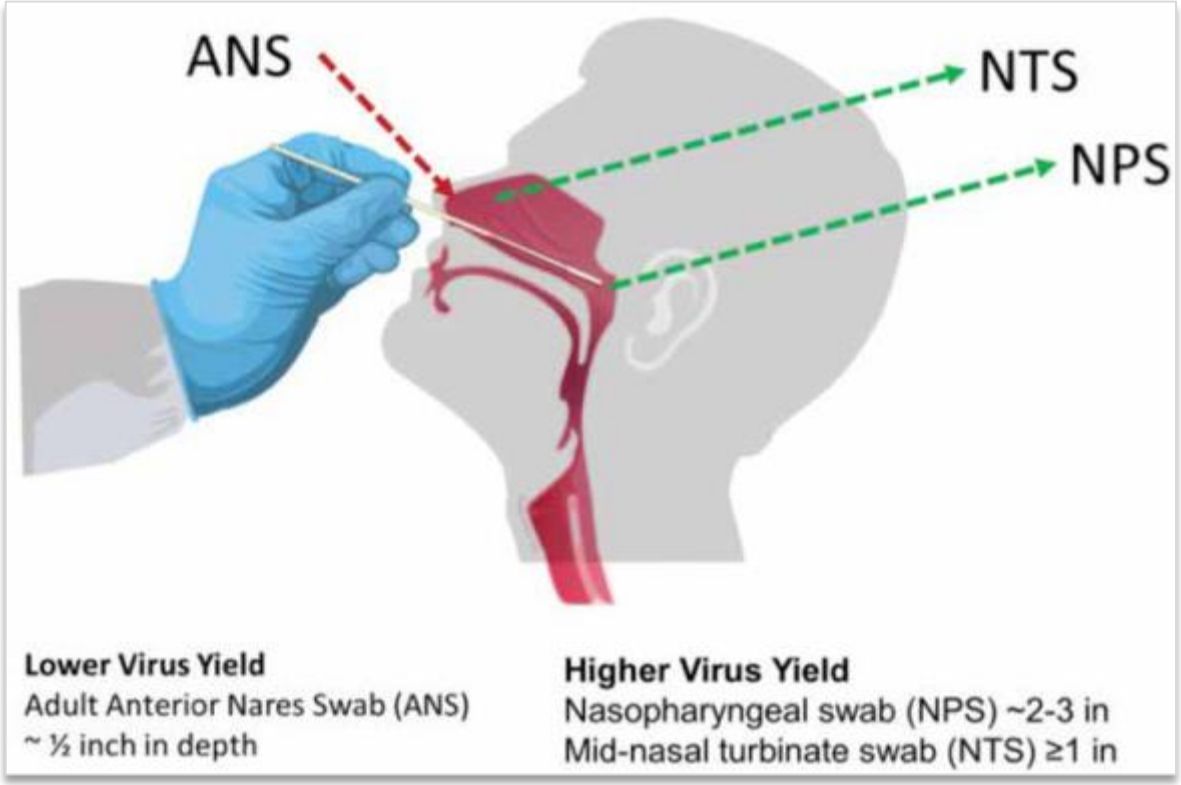
Source: Illumina.com

Illumina COVIDSeq™ - Introduction

- **Application/Use**: amplicon-based kit for detection of the SARS-CoV-2 virus genome for confirmatory diagnostics, surveillance and genetic epidemiology.

- **Test Samples Range** – qPCR COVID positive RNA from:
  - Nasopharyngeal (NP), Oropharyngeal (OP)
  - Optimised for sputum

- It is a **high throughput protocol** capable of multiplexing --> NextSeq 500/550 (n=384) and MiSeq (n=96).



Differences among clinical samples collected from different areas of the nasal cavity
ANS - Anterior Nares Swab
NTS – Nose and Throat Swab

Martinez, 2020, Clin. Microb. News, Vol 42, 15,, Pg 121-127

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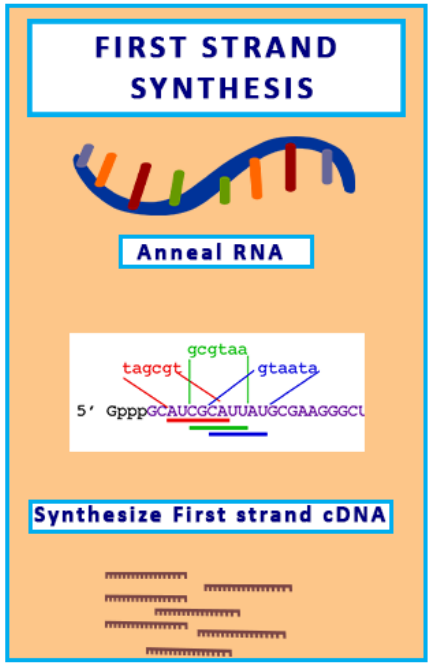
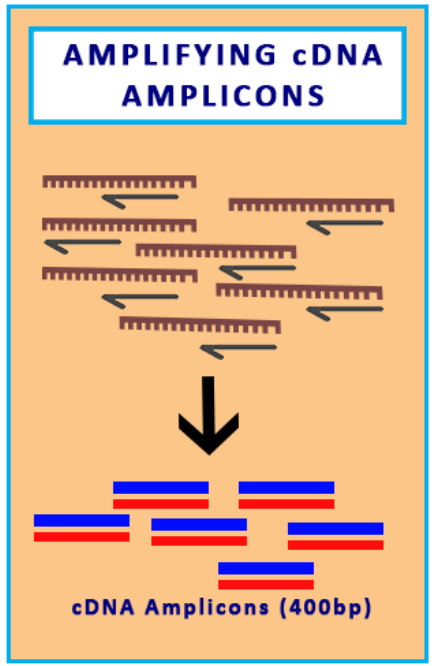
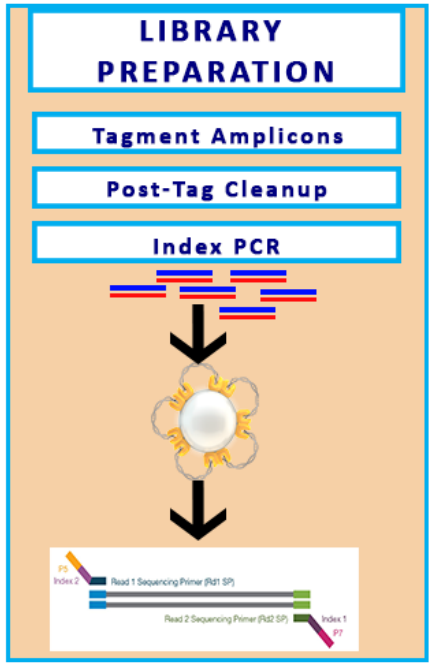
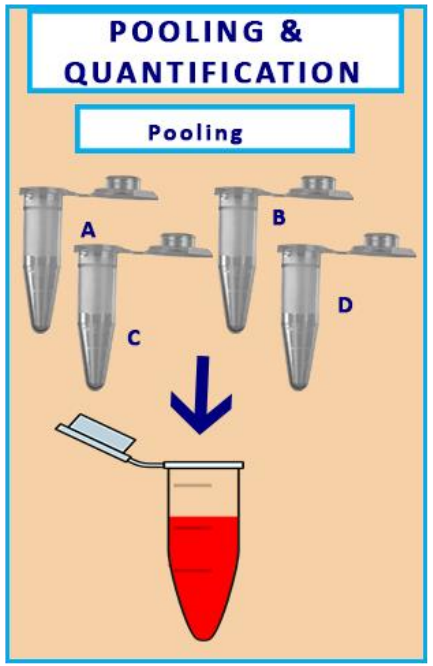
Illumina COVIDSeq™ Test Workflow

The workflow is divided into **five** sections:







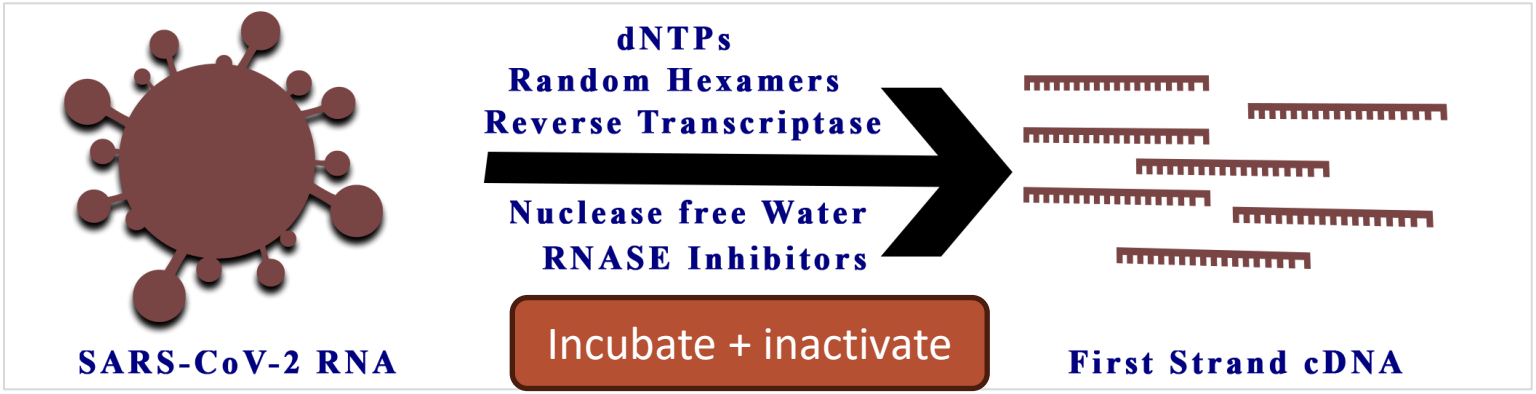


Source: Illumina.com

Step 1: First Strand Synthesis

- RNA is converted to **complementary DNA** (cDNA) through **reverse transcription** (RT).
- RT is the enzyme-mediated synthesis of a DNA molecule from an RNA template. The cDNA can be used as a template for PCR amplification.
- The reaction happens in the presence of:
  - ✓ RNA template - purified
  - ✓ Random Hexamers – target both poly- and non-polyAdenylated RNA
  - ✓ Reverse Transcriptase
  - ✓ Nucleotides – the four dNTPs - – dATP, dTTP, dCTP, and dGTP – are the building blocks of the cDNA strands
  - ✓ RNase Inhibitor – protect RNA from RNA-degrading ribonucleases in the environment
  - ✓ Reaction Buffer - provides optimal conditions for enzyme activity
  - ✓ Nuclease free Water

COVIDSeq™ Kit contains First strand mix (FSM)
Add RTase



Source: Google.com

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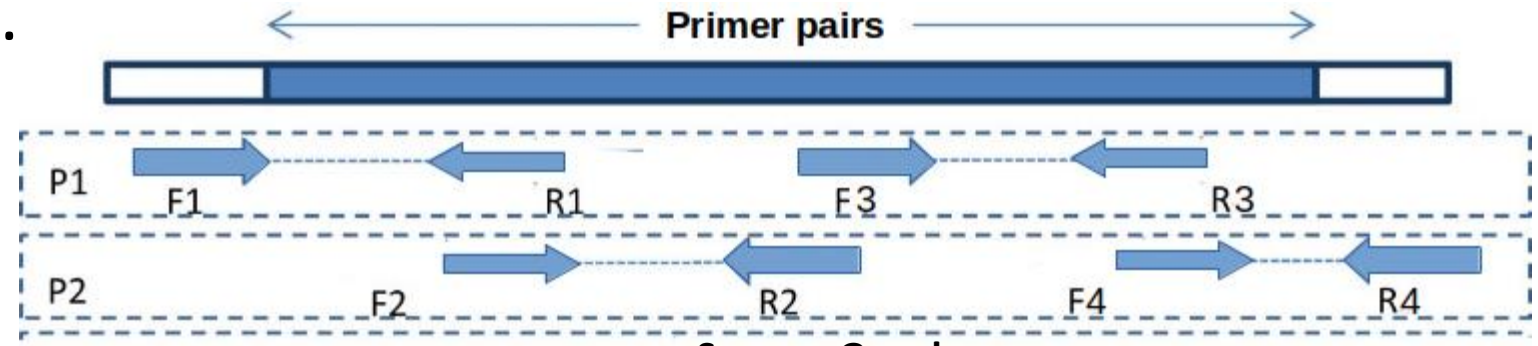
Step 2: Amplifying cDNA a.k.a. Tiling

- Amplification or enrichment of the target virus is done to the first strand **cDNA** using **Artic v3 primers** in the presence of a PCR master mix. (New kits have v4.1 primers – pick Omicron and newer variants).
- The **primers overlap to ensure coverage of the genome**.

- There are a total of **98 primers pairs** designed into **two pools. Why?**
- To prevent short overlap products being produced between neighbouring amplicons, 2 primer pair pools are used to alternate the pairs.

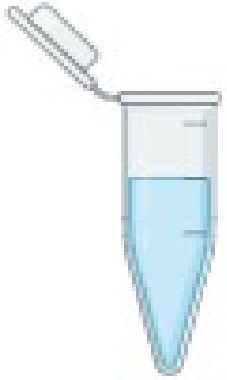


Source: Google.com

Step 2: Amplifying cDNA a.k.a. Tiling

- This step uses **two separate PCR reactions** to prepare and amplify cDNA.
- **Two reactions** (tubes/plates) must be prepared **for each sample.**

1



2

Source: Google.com

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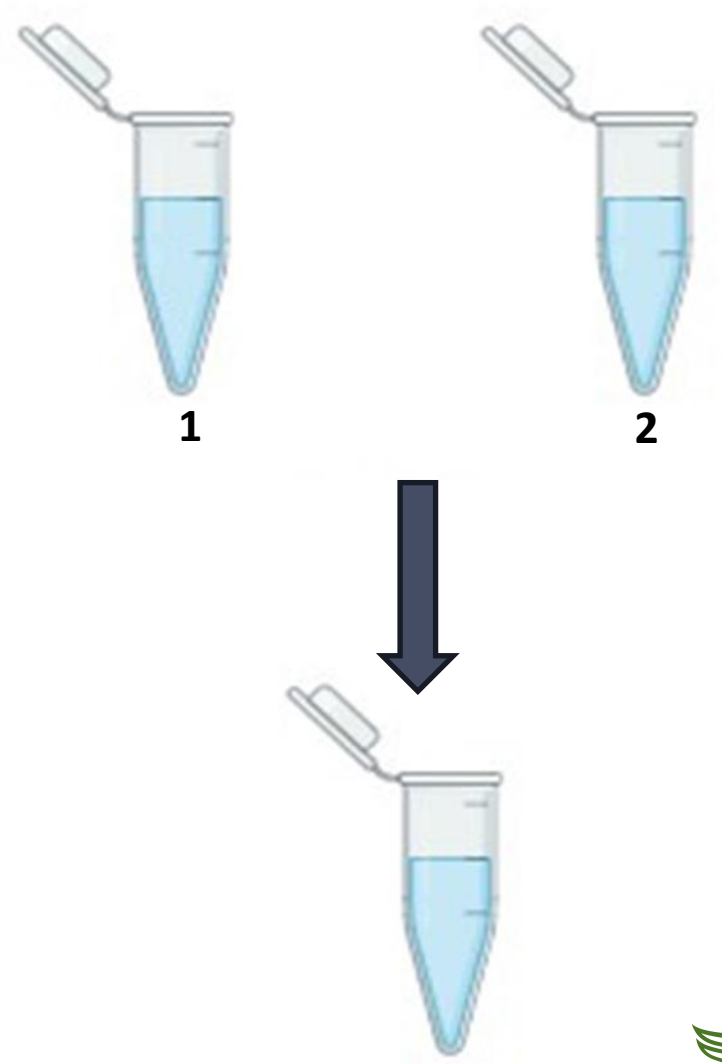
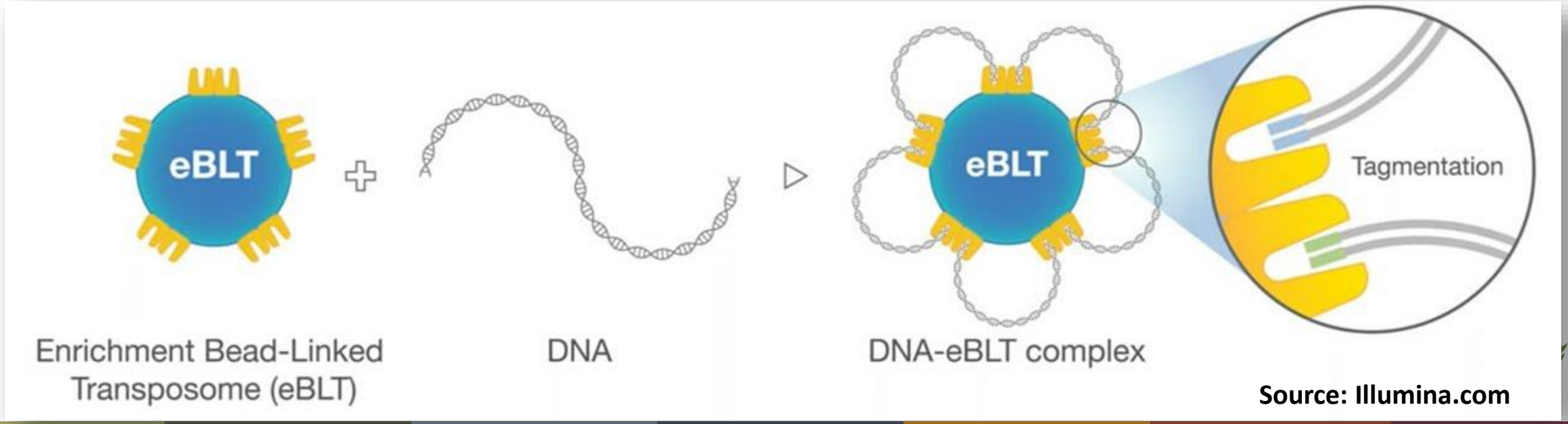
Step 3: Combine Pool 1 and 2 of each sample

- Check on gel/TapeStation if tiling worked!
- Then combine pool 1 and pool 2 into one sample after tiling step.



Step 4: Tagment PCR Amplicons:

- Enrichment Beads-Linked Transposomes (**eBLTs**) are used to **tagment PCR amplicons.**
- Tagmentation is an enzymatic process that **fragments** and **tags** the PCR amplicons with **adapter** sequences in a single reaction.
- Transposomes are bound to beads, fragment and tag DNA for subsequent enrichment step.
- Transposome-based technologies have streamlined production of sequencer-ready DNA libraries: **save time in reduced reaction steps, normalisation and plastics used**.



Step 5: Cleanup of Tagmented amplicons

- Adapter-tagged amplicons are **washed** before index PCR.
- **Tagment wash buffer** is used in two wash rounds.
- Leave the tagmented amplicons in 2nd round wash buffer till ready for next step. **Why?**

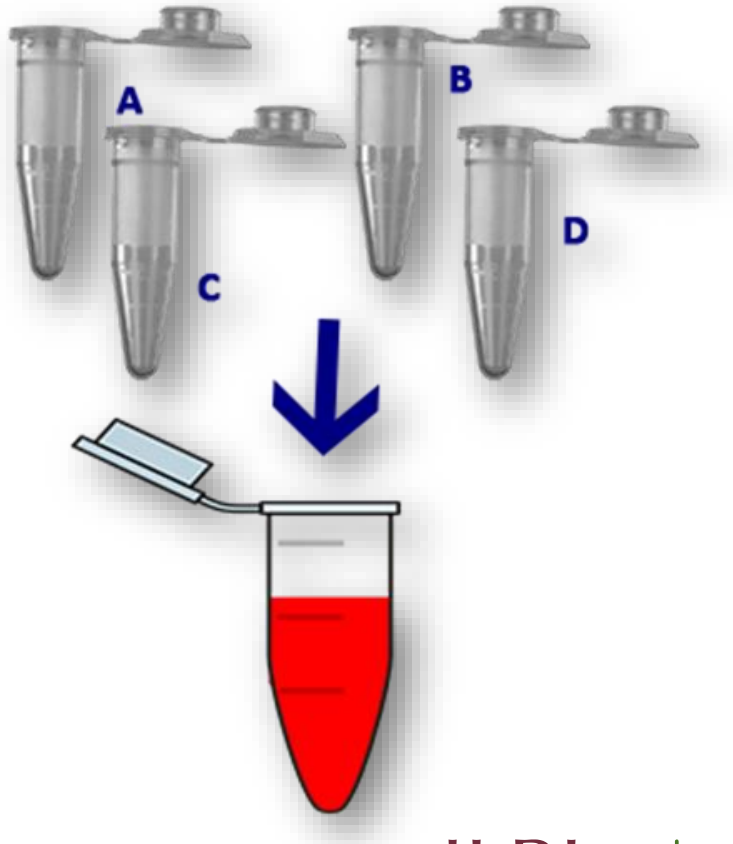
Step 6: Amplify Tagmented Amplicons

- 10 base pair **Index 1** (i7) adapters & **Index 2** (i5) **adapters** are added to amplicons for **multiplexing** and **cluster generation**. **Functions?**
- **P5** (i5) and **P7** (i7) are complementary to DNA sequences found on the **flow cell**, allowing for each molecule to be **captured and amplified**.
- **Index 1** and **Index 2** are **sample-specific indexes** that allow for multiple samples to be pooled prior to sequencing. By reading each index, each DNA insert can be assigned to its sample of origin at the end of the sequencing run.

Source: Illumina.com

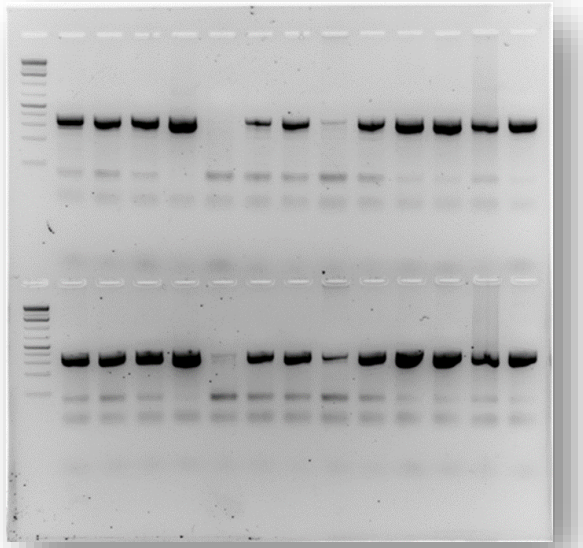
Step 7: Pooling and Clean-up 2

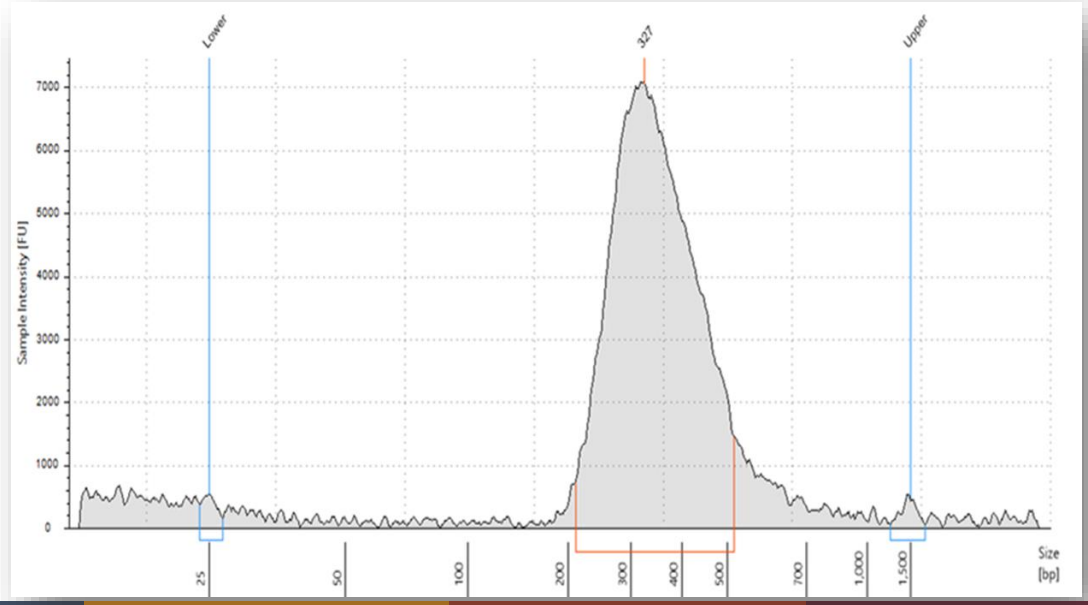
- **Equal volumes (5µl)** of each library are **pooled**/combined into **one sample tube** for clean-up.
- The pooled sample is **cleaned with Illumina Tune Beads (AMPure XP)**
- Next step is to **QC pool** on agarose gels or TapeStation or Bioanalyzer



Step 8: Library QC

- **Quantification:**
  - qPCR Kits (KAPA Library Quantification Kit etc)
  - Qubit **fluorometer** e.g. **4.2ng/ul**
    - Use qubit dsDNA High sensitivity Kit (0.1 to 120 ng)
    - If the readings are out of range repeat with dilution (e.g. 1: 100).
- **Sizing:** The **band/fragment size** is determined using either **gel electrophoresis** or Agilent **TapeStation/Bioanalyzer.** **Expected peak height is 400bp** (inclusive of adapters, indices and insert)





Step 9: Sequencing

Prepare library for loading on Illumina sequencer available at your facility.

Key Considerations for Library Preparation - 1

1) **RNA quality**: The quality of the RNA to be sequenced is critical to obtaining reliable results. Follow input DNA/RNA recommendations. Dilute using nuclease-free water.

2) **Fragmentation**: Being an enzymatic reaction, any **contaminants and inhibitors can inhibit this reaction**. Use recommended input DNA/RNA to ensure complete saturation of the beads and tagmentation for a consistently high yield library.

3) **Library clean up and size selection**: Don't allow the beads to dry out (reduces efficiency and low lib yields). Make fresh 80% Ethanol.

4) **Protocol complexity**: Library preparation can be complex, with some protocols requiring multiple steps to attach adapters and barcodes to nucleotides.

Key Considerations for Library Preparation - 2

6) **Library quantification**: Before loading the libraries onto the sequencer, they should be **adequately quantified** and **normalized** so that each library is sequenced to the desired depth. eBLT makes it easier to normalise libraries.

7) **Contamination**: Sample-sample contamination is a problem because libraries are often prepared in parallel.

8) **Cost**: Library preparation can be expensive.

Accurate library preparation leads to better quality sequencing reads and results

Quiz

1) What is a library?

A **collection of DNA or RNA fragments** that have been **prepared for sequencing**.

2) What are the **four steps** of next generation sequencing (NGS)?

   a) Nucleic acid isolation
   b) Library preparation
   c) Clonal amplification + sequencing
   d) Data analysis.

3) What are the steps in library preparation?

NA extraction & QC, Fragmentation, End Repair, Adapter Ligation, Size Selection, Amplification and QC

3) How do you QC your final library in the COVIDSeq™ workflow?

   a) Quantity - Fluorometer like Qubit
   b) Size – TapeStation, Bioanalyzer, agarose gel

4) What is the size of the final library in the COVIDSeq™ workflow?

- **400bp**

References

1. https://knowledge.illumina.com/library-preparation/general/library-preparation-general-reference_material-list/000003342
2. https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/Illumina-COVIDSeq-Test/illumina-covidseq-test-reference-guide-ruo-1000000126053-04.pdf
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5. Carpenter RE, Tamrakar VK, Almas S, Brown E, Sharma R. COVIDSeq as Laboratory Developed Test (LDT) for Diagnosis of SARS-CoV-2 Variants of Concern (VOC). Arch Clin Biomed Res. 2022;6(6):954-970. doi: 10.26502/acbr.50170309. Epub 2022 Nov 28. PMID: 36588916; PMCID: PMC9802674.

Shukran! Thank you!

Merci beaucoup pour votre attention!

Acknowledgements

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