Adaptive Protocol for Cholera Whole-Genome Sequencing during the Malawi Epidemic

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Climade

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Outline

- Introduction
- Sample Preparation
- Whole-Genome Sequencing Method

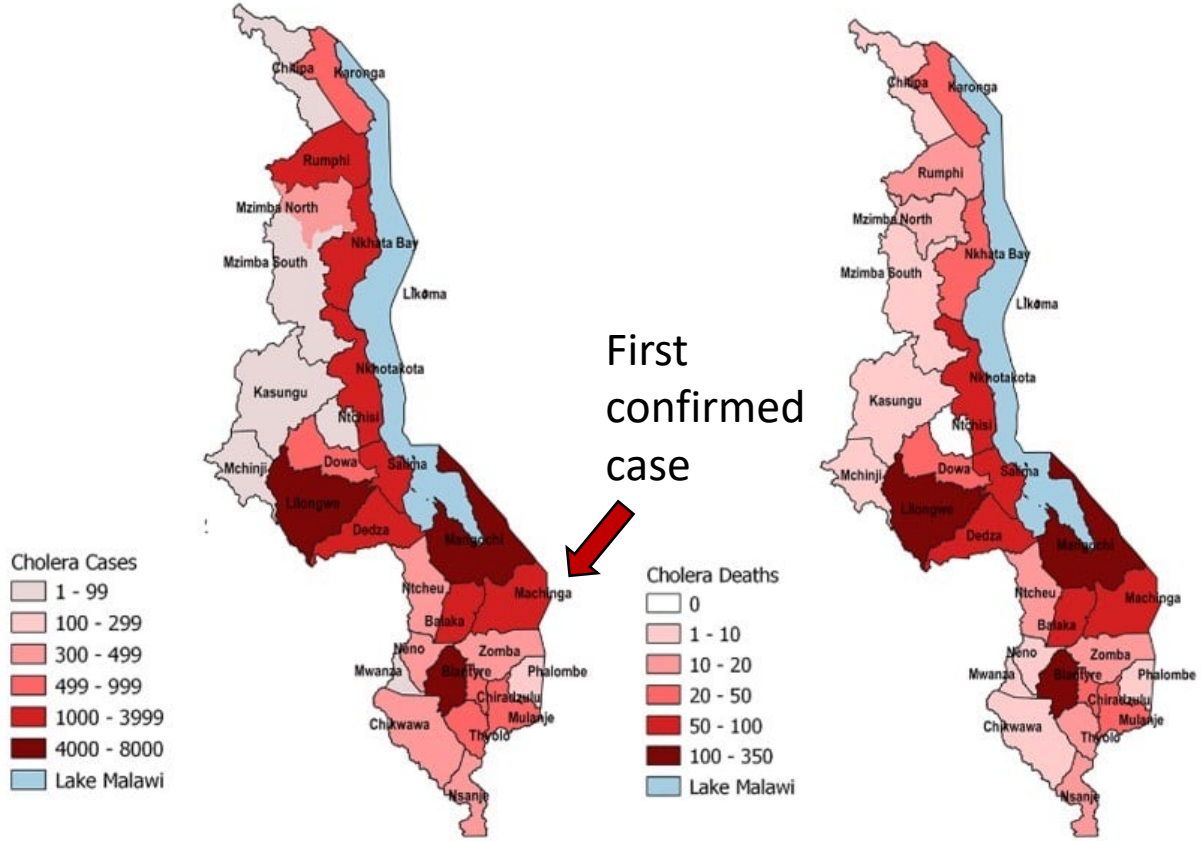
Introduction

- Malawi is in South-East Africa
- 118 480 km^2
- Population size over 19 million
- Divided into 4 regions; Southern, Eastern, Central and Northern
- 29 Districts

Cholera Outbreak Comparison

Year	Cases	Deaths	Case Fatality Rate
1998-1999	25 000	860	3.4%
2001-2002	33 546	968	2.3%
2008-2009	5 751	125	2.2%
2022-Ongoing	>58 000	1 753	>3%

Cholera Outbreak Malawi 2022/2023



Widespread outbreak, involving all **29 districts**

First confirmed case in **Machinga** district

As of 4 May 2023, 24 districts continue to report cases (last 14 days)

Sources: Malawi Ministry of Health and Population

https://www.who.int/emergencies/disease-outbreak-news/item/2022-DON435
https://www.facebook.com/malawimoh

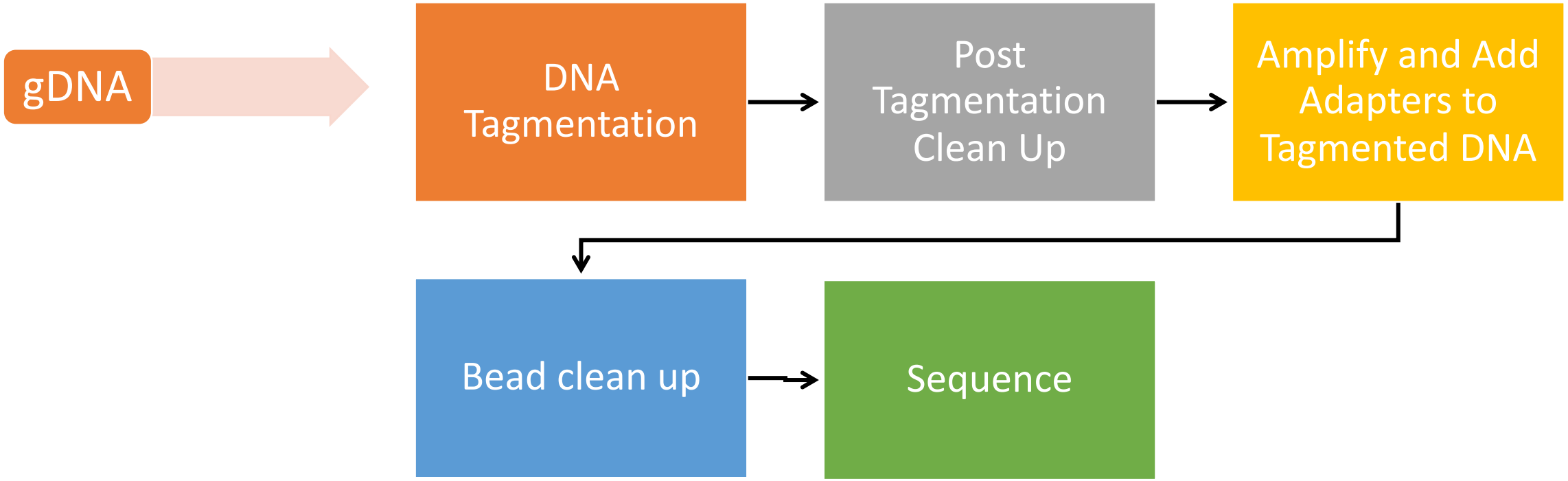
Sample Preparation

- Sub-cultured Isolates
- Colonies were emulsified in Phosphate Buffer Saline

Extraction

- DNA isolation was done using Qiagen QIAmp DNA mini kit

Whole Genome Sequencing using Illumina DNA Prep kit



DNA INPUT RECOMMENDATION

- Quantify using fluorometric assays i.e. Qubit or Picogreen

Post Tagmentation Clean-Up

Handling Reagents

- **Tagmentation Stop Buffer (TSB)**
  - Store TSB at room temperature 15°C to 30°C
  - If precipitants are present, heat the buffer at 37°C for 10 minutes, and vortex until they dissolve
- **Tagmentation Wash Buffer (TWB)**
  - Store TWB properly at room temperature 15°C to 30°C

Reagents For DNA Tagmentation

Handling Bead-Linked Transposomes (BLT) and Tagmentation Buffer 1

- Store BLT properly at 2°C to 8°C
- Store TB1 Properly at -25 °C to -15°C
- Vortex beads thoroughly
  - If beads stick to the side of tube or wells spin down and then gently pipet to mix thoroughly

TAGMENTATION

- Add **5 µl DNA** to each well of a 96-well PCR plate
- Transfer **2 µl tagmentation master mix** to each well of the plate containing a sample

Temperature (°C)	Time
55	15 minutes
10	Hold

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Stop Tagmentation

- Add **1µl TSB** to the tagmentation reaction

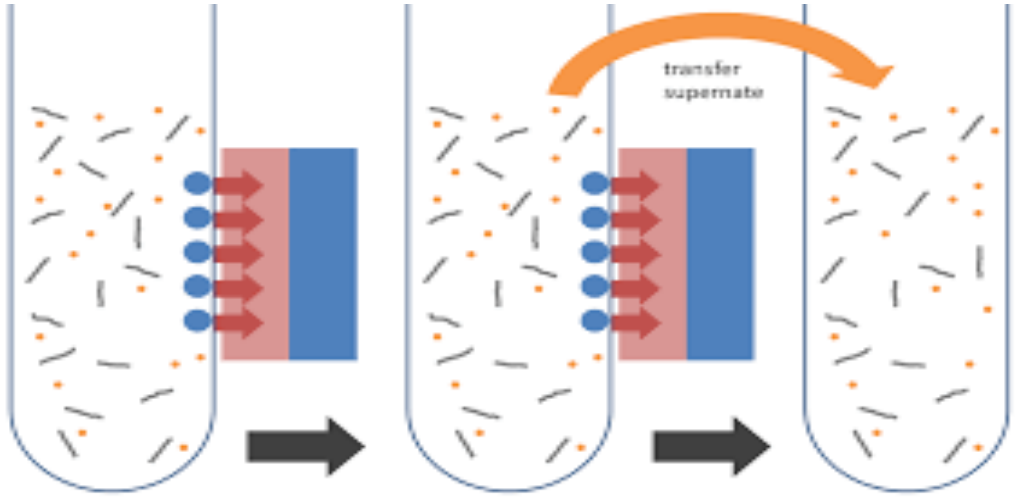
Temperature (°C)	Time
37	15 minutes
10	Hold

Post Tagmentation Clean-Up

- Place the plate on the magnetic stand for approximately **1 minute** until liquid is clear
- Using a multichannel pipette, remove and discard supernatant
- Remove the sample plate from the magnetic stand and use a deliberately slow pipetting technique to add **10µl TWB** directly onto the beads
- This slow pipetting technique minimizes the potential of TWB foaming to avoid incorrect volume aspiration and incomplete mixing
- Slowly pipette until beads are fully resuspended
- Place the plate on the magnetic stand for approximately **1 minute** until liquid is clear

Post Tag Clean-Up

- Using a multichannel pipette, remove and discard supernatant
- Repeat 3 times
- Make sure all supernatant is removed during last wash



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Amplify DNA

Avoid overdrying bead pellet

- Keep post tagmentation clean-up wash pellet in 100 µl TWB on magnet while preparing PCR master mix

Avoid cross-contamination

- Change tips between any sample or reagent addition
- Remove any unused index adapter tubes from the working area

PCR Reagents

- EPM (Enhanced PCR Mix) should be stored -25°C to -15°C
- Index Adapters (Plates) should be stored -25°C to -15°C

Amplify DNA

- Immediately add **4µl PCR master mix** directly onto the beads in each sample well/ tube
- Pipette mix until the beads are fully resuspended
- Add **1µl** of the appropriate index adapters to each sample
- Using a pipette set to **4µl**, pipette 10 times to mix

TDNA PCR CYCLING CONDITIONS

Temperature (°C)	Time	
68	3 minutes	
98	3 minutes	
98	45 seconds	8 cycles
62	30 seconds	
68	2 minutes	
68	1 minute	
10	Hold	

Clean Up Amplified DNA

Handling Reagents

- Store Sample Purification Beads **(SPB)** properly at **2°C** to **8°C**
  - Do NOT freeze
- Prior to use, make sure beads have come to room temperature
- Make sure that beads are fully resuspended
- Use freshly made **80%** ethanol
- Do not over-dry beads

Double SPRI Size Selection

- Dilute **(SPB)** accurately for first SPRI
- Transfer supernatant to new well- **Do NOT discard!**

Beads Selection

Dilute Beads

Dilute **4.5µL** SPB with **4.0µL** of NFW

Add Beads

Add **8.5µL** diluted SPB to **4.5µL** PCR reaction

Transfer Supernatant

Transfer/Pool **12.5µL** of supernatant to new **1.5ml** tube

Add Beads

Add **144µl** SPB (Undiluted) to the Pooled Sample.

Amplified DNA Clean-Up

- Incubate at **room temperature** for approximately **1 minute**
- Place on the magnetic stand for approximately **1 minute** until the liquid is clear
- Without disturbing the beads, remove and discard the supernatant
- Wash two times as follows:
  - Add **200µl freshly prepared 80% ethanol** with the tube on the magnetic stand
  - Incubate for **30 Seconds**
  - Without disturbing the beads, remove and discard the supernatant

Library Elution

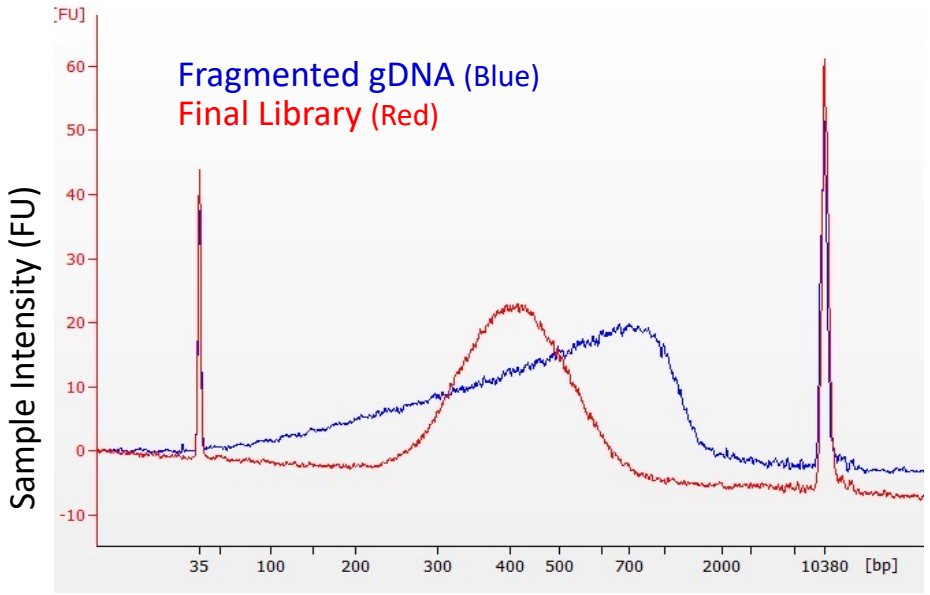
- Use a **200µl** pipette to remove any residual ethanol
- Air-dry on the magnetic stand for approximately **1 minute**
- Remove from the magnetic stand
- Add **30µl RSB** to the tube
- Resuspend by pipette mixing
- Incubate at **Room temperature** for approximately **1 minute**
- Place the tube on the magnetic stand for approximately **1 minute**
- Transfer **30µl supernatant** to a new tube

Size Selection Workflow

Key Steps

First Size Selection Step:
Removes large fragments

- Uses diluted beads
- DNA of interest remains in supernatant



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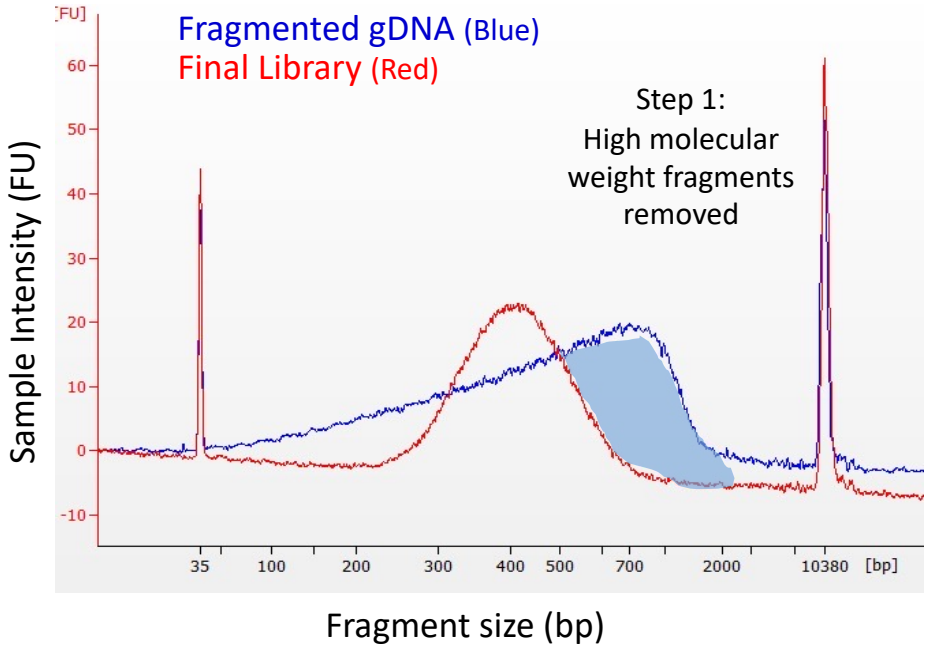

Size Selection Workflow

Key Steps

First Size Selection Step:
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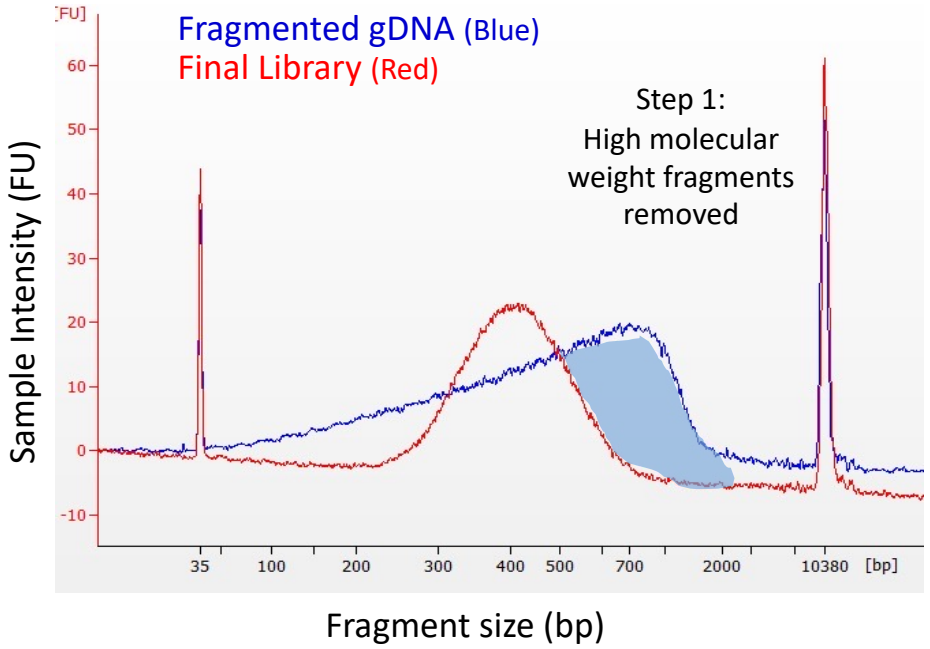
Second Size Selection Step:
Removes small fragments

- Uses undiluted beads
- Desired DNA binds to beads
- DNA eluted with RSB



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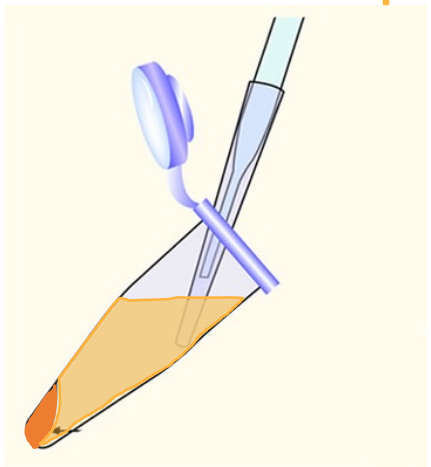
Discarded in Step 2



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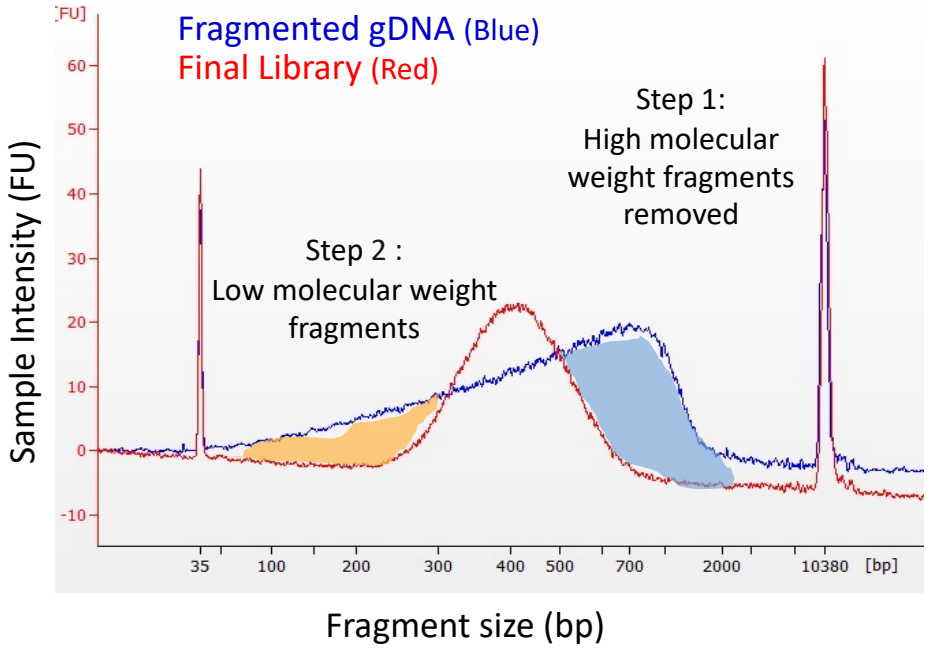

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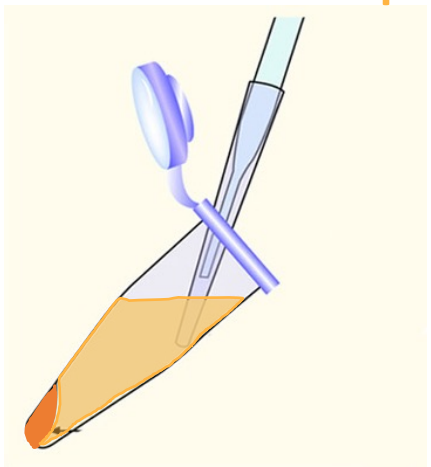

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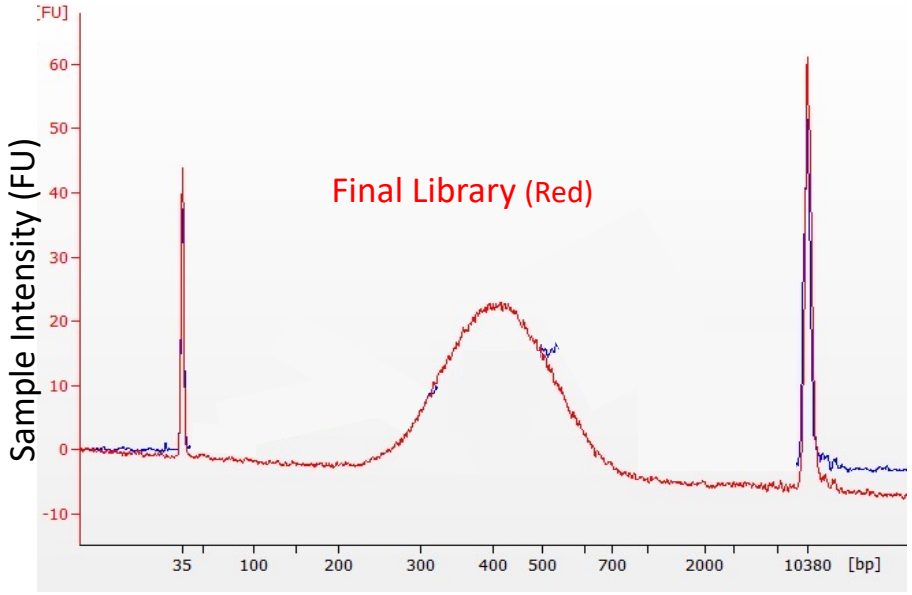

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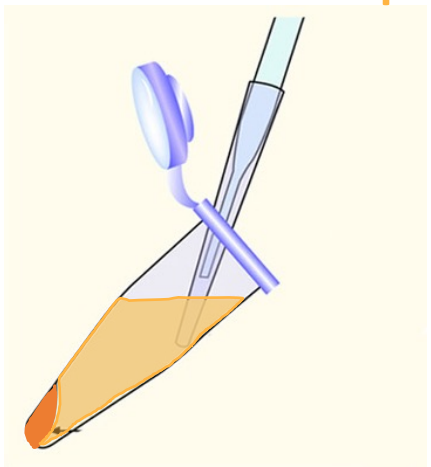

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Normalization of DNA

- Quantify the DNA using the Qubit or Picogram and determine the fragment length using the LabChip/Tape Station/ Agarose Gel Electropheresis
- Using the Qubit concentrations and fragment length normalize the libraries to equimolar 4nM by diluting with RSB buffer

(ng/µl /660 x 500bp x 10^6)

Normalisation

- Dilute 4nM to 750pM using RSB
- Take 24µl from 750pM and add to a new Eppendorf tube
- Add 1µl of 1% Phix to 24µl sample (making total volume of 25µl).
- Mix this solution well and briefly centrifuge.
- Keep on ice or at 4°C until it is ready to be loaded onto the P2 reagent cartridge

SUMMARY

- Introduction
- Sample Preparation
- Whole-Genome Sequencing Method

Acknowledgements

- National Genomic Sequencing Reference laboratory in Malawi
- KRISP & CERI laboratory teams in South Africa
- CLIMADE Africa
- University of Stellenbosch & University of Kwa-Zulu Natal

- THANK YOU SO MUCH FOR YOUR ATTENTION