DukeNUS Medical School | Emerging Infectious Diseases

Amplicon purification & quantification, Illumina Library Preparation

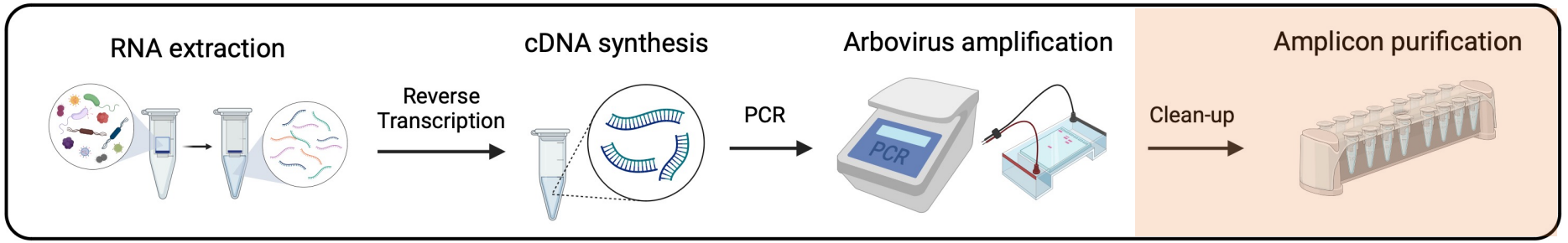
Date : 4th July 2024, 09:00 – 09:30

Venue : Rm L2-S2, Academia

SingHealth DukeNUS ACADEMIC MEDICAL CENTRE

Arbovirus amplicon purification and quantification

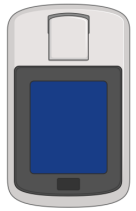
1. Arbovirus RNA amplification & purification



Arbovirus amplicon purification:

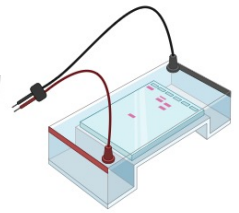
- Combine PCR Pool1 and Pool2 (100uL) of same sample
- Use 0.9X ratio of beads (90uL) to PCR volume (100uL)
    - Size selects for 200bp and above
- Place on magnetic stand, discard supernatant
- Wash twice with **fresh** 80% ethanol
- Elute with 42uL NFW
    - Transfer 40uL to new tube for library preparation
    - Quantify DNA with 1uL

Arbovirus amplicon quantification:



- Qubit 1X dsDNA HS Assay
- Standards: 190uL Reagent + 10uL standard
- Samples: 199uL Reagent + 1uL sample

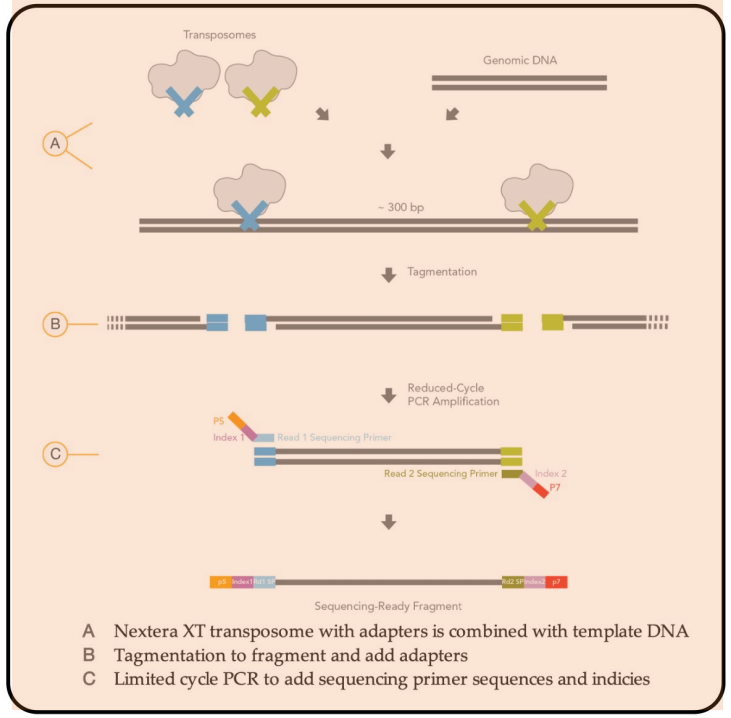
Arbovirus purified amplicon visualization:



50-100ng DNA

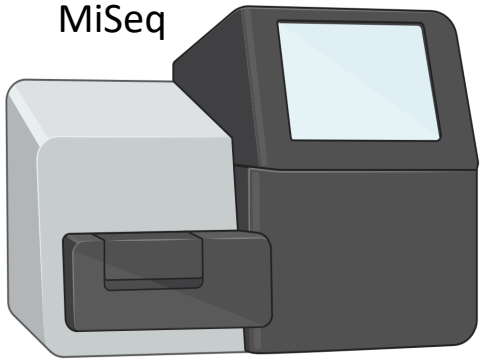
Illumina – Nextera Library Preparation

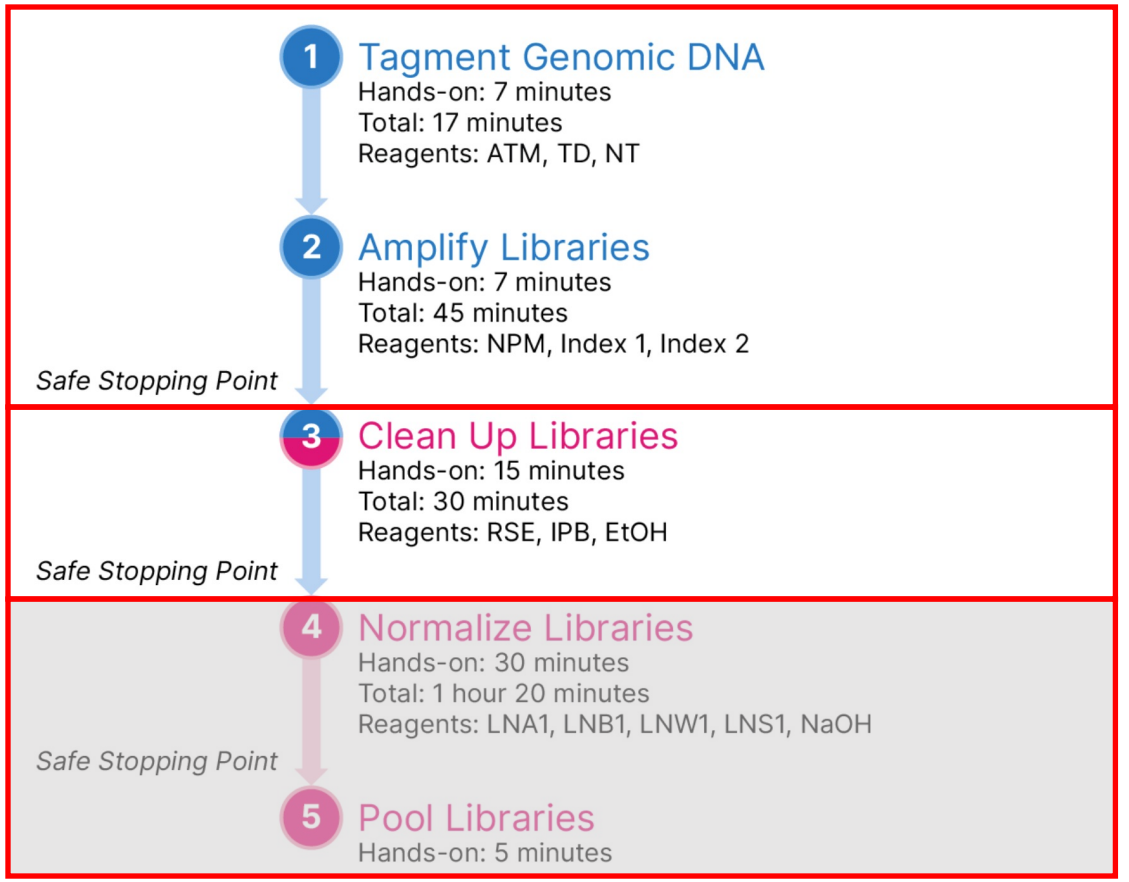
2. Illumina - Nextera Library Prep



A Nextera XT transposome with adapters is combined with template DNA
B Tagmentation to fragment and add adapters
C Limited cycle PCR to add sequencing primer sequences and indicies

MiSeq





Before lunch

After lunch

Not doing

Illumina – Nextera Library Preparation

Step 1

Tagment Genomic DNA:

1. Prepare 1ng DNA in 5ul water (PCR tubes or strips) ← Calculate from Qubit results
2. Thaw reagents ATM (Amplicon Tagment Mix), TD (Tagment DNA Buffer) and NT (Neutralize Tagment Buffer)
3. Add 10ul TD (Tagment DNA Buffer) to DNA and pipette mix thoroughly
4. Add 5ul ATM (Amplicon Tagment Mix) to DNA and pipette mix 10 times. Quick spin down
5. Place tubes in thermal cycler and run TAG program
    a. Lid 100°C, reaction volume 50ul
    b. 55°C, 5mins
    c. Hold 10°C

Fragment amplicons + tag adapters

6. Add 5ul NT (Neutralize Tagment Buffer) to each tube
7. Pipette mix 10 times and quick spin down
8. Incubate at room temperature for 5mins

Stop tagmentation

Step 2

Amplify Libraries:

1. Thaw NPM (Nextera PCR Master Mix) and Index Adapters (i7 and i5 tubes)
2. Add 5ul of i7 index adapter to each tube
3. Add 5ul of i5 index adapter to each tube

Add indexes

Note: Replace each index adapter with new caps (provided in kit) after opening

4. Add 15ul NPM (Nextera PCR Master Mix) to each tube
5. Pipette mix 10 times and quick spin down
6. Place in thermal cycler and run NXT PCR program

Add PCR master mix for amplification

Lid temp. = 100°C, Reaction volume = 50ul		
1	72°C	3 mins
2	95°C	30 secs
3 (12 cycles)	95°C	10 secs
	55°C	30 secs
	72°C	30 secs
4	72°C	5 mins
5	10°C	Hold indefinitely

Note: Safe stopping point. Store at 4°C for up to 2 days.

Illumina – Nextera Library Preparation

Step 3
Clean Up Libraries

Note: Thaw magnetic beads at room temperature for 30mins before starting. Resuspend frequently to ensure even distribution.

1. Thaw RSB (Resuspension Buffer)
2. Prepare fresh 80% EtOH (500ul per sample)
3. Transfer 50ul DNA from PCR tube to a new DNA LoBind 1.5ml tube
4. If using small PCR amplicon sample input, add the magnetic beads volume according to input size

Input size (bp)	Beads Recommendation	Beads volume (ul)
300 - 500	1.8x Beads	90
>500	0.6x Beads	30

Target size is 300-500bp

DNA capture

5. Pipette mix 10 times and quick spin down
6. Incubate at room temperature for 5mins
7. Place on magnetic stand ~ 2mins (wait till liquid is clear and colorless)
8. Remove and discard supernatant without disturbing beads

Discard supernatant

9. Wash 2 times with 200ul of freshly prepared 80% EtOH as follows:
    a. With tube on magnetic stand, add 200ul fresh 80% EtOH without mixing
    b. Incubate for 30s
    c. Remove and discard supernatant without disturbing beads

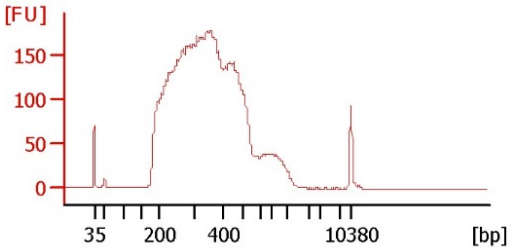
Wash twice

10. Use 20ul pipette to remove and discard residual supernatant
11. Air-dry on magnetic stand ~ 2 – 5mins (do not over-dry as it makes resuspension of beads difficult)
12. Remove from magnetic stand and resuspend beads with 52.5ul RSB (Resuspension Buffer)
13. Pipette mix 10 times and quick spin down
14. Incubate at room temperature for 2mins
15. Place on magnetic stand ~ 2mins (till liquid is clear and colorless)
16. Transfer 50ul supernatant to a new DNA LoBind 1.5ml tube

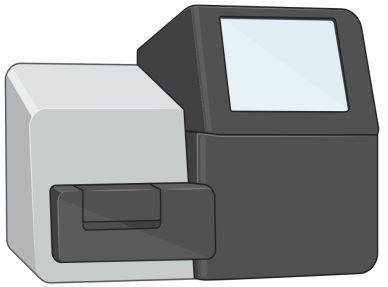
Elute purified libraries

Note: Safe stopping point. Store at - 20°C for up to 7 days.

- Send for library validation on Bioanalzyer



- Pool all libraries for sequencing on MiSeq



DukeNUS Medical School | Emerging Infectious Diseases

SingHealth DukeNUS ACADEMIC MEDICAL CENTRE

Dengue virus amplicon purification and quantification

1. Arbovirus RNA amplification & purification