MONDAY	ACTIVITY	PRESENTER
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9.00 – 9.15	Overview of the Doherty Institute/CPG/VIDRL/MDU	Lisa
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12.30 – 13.30	Lunch	
13.30 – 15.30	**LAB: TILED AMPLICON GENERATION**	**Louise**
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16:00 – 16.30	**LECTURE:** Introduction to ONT sequencing viruses	Louise
16.30 – 17:00	Group discussion: Opportunity for Q&A and further discussion	Nicole

WET LAB DAY 1:
TILED AMPLICON GENERATION

Dr. Louise Judd
Centre for Pathogen Genomics- Innovation Hub
Lead Laboratory Scientist and Genomics Trainer

CENTRE FOR PATHOGEN GENOMICS

World Health Organization
Western Pacific Region

IPSN International Pathogen Surveillance Network
WHO hosted network

LAB REQUIREMENTS

Avoiding Contamination: Lab Design

Library preparation and Illumina sequencing by synthesis involve PCR amplification. It's important to avoid contamination to prevent the wrong template from being amplified which leads to inaccurate results.

- Separate laboratory spaces for pre- and post- PCR steps with dedicated equipment and PPE in each
- At a minimum, perform pre-PCR in a sterile cabinet (PCR, laminar flow or biosafety)
- Work in a unidirectional manner
- We do not have the resources to run the training this way, but this is a very strong recommendation for your lab set up

Avoiding Contamination: Pre- & Post- PCR

Pre-PCR

Master Mix Room

- For reagent preparation only
- Samples and nucleic acid should not be handled here

Sample Preparation Room

- Samples and nucleic acid should be handled here
- Nucleic acid extractions performed here (kits stored here)

Post-PCR

Amplification Room

- Thermocyclers

Post-Amplification Room

- Post-PCR analysis equipment
- Sequencers

Image Source: https://www.labclinics.com/2019/07/22/six-ways-to-minimize-contamination-during-pcr/?lang=en

Avoiding Contamination: Lab Work

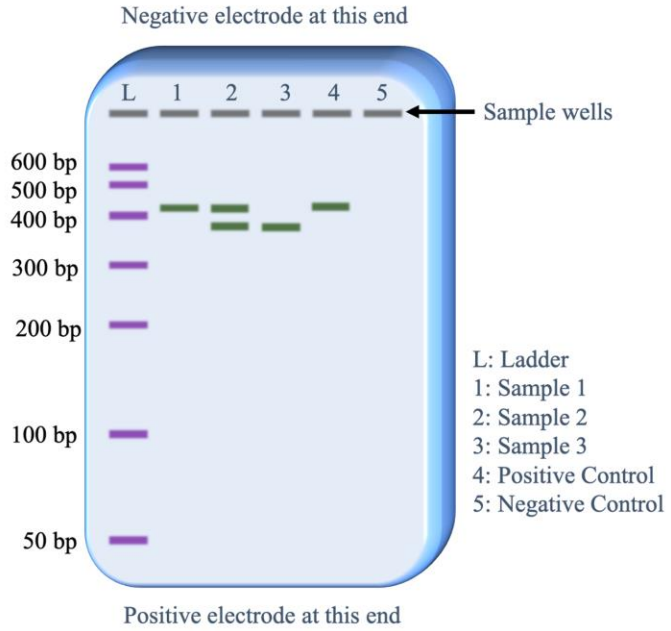
- Have a dedicated fridge/freezer for samples and a separate one for reagents --> or at least dedicated spaces
- Aseptic cleaning practises before and after work
  - 70-80% ethanol (EtoH), 5-15% Sodium Hypochlorite(NaOCl), DNAase Away
- Only use barrier tips to avoid pipette contamination
- Always use fresh tips for each pipetting step and for each different sample
- Use correct pipetting techniques
- Pulse-spin tubes and plates before opening to collect contents at bottom
- Open tubes and plates carefully, making sure to avoid any splashes
- Seal all plates and recap all tubes immediately after use

Avoiding Contamination: NTC & PTC

Remember to treat your controls in the same manner you treat your samples (i.e. if you add a reagent to your sample, add the same reagent to your controls). Try to follow the same control samples through your entire workflow.

NTC (Negative Template Control)

- Do not add any template DNA
- Can show:
  - reagent contamination
  - cross-contamination from samples



PTC (Positive Template Control)

- Use a sample known to give a specific result
- Shows if our reagents and assays are working as expected

Image Source: https://iastate.pressbooks.pub/genagbiotech/chapter/pcr-and-gel-electrophoresis/

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Mpox SEQUENCING

Mpox DNA requirements for Mpox sequencing

- DNA extracted from clinical swabs

- Quantitation of DNA is NOT required and is not informative
  - DNA from host and other microbes will be extracted alongside the Mpox DNA
  - The concentration of the extracted DNA does not correlate with the viral load in the sample

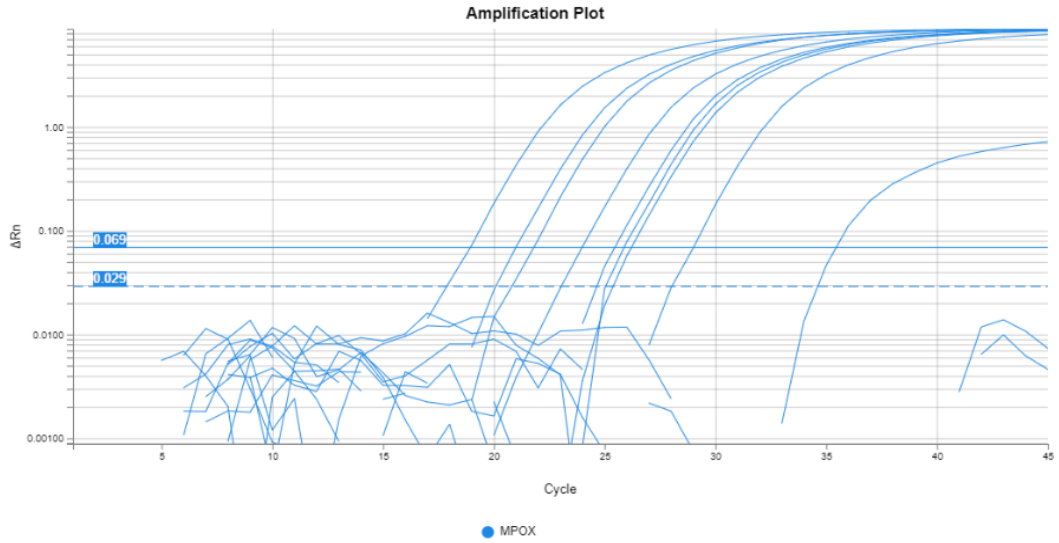
- Mpox is detected in the samples and viral load approximated with Mpox QPCR

- **Samples should have a Mpox QPCR cycle threshold (Ct) less than 30 to proceed with sequencing**

- Minimum volume of DNA extract required for sequencing 10µl

Mpox DNA samples to be used for training

Sample number	Mpox Realtime Ct
1	25.6
2	21.9
3	Not detected
4	21
5	29.1
6	19
7	Not detected



PREPARING TILED AMPLICONS

Mpox Panel for trainees

A joint venture between The University of Melbourne and The Royal Melbourne Hospital

a) Specimen collection ✅ - MPXV Panel for trainees.

PANEL OF CLINICAL SWABS RECEIVED AT VIDRL FOR MPXV TESTING

DNA EXTRACTED FROM SWAB

REAL TIME PCR SCREEN

Extracting Mpox DNA

Kits we have tested

Automated:

- Qiagen EZ2
- **MagMAX Viral/Pathogen Nucleic Acid Isolation Kit (MVP II)**
- QIAamp DNA QIAcube Kit
- TANbead OptiPure Viral Kit

Manual:

- Qiagen QIAamp MinElute Virus Spin Kit

Mpox Amplicon Scheme

Scheme	Amplicon size [bp]	# Primer pairs	Optimal Ct	Reference for primer design
ARTIC / INRB	400 (wastewater surveillance) **2500 (clinical surveillance)**	147	<30	I and II (KJ642613.1)

Pool 1 = 76 primers

Pool 2 = 71 primers

PCR 1 strip & PCR2 Strip
(Pool 1 & Pool 2)

Use of Strip tubes

A joint venture between The University of Melbourne and The Royal Melbourne Hospital

******IMPORTANT REMINDER******

- Always number the first of your tubes (the tube NOT the lid) of your strip with a number 1
- Strip tubes are symmetrical, it is visually not possible to know which is tube 1 and which is 8
- It is a very common error to flip the strip around during an experiment and get samples back to front
- Please make sure you always label the first tube is some way so you always know the correct orientation of your strip

Use of strip tubes

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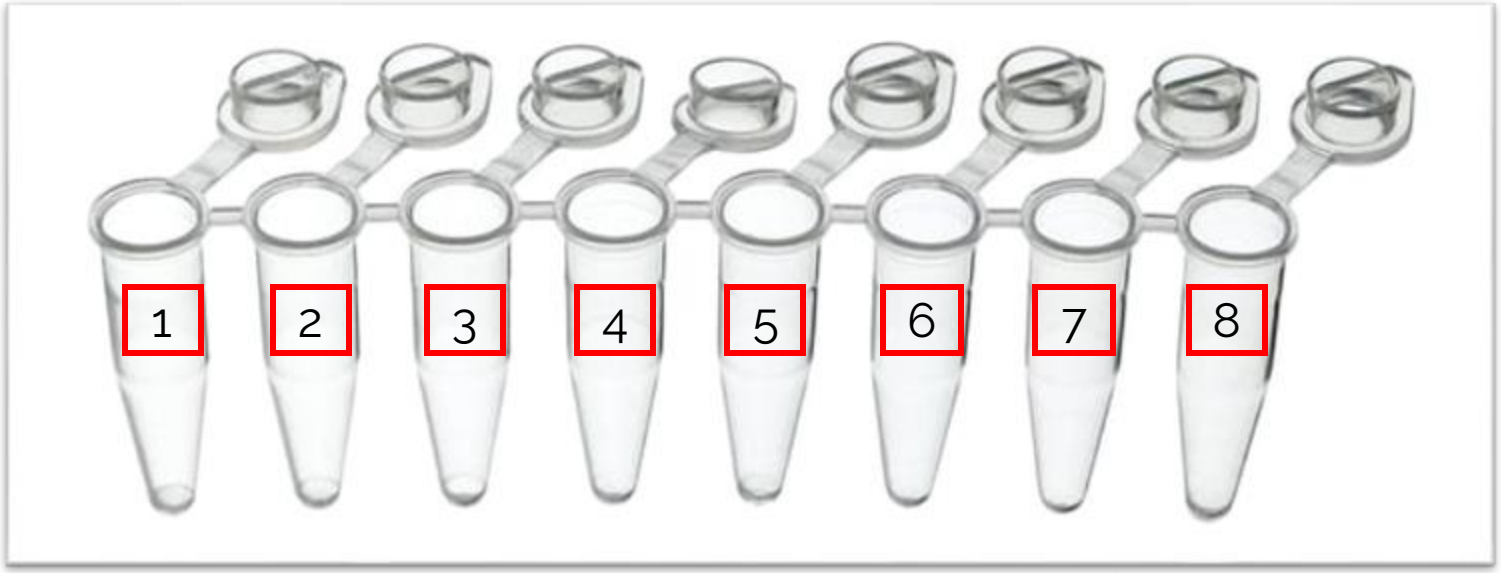
Always number the first of your **tubes** (the tube NOT the lid) of your strip with a number 1

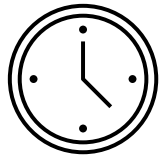


Amplicon PCR Overview

Reagents

Q5 Hot Start HF 2 x Master mix

New England Biolabs M0494



Time

~ 4 hours

~ 30 minutes hands on

Primer Pools 1 and 2

INRB 2500 bp Amplicon Scheme

Stock 100 mM- dilute 1:10 to generate working stock at 10 mM

Nuclease-Free Water (NFW)

2 x PCR strips (PCR1 and PCR2)

Amplicon PCR- pool 1 mastermix

10 mins

1. Label an Eppendorf tube "**PCR1 MM**" for your Amplicon Pool 1 Master Mix
2. Combine the following reagents in the PCR1 MM tube (amounts listed already account for overage)

Reagent	Single reaction	For 8 reactions (x9)
Nuclease free water	7.1 µl	63.9 µl
Mpox primer pool 1 (10 mM)	2.9 µl	26.1 µl
Q5 Master mix (2x)	12.5 µl	112.5 µl
Total	**22.5 µl**	**202.5 µl**

3. Pipette to mix

Amplicon PCR- pool 2 mastermix

10 mins

1. Label an Eppendorf tube "**PCR2 MM**" for your Amplicon Pool 1 Master Mix
2. Combine the following reagents in the PCR1 MM tube (amounts listed already account for overage)

Reagent	Single reaction	For 8 reactions (x9)
Nuclease free water	7.3 µl	65.7 µl
Mpox primer pool 2 (10 mM)	2.7 µl	24.3 µl
Q5 Master mix (2x)	12.5 µl	112.5 µl
Total	**22.5 µl**	**202.5 µl**

3. Pipette to mix
4. Clearly label 2 strip tubes as "PCR 1" and "PCR 2" with your name and initials

Amplicon PCR – pool 1 PCR

10 mins

1. Transfer **22.5 µL of PCR1 MM** to each well in the **PCR1** strip tube
2. Add 2.5 µL of DNA sample 1 to the well 1 of PCR1strip tube
3. Mix gently by pipetting up and down
4. Change pipette tips between samples
5. Add 2.5 µL of DNA sample 2 to the well 2 of PCR1strip tube
6. Repeat this with remaining DNA samples 3-8 into appropriate well
7. Ensure each tube is capped
8. Spin down briefly

Amplicon PCR –pool 2 PCR

10 mins

1. Transfer **22.5 µL of PCR2 MM** to each well in the **PCR2** strip tube
2. Add 2.5 µL of DNA sample 1 to the well 1 of PCR2 strip tube
3. Mix gently by pipetting up and down
4. Change pipette tips between samples
5. Add 2.5 µL of DNA sample 2 to the well 2 of PCR2 strip tube
6. Repeat this with remaining DNA samples 3-8 into appropriate well
7. Ensure each tube is capped
8. Spin down briefly

Amplicon PCR – running PCR

3 ~~4~~ hours

1. Run the Mpox PCR program on the thermocycler

Mpox PCR Program

Preheat Lid: 105˚C

Reaction Volume: 25 µL

.....

98˚C for 30 secs minutes

35 Cycles of:

98˚C for 15 seconds

65˚C for 5 minutes

Hold at 10˚C

Amplicon PCR – running PCR

3 ¼ hours

1. Run the Mpox PCR program on the thermocycler

Mpox PCR Program

Preheat Lid: 105˚C

Reaction Volume: 25 µL

.....

98˚C for 30 secs minutes

35 Cycles of:

98˚C for 15 seconds

65˚C for 5 minutes

Hold at 10˚C

Run PCR Program overnight

PCR 1 strip & PCR2 Strip
(Pool 1 & Pool 2)

Day 2
Qubit QC of Amplicons

THANK YOU

centre-pathogen@unimelb.edu.au

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