Wet Lab schedule DAY 1

MONDAY	ACTIVITY	PRESENTER
8:45 – 9.00	Registration	Lisa
9.00 – 9.15	Overview of the Doherty Institute/CPG/VIDRL/MDU	Lisa
9.15 – 9:45	Welcome and introductions	
9:45 –10.00	Training overview	Jean
10.00– 10.30	LAB: Pipetting exercise	Louise
10.30 – 11.00	Morning tea	
11.00 – 11.30	LECTURE: Introduction to MPXV genomics and VIDRL	Jean
11.30 – 12.30	**LECTURE: TILED AMPLICON FOR MPOX**	**Jean**
12.30 – 13.30	Lunch	
13.30 – 15.30	LAB: Tiled amplicon PCR	Louise
15.30 – 16:00	Afternoon tea	
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

Jean Moselen
Senior Medical Scientist
Victorian Infectious Disease Laboratory (VIDRL)

Viral detection & sequencing methods

Real time PCR

Basics of using PCR as a **detection method** are very similar for different viruses

Sanger sequencing

Sequencing only the **gene/s of interest** for identification of pathogen & it's strain/lineage

Whole-genome sequencing (WGS)

WGS is a comprehensive method for **analysing entire genomes**.

Next Generation Sequencing (NGS)

Technology for sequencing any human, animal, plants, or pathogen microbes.

Mpox surveillance workflow.

The core stages of this workflow are:

a) specimen collection
b) sample preparation – AMPLICON GENERATION
c) genome sequencing – ONT PLATFORM
d) processing of sequencing results
e) sequence data interpretation and data sharing.

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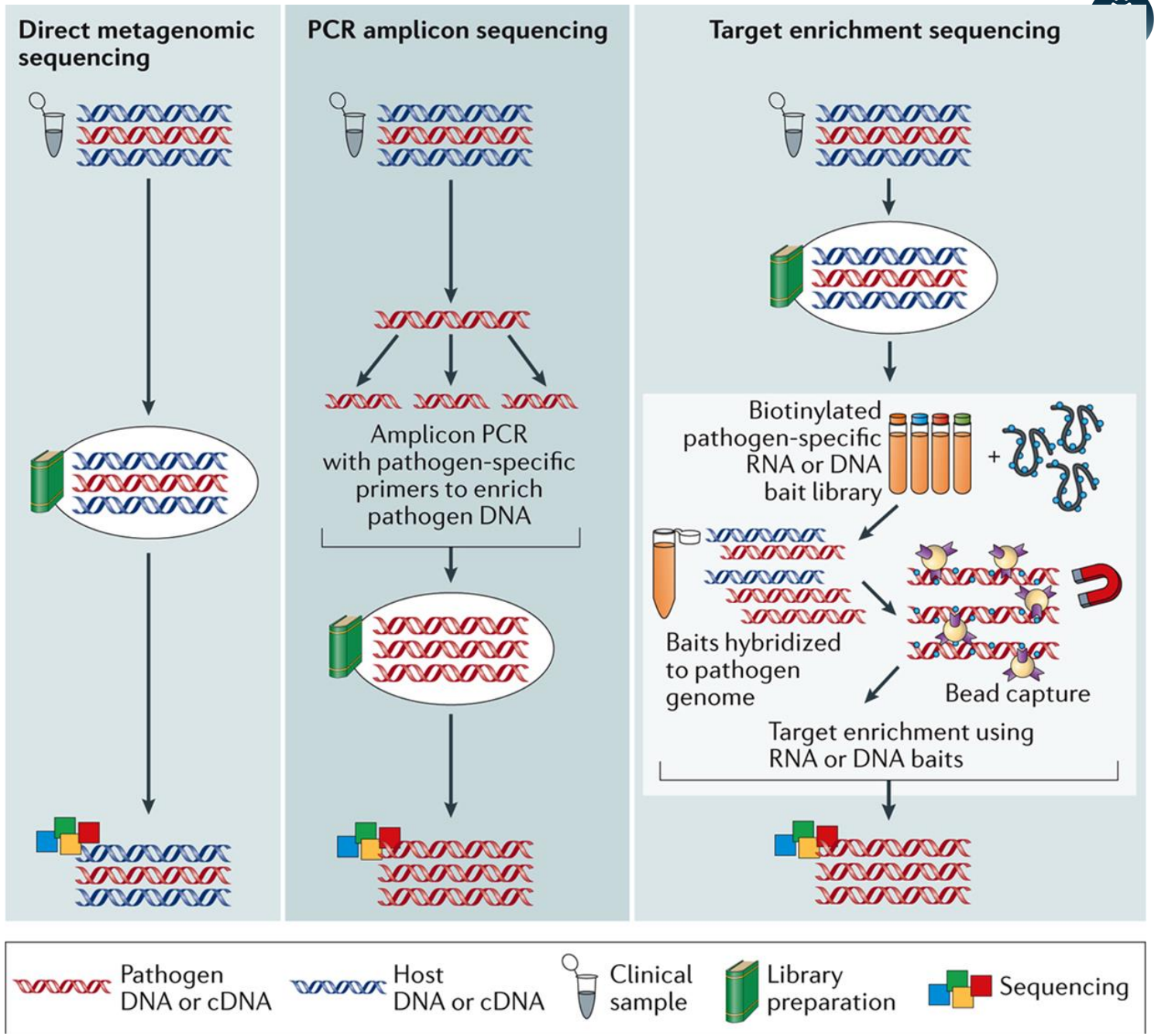
VIRAL SEQUENCING METHODS



Pathogen NGS

Why so many options?

Metagenomics

Amplicon

Target Enriched

Pathogens make up a small proportion of the sample

Viral Diversity

"Needle in the haystack" scenario

Variance in sequencing capacity of laboratories

Different research goals and throughput

Bioinformatic capacity

Emerging field and new technologies

NGS Schemes
Mpox Training

Abundance of host and other non-target sequences in NGS libraries results in low sensitivity

How can we get the best result?

GOAL= improve # of target specific reads

Prepare

- Sample type
- Extraction method
- Concentration/cleanup
- Library Input

Sequence

- Metagenomics
- Amplicon
- Target Enriched
- Sequencer platform

Analyze

- Bioinformatics tools
- Reference sequences
- In house vs online pipelines

Viral NGS
Technique considerations

WET LAB

Virus structure
Sample type & volume
Extraction technique
Pre-treatments
Amplification options

Library Prep Kit
Scalability
Sequencer Selection

Budget

DRY LAB

Data Set size produced
Number of targets
Computational capacity
Coverage & depth required

All these considerations are linked

Viral Genomics
Methodology

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Clinical sample

Extraction of genetic material

Real time PCR

Conventional PCR

AMPLICON

Tiling PCR

Library Prep

METAGENOMICS

TARGET ENRICHED

Amplicon-based WGS

Developed to support genomic surveillance efforts.

Used in different public health emergencies, Zika virus and Ebola.

Broadly applied during the SARS-CoV-2 pandemic.

Primary method in viral genomic epidemiology with clinical case-derived samples due to its:

high sensitivity

multiplexing capability

cost-effectiveness

scalability

Pan Approach vs Targeted Scheme Design

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GOAL= improve # of pathogen specific reads

Single target = Greater specificity and sensitivity.

Higher cost and not useful for detection of dual infections

VS

PAN approach = multiple targets (viruses) of interest

e.g Flavi viruses, MMR, respiratory

“PANELS” of viruses tested at once

Useful when looking at among human, animal, and environmental specimens.

Sensitivity is lower

Outbreak schemes
Amplicon WGS

Workflows were optimized for **simple adaptation of workflows** in laboratories previously involved in SARS-CoV-2 genomic surveillance.

Amplicon-based WGS approaches are highly sensitive due to targeted amplification of the viral DNA, making them particularly suitable to analyze **clinical, environmental and wastewater** samples with low viral loads (up to Ct ~32).

Labs can order in primer sets and start testing within a short time frame.

Sequencing Approaches Mpox

EXAMPLE	SEQUENCING APPROACH
Clinical samples with low viral load (Ct > 28)	Amplicon-based WGS OR Target capture
Detection of structural variants, ITR variation	Amplicon-based WGS or Metagenomic sequencing with long-read technology
Accurate SNP calling and clade typing	Amplicon-based WGS
Validation of amplicon dropout or suspected deletions	Metagenomic sequencing or Target capture
High-throughput routine surveillance	Amplicon-based WGS
Field deployment with minimal infrastructure	Amplicon-based WGS with long-read technology
Wastewater or environmental samples	Metagenomic sequencing or Target capture with short amplicons Possibly: amplicon-based sequencing with short amplicons
Resource-limited labs with basic capacity	Amplicon-based WGS

Community Designed Amplicon Schemes

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protocols.io

VERSION 2

FEB 01, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.kqdg39xxeg25/v2

DengueSeq: A pan-serotype whole genome amplicon sequencing protocol for dengue virus V.2

Chantal Vogels[1], Chrispin Chaguza[1], Emma Taylor-Afeez Sodeinde[1], Salmon[1], Mallery I Breban[1], Abigail J. Porzucek[1], Nathan D Grubaugh[1]

[1]Department of Epidemiology of Microbial Diseases, Yale School of Public Health

Nathan D Grubaugh
Department of Epidemiology of Microbial Diseases, Yale Schoo...

ABSTRACT

Version 2 updates:

- Updated the DENV3 primer file (DENV3_Primer-Scheme.xlsx) with correct version

FEB 23, 2022

SHARE

WORKS FOR ME 1

Poliovirus direct detection and nanopore sequencing (DDNS) FAQs

DOI
dx.doi.org/10.17504/protocols.io.b5ggq3tw

Alex Shaw[1], Catherine Troman[1], Joyce Akello[1], Erika Bujaki[2] Manasi Majumdar[2], Javier Martin[2], Nick Grassly[1]

[1]Imperial College London;
[2]National Institute for Biological Standards and Control

Poliovirus Sequencing Consortium

Nick Grassly
Imperial College London

Sequencing Enrichment Approaches for Viruses

Tiled Amplicon PCR

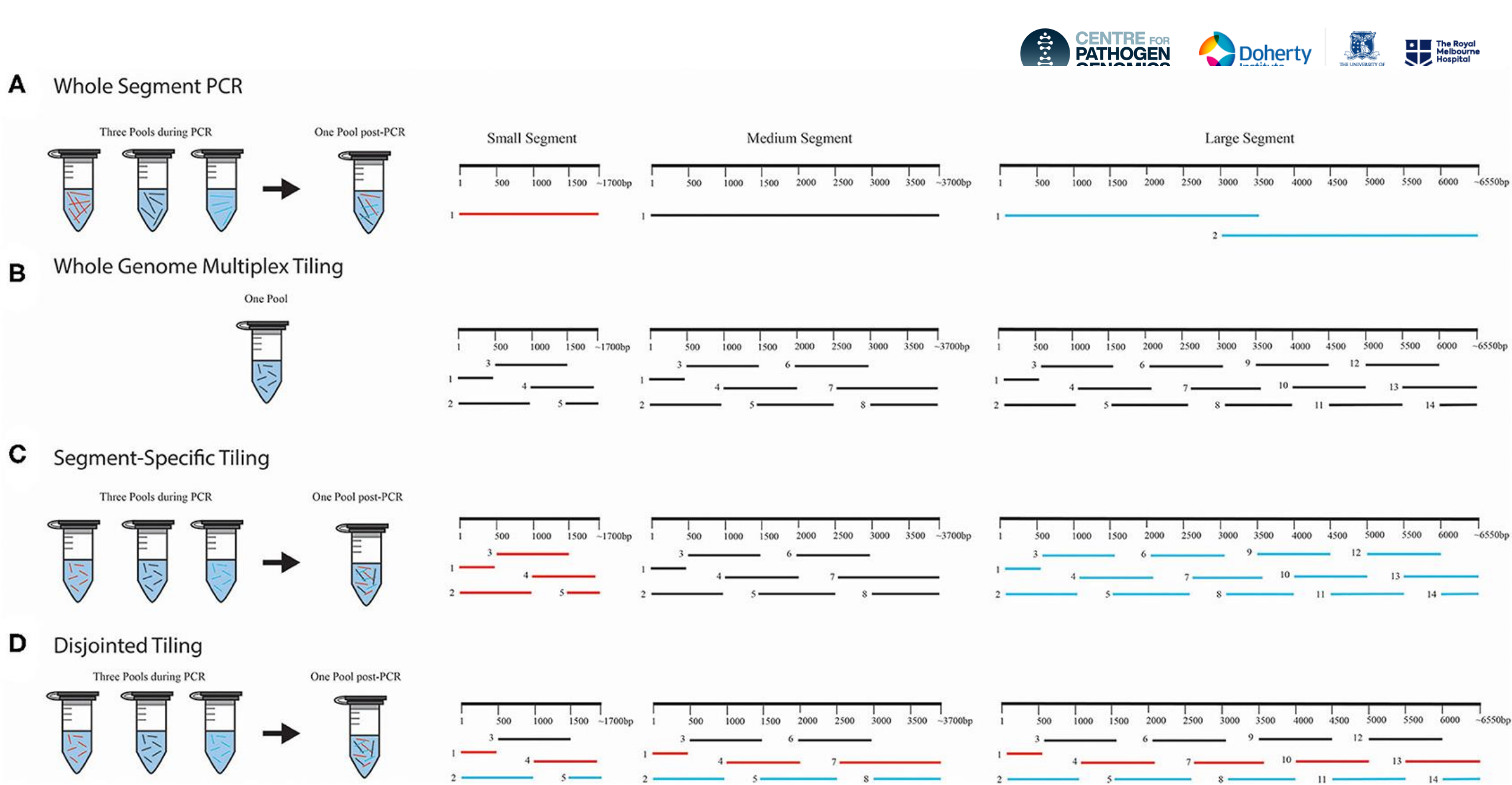
- Multiple primers designed that span the entire genome --> except 3' and 5' region
- Primer pools to allow overlapping regions amplification while reducing any unwanted primer interactions
- Simple to use
- Lower cost
- Widely used

Advantages of Tiled Amplicon

- Relatively low cost to sequence
- Works on low viral copy numbers
- Simple workflows
- Faster turn-around-times for results
- Short amplicons (e.g. ~400 bp) more likely to sequence even when genomic material is degraded
- If a region fails to amplify, less coverage is lost compared to if the amplicon were longer
- **Works directly from clinical samples**

Disadvantages of Tiled Amplicon

- Unable to sequence ends of viral genome --> slightly less complete genome coverage
- Mutations in primer binding sites can cause amplicon drop-outs
- PCR step can amplify small amounts of cross-contamination
- Not ideal for highly diverse or recombinant viruses or new emergence
- Creation of the Primer pools = prone to user error = lots of primers!



https://www.frontiersin.org/articles/10.3389/fcimb.2020.565591/full

Sequencing Enrichment Approaches for Viruses

Tiled Amplicon PCR

Forward and Reverse Primers

Range of dilutions and concentrations

Combinations of primers

Different amplification reactions

= "Pools" of PCR master mixes

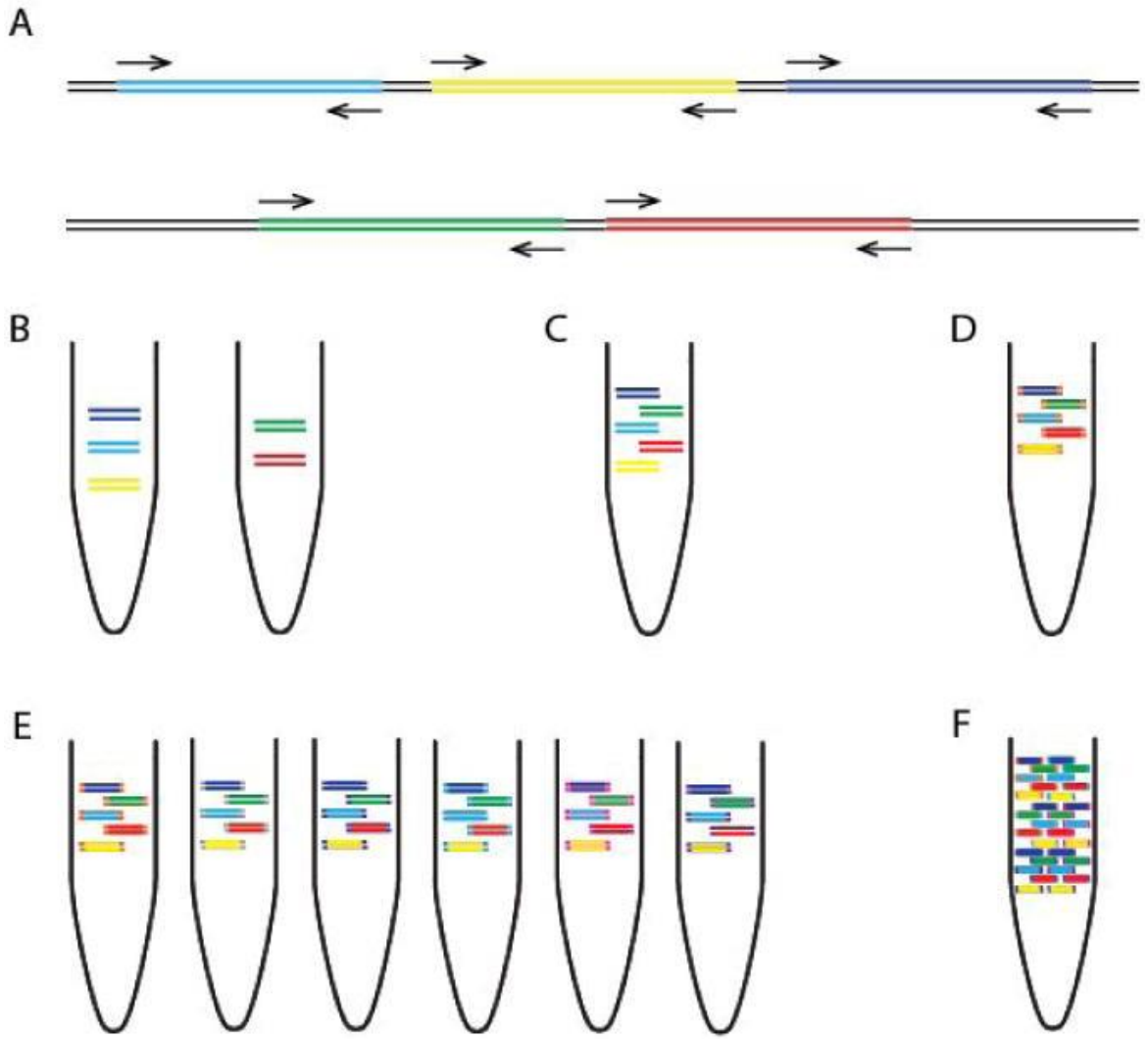


Image Source: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5902022/

Mpox Amplicon schemes WGS of MPXV

	Scheme	Amplicon size [bp]	# Primer pairs	Optimal Ct	Reference for primer design	Sequencing Platform (validated)	Clade Ib coverage
★	**Chen et al. 2023**	Average 200	163	<31	IIb (MT903345)	Illumina	85-90% (43)
	Brinkmann et al. 2024	375	682	<30	IIb (ON585033.1)	ONT	
★	**Welkers et al. 2022**	2500	88	<25	IIb (ON563414.3)	ONT	>93.5 %
	Bosmeny et al. 2023	3000	73	<32	IIb (NC_063383.1)	ONT	
	Isabel et al. 2023	5000	43	<27.9	IIb (ON563414.3)	Illumina	
	ARTIC / INRB	400 (wastewater surveillance) ★ 2500 (clinical surveillance)	147	<30	I and II (KJ642613.1)		
	ARTIC / BCCDC	5000	98		IIb (ON563414.3)		
	Yinda et al. 2023	12,500		<~27		ONT	

★ VIDRL have used these schemes

Mpox Amplicon schemes WGS of MPXV

Majority of MPXV primer schemes were developed using reference genomes from Clade IIb resulting in mismatches when applied to Clade I/Ib genomes.

A pan-clade primer scheme has been developed for optimized primer binding across clades

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)

Details of Amplicon Schemes

Named via publication or authors or lab group

Pool 1 = 76 primers

Pool 2 = 71 primers

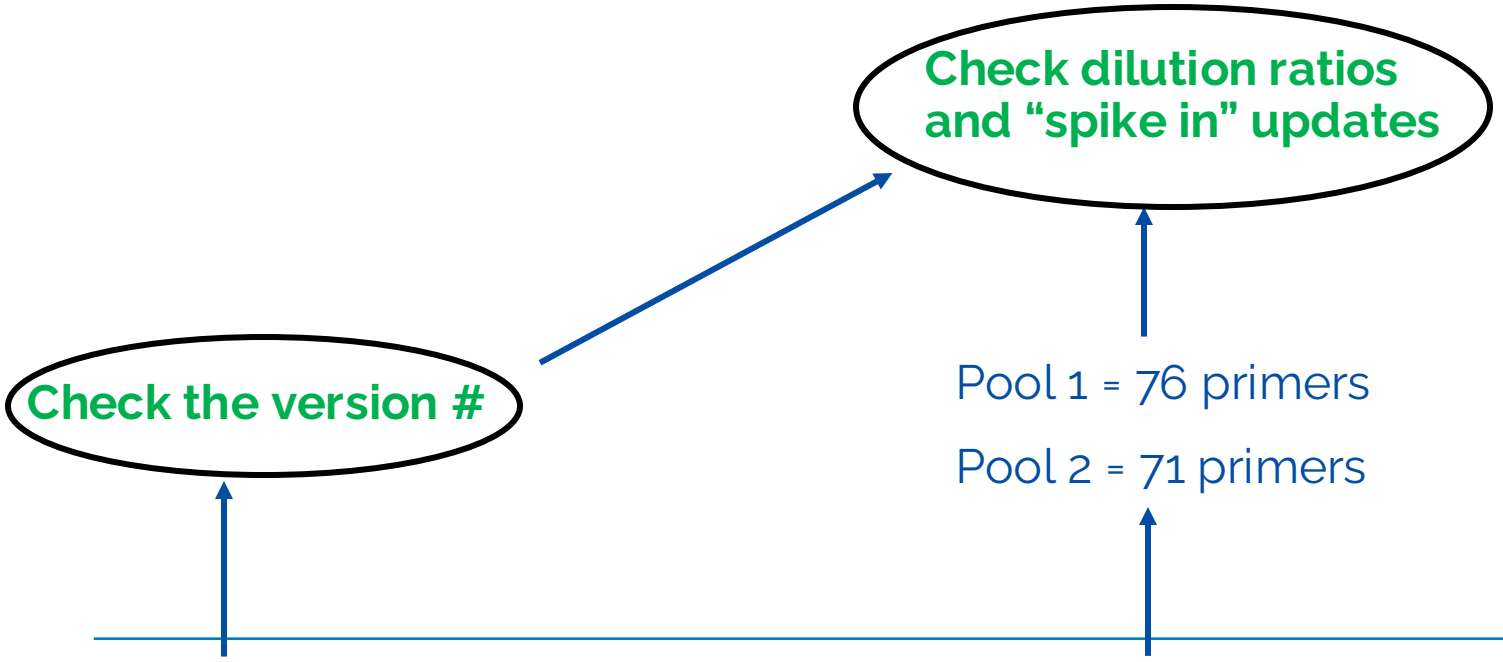
Important to know for data analysis and bioinformatics

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)

same scheme name but different amplicon options/uses

Guideline for ideal sample quality

Amplicon Schemes Updates



Scheme	Amplicon size [bp]	# Primer pairs	Optimal Ct	Reference for primer design
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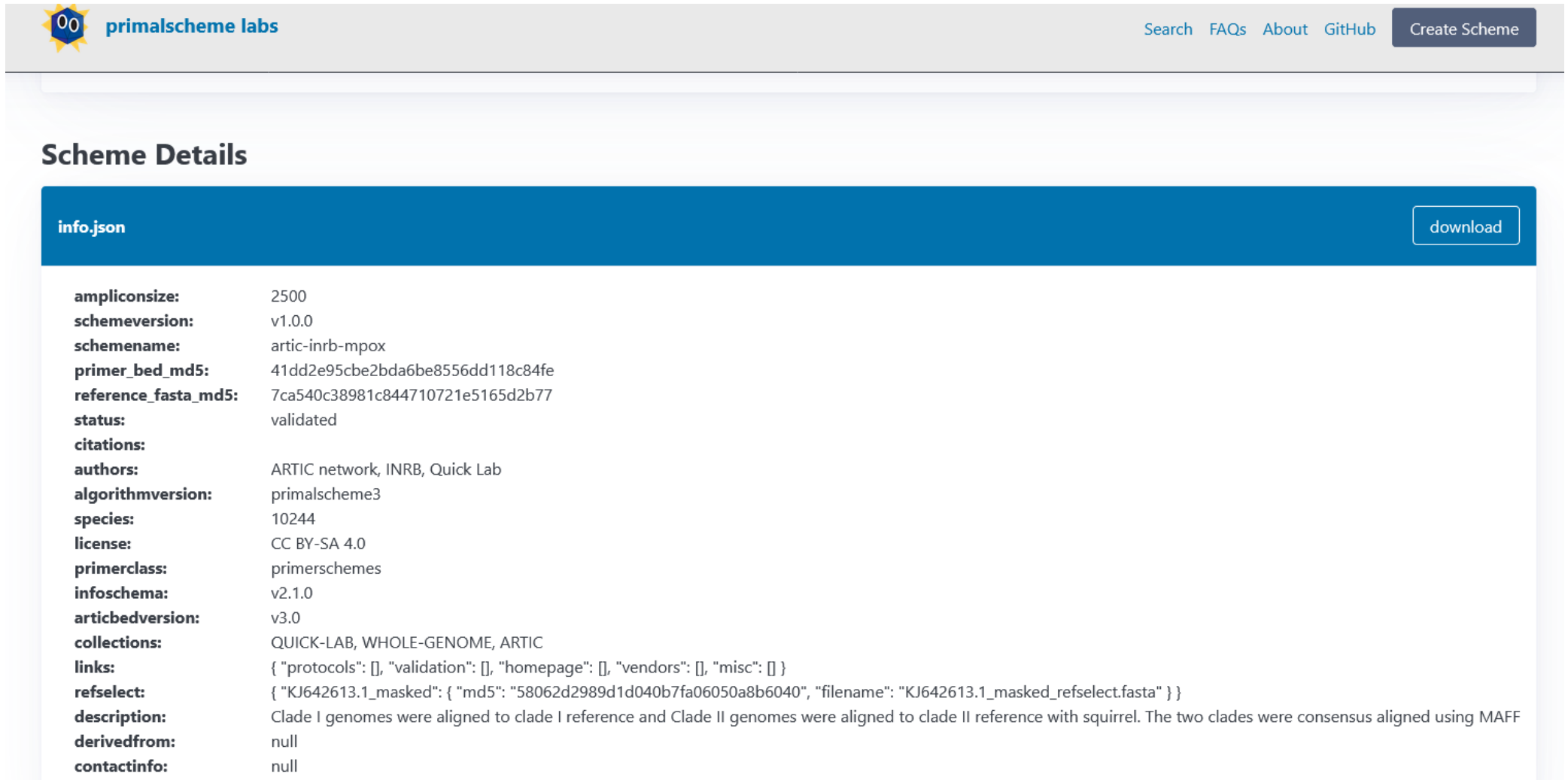
Amplicon Schemes Updates



https://labs.primalscheme.com/detail/artic-inrb-mpox/2500/v1.0.0/?q=

Amplicon schemes dilutions

- Typically diluted to a working concentration of 10 µM **– but not always!!!!!**

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)

When creating your primer pools check:

1. Primer dilution - by volume or concentration?
2. Pools – does the pooling parameters change? E.g Pan scheme vs Genotype specific PCR
3. What the stock concentration vs working concentration.

4. Clearly label your stock and working tubes!

Amplicon Size

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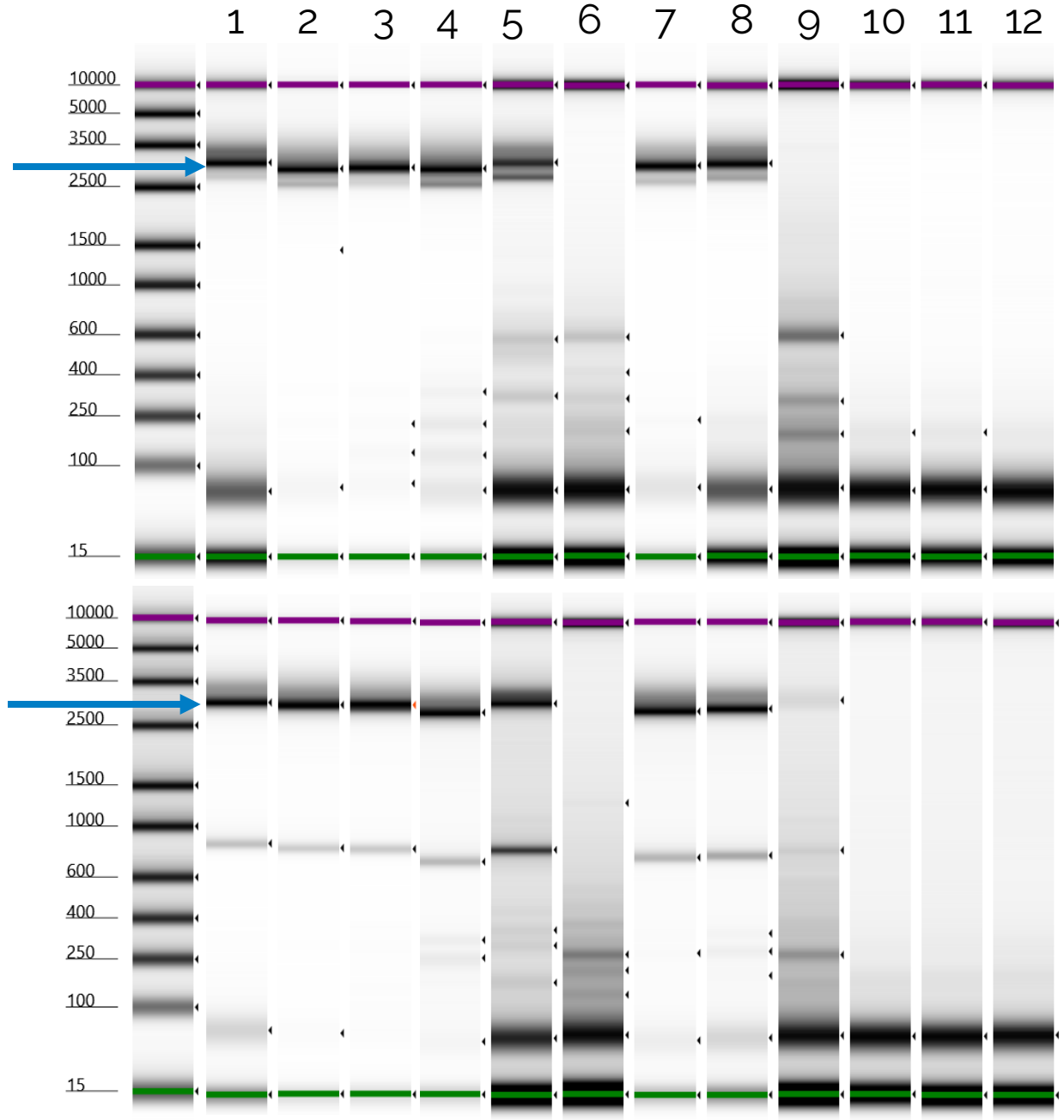
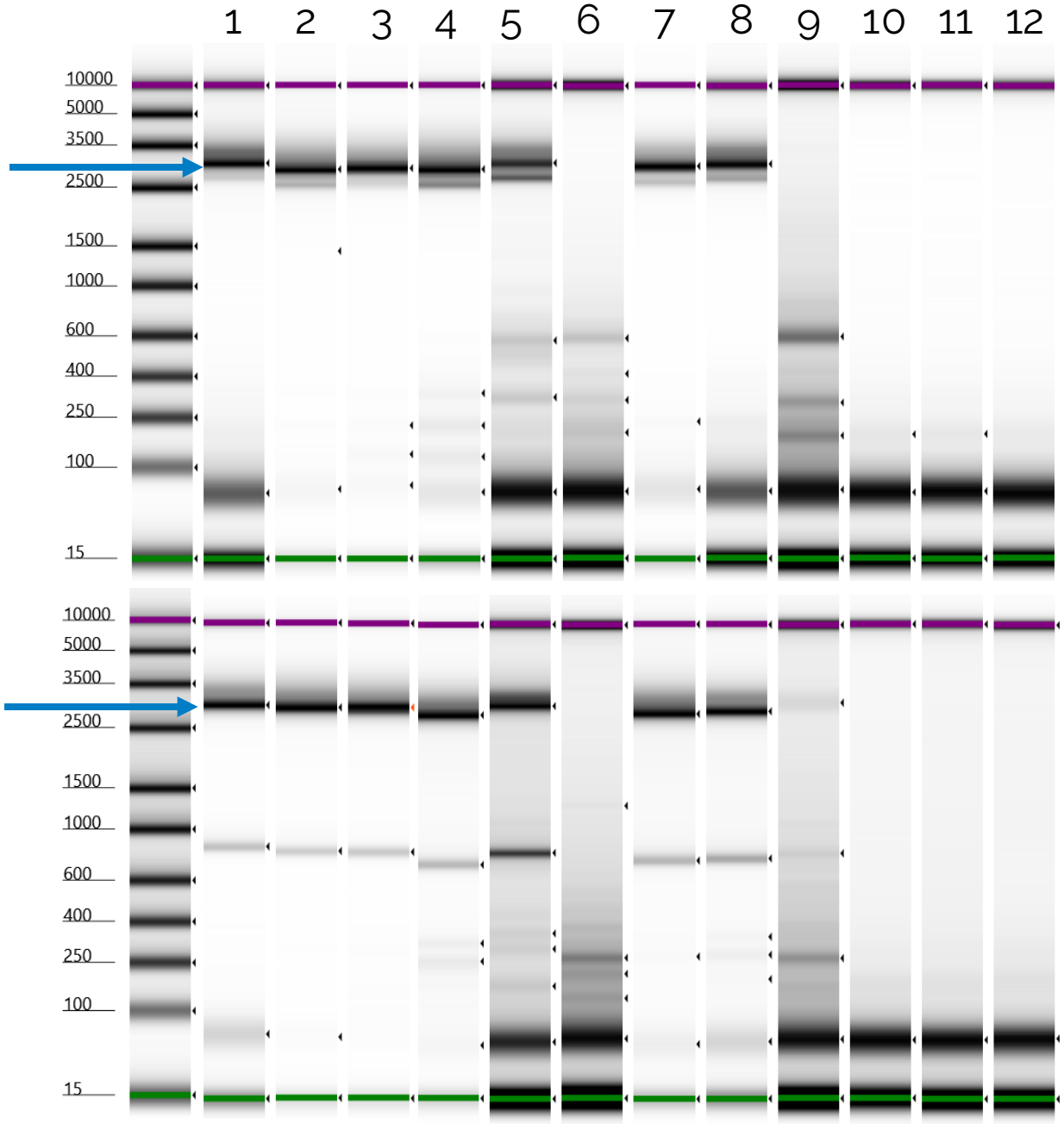
POOL 1

POOL 2



Sample	Mpox Ct
1	25.6
2	21.9
3	19
4	21
5	29.1
6	Not Det
7	24
8	26
9	35.3
10	Not Det
11	Not Det
12	Not Det

Amplicon Concentration



POOL 1

POOL 2

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QuBit Assay

Sample	Mpox Ct	POOL (ng/ul)	
		Pool 1	Pool 2
1	25.6	22.5	34.6
2	21.9	73.4	57.7
3	19	82.8	76.2
4	21	42.9	61.2
5	29.1	14.5	13.8
6	Not Det	13.8	10
7	24	42.2	49
8	26	25.3	31.2
9	35.3	13.8	14.3
10	Not Det	6.98	6.21
11	Not Det	9.08	50.6
12	Not Det	7.48	6.47

Amplicon
Summary

https://www.nature.com/articles/nrmicro.2016.182#Sec6

Method	Advantages	Disadvantages
PCR amplification sequencing	• Tried and trusted well-established methods and trained staff • Highly specific; most sequencing reads will be pathogen-specific, which decreases sequencing costs • Highly sensitive, with good coverage even at low pathogen load • Relatively straightforward design and application of new primers for novel sequences	• Labour-intensive and difficult to scale for large genomes • Iterating standard PCRs across large genomes requires high sample volume • PCR reactions are subject to primer mismatch, particularly in poorly characterized or highly diverse pathogens, or those with novel variants • Limited ability to sequence novel pathogens • High number of PCR cycles may introduce amplification mutations • Uneven amplification of different PCR amplicons may influence minor variant and haplotype reconstruction

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