Schedule DAY 2

TUESDAY	ACTIVITY	PRESENTER
9.00 – 9.10	Review of Day 1	Jean
9.10 – 10.00	**Lab:** Amplicon QC	Louise
10.00 – 10.30	Morning tea	
10.30 – 12.30	**Lab:** Library Preparation & Loading	Jean
12.30 – 13.30	Lunch	
13.30 – 14.15	**Lab:** Flow cell loading practice and Run Reviewing	Louise
14.15 – 14.30	**Lecture**: Review of laboratory workflow	Jean
14.30 – 15.30	Panel: Ask us anything!	
15.30 – 16.00	Afternoon Tea	
16.00 – 17:00	**Lecture: ILLUMINA SEQUENCING MPXV OPTIONS**	**Jean**

WET LAB DAY 2: ILLUMINA SEQUENCING OF MPOX

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

CENTRE FOR PATHOGEN GENOMICS

World Health Organization
Western Pacific Region

IPSN International Pathogen Surveillance Network
WHO hosted network

Viral genomics – brands and machines

Regardless of the ‘commercial’ brand of machine or test kits,
they are all designed to detect **GENETIC MATERIAL** of pathogens.







Sequencing platforms - Illumina and Nanopore

technologies use different chemistries

illumina

Viral NGS
Technique considerations

WET LAB

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

Library Prep Kit
Scalability
Sequencer Selection

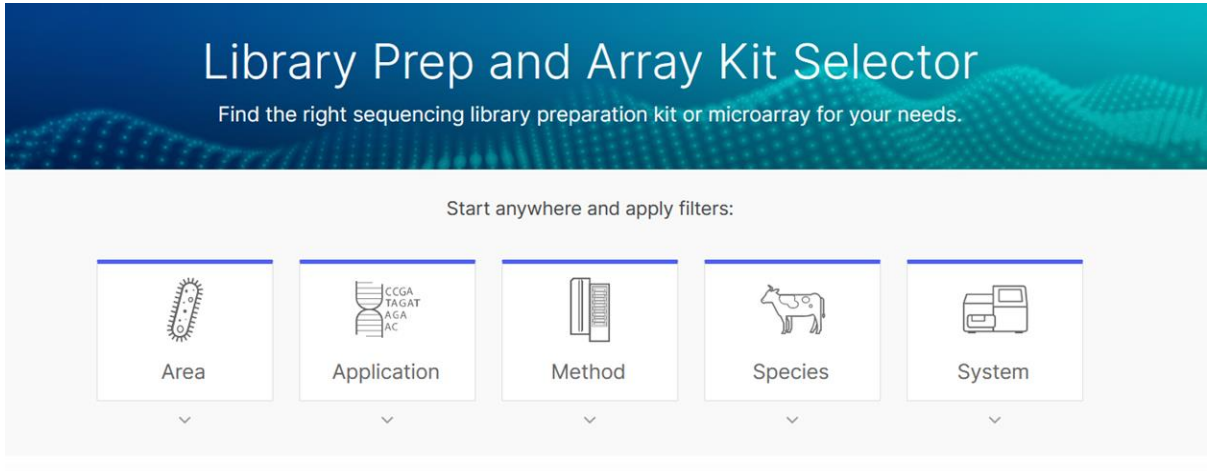
Budget

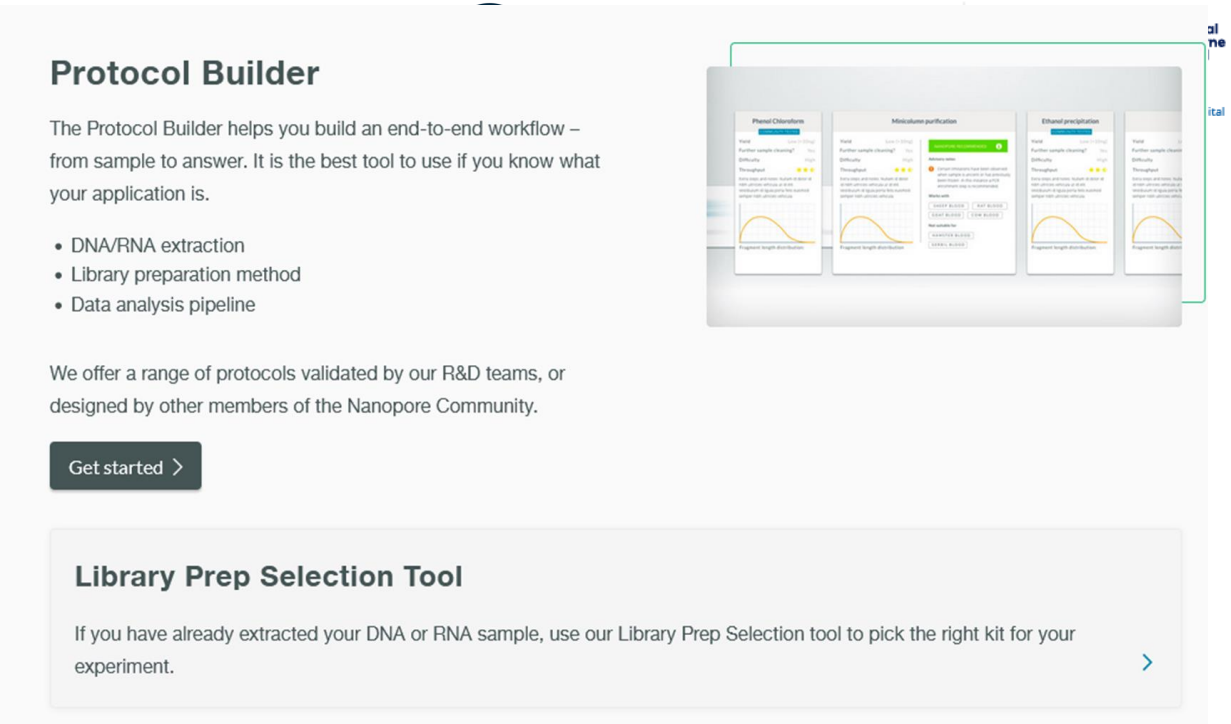
DRY LAB

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

All these considerations are linked

Library Preparation Kits





Choice of the library prep will be determined by the research goal

WGS or targeted approach

Sequencer choice

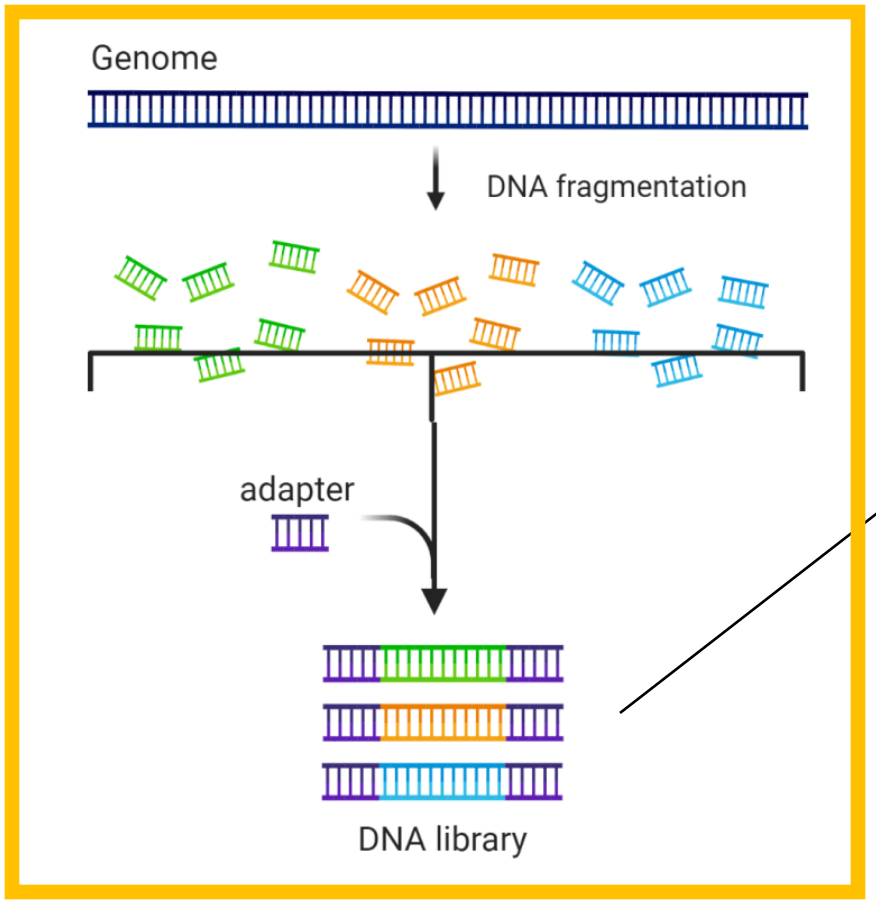
Depth of sequencing and throughput

Data analysis

In house or Illumina cloud-based analytics (DRAGEN)

Library Preparation

NGS Library = a pool of same sized fragments with adapters attached.



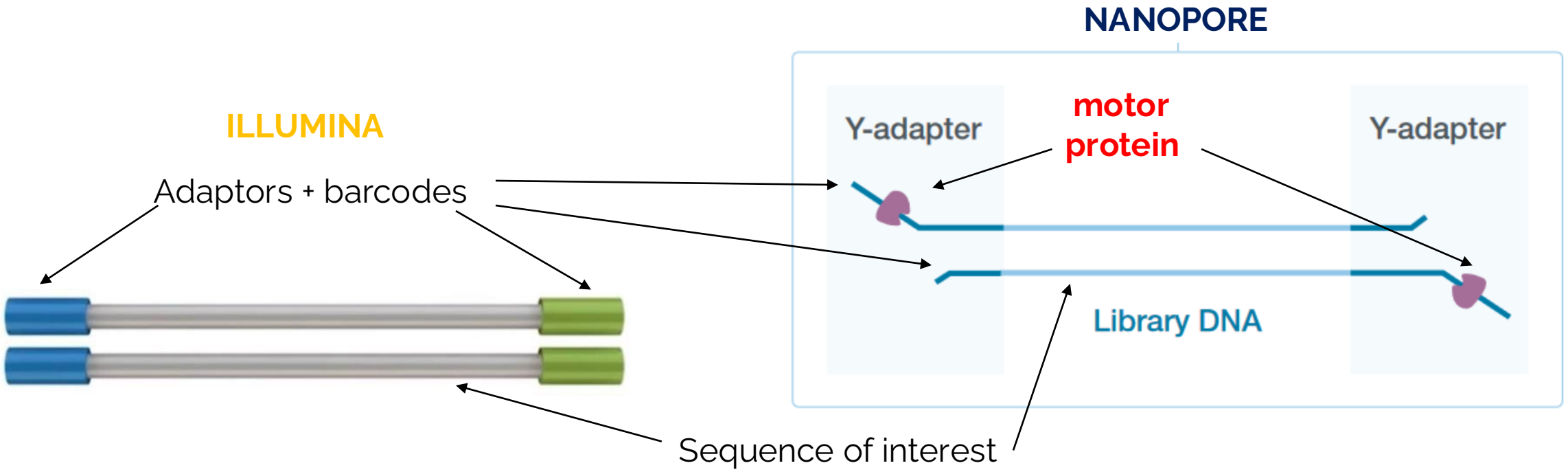
Lib prep complete, the samples pooled in a tube

Quantification of pool and dilute to load on flow cell

NGS Library Preparation

Adapters are designed to interact with a specific sequencing platform, either the flow-cell surface (Illumina) or pore (Nanopore)

The goal of library prep is to add sequencing adaptors to molecules (genetic material) you want to sequence on a NGS platform.



ONT Sequencing

Three distinct steps

1. Library preparation – Barcoding options
2. Sequencing – in real time
3. Data analysis

Choice of the library prep will be determined by the research goal

WGS or targeted approach (amplicon)

Sequencer choice

Depth of sequencing and throughput

Data analysis

Epi2Me, Community Tools or Custom in house pipelines

Illumina Sequencing

Four distinct steps

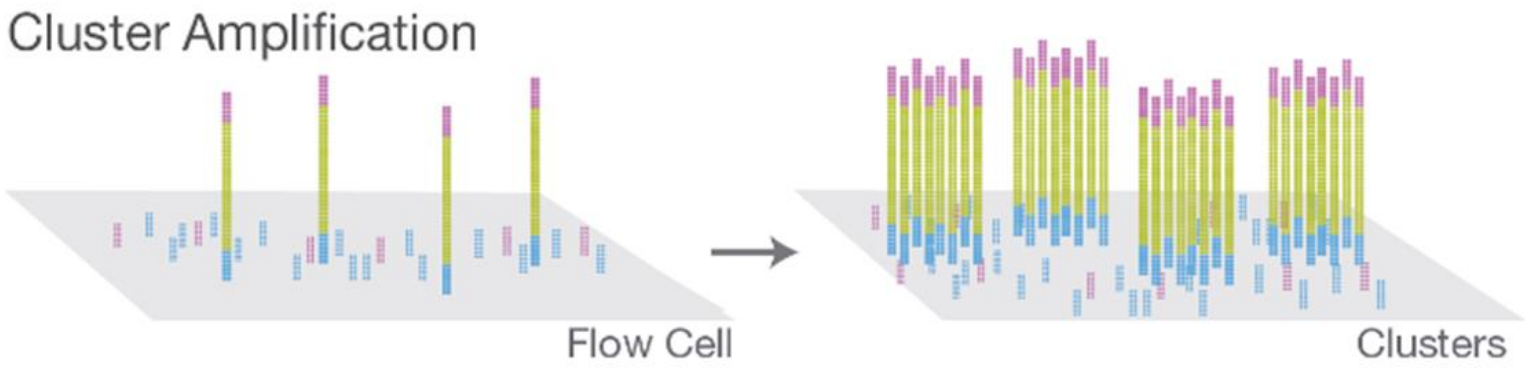
1. Library preparation
2. Cluster Generation
3. Sequencing by Synthesis (SBS)
4. Data analysis

Illumina Sequencing

Cluster generation – cluster density creation

Sequencing by synthesis – cluster intensity measured (photographs)

Base calling – quality scores



Illumina Sequencing by Synthesis

Tagged nucleotides are added to the DNA strand.

Each of the four nucleotides have an identifying label that can be excited to emit a characteristic wavelength.

A computer records all the emissions, and from this data, base calls are made

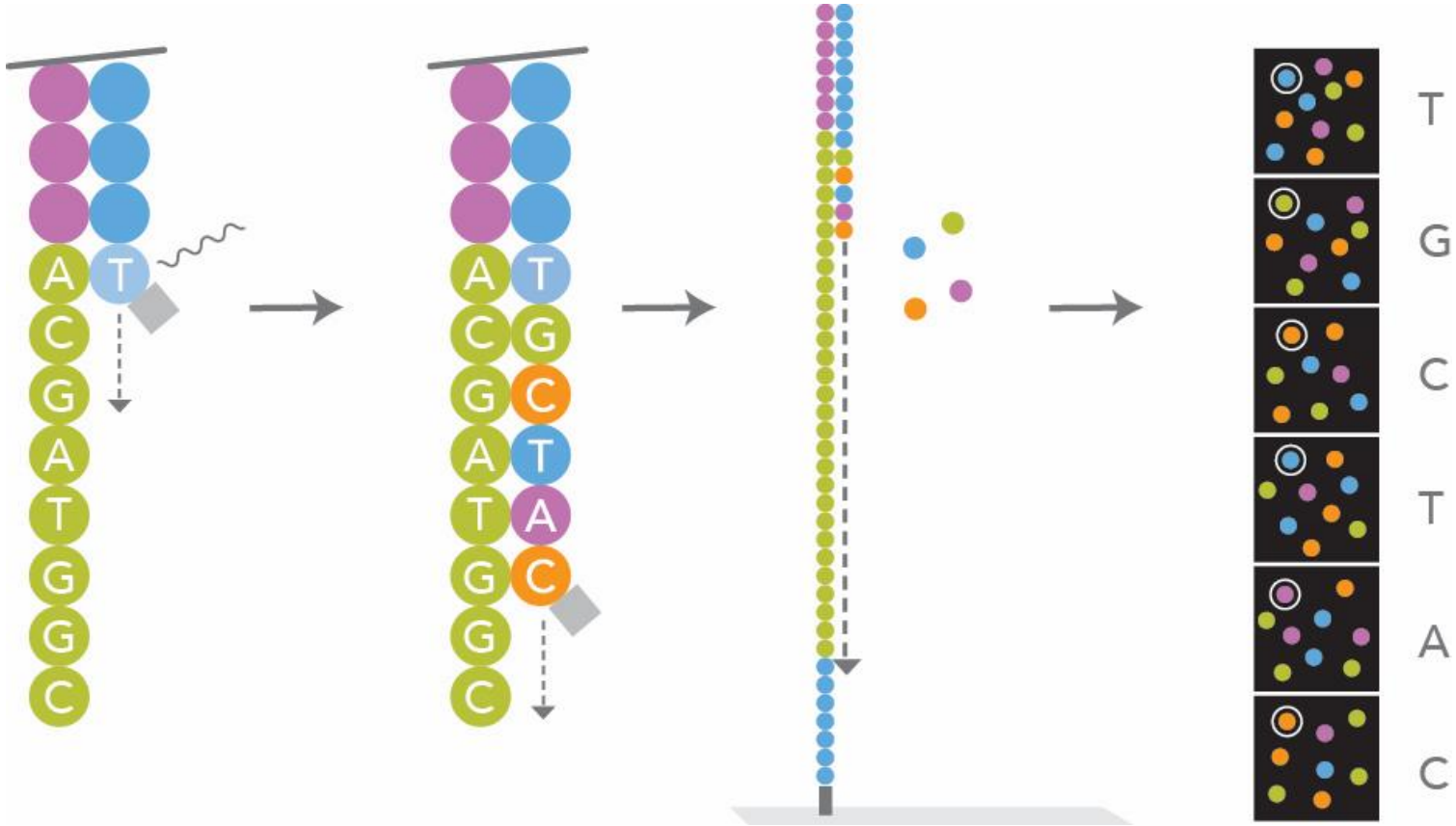


Image source: www.illumina.com

Illumina Sequencing Library

Illumina Library Prep
P5 & P7 + Index

large numbers of libraries with unique indexes can be pooled together on one flow cell and sequenced in the same run

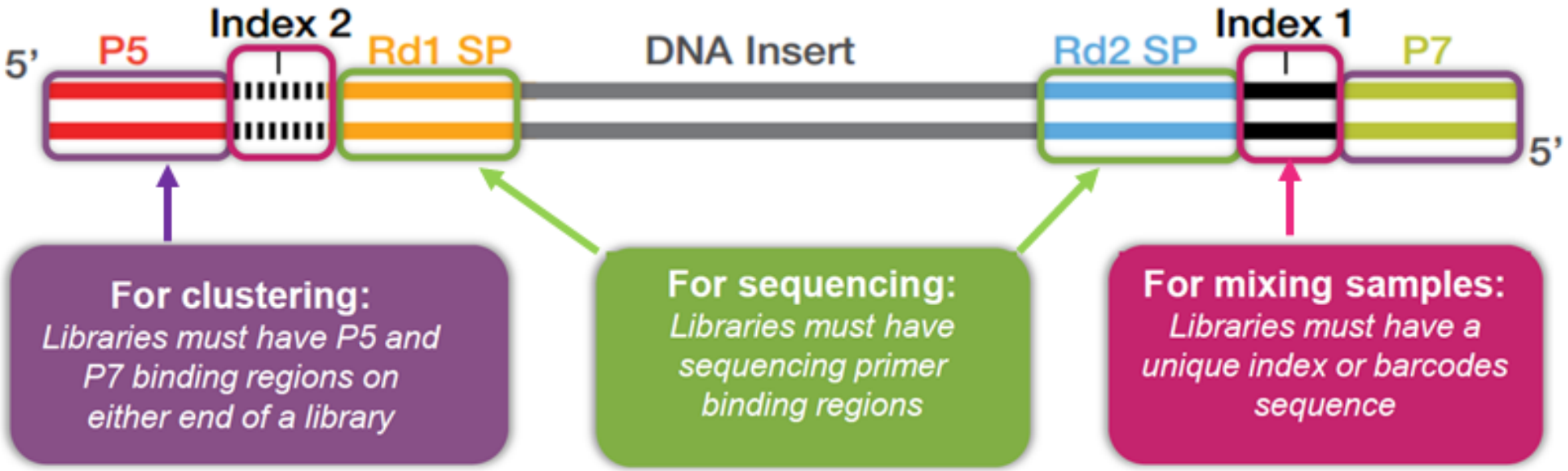


Image source: www.illumina.com

Illumina Sequencing Library

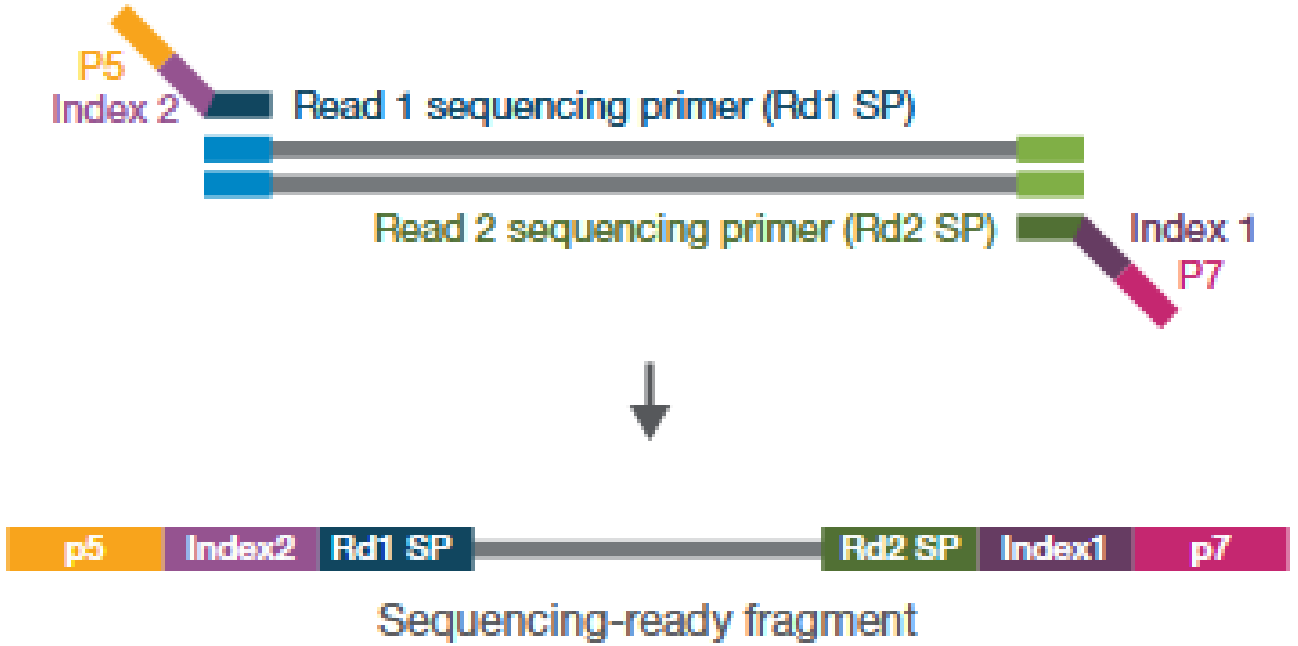
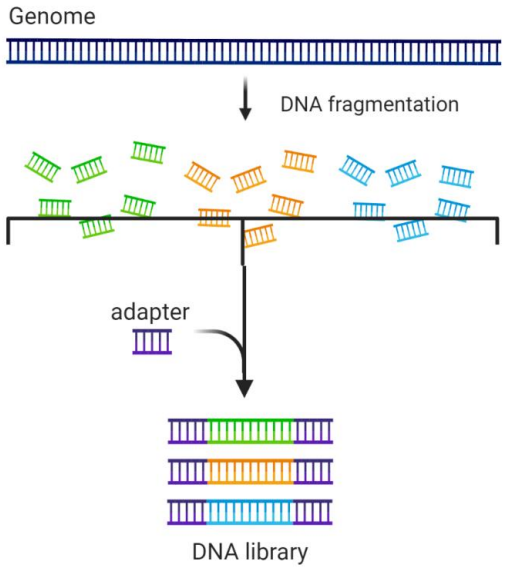


Image source: www.illumina.com

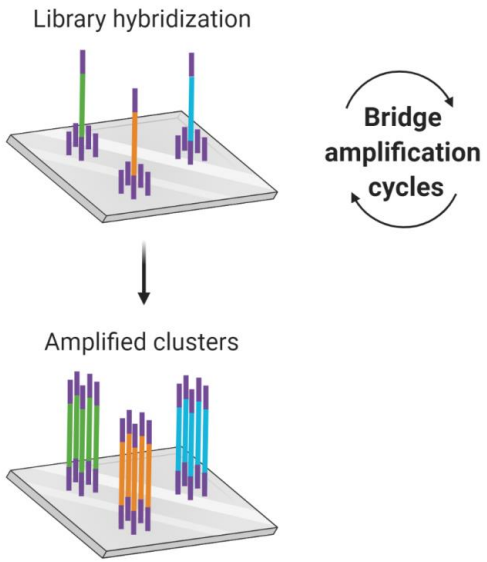
Summary of Illumina Sequencing

CENTRE FOR PATHOGEN GENOMICS
Doherty Institute
THE UNIVERSITY OF MELBOURNE
The Royal Melbourne Hospital
A joint venture between The University of Melbourne and The Royal Melbourne Hospital

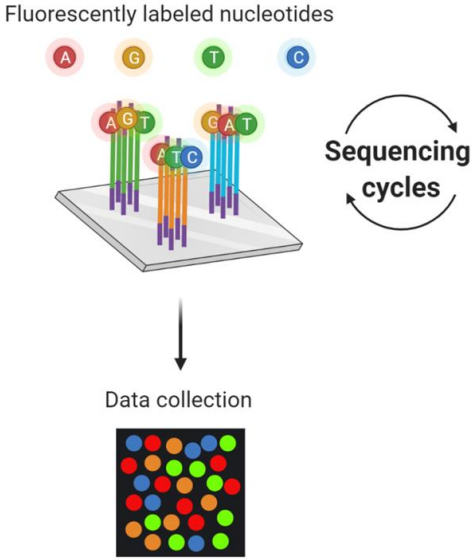
1 Library preparation



2 DNA library bridge amplification



3 DNA library sequencing



4 Alignment and data analysis

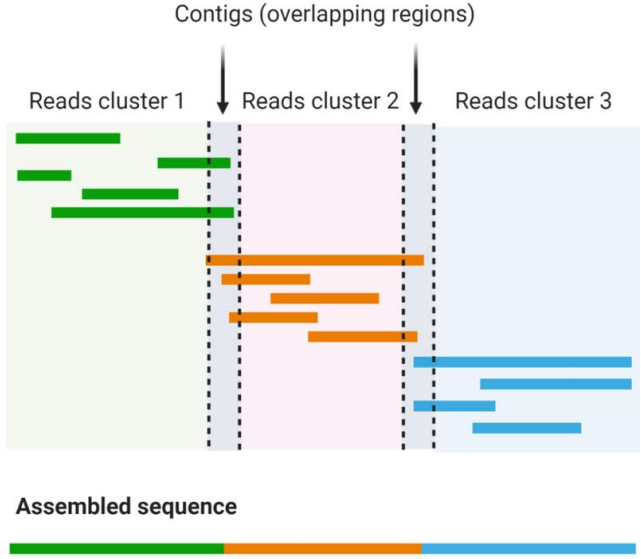


Image source: www.illumina.com

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

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%
	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

Illumina Microbial Amplicon Prep (IMAP)

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

"Versatile library preparation solution for public health surveillance and microbiology Research"

Supports user-designed primers to sequence pathogens of public health concern

- Enables high-quality genome-wide coverage across multiple microbial species
- Accommodates DNA and RNA inputs from a range of sample sources and type

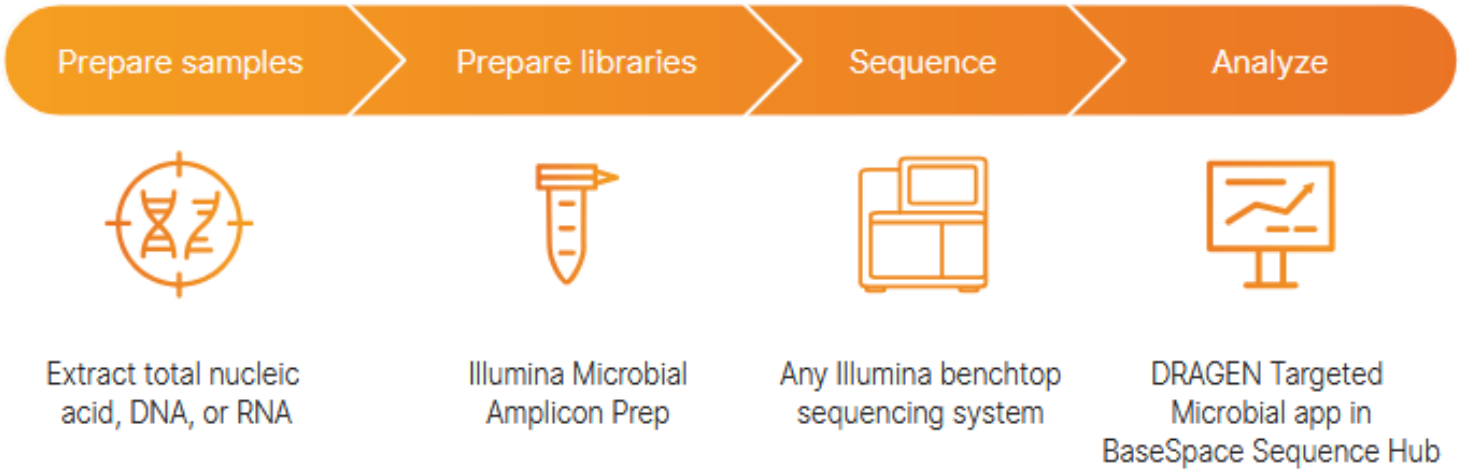


Image source: www.illumina.com

Illumina Microbial Amplicon Prep (IMAP)

This kit is based on Illumina COVIDSeq Assay workflow:

cDNA conversion, amplification and library preparation

Amplicon lengths of 400 base pairs are recommended.
but longer amplicons may be necessary with some targets.

Illumina Tested IMAP protocols:

- Chikungunya
- Dengue
- **Mpox**
- RSV
- Zika

Product	Catalog no.
Illumina Microbial Amplicon Prep (48 samples)	20097857

IMAP and Mpox Scheme

The kit is compatible with lab-designed primers or commercially available **primer sets which are purchased separately.**

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%

Also known as the “Yale” or “Vogels” scheme

Development of an amplicon-based sequencing approach in response to the global emergence of mpox

Nicholas F. G. Chen, Chrispin Chaguza, Luc Gagne, Matthew Doucette, Sandra Smole, Erika Buzby, Joshua Hall, Stephanie Ash, Rachel Harrington, Seana Cofsky, Selina Clancy, Curtis J. Kapsak, Joel Sevinsky, [...], Chantal B. F. Vogels

[view all]

Published: June 13, 2023 • https://doi.org/10.1371/journal.pbio.3002151

Illumina DNA Prep Kit Mpox Sequencing

A fast, integrated library prep workflow for a wide range of sequencing applications

Support a broad DNA input range (1–500 ng) and multiple DNA input types

Access a wide range of applications with the ability to sequence large and small genomes and **amplicons**

☆ **Illumina DNA Prep, (M) Tagmentation (24 Samples, IPB)**

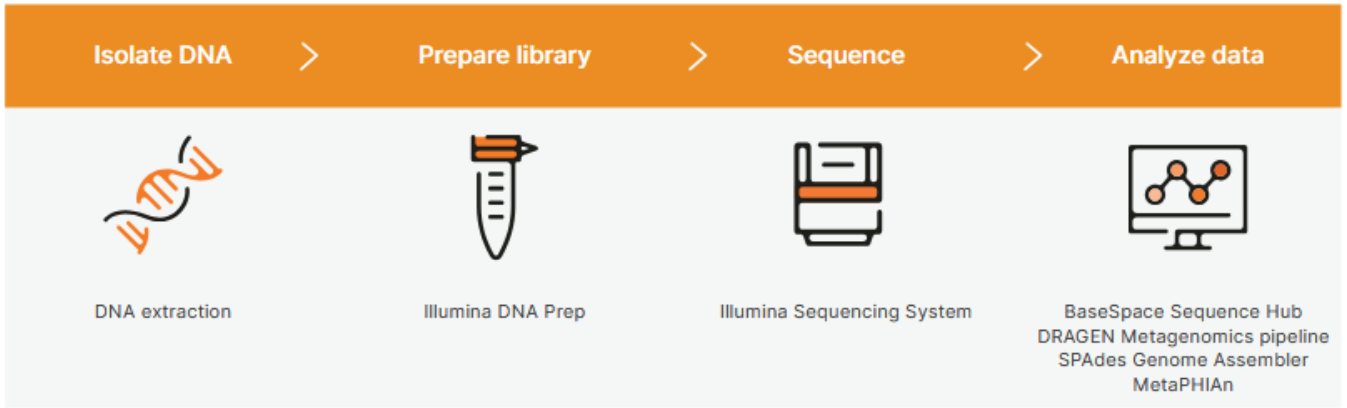
20060060

Includes reagents for preparing 24 libraries. Illumina Purif... View more

☆ **Illumina DNA Prep, (M) Tagmentation (96 Samples, IPB)**

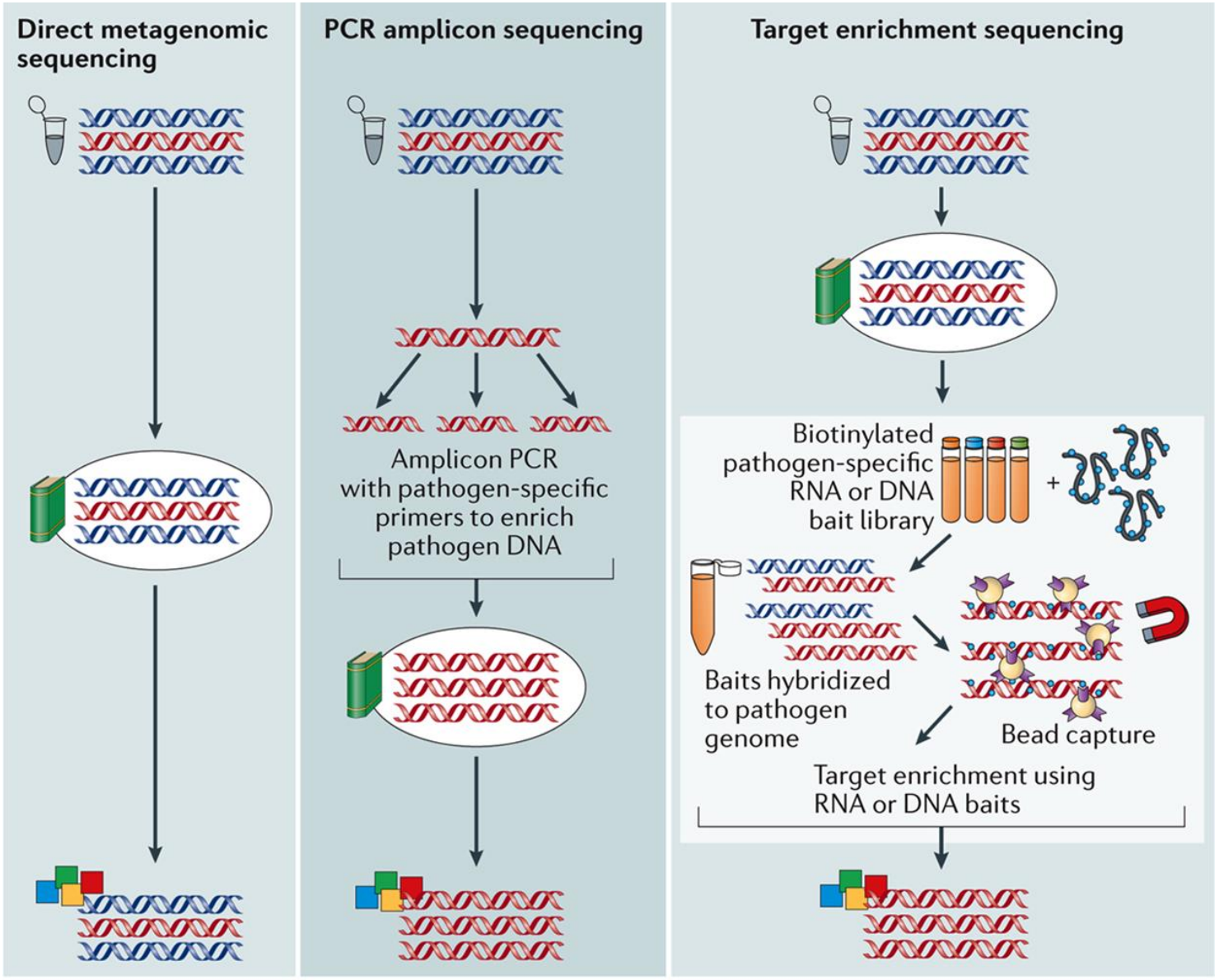
20060059

Includes reagents for preparing 96 libraries. Illumina Purif... View more

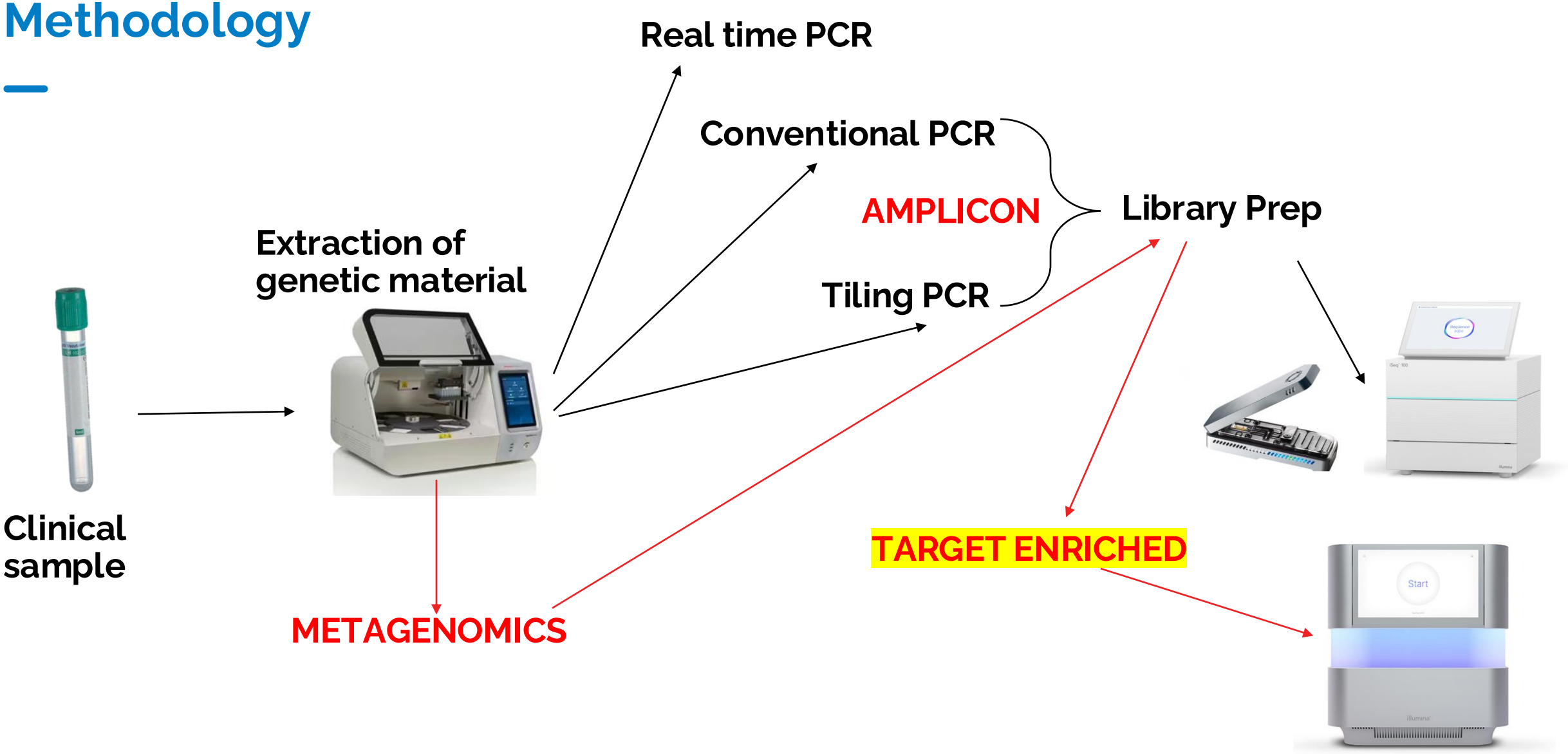


✘ cDNA conversion or PCR scheme/amplification steps included in this kit

Image source: www.illumina.com



Viral Genomics
Methodology



Comprehensive Virus Research Panel
Hybridization Target enrichment at VIDRL

Hybridization capture is a **targeted** “metagenomics” NGS method

Contains over 1M unique probes to screen for 3,153 viral human and nonhuman pathogens

Requires additional NEB reagents for cDNA amplification

Automated library prep procedures on Biomek i5 & i7

585 runs since 2022

In-house bioinformatics pipeline

Dengue, Enterovirus, FluA, FluB, HAV, HBV, Herpes, HEV, HIV-1, JEV, MEV, Measles, Monkeypox,

Mumps, Poliovirus, RSV, SARS-CoV-2, Zika, Seasonal Coronaviruses, Rabies, Parvo, Adenovirus, Norovirus, Zeptometrix

Brain, urine, whole blood, wastewater, isolates, swabs, serum, QAP confirmation

Sequencing Enrichment Approaches for Viruses

Hybridisation Capture (a.k.a. Target Enrichment)

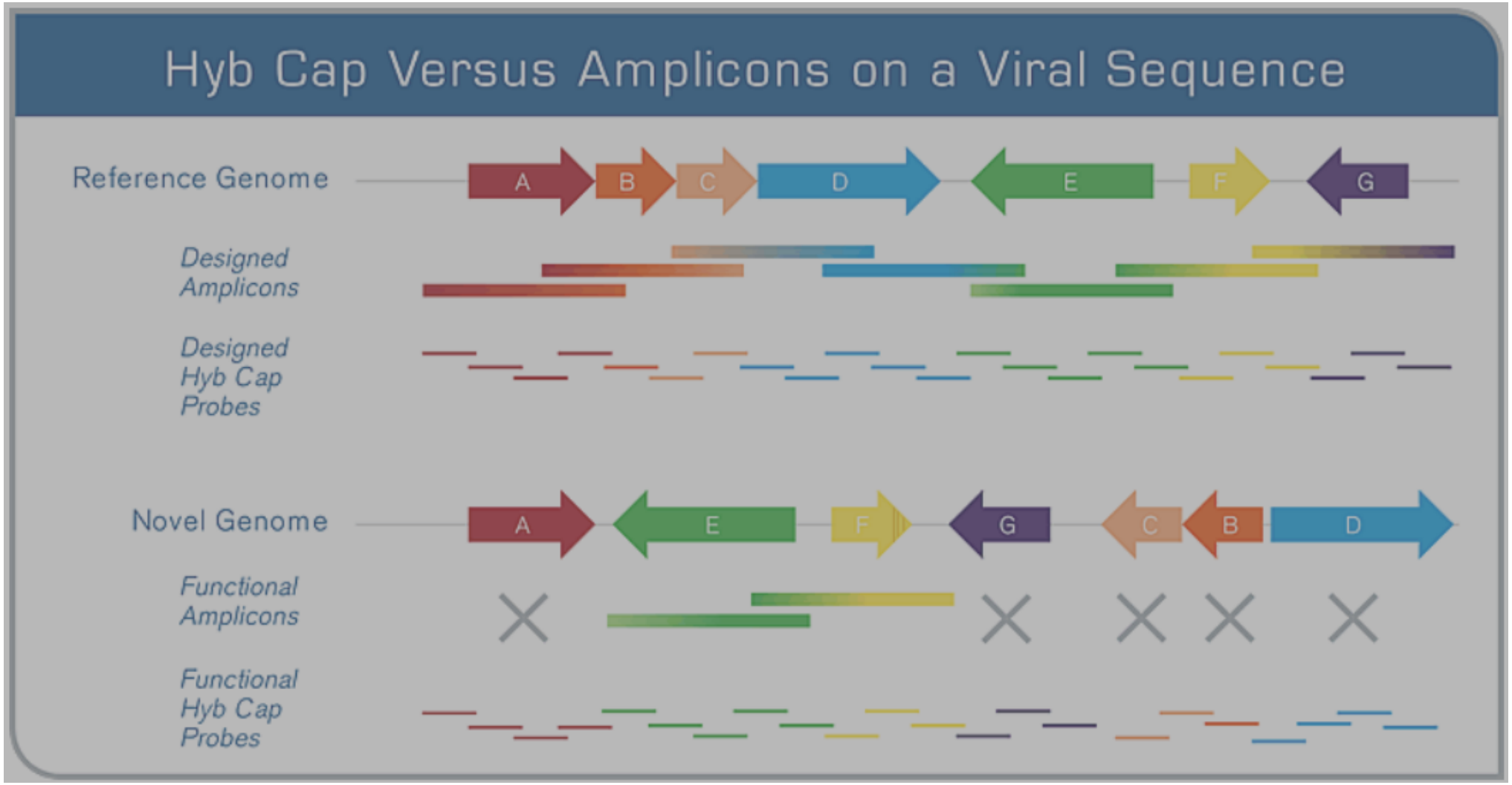


Image Source: https://www1.biocat.com/products/308716.v5-ARB

Sequencing Enrichment Approaches for Viruses

Hybridisation Capture (a.k.a. Target Enrichment)

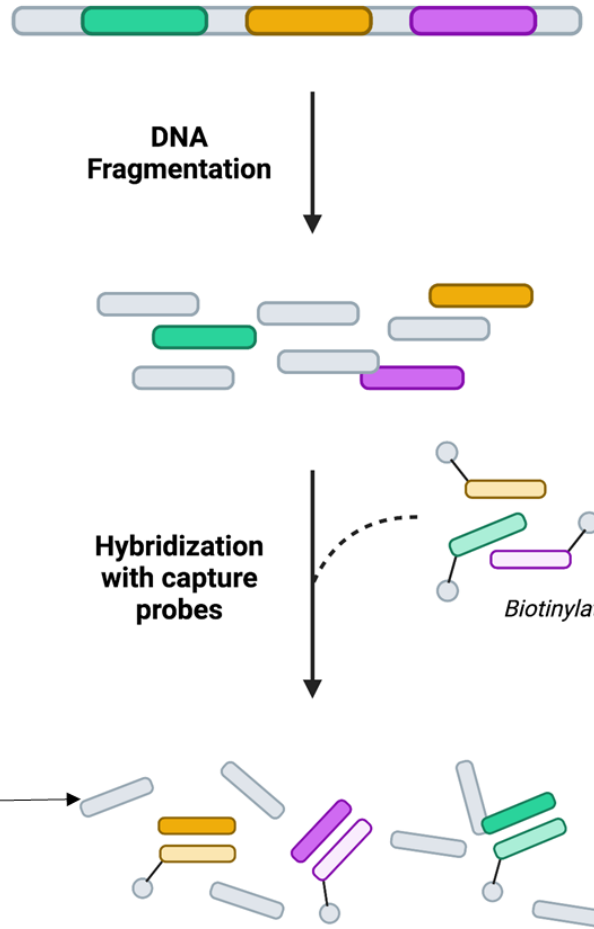
- Better performance than tiled amplicon approach for degraded samples
- Long and complex workflow
- Requires higher input amount
- Does not require PCR primer design
- More flexibility
  - Novel variants
  - Point mutations
  - Small and large indels
  - Indels = insertions and deletions
  - Can tolerate large sequence differences (10-20%)

Sequencing Enrichment Approaches for Viruses

Hybridisation Capture (a.k.a. Target Enrichment)

- Baits made of biotinylated DNA or RNA probes --> complementary to target region of interest
  - Biotinylated = labelled with biotin molecule --> non-fluorescent
  - ~120 bp long
- **Can have millions of targets in a single panel**
- Dozens to hundreds of overlapping probes --> higher specificity for covering entire genome
- More uniform coverage compared to tiled amplicon

Hybridization
Target enrichment



Choice to hybridize as a **POOL** or single sample

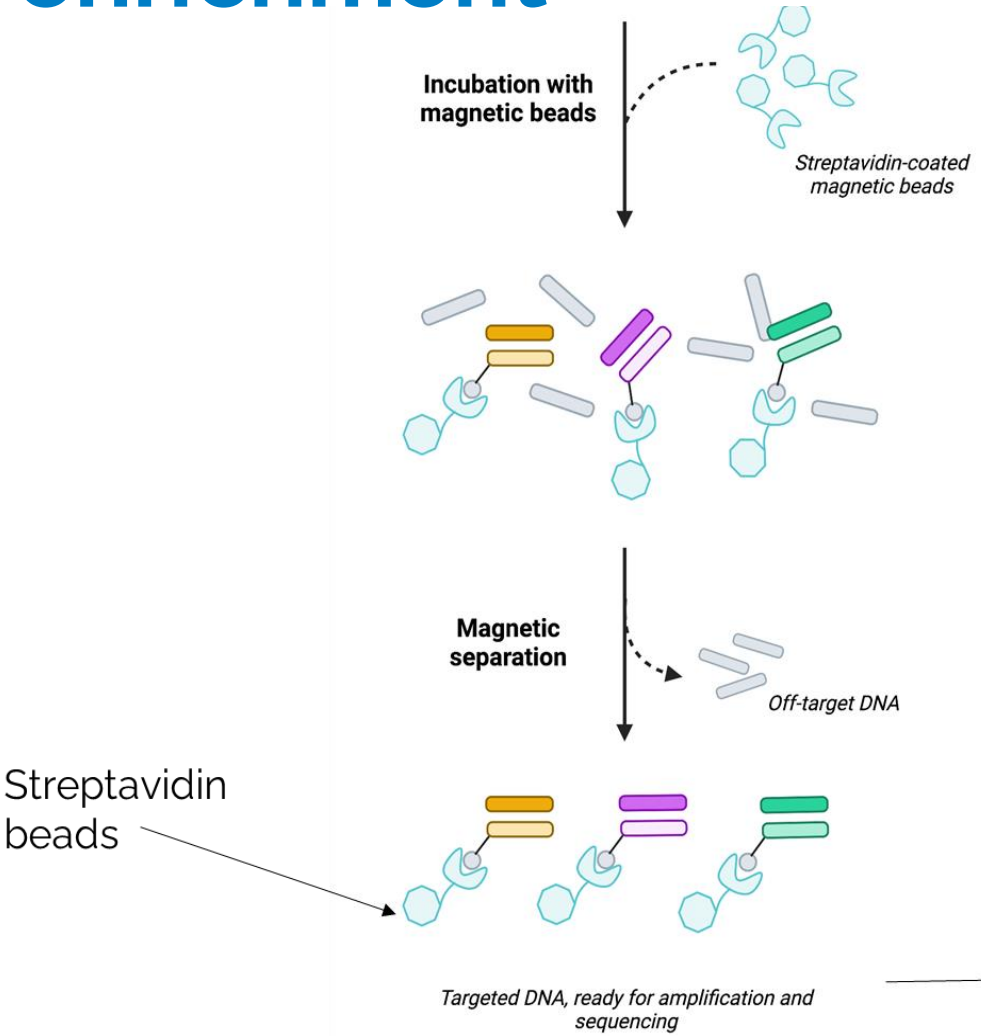
Off target DNA

Hybridization capture is a **targeted** "metagenomics" NGS method

Biotinylated oligonucleotide baits **(probes)** to hybridize to the **regions of interest (target)**

Enrichment = selectively isolating (and amplifying) regions of interest

Hybridisation Target enrichment



Purify, elute, dilute

TWIST

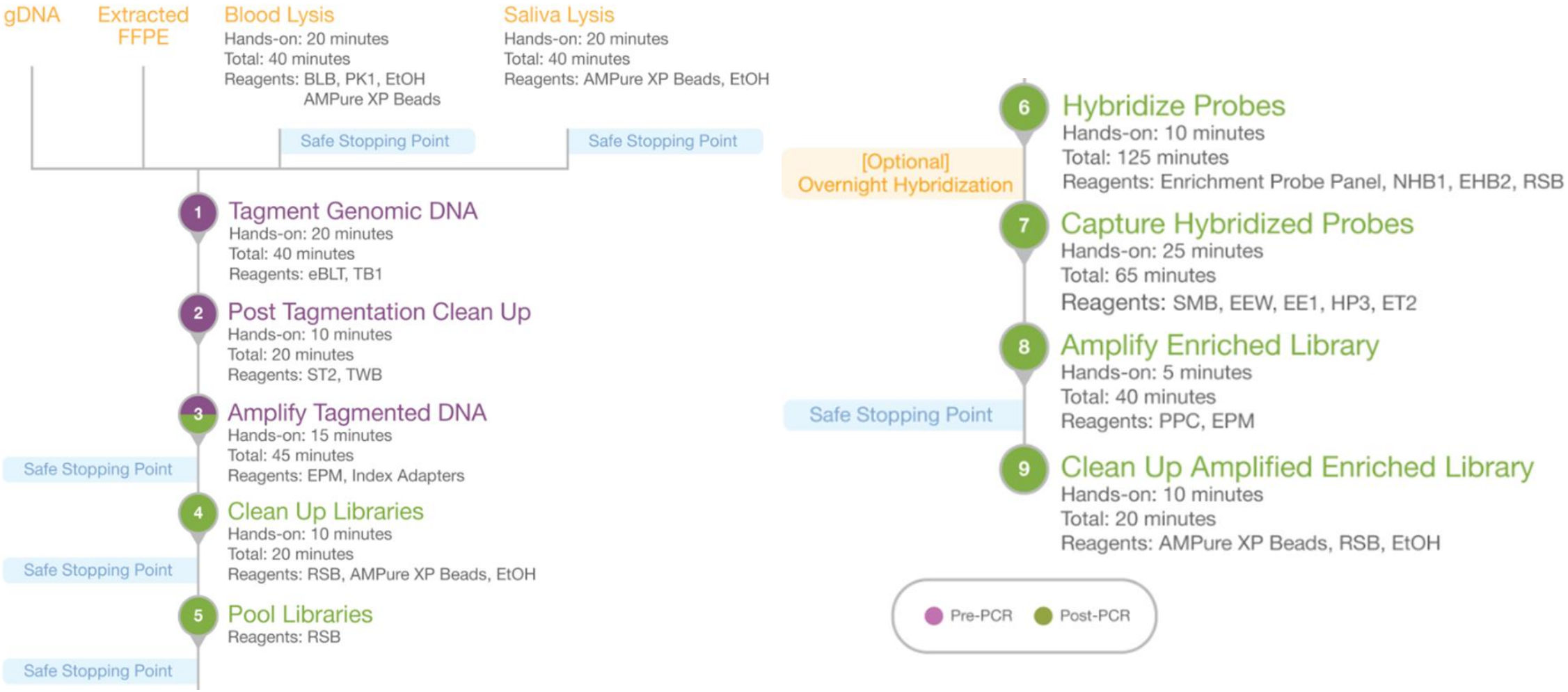
Sequence on an Illumina platform

iSeq100

NextSeq1000/2000

Illumina Enrichment Viral Surveillance Panel

CENTRE FOR PATHOGEN GENOMICS
Doherty Institute
THE UNIVERSITY OF MELBOURNE
The Royal Melbourne Hospital
A joint venture between The University of Melbourne and The Royal Melbourne Hospital



Hybridisation
Target enrichment

Method	Advantages	Disadvantages
Target enrichment sequencing	• Single tube sample preparation that is suited to high-throughput automation and the sequencing of large genomes • Higher specificity than metagenomics decreases sequencing costs • Overlapping probes increases tolerance for individual primer mismatches • Fewer PCR cycles (than PCR amplification) limits the introduction of amplification mutations • Preservation of minor variant frequencies reflects *in vivo* variation	• High cost and technical expertise for sample preparation • Unable to sequence novel pathogens and requires well-characterized reference genomes for probe design • Sensitivity is comparable to PCR, but coverage is proportional to pathogen load; low pathogen load yields low or incomplete coverage • Cost and time to generate new probe sets limit a rapid response to emerging and novel viruses

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

THANK YOU

centre-pathogen@unimelb.edu.au

CENTRE FOR PATHOGEN GENOMICS

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13.30 – 14.15	**Lab:** Flow cell loading practice and Run Reviewing	Louise
14.15 – 14.30	**Lecture:** Review of laboratory workflow	Jean
14.45 – 15.30	**Lecture:** Genomics pathogen topic to be chosen from participants. Questions from the day before	Jean + Louise
15.30 – 16.00	Afternoon Tea	
16.00 – 16.30	Lecture: Illumina Sequencing Mpox Options	Jean
16.30 – 17.00	**Group discussion: Opportunity for Q&A and further discussion**	**Nicole**