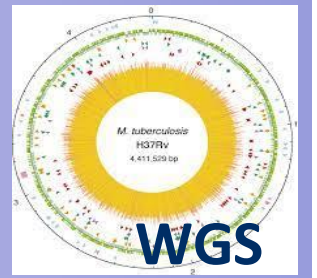
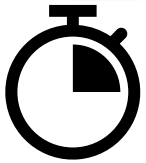
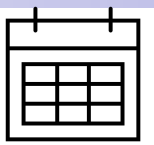
Molecular approaches: TB day

Monday 2 September

University of Antwerp
Faculty of Medicine and Health Sciences

MOLECULAR ASSAYS FOR TUBERCULOSIS: increased levels of complexity

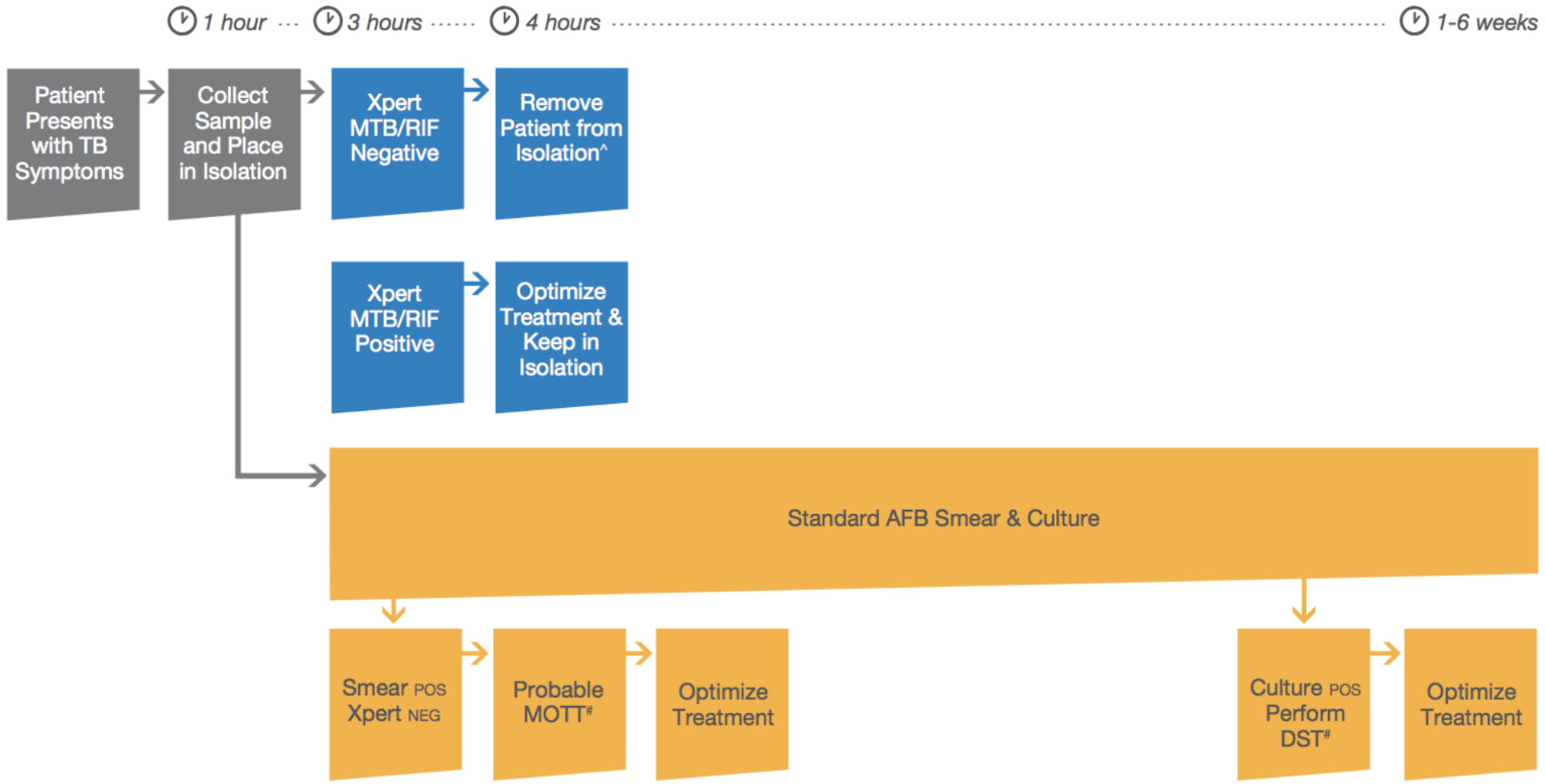
	Simple NAATs	tNGS	WGS
INFORMATION	*Mtb*/no *Mtb* RIF	*Mtb*/no *Mtb* 15 drugs Genotype	*Mtb*/no *Mtb* all drugs Genotype Transmission
TARGETS	MTB-specific 1 (4) drug-target(s)	MTB-specific 18 drug-targets	4.4Mb All drug-targets
SAMPLE TYPE	Raw specimen	Treated Specimen ***	Cultured isolate
MOLECULAR ASSAY		DEEPLEX	
			

Cepheid GeneXpert® System

Nucleic acid amplification tests (NAATs)

The impact of Xpert on TB diagnosis and care

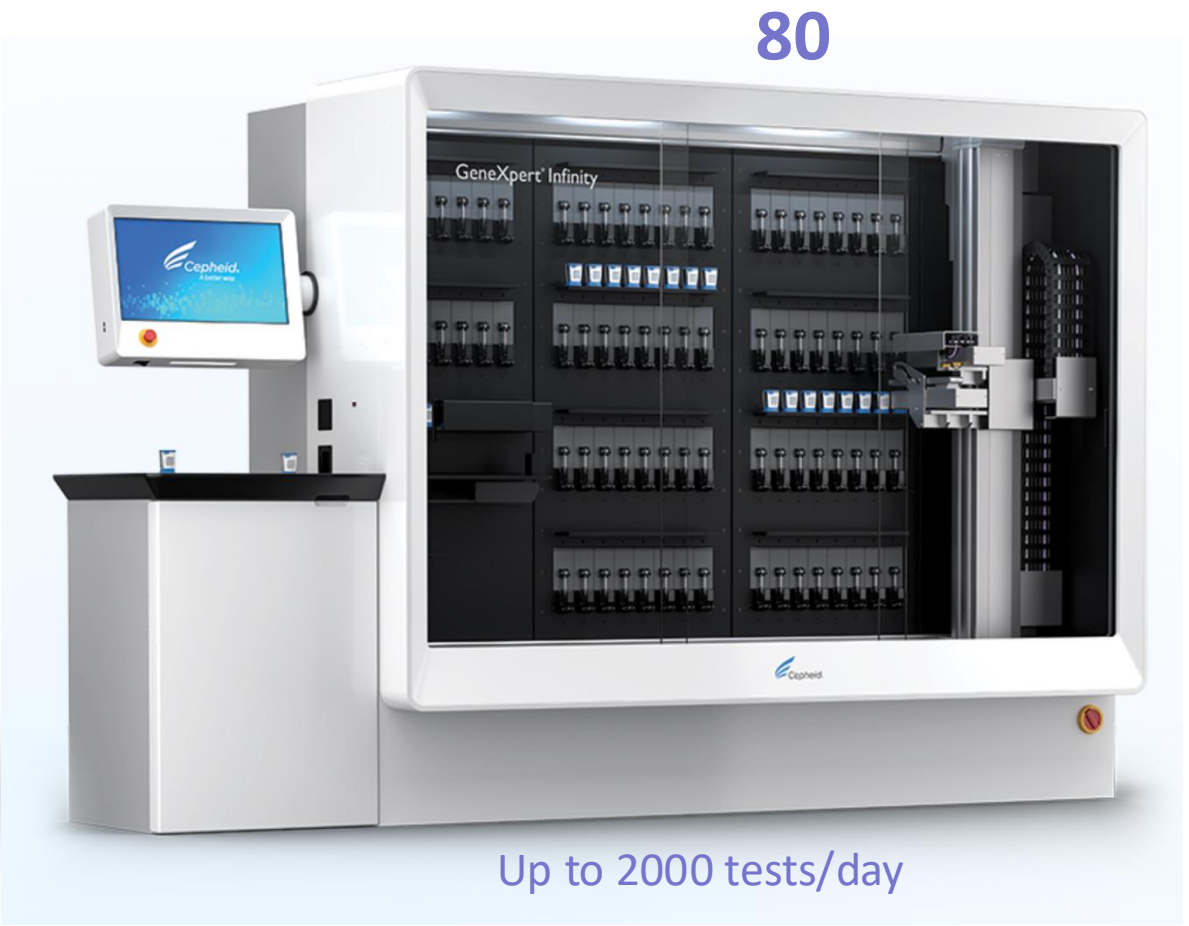
Impact on Patient Isolation Pathway
"Test and Release"or "Test and Treat"



System vs. Assays - semantics

- **THE SYSTEM: GeneXpert® System**
  - "The system is designed to purify, concentrate, detect and identify targeted nucleic acid sequences thereby delivering answers directly from unprocessed samples."
- **THE ASSAY: Xpert <<your favourite assay>>**
  - Xpert® MTB/RIF
  - Xpert® MTB/XDR
  - Xpert® Xpress Flu
  - Xpert® Xpress Strep A

GeneXpert systems for different throughput needs



GeneXpert Infinity

How does it work?

How does the PCR detection used by Xpert MTB/RIF Ultra work?

- **TB or no TB? (That is the question)**
  - Targets:
- **Xpert MTB/RIF:**
  - RIF susceptible or RIF resistant?
    - Target: *rpoB* – RNA polymerase
    - Rifampicin resistance determining region – 82 bp genomic region in *rpoB*
    - Wild type and mutant probes
- **Xpert MTB/XDR**
  - 10 colour-assay
  - Extended drug resistance:
    - INH
    - FQs
    - SLIDs
    - ETH

Xpert assays for TB

2006

Cepheid, FIND, Rutgers University, and NIAID begin development of **Xpert® MTB/RIF** with funding from the Bill & Melinda Gates Foundation

2009

Xpert MTB/RIF launches

2017

Xpert MTB/RIF Ultra launches and gets WHO endorsement

2020

Xpert MTB/XDR launched on new 10-color multiplexing technology

2021

Xpert MTB/XDR gets WHO endorsement

Interpreting results

110008945 ITG Antwerpen 12/23/22 11:17:22

Test Report

Patient ID:
Sample ID: CT202201273
Test Type: Specimen
Sample Type:

Assay Information

Assay	Assay Version	Assay Type
Xpert MTB-XDR	1	In Vitro Diagnostic

Test Result:

MTB DETECTED;
INH Resistance NOT DETECTED;
FLQ Resistance NOT DETECTED;
AMK Resistance NOT DETECTED;
KAN Resistance NOT DETECTED;
CAP Resistance NOT DETECTED;
ETH Resistance NOT DETECTED

Interpreting results

110008945 ITG Antwerpen 12/23/22 11:18:13

Test Report

Patient ID:
Sample ID: CT202201279
Test Type: Specimen
Sample Type:

Assay Information

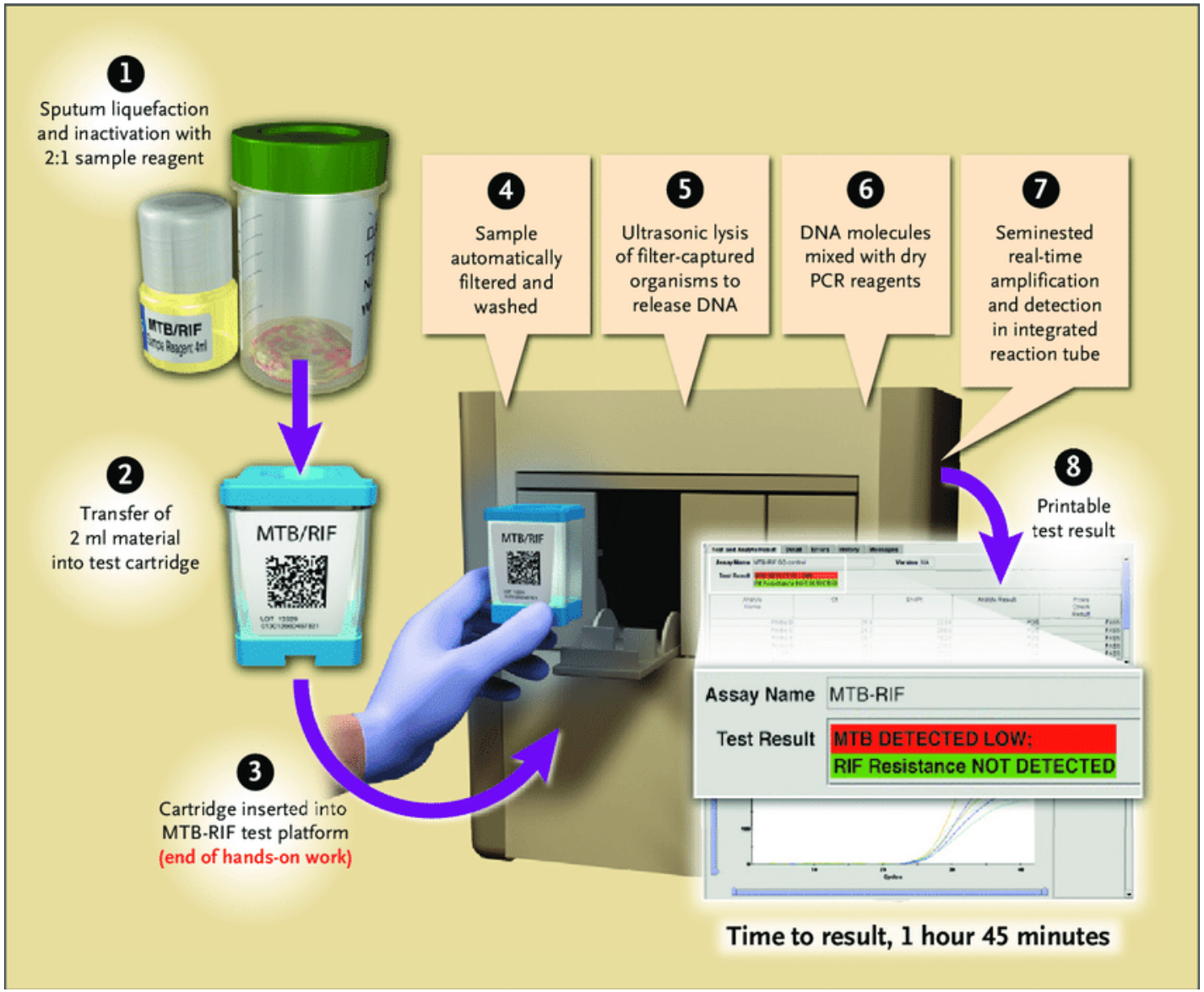
Assay	Assay Version	Assay Type
Xpert MTB-XDR	1	In Vitro Diagnostic

Test Result:

MTB DETECTED;
INH Resistance NOT DETECTED;
FLQ Resistance DETECTED;
AMK Resistance NOT DETECTED;
KAN Resistance NOT DETECTED;
CAP Resistance NOT DETECTED;
ETH Resistance NOT DETECTED

PRACTICAL

- Work in groups of 2
- Eyeball sample volume
- Add 2x volume sample reagent
- Mix well
- Incubate at RT for 15 min
- Mix again at 10 min mark
- Add 2 ml to cartridge
- Load into machine
- Follow software prompts
- Wait for assay to run
- Interpret results



LAB MANUAL: P16

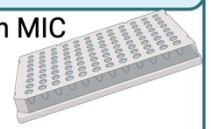
New assays: testing the limits

Prepare bacterial mixtures at various ratios of wild type (S) to mutant (R)

- S100:R0
- S99,5:R0,5
- S99:R1
- S97:R3
- S95:R5
- S90:R10
- S85:R15
- S80:R20
- S70:R30
- S60:R40
- S50:R50
- S40:R60
- S100:R0

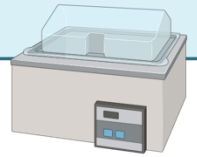
Phenotypic validation of mixtures

EUCAST broth microdilution MIC determination:
LVX and MXF



Each mixture tested in triplicate

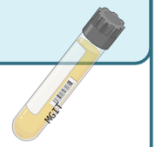
Heat inactivation



Creat thermolysates by incubating tubes containing the bacterial mixtures for 10 mins in a boiling waterbath

Viability

(Non-)viability check
MGIT inoculation
(42 days)



Genotypic validation of mixture ratios

Targeted deep NGS: Deeplex Myc-TB

DEEPLEX

Xpert MTB/XDR

100x dilution of each mixture + 1,5 ml Xpert sample buffer

Each mixture tested in duplicate

Inclusion of mixtures in BCCM

Store 10 x aliquots of 100ul at -20 °C

BCCM
BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS

New assays: testing the limits

- **Xpert MTB/XDR:**

  Determining the limit of
  FQ heteroresistance detection

Proportion *gyrA*_Asp94Gly *Mtb* population	Deeplex Myc-TB	BMD LVX/MXF	Xpert MTB/XDR
0%	0%	S	S
0,5%	0,4%	R	S
1%	0,9%	R	S
3%	3%	R	S
5%	4%	R	S
10%	9%	R	S
15%	13%	R	S
20%	17%	R	R
30%	24%	R	R
40%	35%	R	R
50%	47%	R	R
60%	54%	R	R
100%	100%	R	R

Deeplex Myc-TB

Targeted Next-Generation Sequencing

University of Antwerp
Faculty of Medicine and Health Sciences

tNGS & WGS

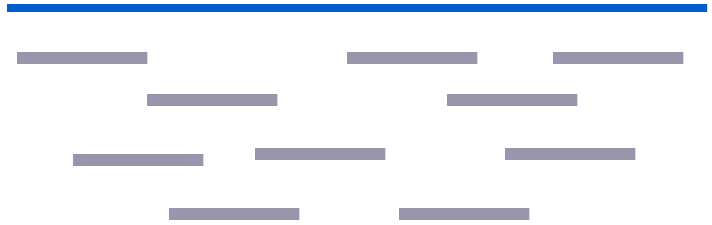
Two NGS approaches for DR-TB control

tNGS

Focus on genomic regions of interest

WGS

Comprehensive analysis of the entire genome



tNGS & WGS

Two NGS approaches for characterisation of DR-TB

tNGS

Focus on genomic regions of interest

DEEPLEX Myc-TB

WGS

Comprehensive analysis of the entire genome

Oxford NANOPORE Technologies

TB Profiler

tNGS & WGS: Head to head

Characteristic	tNGS - *Deeplex® Myc-TB*	WGS
Input sample	Sputum	Culture
Batching	Min 20	Min 4 (Illumina), Min 1 (ONT)

tNGS & WGS: Head to head

Characteristic	tNGS - *Deeplex® Myc-TB*	WGS
Input sample	Sputum	Culture
Batching	Min 20	Min 4 (Illumina), Min 1 (ONT)
bp investigated	10,000 - 13000 bp	~ 4 million bp
Drug resistance	12 – 15 drugs fixed	23 drugs flexible
Limit of variant detection	1-3%	1-3% if 1000x depth

tNGS & WGS: Head to head

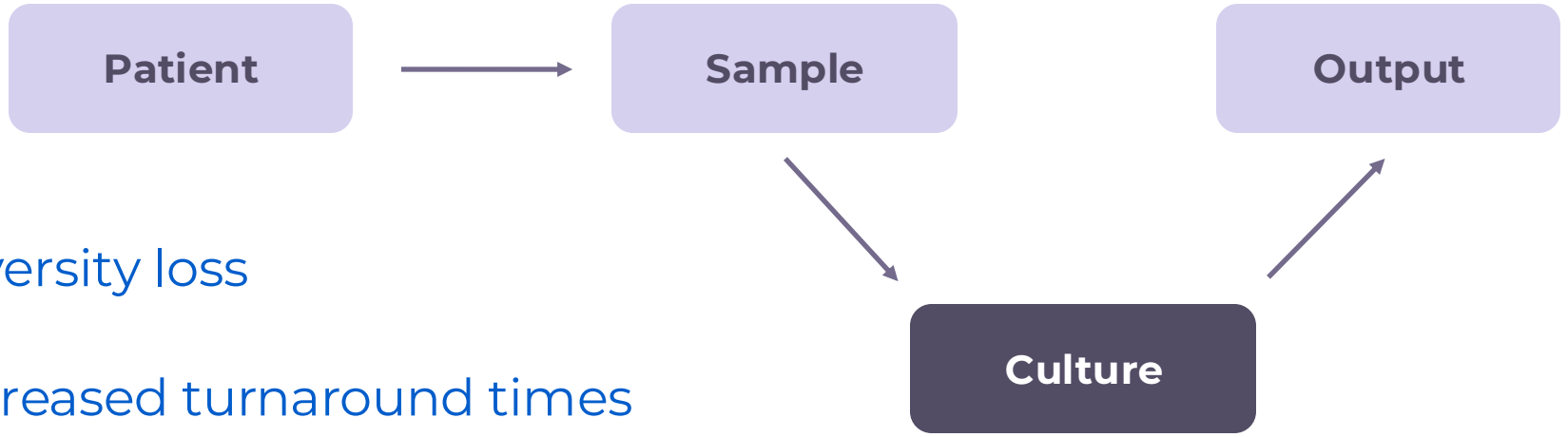
Characteristic	tNGS - *Deeplex® Myc-TB*	WGS
Input sample	Sputum	Culture
Batching	Min 20	Min 4
bp investigated	10,000 - 13000 bp	~ 4 million bp
Drug resistance	12 – 15 drugs fixed	23 drugs flexible
Limit of variant detection	1-3%	1-3% if 1000x depth
Phylogenetic Typing	Moderate resolution (SNPs/spoligotyping)	High resolution
Transmission Inference	Limited	Accurate

Implications of a culture step

tNGS - *Deeplex® Myc-TB*

Implications of a culture step

WGS



- Diversity loss
- Increased turnaround times
- Biosafety and infrastructural needs

A comprehensive approach: Drug resistance

RIF	INH	EMB	PZA	BDQ	LZD	MFX	LFX	STM	AMK	KAN	CAP	CFZ	ETO
rpoB	*inhA*	*embB*	*pncA*	*mmpR5*	*rplC*	*gyrA*	*gyrA*	*rrs*	*rrs*	*rrs*	*rrs*	*mmpR5*	*inhA*
	ahpA				*rrl*	*gyrB*	*gyrB*	*rpsL*		*eis*	*tlyA*		*ethA*
	katG							*gid*					

- 13-15 drugs
- 18 targets

DEEPLEX Myc-TB

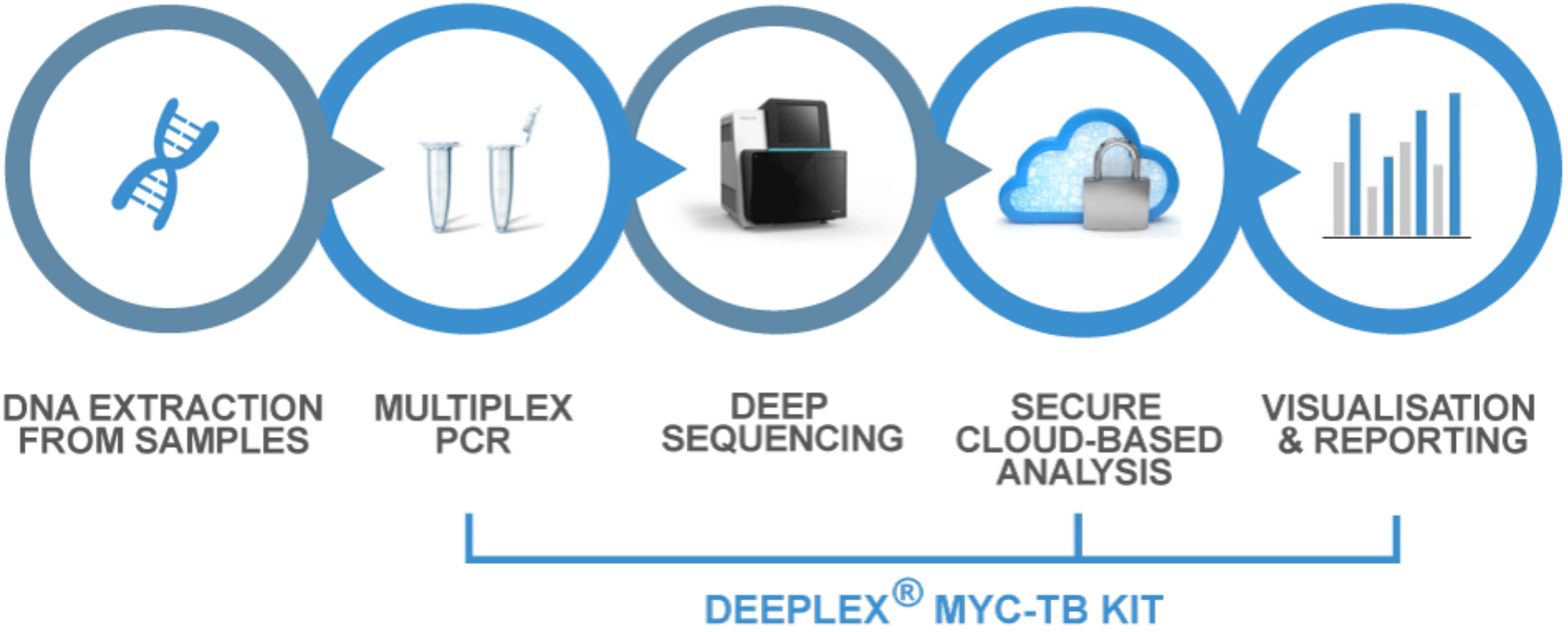
A comprehensive approach: Drug resistance

RIF	INH	EMB	PZA	BDQ	LZD	MFX	LFX	STM	AMK	KAN	CAP	CFZ	ETO
rpoB	*inhA*	*embB*	*pncA*	*mmpR5*	*rplC*	*gyrA*	*gyrA*	*rrs*	*rrs*	*rrs*	*rrs*	*mmpR5*	*inhA*
rpoA	*ahpA*	*embA*	*clpC1*	*mmpS5*	*rrl*	*gyrB*	*gyrB*	*rspL*	*whiB7*	*eis*	*tlyA*	*mmpS5*	*ethA*
rpoC	*katG*	*embC*	*panD*	*mmpL5*				*gid*	*eis*	*whiB7*	*whiB6*	*mmpL5*	*ethR*
Rv2752c	*mshA*	*embR*	*Rv1258c*	*atpE*				*whiB7*	*whiB6*		*ccsA*	*atpE*	*mshA*
	Rv1258c	*ubiA*	*PPE35*	*pepQ*				*Rv1258c*	*ccsA*		*fprA*	*pepQ*	*Rv3083*
	ndh		*Rv3236c*	*Rv1979c*				*whiB6*	*fprA*		*aftB*	*Rv1979c*	*ndh*
	Rv2752c								*aftB*				

DLM
fdg1
ddn
fbiA
fbiB
fbiC
fbiD

World Health Organization

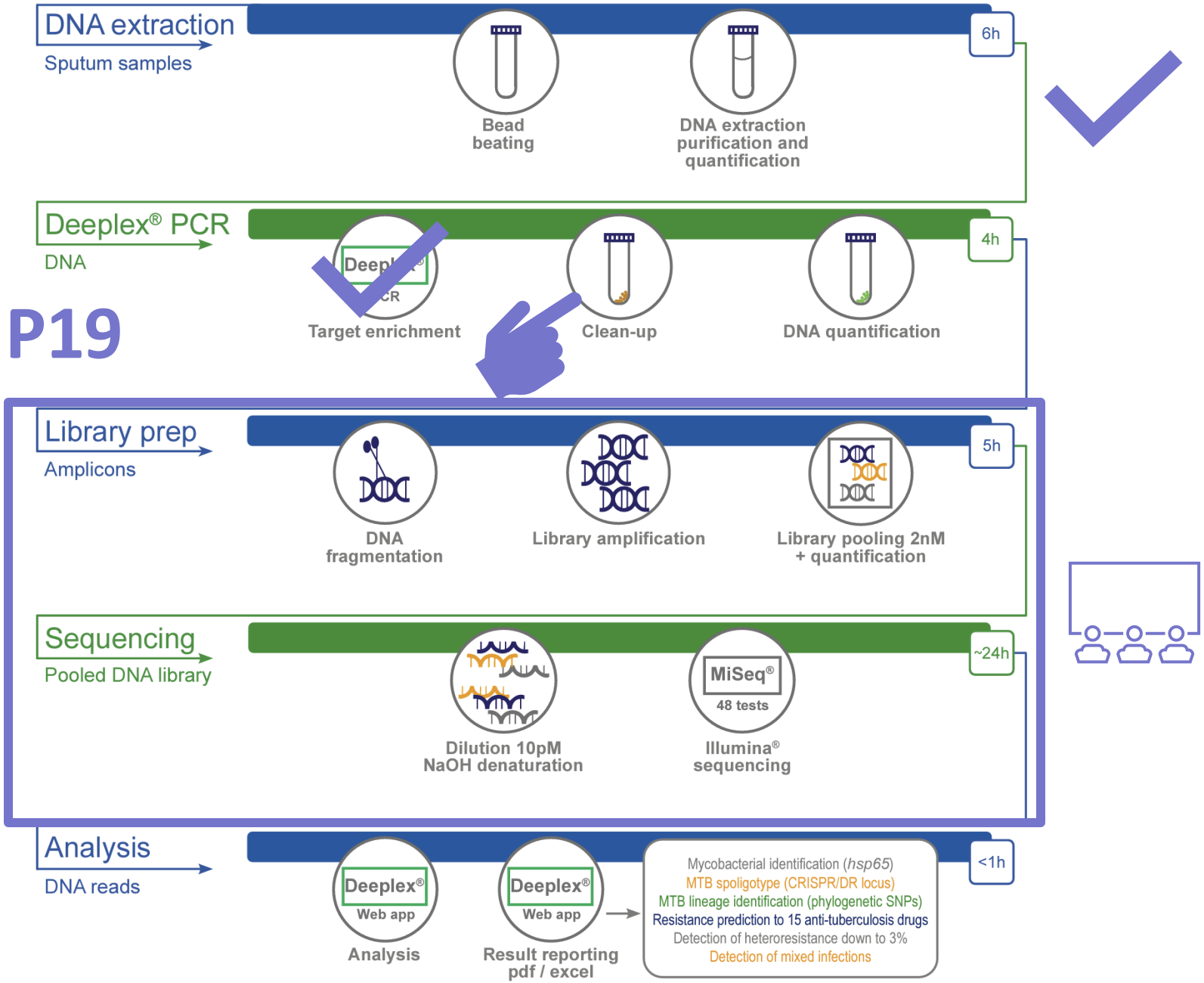
PRACTICAL – Deeplex Myc-TB



Deeplex® Myc-TB workflow

PRACTICAL

LAB MANUAL: P19



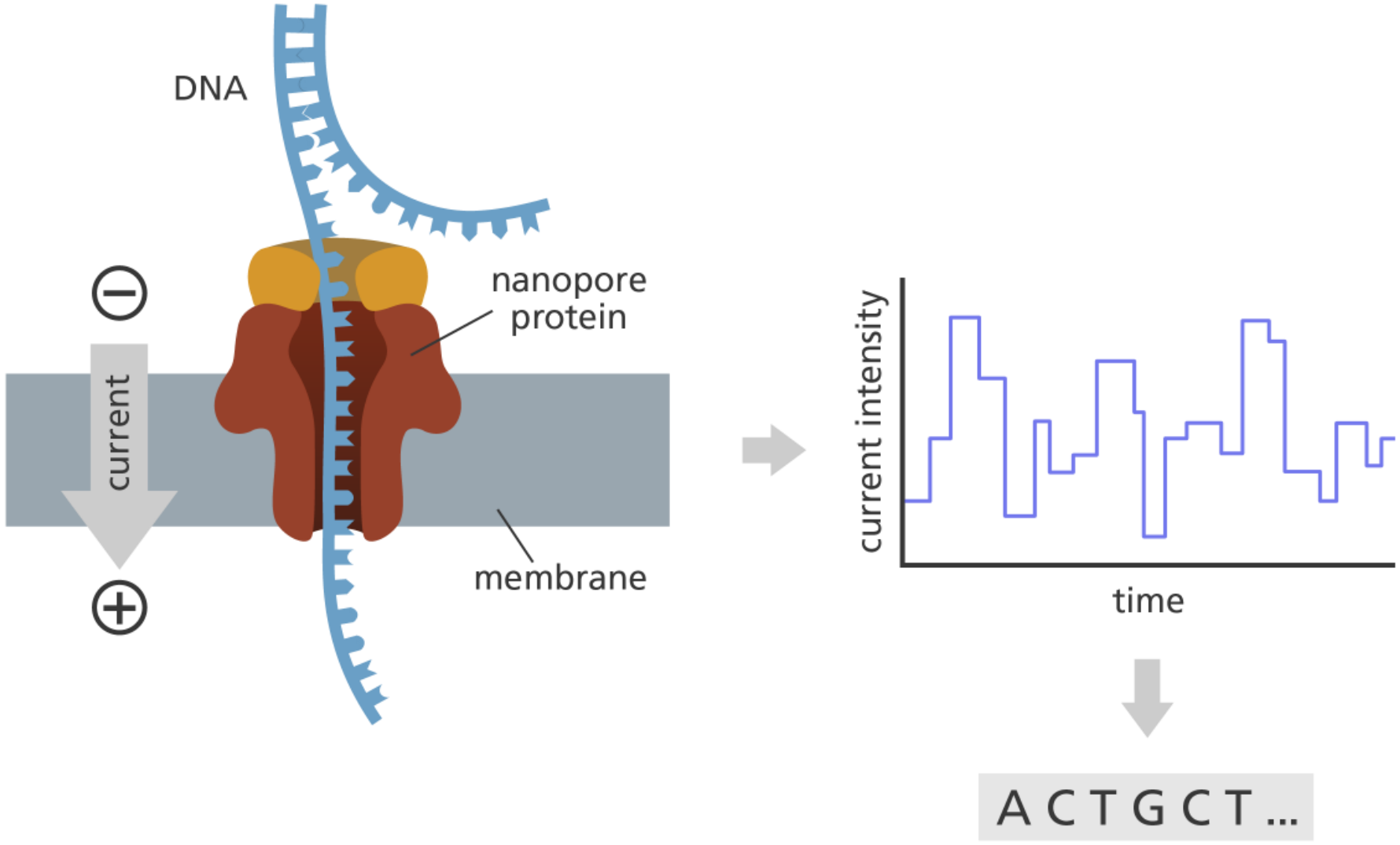
Oxford Nanopore Technologies

Whole genome sequencing

Please make an account on Galaxy before Thursday afternoon's session

https://usegalaxy.org/

How ONT sequencing works - recap



https://www.yourgenome.org/facts/what-is-oxford-nanopore-technology-ont-sequencing/

Overview of ONT sequencing approaches

Investigations

- Structural variation
- Gene expression
- Splice variation
- Fusion transcripts
- Single cell
- SNVs and phasing
- Identification
- Assembly
- Epigenetics
- Chromatin conformation

Techniques

- Whole genome
- Targeted
- Whole transcriptome
- Metagenomics
- Short fragment mode

ONT solutions for WGS

	Application-free, native DNA sequencing and retained base modification			Amplification-based for low DNA amounts or quality
	Ligation Sequencing Kit	Rapid Sequencing Kit	Ultra-Long Sequencing Kit	Rapid PCR Barcoding Kit
Preparation time	60 min	10 min	200 min + O/N elution	15 min + PCR
Input requirement	1,000 ng gDNA; 100–200 fmol amplicons or cDNA	200 ng gDNA (<30 kb)	6 M cells	1–5 ng gDNA
Fragmentation	Optional	Transposase based	Transposase based	Transposase based
Read length	Equal to fragment length	Random distribution, dependent on input fragment length	50–100+ kb N50	~2 kb
Typical throughput	●●●	●●○	●●○	●●○
Multiplexing options	24 plex, 96 plex	24 plex, 96 plex	-	12 plex
Methylation included	Yes	Yes	Yes	-
Overview	Optimised for output; retained base modifications; control over read length	Simple and rapid; retained base modifications	Optimised for production of ultra-long reads (N50 ≥50 kb); retained base modifications	Simple and rapid

Rapid barcoding kit overview

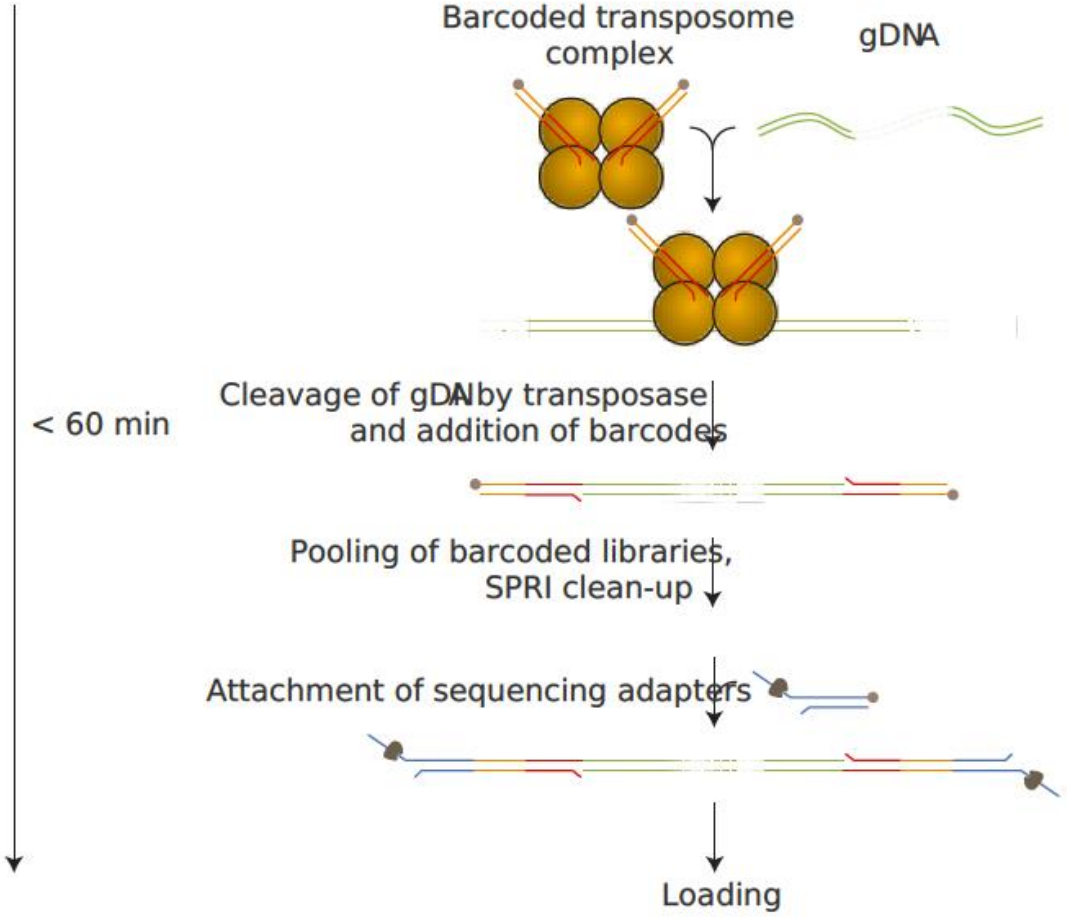
Starting material

Tagmentation

Fragmentation and adapter ligation in a single step

Sample pooling

Sequencing adapter ligation



https://store.nanoporetech.com/eu/rapid-sequencing-kit.html

Rapid barcoding kit overview

Library preparation step	Process	Time	Stop option
DNA barcoding	Tagmentation of the DNA using the Rapid Barcoding Kit V14	15 minutes	4°C overnight
Sample pooling and clean-up	Pooling of barcoded libraries and AMPure XP Bead clean-up	25 minutes	4°C overnight
Adapter ligation	Attach the sequencing adapters to the DNA ends	5 minutes	We strongly recommend sequencing your library as soon as it is adapted
Priming and loading the flow cell	Prime the flow cell and load the prepared library for sequencing	5 minutes	

PRACTICAL – stations and sequencing

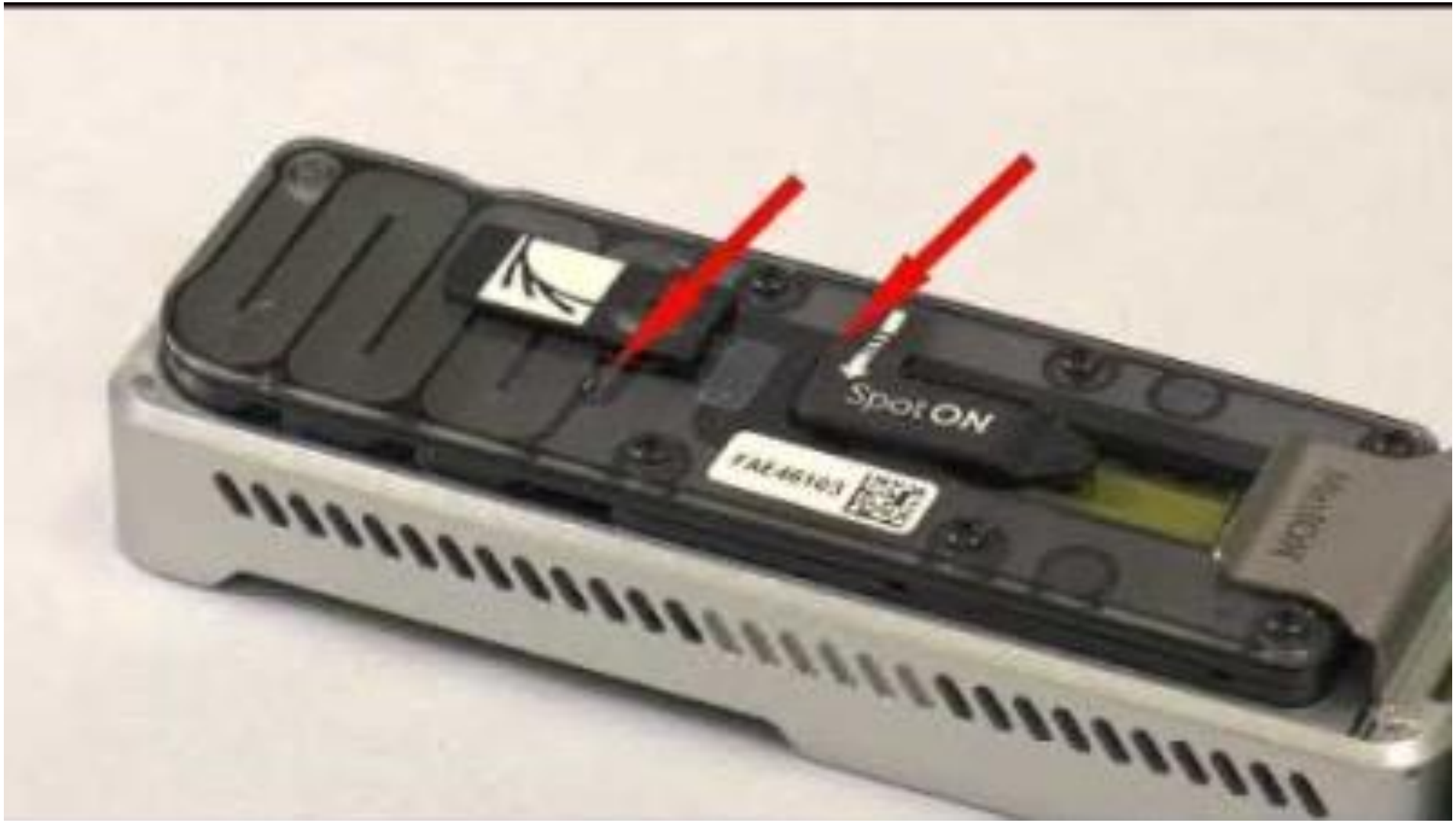
- **24 samples to be prepared** (200 ng gDNA aliquoted)
- **3 library pools: 3 x stations for library preparation**
  - Each group to pool their samples – 8 samples per pool
  - Each sample = one *Mycobacterium tuberculosis* clinical isolate

- **3 x libraries will be loaded for sequencing – training room**
  - 3 x Laptops set up with MinKNOW software and MinION sequencers
  - 3 volunteers to load flowcells

PRACTICAL – flowcell check and loading (seminar room)

- **Quality control of flow cells – flowcell health (done)**
  - Check how many active pores:
    - Up to 2048 pores
    - Ideally more than 800 active pores
- **Follow in instructions in lab manual: p25 - 26**
  - Prime flowcell
  - Prepare library for loading for loading
  - Load the flow cell and start the sequencing

PRACTICAL – priming and loading the flow cell



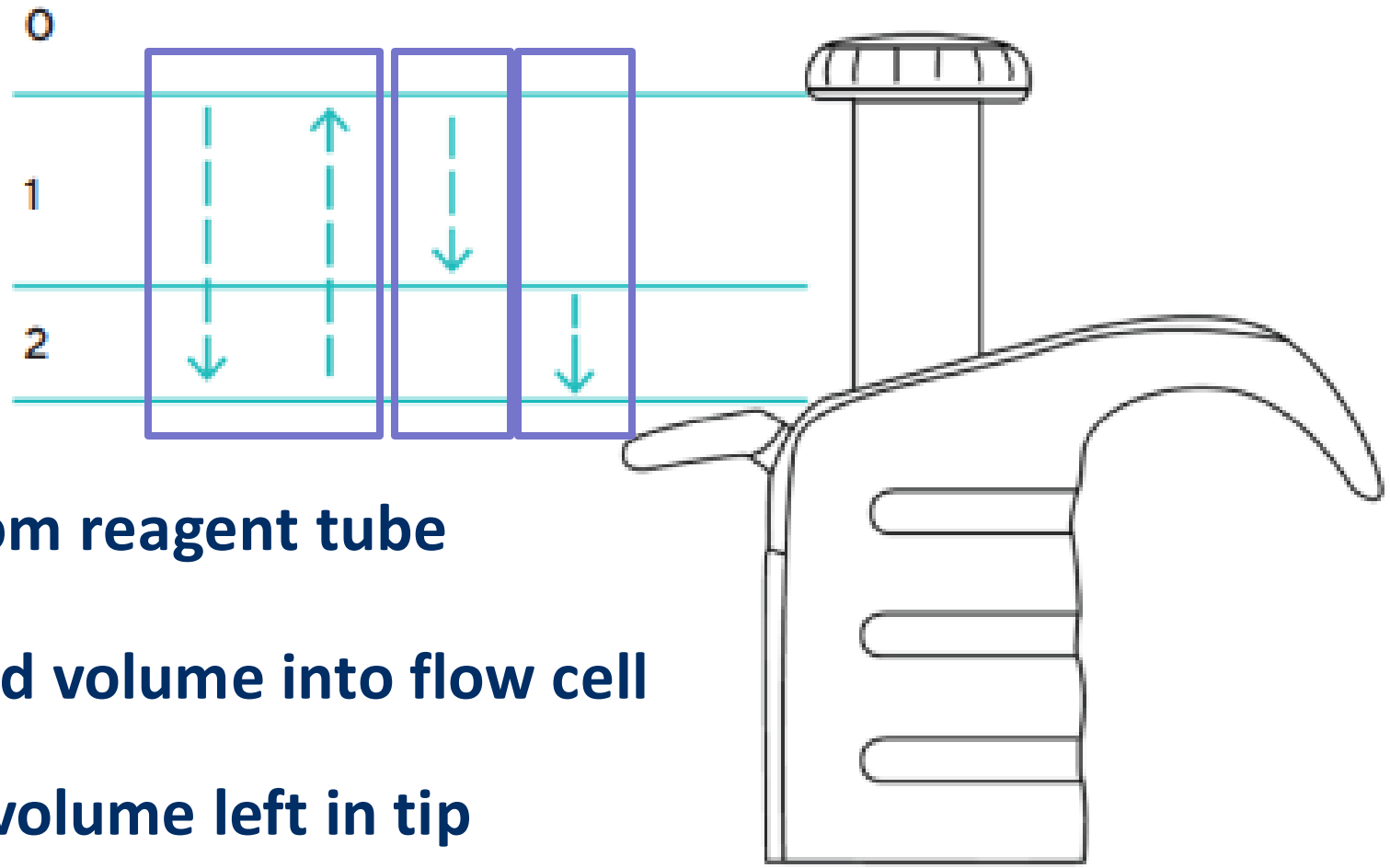
Tips and tricks for loading a flow cell

- **Removing liquid from the priming port to ensure that there are no bubbles in the sequencing channel**
  - Insert the tip of a P1000
  - Slowly turn the dial to remove ±200ul liquid

- **Loading the flowcell: work quickly and accurately**
  - Loading beads precipitate quickly
  - The order in which to open/close the ports matter

- **Reverse pipetting**
  - Use RP to add all reagents to the flow cell

Reverse pipetting



Collect reagent from reagent tube

Load only indicated volume into flow cell

Empty remaining volume left in tip in reagent tube