

A self-paced learning resource

Molecular Diagnostics in Infection and Immunology

A learning resource for trainees in infectious diseases, microbiology, virology, mycology, parasitology and immunology



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Date	23 October 2026
Contents	Part A: 18 units • 26 figures • exercises, 15 case studies self-assessment • glossary and formulas Part B: self-study reading list (free resources)

Figures are original schematics drawn for teaching. Figures, thresholds and guideline details should be checked against current sources before use.

Molecular Diagnostics in Infection and Immunology: A Learning Resource

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First edition, October 2026

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Third-party links. Part B links to external resources that remain under their owners' terms; listing them does not imply endorsement.

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How to use this resource

This is a self-paced learning resource on molecular biology and molecular diagnostics for trainees in infectious diseases, medical microbiology (bacteriology, mycology and parasitology), virology and immunology. It is not tied to a fixed placement: work through it in order, or go straight to the units you need for your current clinical work.

Each unit contains:

- **Learning outcomes** — what you should be able to do at the end
- **Core content** — study notes, tables and figures
- **In practice** — how the technique appears in clinical infection and immunology work
- **Bench and desk activities** — tasks to do in a molecular laboratory or at a computer
- **Check your understanding** — short questions; answers are in the self-assessment section

Part B at the end is a reading list of free, open-access resources mapped to each unit.

Learning pathway

The units are grouped into five stages. Foundation units are needed by everyone; intermediate and advanced units can be chosen by specialty.

Stage	Units	Level	Suggested time
A. Foundations	1 Nucleic acids and microbial genomes; 2 Laboratory organisation and biosafety; 3 Specimens and pre-analytics; 4 Extraction	Foundation	12–16 h
B. Amplification-based testing	5 PCR and RT-PCR; 6 Real-time PCR; 7 Viral load testing; 8 Syndromic panels, point-of-care and digital PCR; 9 Amplification methods, LAMP and CRISPR	Foundation to intermediate	20–25 h
C. Resistance, sequencing and specialist areas	10 Molecular detection of antimicrobial resistance; 11 Sequencing technologies; 12 Pathogen bioinformatics; 13 Clinical metagenomics and 16S/ITS sequencing; 14 Genomic epidemiology; 15 Molecular mycology and parasitology	Intermediate to advanced	30–40 h
D. Molecular immunology	16 HLA typing, chimerism, TREC/KREC, inborn errors of immunity, host response	Intermediate	10–12 h
E. Quality and reporting	17 Quality, verification and validation; 18 Reporting and interpretation	Foundation to intermediate	8–10 h
Applied practice	Exercises, case studies, self-assessment	All	10–15 h

Suggested routes by specialty

Specialty focus	Priority units
Virology	1–9, 10 (antiviral resistance), 11, 12, 14, 17, 18
Bacteriology / medical microbiology	1–6, 8, 9, 10 (bacterial and TB resistance), 11–14, 17, 18
Mycology	1–6, 8, 10 (antifungal resistance), 11–13, 15, 17, 18
Parasitology	1–6, 8, 9 (LAMP), 10 (antimalarial resistance), 11, 13, 15, 17, 18
Infectious diseases (clinical)	1, 3, 6, 7, 8, 10, 13, 15, 18, case studies
Immunology	1–6, 11, 12, 16, 17, 18

Specialty focus	Priority units
Public health / infection prevention	3, 6, 8, 11, 12, 14, 17

Self-rated competency record

Rate yourself at the start and end: **1** = aware of the concept, **2** = can explain it, **3** = can do or interpret it under supervision, **4** = can do or interpret it independently.

Competency	Unit	Start	End
Explain molecular laboratory workflow and contamination control	2		
Choose the right specimen, container and transport for a molecular test	3		
Compare extraction methods and recognise inhibition	4		
Interpret amplification curves, Ct values and controls	6		
Interpret a viral load result and a change between results	7		
Interpret a syndromic panel result in clinical context	8		
Explain LAMP and CRISPR-based detection	9		
Interpret genotypic resistance results (bacterial, TB, viral, fungal)	10		
Describe Sanger, short-read and long-read sequencing	11		
Follow a pathogen bioinformatics pipeline and read a phylogenetic tree	12, 14		
Decide when metagenomic or 16S sequencing is appropriate	13		
Interpret SNP distances in an outbreak investigation	14		
Interpret fungal and parasite PCR results (<i>Aspergillus</i> , <i>Pneumocystis</i> , malaria, stool protozoa)	15		
Read HLA nomenclature; interpret chimerism and TREC results	16		
Plan a verification of a molecular assay; review IQC and EQA	17		
Write a clear molecular microbiology report	18		

Unit 1 — Nucleic acids, microbial genomes and pathogen variation

Every molecular test detects a sequence; to choose targets and interpret results you need to know how DNA and RNA behave, how different pathogens store their genomes, and how fast those genomes change.

Learning outcomes

1. Describe DNA and RNA structure and the properties that molecular tests exploit.
2. Classify viruses by genome type (Baltimore classification) and relate this to test design.
3. Describe bacterial, fungal and parasite genomes, including plasmids and mobile elements.
4. Explain mutation, quasispecies, recombination and reassortment, and why they matter for assays.

1.1 Nucleic acid essentials

- **DNA** is double-stranded with antiparallel strands (5'→3' paired with 3'→5'). A pairs with T (two hydrogen bonds), G with C (three), so GC-rich sequences melt at higher temperatures.
- **RNA** is usually single-stranded, uses uracil instead of thymine and ribose with a 2'-OH, which makes it chemically less stable. RNases are stable and ubiquitous, so RNA targets need careful handling.
- **Denaturation and annealing.** Heat (94–98 °C) separates strands; on cooling, complementary sequences re-pair. The melting temperature (T_m) is the basis of PCR, probes and melt-curve analysis.
- **Polymerases** extend only from a free 3'-OH in the 5'→3' direction and need a primer. **Reverse transcriptases** copy RNA into complementary DNA (cDNA) — essential for RNA viruses and for measuring RNA.
- **Copy number matters.** Multi-copy targets (bacterial 16S rRNA genes, IS6110 in *M. tuberculosis*, mitochondrial genes, ribosomal RNA itself) give more sensitive assays than single-copy genes.

1.2 Viral genomes: the Baltimore classification

Viruses are grouped by how their genome is converted to mRNA. This decides whether a test needs reverse transcription, what the “viral load” measures, and how fast the virus mutates.

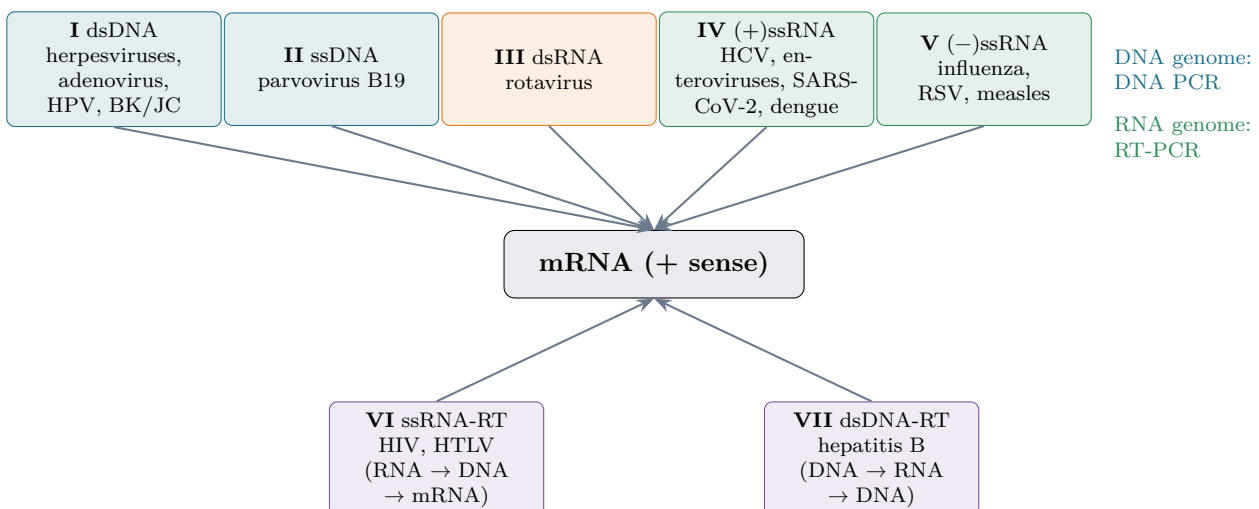


Figure 1: Baltimore classification of viruses by genome type and route to mRNA, with clinically important examples. RNA genomes need reverse transcription before PCR.

Group	Genome	Examples	Molecular test implications
I	dsDNA	Herpesviruses (HSV, VZV, CMV, EBV, HHV-6), adenovirus, polyomaviruses (BK, JC), HPV, poxviruses	DNA PCR; latent DNA can be detected without active disease — quantification and specimen choice matter
II	ssDNA	Parvovirus B19	DNA PCR; low-level DNA can persist for months after infection
III	dsRNA	Rotavirus	RT-PCR
IV	+ssRNA	Enteroviruses, HCV, flaviviruses (dengue, Zika), coronaviruses, norovirus, HAV, HEV	RT-PCR; high mutation rates
V	−ssRNA	Influenza, RSV, measles, mumps, rabies, hMPV	RT-PCR; segmented genomes (influenza) allow reassortment
VI	ssRNA with reverse transcription	HIV-1, HIV-2, HTLV	RT-PCR for plasma RNA; DNA PCR for integrated proviral DNA (infant diagnosis, reservoir)
VII	dsDNA with reverse transcription	Hepatitis B virus	DNA PCR; cccDNA persists in hepatocytes

Free reference: ViralZone (Part B) has a one-page summary and genome diagram for every virus family.

1.3 Bacterial, fungal and parasite genomes

- **Bacteria:** usually one circular chromosome (about 0.6–10 Mb; *E. coli* about 5 Mb, *M. tuberculosis* about 4.4 Mb) plus **plasmids** that often carry resistance genes. **Mobile genetic elements** — transposons, integrons, insertion sequences — move resistance genes between plasmids and chromosomes. Resistance can therefore spread between species, not only by clonal expansion.
- **Universal targets:** the 16S rRNA gene (about 1,500 bp, with nine hypervariable regions V1–V9) identifies bacteria; the internal transcribed spacer (ITS) region identifies fungi (Unit 13).
- **Fungi:** eukaryotic, about 10–40 Mb, tough cell walls (chitin, glucans) that need mechanical or enzymatic lysis before extraction.
- **Parasites:** large eukaryotic genomes (*Plasmodium falciparum* about 23 Mb); targets are often multi-copy (18S rRNA genes) for sensitivity.
- **Host DNA:** a clinical sample usually contains far more human DNA than pathogen DNA — the central problem in metagenomics (Unit 13).

1.4 How pathogens change

Process	What happens	Examples	Why it matters for diagnostics
Point mutation	Single-base errors during replication; RNA viruses (no proofreading) have rates of roughly 10^{-6} – 10^{-4} per base per replication, DNA viruses and bacteria far lower	HIV, HCV, influenza drift	Primer/probe mismatches cause false negatives or under-quantification; resistance emerges
Quasispecies	A swarm of related variants in one patient	HIV, HCV	Minor resistant variants can be selected by treatment

Process	What happens	Examples	Why it matters for diagnostics
Recombination	Exchange of genome segments between strains	HIV circulating recombinant forms, SARS-CoV-2 recombinants, enteroviruses	Typing and lineage assignment become complex
Reassortment	Exchange of whole genome segments	Influenza A (antigenic shift)	New subtypes; subtype-specific assays may fail
Horizontal gene transfer	Plasmids and mobile elements move genes between bacteria	Carbapenemase genes (KPC, NDM, OXA-48)	Resistance spreads across species; “plasmid outbreaks”
Gene deletion	Loss of a target gene	<i>pfhrp2</i> deletions in <i>P. falciparum</i> (rapid antigen test failure); SARS-CoV-2 S-gene deletion (S-gene target failure)	Single-target assays fail; dual-target designs are safer

In practice

- An HIV viral load assay that targets one region may under-quantify a patient whose virus has a mismatch under the probe. Dual-target assays reduce this risk.
- A negative HSV PCR in CSF early in encephalitis may be a timing problem, not absence of infection (Case C).
- Detecting herpesvirus DNA does not equal disease: latent virus in leukocytes can be amplified from whole blood.

Check your understanding

1. Why does an assay for HIV RNA need a reverse transcription step, but an assay for HBV DNA does not?
2. Give two mechanisms by which a pathogen’s genome change can cause a false-negative PCR.
3. Why are multi-copy targets more sensitive?

Unit 2 — Laboratory organisation, biosafety and contamination control

Amplification can turn one molecule into billions, so molecular labs are designed around preventing contamination, while infection labs must also protect staff from the pathogens in the samples.

Learning outcomes

1. Describe a unidirectional molecular workflow and why it is needed.
2. Apply risk-based biosafety to molecular work with infectious samples.
3. Write a contamination-control plan and investigate a contamination event.

2.1 Laboratory layout and workflow

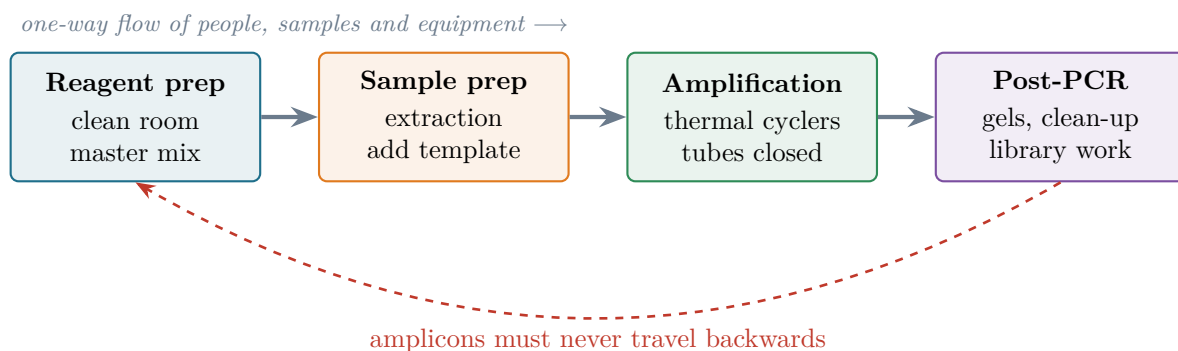


Figure 2: Unidirectional workflow in a molecular laboratory. Separate rooms (or cabinets) each have their own coats, pipettes and consumables.

Area	Activities	Rules
Reagent preparation (“clean room”)	Master mix preparation, aliquoting primers and probes	No samples or amplicons ever enter; dedicated pipettes, coats, consumables
Sample preparation	Opening primary samples, lysis, extraction, adding template	Biosafety cabinet for infectious material until lysed
Amplification	Thermal cyclers, real-time instruments	Tubes stay closed
Post-amplification	Gel electrophoresis, Sanger clean-up, NGS post-PCR steps	Nothing returns to earlier areas

Closed, cartridge-based “sample-to-answer” systems (Unit 8) combine all areas in a sealed cartridge and greatly reduce contamination risk.

2.2 Biosafety for molecular work

- **Risk-based approach.** The WHO *Laboratory Biosafety Manual* (4th edition, free — Part B) replaces fixed levels with risk assessment of the agent, procedure and setting. Many countries still classify agents into hazard groups (1–4) handled at matching containment levels.
- **Typical classification:** most respiratory and enteric viruses — hazard group 2; HIV, HBV, HCV, *M. tuberculosis*, SARS-CoV-2 (in many jurisdictions) — hazard group 3 for culture work; Ebola and other haemorrhagic fever viruses — hazard group 4. Diagnostic (non-culture) molecular work on hazard group 3 samples is often permitted at lower containment with additional precautions, according to national rules and local risk assessment.

- **Inactivation.** Guanidinium-based lysis buffers inactivate enveloped viruses and most vegetative bacteria; mycobacteria and spores are more resistant and may need heat inactivation. Validate inactivation steps before moving work out of the biosafety cabinet.
- **Chemical safety.** Never mix guanidinium-containing waste with bleach (toxic gases).

2.3 Contamination control

- **Physical separation** and unidirectional flow (above).
- **Filter tips** for all molecular pipetting.
- **Decontamination:** freshly diluted bleach (about 10% household bleach) followed by water or 70% ethanol — bleach destroys DNA; ethanol alone does not. UV exposure of cabinets between sessions.
- **dUTP/UNG system:** dUTP replaces dTTP in reactions; uracil-N-glycosylase destroys carry-over amplicons before the next PCR.
- **Controls on every run:** no-template control (NTC), extraction negative, positive control, internal control per sample.
- **Environmental monitoring:** periodic swabs of benches, pipettes and equipment tested by PCR, trended over time.
- **Contamination event:** stop reporting affected targets; define scope (runs, targets, dates); decontaminate; replace reagents; retest affected patient samples; record a non-conformity with root-cause analysis (Case J).

In practice

Contamination risk is highest for high-titre targets in the lab (positive controls, outbreak samples, amplicons from sequencing). A sudden run of low-level positives for the same target across unrelated patients is a contamination signal until proved otherwise.

Bench and desk activities

- ☐ Draw your laboratory's layout and mark the direction of workflow.
- ☐ Read the local risk assessments for HIV, HBV, *M. tuberculosis* and SARS-CoV-2 molecular work.
- ☐ Review the last 6 months of environmental monitoring results.

Unit 3 — Specimens and pre-analytical factors in infection

The best assay cannot rescue the wrong specimen: the specimen must come from where the pathogen is, at a time it is present, and reach the laboratory with its nucleic acid intact.

Learning outcomes

1. Choose the appropriate specimen for common infectious syndromes.
2. Specify containers, transport media, temperature and time limits.
3. Recognise pre-analytical causes of false-negative and false-positive results.

3.1 Specimen choice by syndrome

Syndrome	Preferred specimens for molecular testing	Notes
Upper respiratory infection	Nasopharyngeal swab (flocked) in transport medium; combined nose and throat swab	Nasopharyngeal swabs give the highest yield for most viruses
Lower respiratory infection	Sputum, endotracheal aspirate, bronchoalveolar lavage (BAL)	BAL for <i>Pneumocystis</i> , CMV pneumonitis, fungi; quantitative interpretation for <i>Pneumocystis</i>
Meningitis / encephalitis	Cerebrospinal fluid (CSF); plus blood, throat and stool for enteroviruses	Volume matters: send at least 0.5–1 mL for PCR; HSV PCR may be negative in the first 72 h
Bloodstream infection	Blood culture bottle (for panel tests on positive cultures); EDTA whole blood or plasma for direct PCR	Direct blood PCR has lower sensitivity for bacteria than culture
Viral load monitoring	EDTA plasma (HIV, HBV, HCV, BK); plasma or whole blood (CMV, EBV) — use the same specimen type over time	Whole blood and plasma results are not interchangeable
Gastroenteritis	Stool (not rectal swab for some panels)	Multiplex panels detect carriage as well as disease
Sexually transmitted infections	First-void urine (men), vulvovaginal swab (women), rectal and pharyngeal swabs	Self-collected vaginal swabs perform as well as clinician-collected
Tuberculosis	Sputum (two specimens), BAL, gastric aspirate (children), tissue, CSF, urine (HIV)	Xpert MTB/RIF Ultra on sputum; stool in children in some programmes
Ocular infection	Conjunctival swab, corneal scrape, aqueous or vitreous fluid	Very small volumes; prioritise tests
Congenital infection	Neonatal urine or saliva for CMV within 21 days of birth; dried blood spot (retrospective)	After 21 days, a positive result cannot separate congenital from postnatal infection
Tissue and fluids	Fresh tissue, pus, joint fluid, heart valve	Sterile container without formalin; formalin-fixed tissue gives fragmented DNA
Immunology tests	EDTA whole blood (HLA, chimerism, gene panels); dried blood spot (TREC/KREC newborn screening)	See Unit 16

3.2 Containers, transport and stability

Item	Use	Key points
Viral transport medium (VTM/UTM)	Swabs for viruses and many bacteria	Keep at 2–8 °C; most assays accept up to 72 h refrigerated — check the assay insert
Inactivating transport media (guanidinium-based)	Swabs from high-risk patients; postal samples	Inactivate pathogens and stabilise RNA at room temperature; not suitable for culture
Liquid Amies (e.g. eSwab)	Bacterial culture and many PCR assays	Allows culture and PCR from one swab
EDTA tubes	Plasma viral loads, whole blood PCR, human DNA	Separate plasma within the time limit stated by the assay (often 6–24 h)
Plasma preparation tubes (gel)	Some viral load assays	Follow the assay's validated centrifugation steps
Heparin tubes	Avoid	Heparin inhibits PCR
Sterile universal container	CSF, urine, tissue, fluids	No additives or formalin
Dried blood spots	Newborn screening, HIV early infant diagnosis, remote settings	Stable at room temperature; lower sensitivity

Freezing. Plasma and extracts for RNA are best stored at -70 °C or below; avoid repeated freeze–thaw cycles.

3.3 Pre-treatment by specimen type

Specimen	Pre-treatment	Purpose
Sputum, BAL	Liquefaction with N-acetyl-L-cysteine or dithiothreitol	Even lysis of mucoid samples
Mycobacteria, fungi, Gram-positive bacteria	Bead beating; enzymatic lysis (lysozyme, lysostaphin, lyticase); heat	Break tough cell walls
Stool	Dilution in stool buffer, centrifugation, bead beating	Remove inhibitors (bile salts, complex polysaccharides)
Tissue	Mechanical homogenisation + proteinase K	Release nucleic acid from tissue
Plasma for cell-free DNA / metagenomics	Two-step centrifugation; process promptly	Remove cells; reduce human background
Positive blood culture	Dilution or direct loading per assay	Charcoal and resins in some bottles inhibit PCR

3.4 Sample pooling

For screening when positivity is low (blood donor nucleic acid testing, SARS-CoV-2 surveillance, STI screening in some programmes) several samples are combined and tested as one; positive pools are then tested individually. Pooling dilutes each sample, so sensitivity falls by roughly $\log_2(\text{pool size})$ Ct values — about 3 Ct for a pool of 8.

3.5 Pre-analytical causes of error

- **False negatives:** wrong specimen or site; collection too early or too late; poor swabbing technique; delayed transport; heparin; freeze–thaw; inadequate volume.

- **False positives:** cross-contamination during collection or aliquoting; mislabelling; environmental contamination (e.g. *Legionella* in water used for samples; skin flora in 16S tests).

Check your understanding

1. Why is a urine CMV PCR from a 5-week-old baby not enough to diagnose congenital CMV?
2. What specimen should be used to follow EBV DNA load after transplantation, and why must it stay the same?

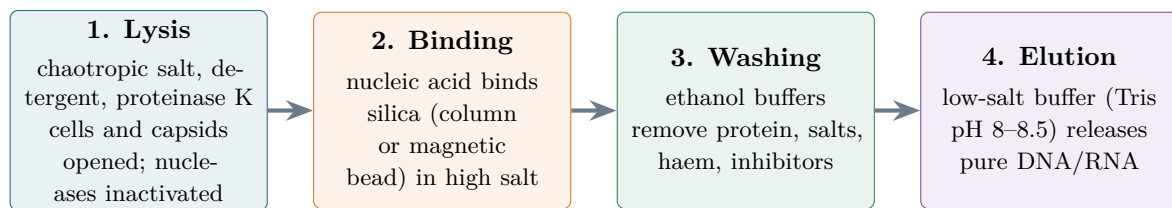
Unit 4 — Nucleic acid extraction

Extraction releases nucleic acid, removes inhibitors and concentrates the target; most invalid or falsely negative results trace back to this step.

Learning outcomes

1. Explain the four steps of silica-based extraction.
2. Compare extraction methods used in infection laboratories.
3. Explain the role of internal and extraction controls, and of carrier RNA.
4. Recognise and manage inhibition.

4.1 The four steps



internal extraction control added at lysis → proves the whole process worked

Figure 3: The four steps shared by silica-based extraction methods (spin column and magnetic bead).

1. **Lysis** — chaotropic salts (guanidinium), detergents and proteinase K open cells, capsids and envelopes and inactivate nucleases.
2. **Binding** — nucleic acid binds silica (membrane or magnetic beads) in high-salt conditions.
3. **Washing** — ethanol buffers remove proteins, salts, haem and other inhibitors.
4. **Elution** — low-salt buffer releases the nucleic acid; smaller volumes give higher concentrations.

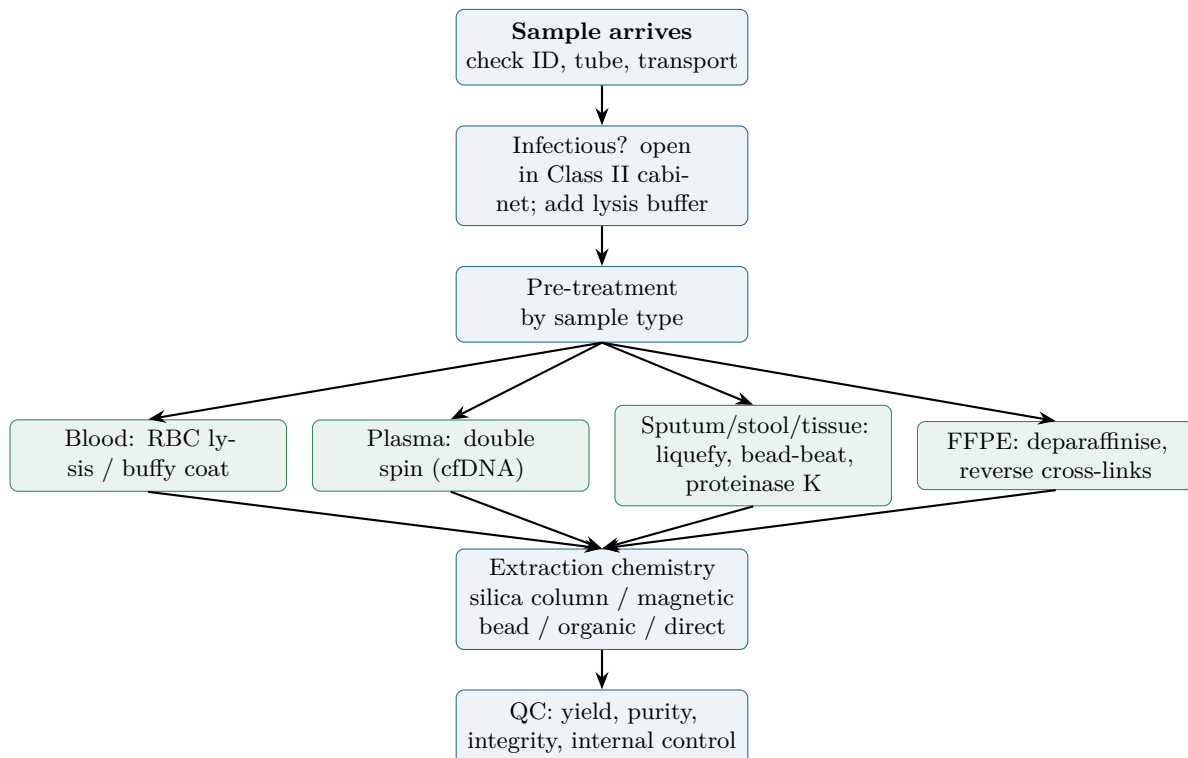


Figure 4: Sample processing pathway from receipt to a quality-checked extract.

4.2 Methods compared

Method	Principle	Strengths	Limitations	Typical use
Silica spin column	Chaotrope binding to silica membrane	Cheap, flexible, pure eluates	Manual, slow, handling errors	Small batches, unusual samples
Magnetic bead (automated)	Silica-coated beads moved by magnets	High throughput, walk-away, fewer errors	Instrument cost	Routine viral loads, respiratory testing
Sample-to-answer cartridges	Extraction, amplification and detection in one sealed cartridge	Minimal hands-on time; low contamination; random access	Cost per test; limited menu	GeneXpert, BioFire, ID NOW, Cobas Liat
Organic (phenol–chloroform)	Phase separation	High yield, long DNA	Toxic, slow	Long-read sequencing, research
Heat / direct lysis	Boiling or buffer lysis, no purification	Fast, cheap	Inhibitors, lower sensitivity	Colony PCR, some point-of-care and LAMP tests
Chelex-100	Chelating resin + boiling	Very fast	Crude, unstable DNA	Quick bacterial identification
Host depletion methods	Selective lysis of human cells + DNase, or methylated-DNA depletion, before extraction	Increases pathogen reads	Can lose pathogens without cell walls; adds steps	Metagenomic sequencing (Unit 13)

Total nucleic acid extraction (DNA + RNA) is standard for viral and syndromic testing so that one extract serves DNA and RNA targets.

Carrier RNA (poly-A) improves recovery of tiny amounts of viral nucleic acid. It interferes with spectrophotometry and dominates sequencing libraries, so it is omitted for metagenomic and some NGS work.

4.3 Internal and extraction controls

- **Internal control:** a non-target organism or synthetic sequence (e.g. MS2 phage, phocine herpesvirus, equine arteritis virus) added to every sample at lysis, or a human gene (e.g. RNase P) that also checks specimen adequacy. A failed internal control means the result is **invalid**, never “not detected”.
- **Extraction negative:** a blank processed alongside samples, to detect contamination introduced during extraction.
- **Positive extraction control:** a weak positive sample processed through the whole method.

4.4 Quality and inhibition

Measure	Method	Notes
Concentration	UV spectrophotometry (A260); fluorometry (Qubit)	Total nucleic acid in a viral extract is mostly human or carrier, not viral
Purity	A260/280 (~1.8 DNA, ~2.0 RNA); A260/230 (2.0–2.2)	Low 260/230 = guanidinium carry-over
Integrity	Gel, TapeStation (DIN/RIN)	Important before sequencing

Measure	Method	Notes
Inhibition	Internal control Ct shift; spiked dilution	Common inhibitors: heparin, haem, bile salts, polysaccharides, ethanol, EDTA, humic acids, mucus

Managing inhibition: dilute the extract 1:5–1:10 and retest (a 10-fold dilution costs about 3.3 Ct); re-extract with an extra wash; request a different specimen.

Check your understanding

1. A respiratory panel internal control fails on a BAL sample. What do you report and what do you do next?
2. Why is carrier RNA avoided before metagenomic sequencing?

Unit 5 — PCR and RT-PCR principles

PCR doubles a defined DNA segment each cycle; reverse transcription first copies RNA into cDNA so that RNA pathogens can be amplified the same way.

Learning outcomes

1. Describe the PCR cycle and the role of each reaction component.
2. Explain one-step and two-step RT-PCR.
3. Apply primer and probe design rules, including for variable pathogens.
4. Describe PCR variants used in microbiology.

5.1 The PCR cycle

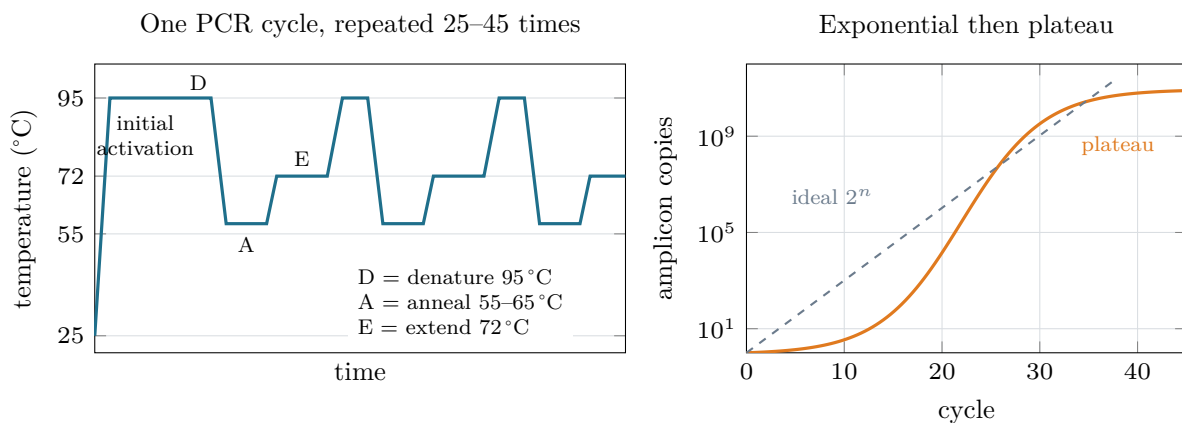


Figure 5: Left: the temperature profile of PCR. Right: product accumulates exponentially, then plateaus as reagents are exhausted, which is why end-point product is not quantitative.

1. **Initial activation** — 95 °C for 2–10 min (hot-start polymerase).
2. **Denaturation** — 94–98 °C, 10–30 s.
3. **Annealing** — 50–65 °C, 15–30 s; primers bind.
4. **Extension** — 72 °C (often combined with annealing at about 60 °C in real-time assays).
5. Repeat 35–45 cycles in diagnostic assays.

In the exponential phase product roughly doubles each cycle (90–105% efficiency); then the reaction plateaus. End-point product is therefore not quantitative — the reason for real-time PCR (Unit 6).

5.2 Reaction components

Component	Role and notes
Template	Extracted nucleic acid; too much human DNA can inhibit or cause non-specific products
Primers (0.1–1 μ M each)	Define the target; must match all relevant strains
Probe (real-time)	Adds specificity and allows multiplexing by colour
dNTPs	Building blocks; dUTP replaces dTTP in UNG systems
MgCl ₂ (1.5–5 mM)	Polymerase cofactor; EDTA in specimens chelates it
Thermostable polymerase	Taq (no proofreading) or high-fidelity enzymes for sequencing
Hot-start modification	Prevents primer-dimers forming before cycling
Reverse transcriptase	For RNA targets; often in the same tube (one-step)

5.3 Reverse transcription

- **One-step RT-PCR** — RT and PCR in one tube with gene-specific primers; fewer handling steps; standard for HIV, HCV, influenza, SARS-CoV-2 and enterovirus assays.
- **Two-step RT-PCR** — cDNA made first with random or oligo(dT) primers; used in research, sequencing and some panels.
- **DNA versus RNA targets of the same virus:** HIV proviral DNA (integrated in cells) versus plasma HIV RNA (replicating virus); HBV DNA versus pregenomic RNA. The choice depends on the clinical question.

5.4 Primer and probe design for pathogens

Parameter	Target
Length; GC; T _m	Primers 18–25 nt, 40–60% GC, T _m 55–65 °C, pair within 2–3 °C
Amplicon size	70–200 bp for real-time PCR
Conservation	Align all available sequences (NCBI Virus, GISAID, PubMLST) and choose conserved regions; check every genotype or lineage the assay claims
Specificity	BLAST against related organisms, human genome and normal flora
Redundancy	Two targets (dual-target design) protect against target failure from mutations or deletions
Ongoing surveillance	Re-check primer and probe sites against new sequences (in silico inclusivity) as pathogens evolve

5.5 PCR variants used in microbiology

Variant	Principle	Example
Multiplex PCR	Several targets in one tube	Respiratory, gastrointestinal, STI panels
Nested PCR	Second-round PCR with internal primers	Historical TB and HCV assays; high contamination risk
Broad-range PCR	Primers in conserved regions of a gene family	16S rRNA (bacteria), ITS (fungi), pan-herpesvirus, pan-enterovirus
Genotype-specific PCR	Primers or probes for one genotype or subtype	HSV-1 vs HSV-2; influenza A subtypes H1/H3/H5
Allele-specific PCR	3' end matches one variant	Resistance mutations (e.g. <i>rpoB</i> hot spots), HLA-B*57:01 screening
PCR + melt curve	Product T _m distinguishes targets or mutations	Xpert MTB/RIF Ultra resistance probes
RT-PCR for mRNA	Detects transcripts of viable organisms	Late mRNA in some HPV assays

Check your understanding

1. Why is a dual-target design recommended for rapidly evolving viruses?
2. What is the difference between detecting HIV RNA and HIV proviral DNA, and when is each used?

Unit 6 — Real-time PCR: detection, quantification and interpretation

Real-time PCR measures fluorescence every cycle; the cycle at which signal crosses a threshold (Ct) falls as the starting amount rises, which makes PCR quantitative and lets results be read without opening tubes.

Learning outcomes

1. Compare dye and probe chemistries.
2. Interpret amplification plots, Ct values and controls.
3. Build and assess a standard curve; explain absolute quantification in IU/mL.
4. Explain why Ct values are not directly comparable between assays.
5. Troubleshoot common failures.

6.1 Detection chemistries

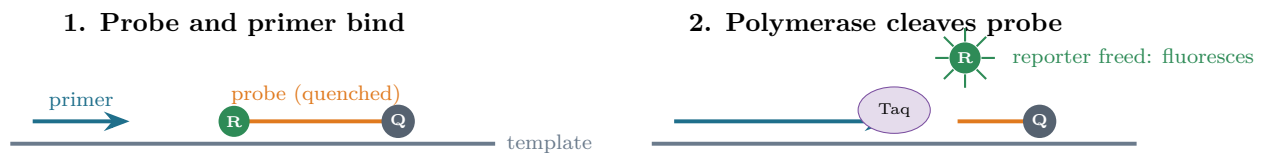


Figure 6: Hydrolysis (TaqMan) probe chemistry. The 5' → 3' exonuclease activity of Taq separates reporter (R) from quencher (Q), so fluorescence rises with each cycle.

Chemistry	How it works	Strengths	Limitations
Intercalating dye (SYBR Green, EvaGreen)	Fluoresces with any dsDNA	Cheap; melt curve	Detects non-specific products; no multiplexing
Hydrolysis probe (TaqMan)	Polymerase exonuclease cleaves probe, separating reporter and quencher	Specific; multiplex by colour	Probe mismatches reduce signal
Molecular beacon / FRET probes	Fluorescence on hybridisation	Mutation detection; melt analysis	Design complexity
MGB probes	Minor groove binder allows short probes	Good mismatch discrimination	Cost

6.2 Reading an amplification plot

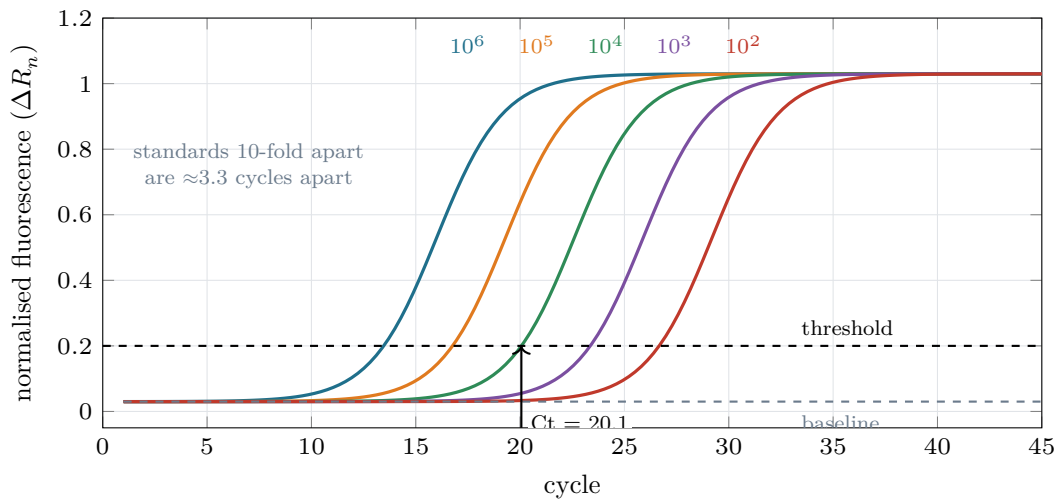


Figure 7: Real-time PCR amplification plot for a 10-fold dilution series (copies per reaction shown). The cycle at which each curve crosses the threshold is its Ct.

- **Baseline** — background fluorescence (cycles 3–15), subtracted by software.
- **Threshold** — set in the exponential phase above background.
- **Ct (Cq)** — cycle at which a curve crosses the threshold. 1 Ct $\approx$ 2-fold; 3.3 Ct $\approx$ 10-fold.
- **Shape check** — true positives are sigmoid; linear drift or a jagged curve is suspicious.
- **Late Ct** (typically > 35–38) — near the limit of detection; Poisson sampling makes results irreproducible. Many labs repeat or report as “detected, low level”.

6.3 Standard curves and quantification

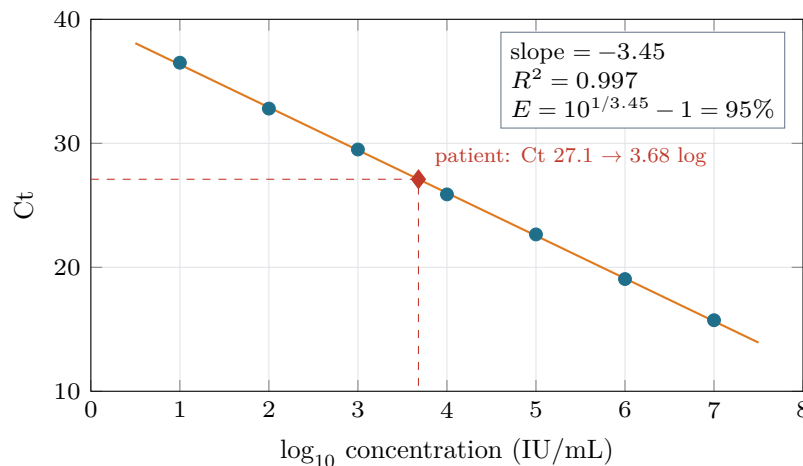


Figure 8: Standard curve (data from Exercise 1). Ct falls linearly with the logarithm of concentration; the slope gives the amplification efficiency.

$$E = (10^{-1/\text{slope}} - 1) \times 100\%$$

Acceptable efficiency is 90–110% (slope -3.58 to -3.10) with $R^2 > 0.98$.

- **Absolute quantification** reads unknowns off a calibration traceable to a **WHO International Standard**, reported in IU/mL and $\log_{10}$ IU/mL (Unit 7).
- **Copies vs IU:** conversion factors are assay-specific (for HBV, 1 IU is roughly 5 copies, but this varies) — never convert between assays with a single factor.

6.4 What a Ct value can and cannot tell you

- Ct depends on the specimen, extraction efficiency, elution volume, input volume, target gene, chemistry, instrument and threshold setting. **The same sample can differ by several Ct between assays.**
- Within one validated assay and specimen type, lower Ct means more target. In SARS-CoV-2 and influenza, very high Ct values often correlate with lower culture positivity, but no single Ct cut-off reliably defines infectiousness.
- Ct values should only be reported or used clinically when the laboratory has defined how (e.g. semi-quantitative categories) and clinicians understand the limits.

6.5 Melt curves and genotyping

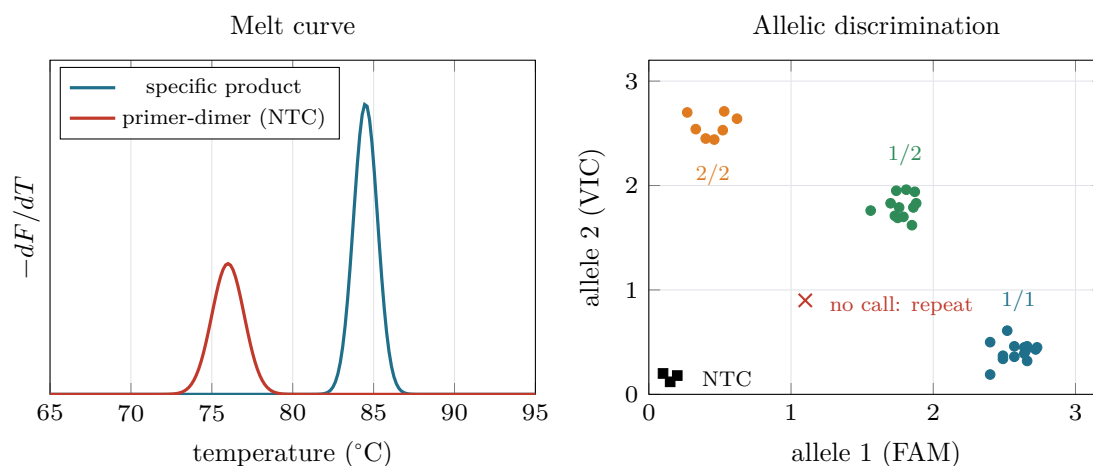


Figure 9: Left: melt-curve analysis shows one specific product and a low- T_m primer-dimer. Right: TaqMan SNP genotyping plot with homozygous, heterozygous and no-template clusters.

- **Melt-curve analysis:** a specific product gives one peak; primer-dimers give a lower- T_m peak. Mutations in a probe-binding region shift the T_m — the basis of some resistance assays (Unit 10).
- **Genotyping with two probes:** used in microbiology for HSV-1/HSV-2 typing, influenza subtyping, and in immunology for single HLA alleles and host genotypes.

6.6 Troubleshooting

Observation	Likely causes	Action
Signal in NTC	Reagent or environmental contamination	Invalidate target; decontaminate; repeat; investigate
Internal control delayed/absent	Inhibition, extraction failure	Dilute and retest; re-extract; report “invalid”
Many weak positives for one target	Contamination (positive control, high-titre sample, amplicon)	Stop, review run layout, retest, environmental swabs
All controls late	Reagent degradation, wrong cycling	Check lot, storage, IQC trend
Target detected, internal control negative	Very high target load competes with control	Result valid for target if assay rules allow
Unexpected drop in quantification for one patient	Primer/probe mismatch in a variant	Test on second assay; sequence target region

Bench and desk activities

- ☐ Analyse one run: check baseline and threshold, controls, standard-curve slope and efficiency.
- ☐ Find three examples of late-Ct positives and decide how each should be reported under local rules.
- ☐ Review your laboratory's policy on reporting Ct values.

Unit 7 — Viral load testing in clinical practice

Quantitative nucleic acid tests guide diagnosis, treatment decisions and monitoring for blood-borne viruses and for opportunistic viruses in immunocompromised patients; interpretation depends on the specimen, the assay and the size of change.

Learning outcomes

1. Explain WHO International Standards, IU/mL, and commutability.
2. Interpret a change between two viral load results.
3. Apply viral load results in HIV, HBV, HCV, CMV, EBV, BK virus and other infections.
4. Recognise special situations such as inherited chromosomally integrated HHV-6.

7.1 Standardisation

- **WHO International Standards** (prepared by NIBSC and others) exist for HIV-1, HBV, HCV, CMV, EBV, BK virus, HHV-6, adenovirus, JC virus, parvovirus B19 and others. Assays calibrated to them report in IU/mL.
- **Commutability:** a standard behaves like a patient sample in every assay only if it is commutable. The first WHO CMV standard showed poor commutability with some assays, so CMV loads still differ between laboratories even in IU/mL (Hayden et al. 2015 — Part B).
- **Practical rule:** monitor each patient with the same assay and specimen type; when changing assays, run parallel testing and re-baseline.

7.2 Interpreting change

Assay imprecision is roughly $0.15\text{--}0.25 \log_{10}$ at mid-range and higher near the limit of quantification. A change of **more than $0.5 \log_{10}$ (about 3-fold)** is generally considered real for HIV, HBV, HCV and CMV; smaller changes may be noise.

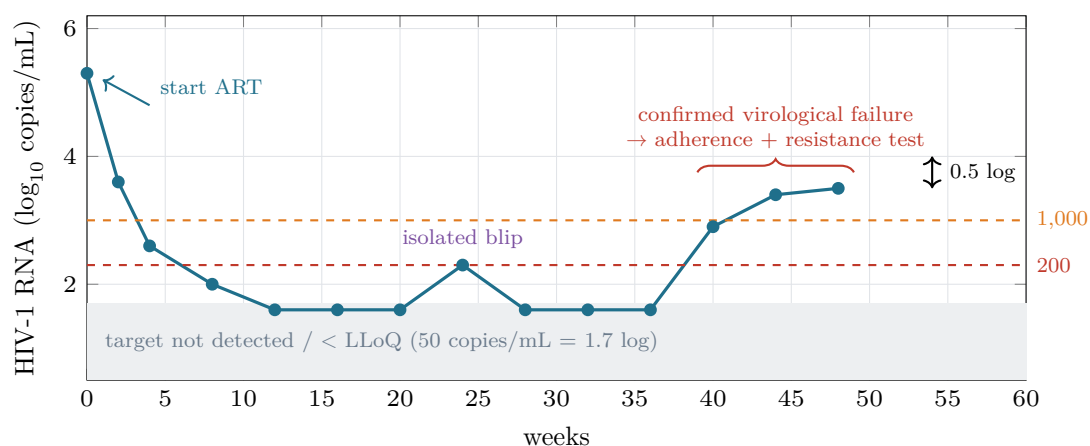


Figure 10: Illustrative HIV-1 viral load course on treatment: suppression below the limit of quantification, an isolated blip, then confirmed virological failure. Thresholds differ between guidelines (e.g. 200 or 1,000 copies/mL).

Reporting terms: “Target not detected”; “Detected, below the lower limit of quantification (< LLoQ)”; a numeric value within the linear range; “> ULoQ” (dilute and retest).

7.3 Clinical applications

Virus	Specimen	Main uses	Key interpretation points
HIV-1	EDTA plasma	Treatment monitoring; early infant diagnosis (nucleic acid test, as maternal antibody persists)	Goal on treatment is suppression (< 50 copies/mL on most assays). Isolated low-level “blips” usually return to undetectable. Persistent viraemia above treatment-failure thresholds (e.g. > 200 copies/mL in some guidelines; ≥ 1,000 copies/mL in WHO guidance) prompts adherence review and resistance testing
HIV-2	EDTA plasma	Monitoring	Needs an HIV-2-specific assay; HIV-1 assays do not quantify HIV-2
Hepatitis B	Serum/plasma	Treatment decisions (with ALT, HBeAg, fibrosis); monitoring; pregnancy	HBV DNA thresholds (e.g. > 2,000 IU/mL) are combined with liver tests; high HBV DNA in pregnancy (e.g. > 200,000 IU/mL in WHO guidance) is an indication for antiviral prophylaxis to prevent transmission
Hepatitis C	Serum/plasma	Confirms active infection after a positive antibody test; cure assessment	Sustained virological response (SVR12) = HCV RNA not detected 12 weeks after treatment ends; HCV core antigen is an alternative where RNA testing is limited
Hepatitis D and E	Plasma	HDV RNA in HBV co-infection; HEV RNA in chronic hepatitis E (immunosuppressed)	Use assays covering all genotypes
CMV	Plasma or whole blood	Pre-emptive therapy in transplant recipients; monitoring response; congenital infection (urine/saliva)	Thresholds are institution- and assay-specific; rising trend matters more than a single value; persistent rise on treatment raises suspicion of resistance (Unit 10)
EBV	Whole blood or plasma	Post-transplant lymphoproliferative disorder (PTLD) monitoring; nasopharyngeal carcinoma	Plasma EBV DNA reflects cell-free (often tumour-derived or lytic) virus; whole blood includes latently infected cells
BK polyomavirus	Plasma (also urine)	Kidney transplant monitoring	Sustained plasma BK DNA above about 10 ⁴ copies/mL suggests presumptive BK nephropathy (international transplant guidance); prompts reduction of immunosuppression
Adenovirus	Plasma/whole blood	Paediatric stem cell transplant	Rising loads predict disseminated disease
JC polyomavirus	CSF	Progressive multifocal leukoencephalopathy	Very low levels may be significant; sensitive assay needed
HHV-6	Whole blood, CSF	Encephalitis after stem cell transplant	Beware inherited chromosomally integrated HHV-6 (below)

Inherited chromosomally integrated HHV-6 (ciHHV-6). About 1% of people carry HHV-6 integrated into a chromosome in every cell, so whole-blood HHV-6 DNA is very high (around one viral copy per human cell) without active infection. Suspect it when HHV-6 DNA is persistently very high in whole blood; confirm with hair follicle or nail testing, or with ddPCR ratio of HHV-6 to a human gene (Case L).

Check your understanding

1. A kidney transplant recipient's CMV load goes from 1,200 to 2,000 IU/mL. Is this a significant change?
2. Why can a CMV load from laboratory A not be compared directly with one from laboratory B, even when both report IU/mL?

Unit 8 — Syndromic panels, point-of-care molecular testing and digital PCR

Automated multiplex panels test for many pathogens from one specimen in about an hour; their value depends on choosing the right patients and interpreting detections in clinical context.

Learning outcomes

1. Describe how syndromic multiplex panels and sample-to-answer systems work.
2. Interpret panel results, including co-detections and colonisation.
3. Describe point-of-care molecular testing and its governance.
4. Explain digital PCR and its uses in infection.

8.1 Syndromic panels

Panel type	Typical targets	Interpretation issues
Respiratory	Influenza A/B, RSV, SARS-CoV-2, hMPV, parainfluenza, adenovirus, rhinovirus/enterovirus, seasonal coronaviruses, <i>M. pneumoniae</i> , <i>B. pertussis</i>	Rhinovirus and adenovirus can be shed for weeks; co-detections common in children
Pneumonia (lower respiratory)	Bacteria with semi-quantitative bins, resistance genes, viruses	Colonisers vs pathogens; resistance gene not linked to a specific organism
Gastrointestinal	Bacteria (<i>Campylobacter</i> , <i>Salmonella</i> , STEC, <i>C. difficile</i> toxin genes), viruses, parasites	Asymptomatic carriage; <i>C. difficile</i> PCR detects colonisation — combine with toxin test
Meningitis / encephalitis	<i>S. pneumoniae</i> , <i>N. meningitidis</i> , <i>H. influenzae</i> , <i>L. monocytogenes</i> , <i>E. coli</i> K1, <i>S. agalactiae</i> , HSV-1/2, VZV, CMV, HHV-6, enterovirus, parechovirus, <i>Cryptococcus</i>	False positives and false negatives have been reported; confirm unexpected results and repeat HSV testing with a dedicated assay when suspicion is high
Blood culture identification	Organism identification and resistance genes from positive blood culture bottles	Speeds targeted therapy; does not replace culture and susceptibility testing
Sexually transmitted infections	<i>C. trachomatis</i> , <i>N. gonorrhoeae</i> , <i>T. vaginalis</i> , <i>M. genitalium</i> ($\pm$ resistance markers)	Test-of-cure timing (RNA/DNA persists after treatment)

Diagnostic stewardship. Panels cost more than single tests, and broad testing increases detections of uncertain significance. Many laboratories restrict panels to defined patient groups (e.g. immunocompromised, critical care) and require clinical criteria for meningitis/encephalitis panels.

8.2 Point-of-care and near-patient molecular testing

- **Platforms:** cartridge-based PCR (e.g. GeneXpert, Cobas Liat), isothermal tests (e.g. ID NOW, which uses NEAR; LAMP-based tests), and emerging CRISPR tests (Unit 9).
- **Advantages:** results within 15–60 minutes for infection control decisions (bed placement), antiviral treatment, and TB/HIV programmes in remote settings.
- **Governance:** the laboratory remains responsible for training, competency, IQC, EQA, connectivity to the patient record and result review — as for point-of-care chemistry.

8.3 Digital PCR

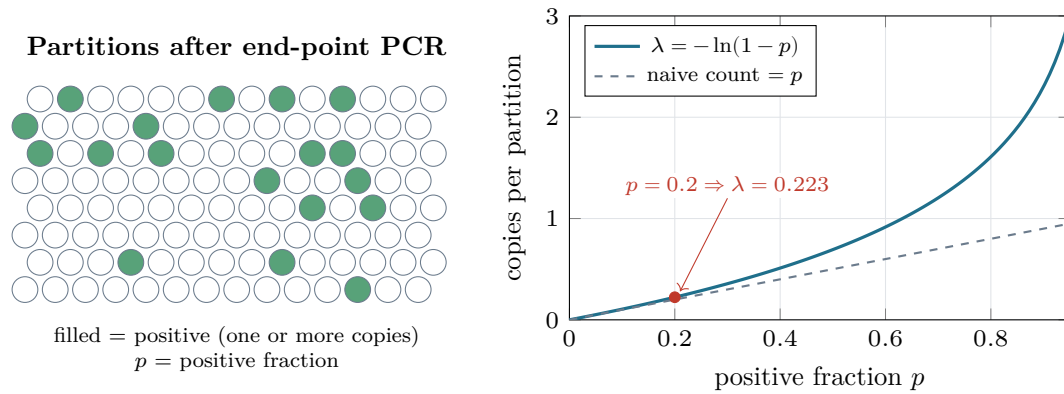


Figure 11: Digital PCR. The sample is split into thousands of partitions; Poisson correction converts the positive fraction into absolute copies.

Digital PCR splits a reaction into thousands of partitions, counts positive partitions after end-point PCR, and uses Poisson statistics for absolute quantification without a standard curve.

$$\lambda = -\ln(1 - p)$$

Uses in infection and immunology: HIV reservoir quantification (proviral DNA), precise CMV/EBV quantification for reference materials, detection of low-level resistance mutations, ciHHV-6 confirmation (viral to human gene ratio), chimerism monitoring (Unit 16), and wastewater surveillance.

Check your understanding

1. A respiratory panel in a 2-year-old detects rhinovirus/enterovirus and adenovirus. How would you interpret this?
2. Why is *C. difficile* PCR alone not sufficient to diagnose *C. difficile* infection?

Unit 9 — Amplification and detection methods beyond PCR, LAMP and CRISPR

Every nucleic acid test copies the **target**, copies a **probe** that has bound the target, or boosts the **signal** from a bound probe; a readout technology then makes the result visible. Many commercial infection assays use non-PCR methods.

Learning outcomes

1. Classify assays as target, probe or signal amplification.
2. Describe the main isothermal methods and the platforms that use them.
3. Explain LAMP and CRISPR-based detection in detail.
4. Match readout technologies to platforms and settings.

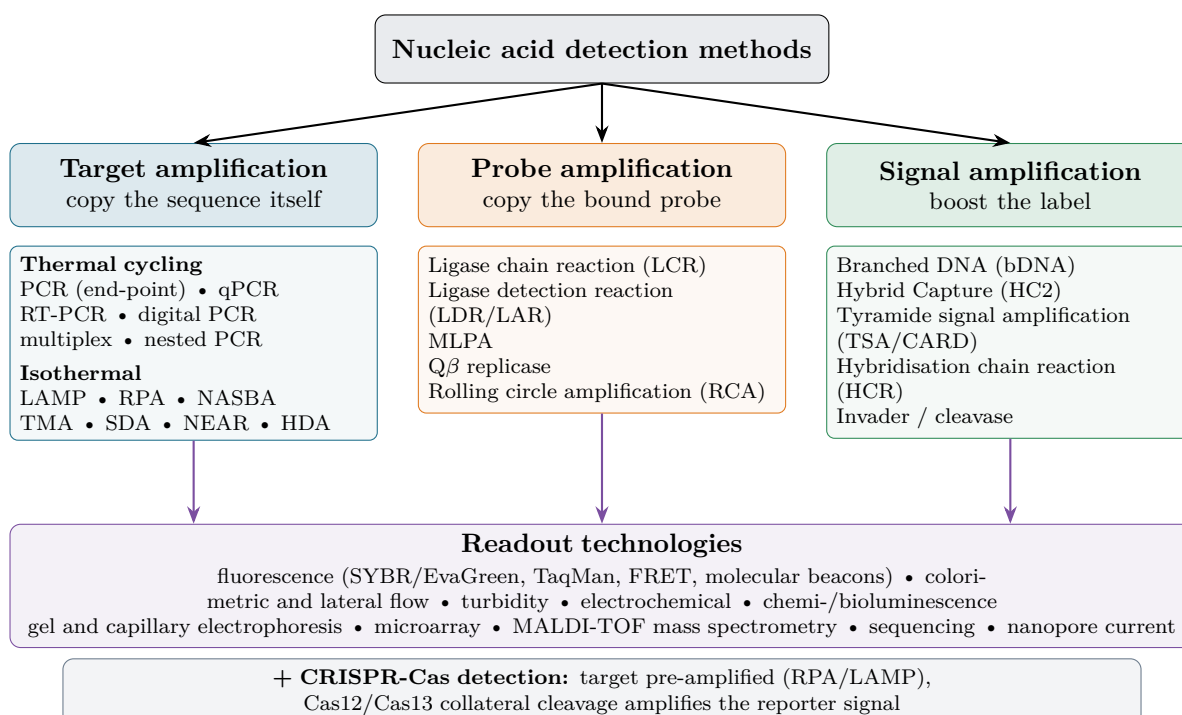


Figure 12: Classification of nucleic acid detection methods by what is amplified, and the readout technologies that make the result visible.

9.1 Target amplification

Method	Principle	Temp.	Example platforms and uses
PCR family	Thermal cycling with DNA polymerase (end-point, real-time, RT, digital, multiplex, nested)	Cycling	Most viral loads and panels
LAMP	4–6 primers recognise 6–8 regions; strand-displacing <i>Bst</i> polymerase	60–65 °C	TB-LAMP, malaria, SARS-CoV-2 home tests
RPA	Recombinase–primer complexes invade duplex DNA	37–42 °C	Field tests; pre-amplification for CRISPR
NASBA	Reverse transcriptase + RNase H + T7 RNA polymerase; RNA-specific	about 41 °C	Older HIV and CMV mRNA assays
TMA	Reverse transcriptase + T7 RNA polymerase; RNA amplicons	about 41 °C	Aptima assays (chlamydia/gonorrhoea, HIV, HCV, HBV, HPV mRNA)

Method	Principle	Temp.	Example platforms and uses
SDA	Restriction-enzyme nicking + strand displacement	50–60 °C	BD ProbeTec (chlamydia/gonorrhoea)
NEAR	Nicking enzyme + polymerase; 5–15 minutes	about 56 °C	ID NOW (influenza, RSV, SARS-CoV-2, group A streptococcus)
HDA	Helicase unwinds DNA instead of heat	about 65 °C	Some simple point-of-care devices

9.2 Probe amplification

Method	Principle	Example
Ligase chain reaction (LCR)	Adjacent probes ligated only when perfectly matched; products become new templates	Former chlamydia/gonorrhoea assays
Ligase detection reaction (LDR/LAR)	Linear ligation for point mutations	SNP and resistance mutation detection
Q β replicase	Replicase copies an RNA reporter probe	Historical
Rolling circle amplification (RCA)	Padlock probe circularises on target; phi29 polymerase copies it continuously	In situ detection, single-molecule assays

9.3 Signal amplification

Method	Principle	Example
Branched DNA (bDNA)	Capture and label extenders build a branched scaffold carrying many alkaline phosphatase labels; chemiluminescence	Older Versant HIV, HCV and HBV viral load assays
Hybrid Capture (HC2)	RNA probes form DNA:RNA hybrids detected by antibodies and chemiluminescence	HPV testing (historical reference test)
Tyramide signal amplification (TSA/CARD)	Peroxidase deposits many labels near the target	In situ hybridisation (e.g. EBER for EBV in tissue)
Hybridisation chain reaction (HCR)	Enzyme-free self-assembly of labelled DNA hairpins	In situ RNA imaging
Invader assay	Flap endonuclease cleavage releases a signal-generating flap	HPV (Cervista), SNP genotyping

Signal amplification produces no amplicons, so carries less contamination risk.

9.4 Readout technologies

Readout	Where used
Fluorescence (dyes, TaqMan, FRET, molecular beacons)	Real-time PCR, dPCR, isothermal assays
Colorimetric and lateral flow (gold nanoparticles, enzyme substrates, pH dyes)	LAMP, CRISPR, point-of-care tests
Turbidity	LAMP (magnesium pyrophosphate)

Readout	Where used
Electrochemical	Some multiplex panels and portable devices
Chemiluminescence and bioluminescence	bdNA, HC2, TMA (acridinium ester probes)
Gel and capillary electrophoresis	Conventional PCR, Sanger, fragment analysis, STR chimerism
Microarray hybridisation	Multiplex identification and resistance arrays
MALDI-TOF mass spectrometry	Mass of PCR or extension products (genotyping); separate from MALDI-TOF protein identification of cultured organisms
Sequencing and nanopore current	Units 11–14

9.5 LAMP in detail

Target: six primer-binding regions (plus loop regions)

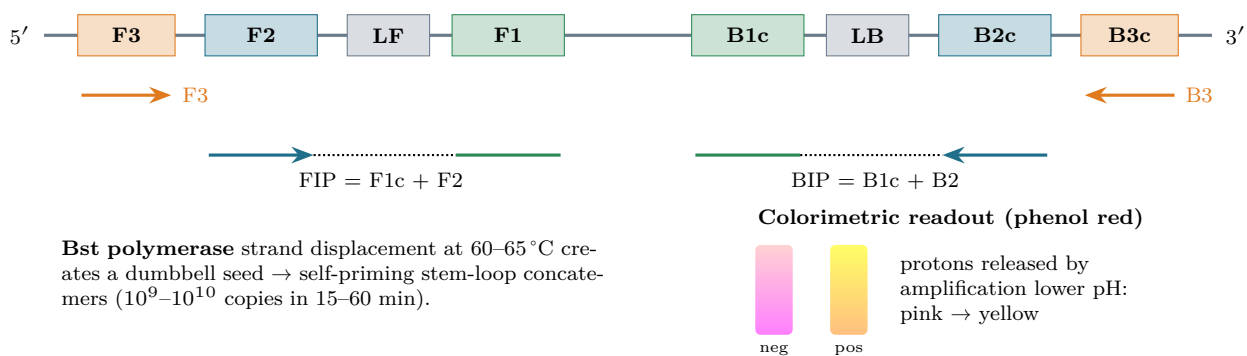


Figure 13: LAMP primer design. Outer primers (F3, B3) and inner primers (FIP, BIP) recognise six regions; optional loop primers (LF, LB) accelerate the reaction. Results can be read by eye.

Primers. Outer primers F3 and B3; inner primers FIP (F1c + F2) and BIP (B1c + B2); optional loop primers LF and LB accelerate the reaction.

Mechanism. *Bst* polymerase displaces strands without heat. Inner and outer primers create a dumbbell-shaped seed, which drives self-priming exponential amplification of long stem-loop concatemers — 10^9 – 10^{10} copies in 15–60 minutes.

Readouts. Turbidity; colour change (phenol red: pink to yellow as protons are released; hydroxynaphthol blue or calcein as Mg^{2+} is consumed); real-time fluorescence; lateral flow. RT-LAMP adds reverse transcriptase for RNA targets.

Advantages	Limitations
No thermal cycler; heat block or water bath	Complex primer design; six regions restrict target choice in variable viruses
Fast (15–60 min); visual readout	Mostly qualitative
Tolerant of inhibitors; works on crude extracts	Huge product yield: carry-over contamination risk — never open tubes after amplification
Suits low-resource and point-of-care settings	Non-specific amplification at long run times; multiplexing difficult

Applications. WHO-endorsed TB-LAMP; malaria; neglected tropical diseases (e.g. leishmaniasis,

trypanosomiasis); SARS-CoV-2 laboratory and home tests; *Legionella* and other environmental testing.

9.6 CRISPR-Cas diagnostics

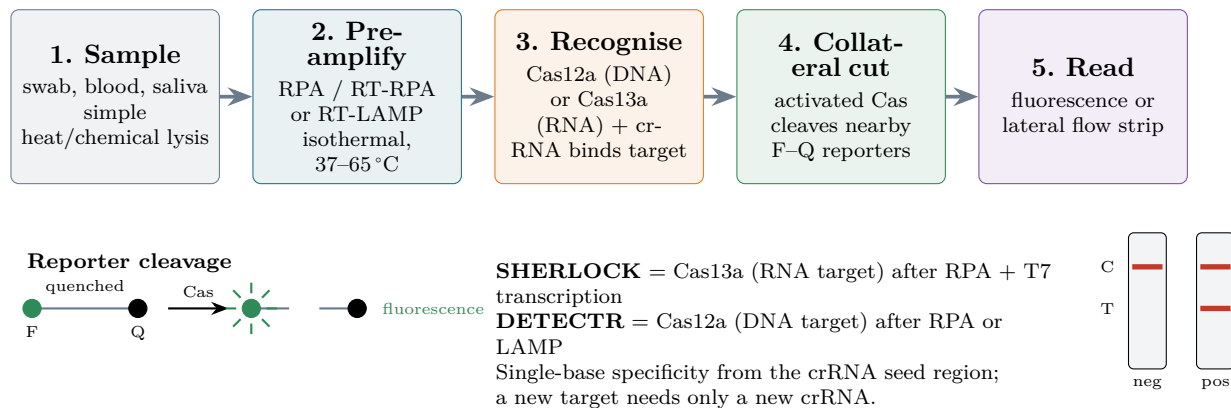


Figure 14: CRISPR-Cas diagnostic workflow. Target recognition activates collateral cleavage of a quenched reporter, read by fluorescence or on a lateral flow strip.

A Cas enzyme guided by a CRISPR RNA (crRNA) recognises a matching target. **Cas12a** (DNA targets) and **Cas13a** (RNA targets), once activated, cut nearby single-stranded reporters non-specifically (**collateral or trans-cleavage**), so one target produces many signal events.

Enzyme	Target	Collateral activity	Platform
Cas9	dsDNA (NGG PAM)	None	Enrichment for sequencing
Cas12a	dsDNA (TTTV PAM), ssDNA	ssDNA	DETECTR
Cas13a	ssRNA	ssRNA	SHERLOCK
Cas12f (Cas14)	ssDNA	ssDNA	Research; high specificity

Workflow. Simple lysis → isothermal pre-amplification (RPA or RT-LAMP) → Cas–crRNA recognition → collateral cleavage of a fluorophore–quencher reporter → fluorescence or lateral flow read-out.

Strengths: single-base specificity (useful for resistance mutations and variant typing), speed, minimal equipment, programmable by changing only the crRNA. **Limitations:** usually needs pre-amplification (contamination risk unless one-pot); mostly qualitative; fewer regulatory approvals than PCR. SHERLOCK and DETECTR received FDA Emergency Use Authorizations for SARS-CoV-2 in 2020; applications in development include TB, HIV drug resistance, HPV and dengue/Zika.

Check your understanding

1. Classify these as target, probe or signal amplification: TMA, bDNA, MLPA, NEAR, HC2.
2. Why does LAMP carry a higher carry-over contamination risk than real-time PCR?
3. Why is CRISPR detection usually combined with RPA or LAMP?

Unit 10 — Molecular detection of antimicrobial resistance

Genotypic tests detect the genes or mutations that cause resistance, often days or weeks faster than phenotypic tests; they predict resistance well when the mechanism is known, but cannot rule out mechanisms they do not look for.

Learning outcomes

1. Describe the main molecular resistance mechanisms in bacteria, mycobacteria, viruses and fungi.
2. Interpret genotypic resistance results and their limitations.
3. Explain how Xpert MTB/RIF and line probe assays detect TB drug resistance.
4. Use online interpretation tools (e.g. Stanford HIVdb, WHO TB mutation catalogue, CARD).

10.1 Genotype versus phenotype

	Genotypic testing	Phenotypic testing
What it measures	Presence of a gene or mutation	Growth (or replication) in the presence of a drug
Speed	Hours (direct from specimen) to days	Days (bacteria) to weeks (TB, viral phenotyping)
Detects	Only known, targeted mechanisms	All mechanisms that affect growth
Problems	Silent or non-expressed genes; unknown mechanisms; mixed populations	Slow; growth needed; some drugs hard to test

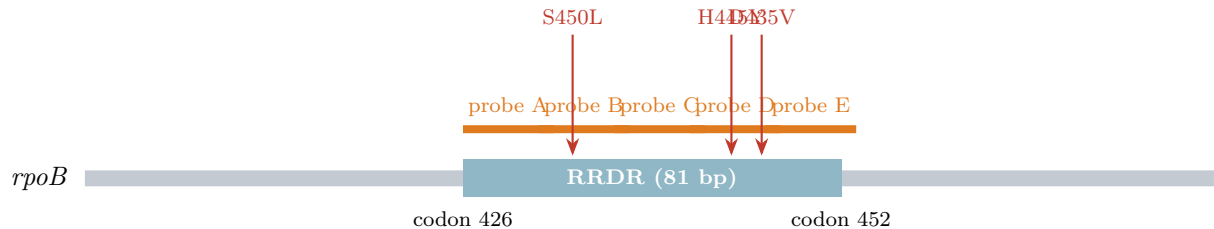
Rule of thumb: a detected, well-characterised resistance mechanism is strong evidence of resistance; failure to detect one does **not** prove susceptibility.

10.2 Bacterial resistance genes

Gene / target	Resistance	Organisms	Molecular test examples
<i>mecA</i> , <i>mecC</i>	Methicillin/oxacillin (all beta-lactams except anti-MRSA cephalosporins)	<i>S. aureus</i> , coagulase-negative staphylococci	Xpert MRSA/SA; blood culture panels
<i>vanA</i> , <i>vanB</i>	Vancomycin	Enterococci	Screening PCR; panels
<i>blaKPC</i> , <i>blaNDM</i> , <i>blaOXA-48</i> -like, <i>blaVIM</i> , <i>blaIMP</i>	Carbapenems (carbapenemases — the “big five”)	Enterobacterales, <i>Pseudomonas</i> , <i>Acinetobacter</i>	Xpert Carba-R; lateral flow (protein); multiplex PCR
<i>blaCTX-M</i>	Third-generation cephalosporins (ESBL)	Enterobacterales	Panels; WGS
<i>mcr-1</i> to <i>mcr-10</i>	Colistin (plasmid-mediated)	Enterobacterales	PCR; WGS
23S rRNA mutations	Macrolides	<i>M. genitalium</i> , <i>M. pneumoniae</i> , <i>H. pylori</i>	Resistance-guided therapy assays
<i>gyrA</i> mutations	Fluoroquinolones	<i>N. gonorrhoeae</i> , <i>M. genitalium</i> (<i>parC</i>), Enterobacterales	Resistance-guided ciprofloxacin use in gonorrhoea

Limitations: resistance due to porin loss, efflux pumps or regulatory mutations may be missed; gene presence does not always mean expression; in a panel from a mixed specimen, the gene cannot always be assigned to a particular organism.

10.3 Tuberculosis



A mutation under a probe stops it binding (or shifts its melt temperature in Ultra) → “rifampicin resistance detected”. More than 95% of rifampicin-resistant *M. tuberculosis* have RRDR mutations; S450L is the most common. Silent mutations can give false resistance calls on probe assays.

Figure 15: The rifampicin resistance-determining region (RRDR) of *rpoB* covered by overlapping probes, as in Xpert MTB/RIF. Mutation positions are shown schematically (*M. tuberculosis* codon numbering).

- **Rifampicin:** more than 95% of rifampicin resistance is caused by mutations in the 81-bp rifampicin resistance-determining region (RRDR) of *rpoB* (codons 426–452 in *M. tuberculosis* numbering; 507–533 in the older *E. coli* numbering). Xpert MTB/RIF uses probes that cover the RRDR; Ultra uses melt-curve analysis and adds multi-copy targets (IS6110, IS1081) for sensitivity, with a “trace” result category.
- **Isoniazid:** *katG* (S315T) and the *inhA* promoter (c.-15C>T); *inhA* mutations also confer ethionamide resistance.
- **Second-line drugs:** fluoroquinolones (*gyrA*, *gyrB*), aminoglycosides (*rrs*, *eis* promoter), bedaquiline and clofazimine (*Rv0678*, *atpE*), linezolid (*rrl*, *rplC*), pretomanid (*ddn* and others).
- **Line probe assays** (GenoType MTBDR*plus*, MTBDR*sl*): PCR then hybridisation to strips with wild-type and mutant probes; absence of a wild-type band suggests a mutation.
- **Targeted NGS and WGS** cover all resistance genes; mutations are interpreted with the **WHO catalogue of mutations in *M. tuberculosis* complex** (2nd edition, 2023 — free, Part B).
- **Pitfalls:** silent (synonymous) RRDR mutations can cause false resistance on probe assays; low positive predictive value in low-prevalence settings — confirm unexpected rifampicin resistance (Case F); heteroresistance (mixed populations) may be missed.

10.4 Antiviral resistance

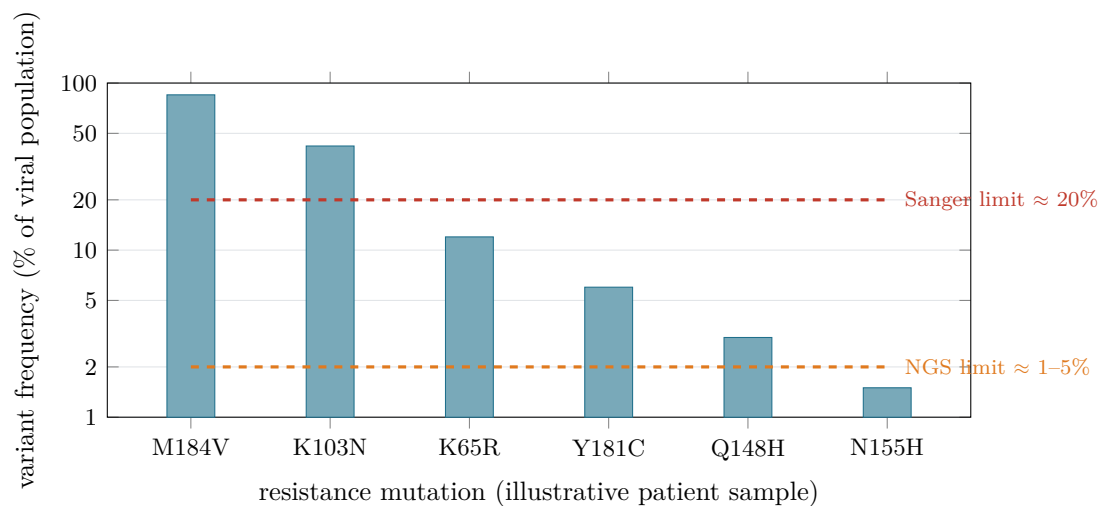


Figure 16: Minority resistance variants: Sanger sequencing reports only variants above about 15–20% of the population; NGS detects variants down to about 1–5%. Values are illustrative.

Virus	Genes	Key mutations (examples)	Notes
HIV-1	Reverse transcriptase, protease, integrase; envelope (tropism)	M184V/I (lamivudine/emtricitabine); K65R (tenofovir); K103N, Y181C (NNRTIs); Q148H/K/R, N155H, R263K (integrase inhibitors)	Interpret with Stanford HIVdb; genotyping usually needs viral load > 500–1,000 copies/mL; archived resistance in proviral DNA
Hepatitis B	Polymerase (reverse transcriptase domain)	rtM204V/I, rtL180M (lamivudine, telbivudine; entecavir with additional mutations)	Tenofovir resistance is rare
Hepatitis C	NS3, NS5A, NS5B	NS5A Y93H and others (resistance-associated substitutions)	Mainly relevant after treatment failure
CMV	UL97 kinase, UL54 polymerase, UL56 terminase	UL97 M460V/I, H520Q, A594V, L595S, C603W (ganciclovir); UL54 (ganciclovir, foscarnet, cidofovir); UL56 (letermovir); UL97 T409M, H411Y (maribavir)	Suspect when loads rise after 2 or more weeks of adequate therapy
HSV, VZV	Thymidine kinase (UL23), DNA polymerase (UL30)	Many TK frameshifts and stop codons (aciclovir)	Mostly in immunocompromised hosts
Influenza	Neuraminidase; PA	NA H275Y (oseltamivir, N1 numbering); PA I38T (baloxavir)	Emerges during treatment of immunocompromised patients (Case K)
SARS-CoV-2	Main protease; RdRp; spike	Mpro E166V (nirmatrelvir); spike changes affect monoclonal antibodies	Surveillance mostly through genomic programmes

Sanger vs NGS: Sanger detects variants present in roughly 15–20% or more of the viral population; NGS detects minority variants down to 1–5%. Clinical significance of very low-frequency variants depends on the drug and is still debated.

10.5 Antifungal resistance

- *Candida auris* and other *Candida*: *FKS1*/*FKS2* hot-spot mutations (echinocandins); *ERG11* (azoles).
- *Aspergillus fumigatus*: *cyp51A* mutations, especially TR34/L98H and TR46/Y121F/T289A, linked to environmental azole use.

10.6 Antiparasitic resistance

- *Plasmodium falciparum*: *kelch13* propeller mutations (e.g. C580Y, R539T, R561H, A675V) — artemisinin partial resistance, first in South-East Asia and now emerging independently in Africa; *pfcr1* K76T — chloroquine; *pfmdr1* copy number and mutations — mefloquine and lumefantrine susceptibility; *dhfr* and *dhps* — sulfadoxine–pyrimethamine.
- Mostly used for surveillance and research rather than individual treatment decisions; WHO publishes the list of validated *kelch13* markers.

10.7 Interpretation tools

Tool	Use
Stanford HIV Drug Resistance Database (HIVdb)	HIV genotype interpretation; also HBV and SARS-CoV-2 programmes
geno2pheno	HIV tropism (co-receptor use) and resistance
WHO TB mutation catalogue	Graded mutation–drug associations for <i>M. tuberculosis</i>
TB-Profiler, Mykrobe	WGS-based TB lineage and resistance prediction
MalariaGEN resources	<i>P. falciparum</i> genomic surveillance data, including resistance markers
CARD (Comprehensive Antibiotic Resistance Database) with RGI	Bacterial resistance genes from sequence
AMRFinderPlus, ResFinder / PointFinder	Bacterial resistance genes and point mutations

Check your understanding

1. An *E. coli* blood culture is negative for all carbapenemase genes on a panel. Can you report it as carbapenem-susceptible?
2. Why should Xpert “rifampicin resistance detected” be confirmed in a low-prevalence setting?
3. Why might Sanger sequencing miss emerging HIV resistance?

Unit 11 — Sequencing technologies

Sequencing reads the order of bases directly; Sanger reads one amplicon accurately, short-read NGS reads millions of fragments in parallel, and long-read platforms read whole genes or genomes in single molecules.

Learning outcomes

1. Explain Sanger chain-termination sequencing and read a chromatogram.
2. Describe NGS library preparation, enrichment and Illumina sequencing by synthesis.
3. Compare short-read and long-read platforms for pathogen work.
4. Interpret run QC metrics.

11.1 Sanger sequencing

1. PCR of the target region; clean-up.
2. Cycle sequencing with fluorescent dideoxynucleotides (ddNTPs) that terminate extension.
3. Capillary electrophoresis separates fragments by size; a laser reads the base colour at each position.

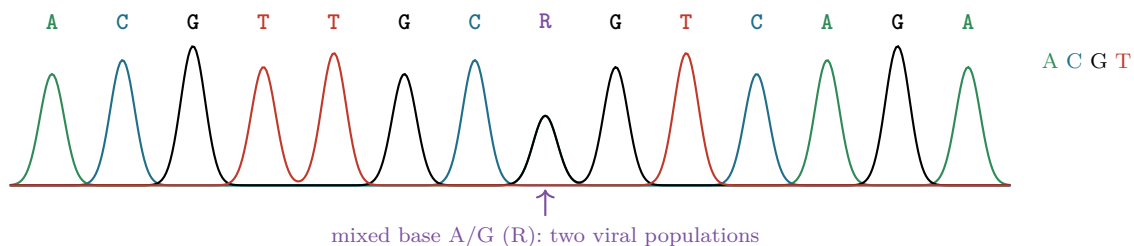


Figure 17: Sanger chromatogram (schematic). Each base gives a coloured peak; a mixed base shows two overlapping peaks (IUPAC code R = A/G) when two variants coexist in a viral population.

Uses in infection: HIV, HBV and CMV resistance genotyping; 16S/ITS identification (Unit 13); HSV typing; outbreak typing by single genes (e.g. norovirus, *emm* typing of group A streptococcus, *spa* typing of *S. aureus*).

Mixed bases: in viruses, two peaks at one position mean a mixture of variants (quasispecies), reported with IUPAC codes (e.g. R = A/G). Sanger detects a minority variant only above about 15–20%.

11.2 Next-generation sequencing workflow

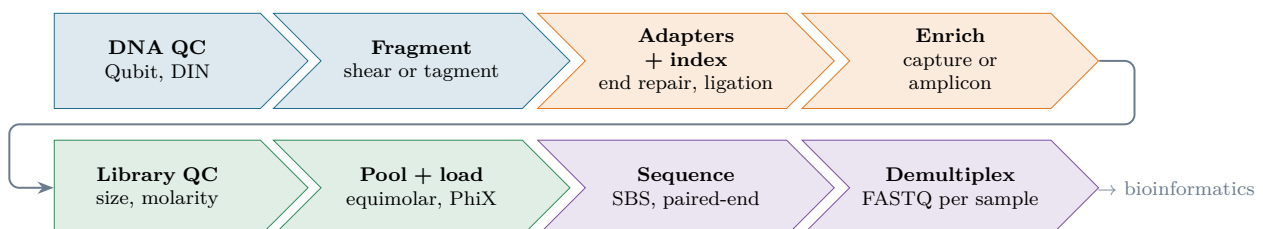


Figure 18: The NGS laboratory workflow from input DNA to demultiplexed reads.

1. Input nucleic acid QC (fluorometric quantity; integrity).
2. Fragmentation (mechanical, enzymatic or tagmentation).
3. End repair, A-tailing, adapter ligation with **indexes** (barcodes) that identify each sample.
4. Enrichment (if needed): amplicon or hybrid capture.

5. Library QC, normalisation and pooling.
6. Sequencing and demultiplexing into FASTQ files per sample.

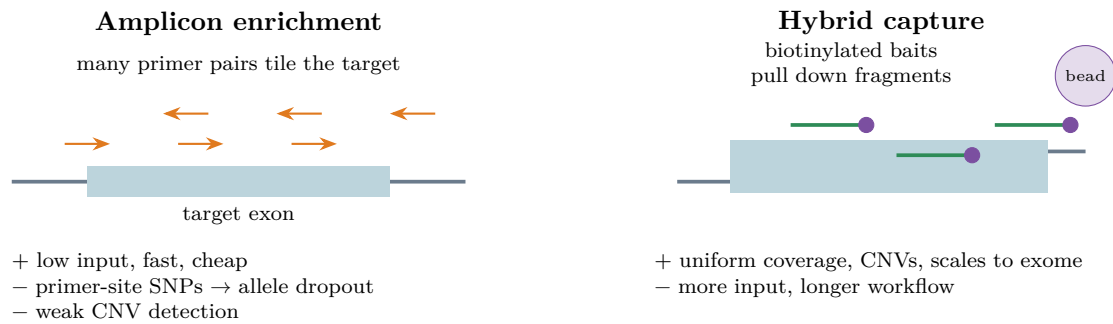


Figure 19: Two ways to enrich target regions before sequencing.

11.3 Approaches for pathogens

Approach	How	Typical use
Isolate whole-genome sequencing (WGS)	Sequence DNA from a cultured isolate	Bacterial typing, outbreak investigation, resistance prediction, TB
Amplicon tiling of viral genomes	Overlapping PCR amplicons cover the whole genome (e.g. ARTIC protocol)	SARS-CoV-2, influenza, HIV, measles, mpox, dengue directly from specimens
Targeted enrichment (hybrid capture)	Probes capture pathogen sequences from a specimen	Viral panels, TB targeted NGS
Targeted amplicon panels	Amplify resistance genes or regions	TB resistance (targeted NGS), HIV drug resistance
Metagenomic (shotgun) sequencing	Sequence all nucleic acid in a specimen	Unknown pathogens (Unit 13)
16S/ITS amplicon sequencing	Broad-range PCR then sequencing	Culture-negative infections (Unit 13)

11.4 Platforms

Platform	Chemistry	Read length	Accuracy	Strengths for pathogen work
Sanger	Dye-terminator capillary	500–1,000 bp	> 99.9%	Single genes; confirmation
Illumina	Sequencing by synthesis	50–300 bp (paired)	> 99.9% (Q30+)	Accurate SNPs; high throughput; outbreak SNP analysis
Ion Torrent	Semiconductor (H ⁺ detection)	200–600 bp	~99%	Fast amplicon panels
Oxford Nanopore	Current change through a protein pore	Up to > 100 kb	~99%+ with current chemistry	Portable, real-time; complete plasmids; field outbreak work (e.g. Ebola, Zika); direct RNA
PacBio HiFi	Circular consensus single-molecule	10–25 kb	> 99.9%	Complete bacterial genomes and plasmids; HLA full-gene typing

11.5 Run QC

Metric	Meaning
% $\geq$ Q30	Proportion of bases with error ≤ 1 in 1,000
Cluster density / pass filter	Flow-cell loading and signal quality (Illumina)
Yield and reads per sample	Enough data for required depth
Genome coverage and depth	E.g. $\geq 90\%$ of a viral genome at $\geq 10\text{--}20\times$ for a consensus
Negative controls	Reads in negative controls show contamination or index misassignment

Phred score: $Q = -10 \log_{10} P$ (Q30 = 99.9% accuracy).

Unit 12 — Pathogen bioinformatics

Bioinformatics turns reads into a consensus genome, variants, a lineage, resistance predictions and relationships between isolates; clinicians and microbiologists need to understand each step well enough to judge the result.

Learning outcomes

1. Name the files and steps in a pathogen genomics pipeline.
2. Distinguish reference mapping from de novo assembly.
3. Explain consensus calling, minority variants, lineage assignment and typing schemes.
4. Use web tools (Nextclade, PubMLST, CARD, Stanford HIVdb) on example data.

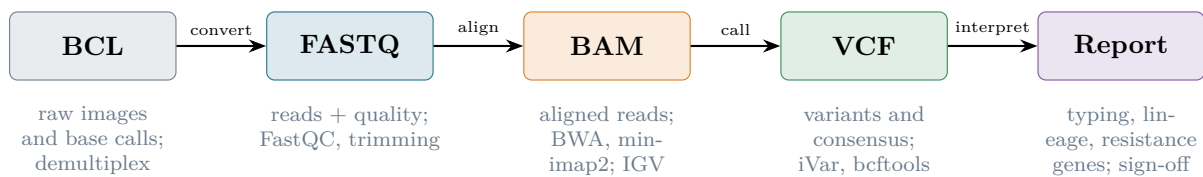


Figure 20: The pathogen bioinformatics pipeline and the file format produced at each step (de novo assembly to FASTA contigs is the alternative to mapping).

12.1 Pipeline steps

1. **Read QC and trimming** (FastQC, fastp); for amplicon data, primer trimming (e.g. iVar).
2. **Host read removal** (map to human genome and discard) — protects privacy and speeds analysis.
3. **Reference mapping** (BWA, minimap2) → BAM file; or **de novo assembly** (SPAdes for short reads; Flye for long reads; hybrid assembly with Unicycler) → contigs.
4. **Variant calling and consensus**: call positions that differ from the reference; positions below a minimum depth are masked as N.
5. **Minority variant calling** (viral): report variants above a frequency threshold (e.g. 2–5%) only when depth and quality support them.
6. **Typing and lineage assignment** (below).
7. **Resistance and virulence prediction** (Unit 10 tools).
8. **Phylogenetics and outbreak analysis** (Unit 14).

12.2 File formats

File	Contains
FASTQ	Reads with quality scores
BAM / CRAM	Reads aligned to a reference
VCF	Variants relative to the reference
FASTA	Sequences — reference genomes, consensus genomes, assembled contigs
Newick / Nexus	Phylogenetic trees
GFF / GenBank	Genome annotations (genes and their coordinates)

12.3 Typing and lineage nomenclature

Organism group	Scheme	Notes
Bacteria	MLST (7 housekeeping genes → sequence type, e.g. ST131 <i>E. coli</i> , ST258 <i>K. pneumoniae</i>)	PubMLST, Pasteur databases
Bacteria	Core-genome MLST (cgMLST, ~1,500–4,000 genes) and SNP-based typing	High resolution for outbreaks
<i>S. aureus</i> ; group A streptococcus	<i>spa</i> typing; <i>emm</i> typing	Single-gene sequence typing
<i>M. tuberculosis</i>	Lineages 1–9; SNP distances	TB-Profiler
SARS-CoV-2	Pango lineages; Nextstrain clades	Pangolin, Nextclade
Influenza	Subtype (HA/NA), genetic clades	Vaccine strain selection
HIV-1	Group M subtypes A–K and circulating recombinant forms (CRFs)	REGA, COMET tools
Hepatitis B	Genotypes A–J	Affects disease course and treatment response
Hepatitis C	Genotypes 1–8 with subtypes	Less important with pangenotypic treatment

12.4 Reading a phylogenetic tree

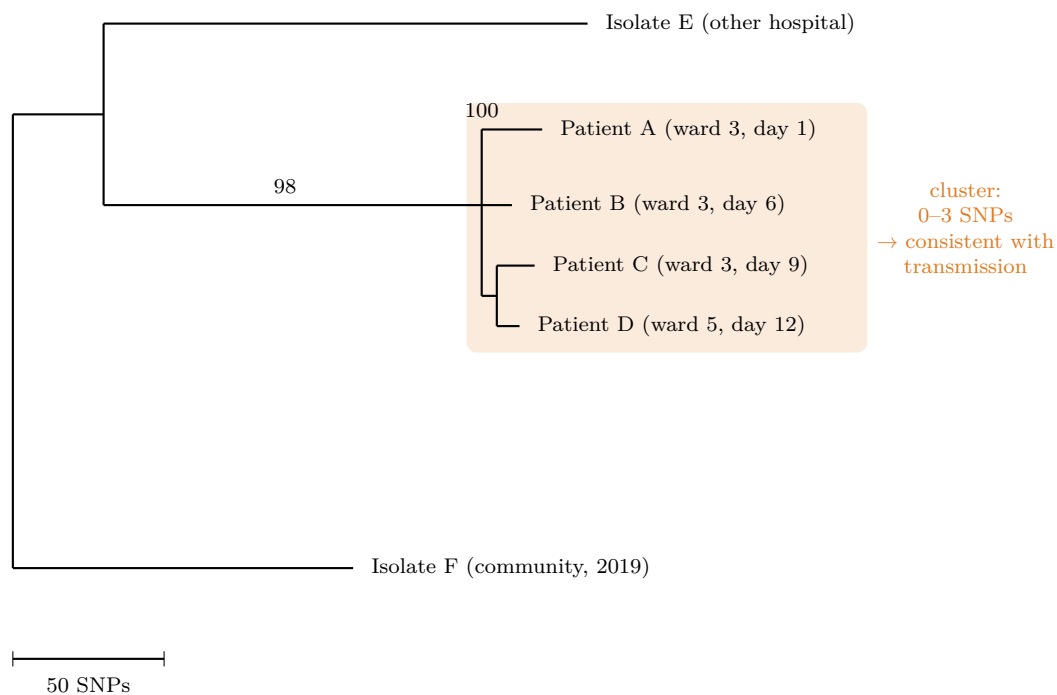


Figure 21: Reading a phylogenetic tree in an outbreak. Patients A–D form a tight cluster (consistent with transmission, to be combined with ward and timing data); E and F are unrelated. Numbers on nodes are bootstrap support values.

- **Tips** are sampled sequences; **internal nodes** are inferred common ancestors; **branch length** shows genetic change (substitutions per site, or SNPs).
- **Clades** are a common ancestor and all its descendants. Closely related isolates cluster together.
- **Support values** (bootstrap, posterior probability) show confidence in a grouping.
- **Rooting** with an outgroup gives direction in time; time-scaled trees use a molecular clock.

- **Do not over-read trees:** close relationship is consistent with transmission but does not prove direct transmission or its direction.

Bench and desk activities

- ☐ Run a public SARS-CoV-2 consensus sequence through Nextclade (web, free) and identify its clade, lineage and mutations.
- ☐ Run a bacterial genome assembly through ResFinder or CARD and list resistance genes.
- ☐ Complete the Galaxy Training tutorial on SARS-CoV-2 variant analysis or bacterial genome assembly (Part B).

Unit 13 — Clinical metagenomics and broad-range (16S/ITS) sequencing

Hypothesis-free sequencing can identify pathogens no targeted test would look for; its main challenges are human background, contamination and deciding what a detected sequence means.

Learning outcomes

1. Describe the metagenomic NGS (mNGS) workflow, including host depletion and controls.
2. Explain 16S rRNA and ITS amplicon sequencing.
3. Interpret results using thresholds, negative controls and clinical context.
4. Identify the clinical situations where these tests add value.

13.1 Metagenomic NGS

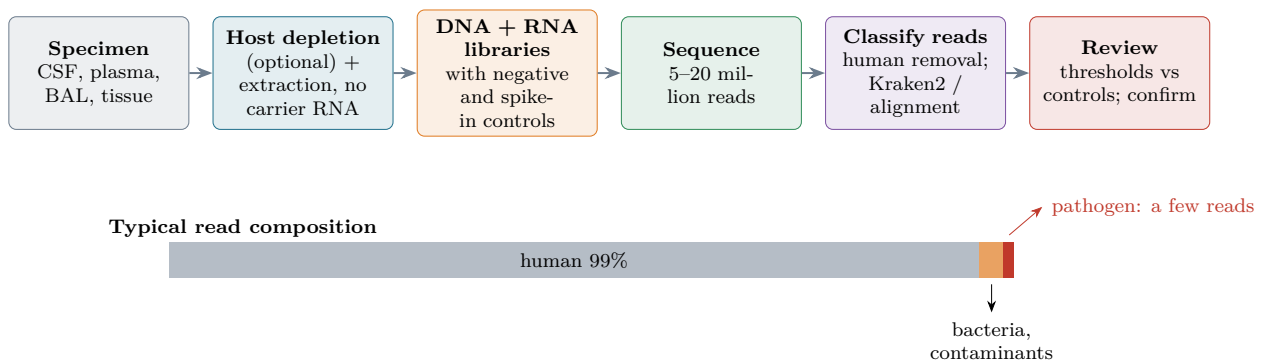


Figure 22: Clinical metagenomic sequencing workflow. Human reads dominate, so pathogen reads must be judged against negative controls and laboratory thresholds.

- **Workflow:** extraction (no carrier RNA) → optional host depletion → DNA and/or RNA library preparation → sequencing (often 5–20 million reads per sample) → bioinformatic classification (e.g. Kraken2, alignment to curated databases) → review.
- **Human background:** commonly > 99% of reads are human; pathogens may be represented by only a few reads.
- **Controls:** negative extraction controls processed with every batch (reveal “kitome” and environmental contaminants such as *Ralstonia*, *Bradyrhizobium*); positive spike-in controls; internal spike-ins for normalisation.
- **Reporting thresholds:** labs define minimum reads, genome coverage across the genome, and fold-excess over negative controls before calling a detection.
- **Confirmation:** confirm unexpected findings with a targeted test on the original or another specimen.

Where it helps: meningitis and encephalitis of unknown cause, culture-negative infections in immunocompromised hosts, unexplained fever, new or emerging pathogens, and outbreak investigation. A large prospective study of meningitis and encephalitis (Wilson et al. 2019 — Part B) showed mNGS identified infections missed by conventional tests, but did not replace them.

13.2 16S rRNA and ITS sequencing

- **16S rRNA gene** (about 1,500 bp, nine hypervariable regions V1–V9): broad-range PCR from tissue or fluid, then Sanger (single organism) or NGS (mixed infections).
- **ITS1/ITS2 regions** for fungi.
- **Uses:** culture-negative endocarditis (valve tissue), bone and joint infections, brain abscess, empyema, and patients who received antibiotics before culture.

- **Pitfalls:** contamination from reagents and skin flora; mixed infections give unreadable Sanger traces (use NGS); some genera are poorly separated by 16S (e.g. some streptococci, *E. coli* vs *Shigella*).

Check your understanding

1. mNGS of CSF detects 3 reads of *Cutibacterium acnes*. The negative control has 2 reads of the same organism. How do you report it?
2. Why is valve tissue better than blood for 16S PCR in culture-negative endocarditis?

Unit 14 — Genomic epidemiology and outbreak investigation

Pathogen genomes accumulate mutations over time, so the number of differences between isolates, together with epidemiological data, can support or exclude transmission and track variants across populations.

Learning outcomes

1. Explain how SNP distances and phylogenies are used in outbreak investigation.
2. Integrate genomic and epidemiological data.
3. Describe genomic surveillance for variants and antimicrobial resistance.
4. Use visualisation tools such as Nextstrain and Microreact.

14.1 SNP distances and transmission

- Isolates from a recent transmission chain differ by few SNPs; unrelated isolates usually differ by many.
- **Thresholds depend on the organism and setting.** For *M. tuberculosis*, ≤ 5 SNPs is consistent with recent transmission and > 12 SNPs makes it unlikely (Walker et al. 2013). For other bacteria (e.g. MRSA, *K. pneumoniae*) thresholds are often in the range of 10–25 SNPs but vary by species, lineage and time between samples.
- **Mutation rates:** SARS-CoV-2 accumulated roughly two substitutions per genome per month during the pandemic; *M. tuberculosis* about 0.5 SNPs per genome per year — so TB needs very long time windows and careful interpretation.
- **Genomics cannot prove direction or direct contact:** an unsampled intermediate may exist. Always combine with time, place and contact data.

14.2 Plasmid outbreaks

Carbapenemase genes can spread on plasmids between different species and sequence types. A hospital “outbreak” of *blaKPC* may involve unrelated bacterial strains carrying the same plasmid — long-read sequencing is often needed to resolve plasmids.

14.3 Genomic surveillance

- **Variant surveillance:** SARS-CoV-2 lineages, influenza clades (vaccine strain selection), mpox clades, measles genotypes for elimination monitoring.
- **AMR surveillance:** national programmes sequence resistant isolates to track high-risk clones and resistance genes.
- **Wastewater sequencing:** population-level variant tracking.
- **Data sharing:** GISAID, NCBI and ENA host shared genomes; national rules govern patient data linked to sequences.

14.4 Tools

Tool	Use
Nextstrain	Real-time phylogenetic tracking of viral and bacterial pathogens
Nextclade	Clade assignment, QC and mutation calling for viral sequences (web)
Microreact	Linking trees, maps and timelines
Pathogenwatch	Genomic surveillance of bacterial pathogens and AMR
PubMLST	MLST and cgMLST schemes and isolate databases

Check your understanding

1. Four ICU patients have *blaKPC*-positive *K. pneumoniae*. Three isolates differ by 0–3 SNPs; one differs by over 400 SNPs and has a different sequence type but carries *blaKPC*. Interpret this (Case E).
2. Why does TB need a different SNP threshold from SARS-CoV-2?

Unit 15 — Molecular mycology and parasitology

Fungi and parasites are often slow or impossible to culture and hard to see by microscopy at low burden; molecular tests add sensitivity, species identification and resistance markers, but need specimen-specific extraction and careful interpretation because of colonisation and environmental contamination.

Learning outcomes

1. Describe the ribosomal DNA targets used for fungal and parasite detection and identification.
2. Interpret *Aspergillus*, *Pneumocystis*, *Candida* and Mucorales PCR in clinical context.
3. Explain the role of PCR in malaria, intestinal protozoa, *Toxoplasma*, *Leishmania* and other parasites.
4. Describe antifungal and antimalarial resistance markers.
5. Recognise extraction and contamination problems specific to fungi and parasites.

15.1 Ribosomal DNA targets

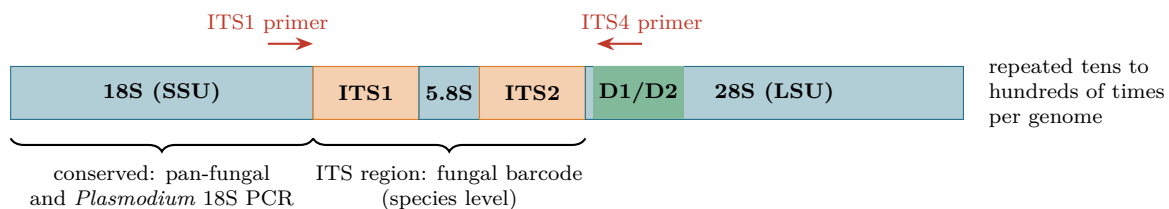


Figure 23: The ribosomal DNA operon. Conserved 18S and 28S genes allow broad-range PCR; the variable ITS regions and D1/D2 identify fungal species. Multiple copies per genome make these sensitive targets.

- The ribosomal DNA (rDNA) operon is present in tens to hundreds of copies per genome, giving sensitive PCR targets.
- **18S** is highly conserved (genus or broader); **ITS1** and **ITS2** are variable (species level); the **D1/D2 region of 28S** is a second identification target. The ITS region is the formal fungal DNA barcode (Schoch et al. 2012 — Part B).
- Some genera need secondary markers for species identification: β -tubulin and calmodulin (*Aspergillus*), *TEF1 α* (*Fusarium*).
- Parasite assays often target 18S rRNA genes (*Plasmodium*), multi-copy repeats (*Toxoplasma* 529-bp repeat element, *Leishmania* kinetoplast minicircle DNA) or species-specific genes.

15.2 Molecular mycology

Pathogen / test	Specimens	Role and interpretation
<i>Aspergillus</i> PCR	Serum, plasma, whole blood, BAL	Included as mycological evidence in the 2020 EORTC/MSGERC definitions of invasive fungal disease; best used with galactomannan; repeated positives in blood are more significant than a single positive; standardised methods (EAPCRI recommendations) improve reliability
<i>Pneumocystis jirovecii</i> quantitative PCR	BAL, induced sputum, upper respiratory specimens	Very high negative predictive value — a negative BAL PCR makes pneumocystis pneumonia unlikely. Low loads may reflect colonisation, especially in non-HIV patients; labs set load thresholds per assay and specimen (Case M)

Pathogen / test	Specimens	Role and interpretation
Mucorales PCR	Serum, tissue	Early detection of mucormycosis in high-risk patients
<i>Candida</i> direct blood detection	Whole blood (e.g. T2 magnetic resonance)	Faster than blood culture for candidaemia
<i>Candida auris</i> screening PCR	Axilla and groin swabs	Infection prevention screening; rapid isolation decisions
<i>Cryptococcus</i>	CSF (panel PCR)	Antigen testing remains first-line; PCR in meningitis panels
Endemic mycoses (<i>Histoplasma</i> , <i>Talaromyces</i> , <i>Coccidioides</i> , <i>Blastomyces</i>)	Tissue, blood, BAL	Reference-laboratory PCR; antigen tests complement
Pan-fungal PCR + sequencing (ITS, D1/D2)	Tissue, sterile fluids	Identifies fungi in culture-negative biopsies; contamination must be excluded
Isolate identification	Cultured isolates	ITS and secondary barcodes; MALDI-TOF is the routine alternative

Resistance markers (Unit 10): *cyp51A* TR34/L98H and TR46/Y121F/T289A in *A. fumigatus* (azoles); *FKS1/FKS2* hot spots in *Candida* (echinocandins); *ERG11* in *Candida* (azoles).

Practical points

- Fungal cell walls need mechanical disruption (bead beating) and enzymatic lysis; extraction efficiency varies widely between methods.
- *Aspergillus* spores are everywhere in air: use clean extraction areas, negative extraction controls on every run, and filtered reagents.
- Pan-fungal PCR of non-sterile specimens often detects environmental or colonising fungi.

15.3 Molecular parasitology

Parasite / test	Specimens	Role and interpretation
<i>Plasmodium</i> species PCR (18S rRNA)	EDTA whole blood	Microscopy and rapid diagnostic tests remain first-line for urgent diagnosis. PCR confirms species, detects mixed and low-density infections, and identifies <i>P. knowlesi</i> (often mistaken for <i>P. malariae</i> on microscopy) (Case N)
<i>pfhrp2/pfhrp3</i> deletion testing	Blood	Deletions cause false-negative HRP2-based rapid tests; surveillance guides choice of rapid test
Malaria resistance markers	Blood	<i>kelch13</i> propeller mutations (artemisinin partial resistance), <i>pfcr1</i> K76T (chloroquine), <i>pfmdr1</i> , <i>dhfr/dhps</i> (sulfadoxine–pyrimethamine) — mainly for surveillance
Intestinal protozoa multiplex PCR	Stool	<i>Giardia</i> , <i>Cryptosporidium</i> , <i>Entamoeba histolytica</i> , <i>Cyclospora</i> , <i>Dientamoeba fragilis</i> ; more sensitive than microscopy. PCR separates pathogenic <i>E. histolytica</i> from non-pathogenic <i>E. dispar</i> , which microscopy cannot (Case O)
Helminths	Stool, urine	<i>Strongyloides</i> and <i>Schistosoma</i> PCR available in reference labs; serology is often more sensitive for <i>Strongyloides</i> ; egg-rich stool needs bead beating

Parasite / test	Specimens	Role and interpretation
<i>Toxoplasma gondii</i> PCR	Amniotic fluid, CSF, aqueous/vitreous humour, blood (transplant)	Congenital toxoplasmosis diagnosis (amniotic fluid after 18 weeks' gestation), ocular toxoplasmosis, reactivation after transplant
<i>Leishmania</i> PCR and species typing	Skin biopsy, bone marrow, blood	Detection more sensitive than microscopy; species identification (e.g. by ITS1 or <i>hsp70</i> sequencing) guides treatment of cutaneous leishmaniasis
<i>Trypanosoma cruzi</i> PCR	Blood	Congenital Chagas disease, acute infection and reactivation after transplant
Free-living amoebae (<i>Naegleria</i> , <i>Acanthamoeba</i> , <i>Balamuthia</i>)	CSF, corneal scrapes, tissue	Rapid diagnosis of rare, often fatal infections
<i>Babesia</i> PCR	Blood	Species identification and low-level parasitaemia
<i>Trichomonas vaginalis</i> NAAT	Vaginal swab, urine	Much more sensitive than wet-mount microscopy; often in STI panels
Microsporidia PCR	Stool, tissue	Immunocompromised patients

Practical points

- Stool contains strong PCR inhibitors; use stool-specific extraction and an internal control.
- Helminth eggs and protozoan cysts are robust — bead beating improves DNA release.
- Molecular detection does not always mean disease: *Blastocystis* and *Dientamoeba* are often found in healthy people.
- For malaria, a positive PCR without microscopic parasites may represent low-density or recently treated infection; PCR can stay positive for weeks after treatment.

Bench and desk activities

- ☐ Review your laboratory's *Pneumocystis* PCR reporting thresholds and the evidence behind them.
- ☐ Compare malaria microscopy, RDT and PCR results for 10 recent samples (or EQA material).
- ☐ Run an ITS sequence from a fungal isolate through NCBI BLAST and a curated fungal database; note the top matches and percentage identity.

Check your understanding

1. Why is a negative *Pneumocystis* PCR on BAL more useful than a low-level positive?
2. Why might an HRP2 rapid test be negative in a patient with *P. falciparum* malaria?
3. Which molecular test distinguishes *E. histolytica* from *E. dispar*, and why does it matter?

Unit 16 — Molecular immunology

Molecular methods in immunology type the HLA genes that govern transplant matching and drug hypersensitivity, track donor cells after stem cell transplantation, screen newborns for severe combined immunodeficiency, and diagnose inborn errors of immunity.

Learning outcomes

1. Describe HLA genes, nomenclature and typing methods.
2. Explain HLA associations with drug hypersensitivity and disease.
3. Interpret chimerism results after stem cell transplantation.
4. Explain TREC/KREC newborn screening.
5. Describe genetic testing for inborn errors of immunity and host-response testing.

16.1 HLA genes and nomenclature

The major histocompatibility complex on chromosome 6p21.3 contains the most polymorphic human genes: class I (HLA-A, -B, -C) and class II (HLA-DR, -DQ, -DP). Tens of thousands of alleles are recorded in the IPD-IMGT/HLA database.

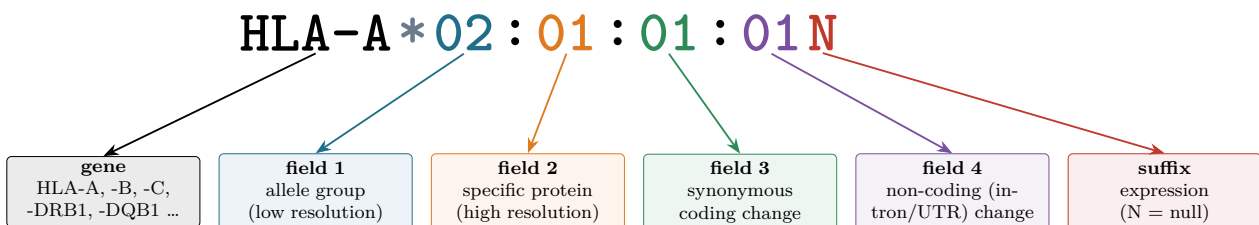


Figure 24: Anatomy of an HLA allele name. Stem cell transplant matching usually needs at least two-field (high) resolution.

Resolution	Example	Meaning
Low (first field)	HLA-A*02	Allele group, close to the old serological antigen
High (two fields)	HLA-A*02:01	Specific protein (antigen recognition domain) — needed for stem cell donor matching
Allele level (three or four fields)	HLA-A*02:01:01:01	Synonymous coding and non-coding differences
Suffix	N, L, S, Q	Null (not expressed), low expression, secreted, questionable

16.2 HLA typing methods

Method	Principle	Resolution	Use
SSO (sequence-specific oligonucleotide, bead-based)	PCR then hybridisation to probe-coated beads	Low to intermediate	Solid organ donors, registries
SSP (sequence-specific primers)	Allele-specific PCR; gel or real-time	Low to intermediate	Rapid typing; deceased donors
Real-time PCR for single alleles	Allele-specific probes	One allele	HLA-B*57:01 screening (present/absent) before abacavir
Sanger sequencing-based typing (SBT)	Sequence exons 2–3 (class I) or 2 (class II)	High, with ambiguities	Historical standard

Method	Principle	Resolution	Use
NGS	Sequence whole genes or long amplicons; phased reads	High to allele level	Current standard for stem cell transplant
Long-read sequencing	Full-length, fully phased alleles	Allele level	Resolving ambiguities; new alleles

HLA antibody testing (single antigen bead assays, crossmatching) detects antibodies, not genes — it is serological, but is interpreted together with molecular HLA types.

16.3 HLA associations in clinical practice

Association	Clinical use
HLA-B*57:01 — abacavir hypersensitivity	Screen all patients before starting abacavir; positive = do not prescribe (Case I)
HLA-B*58:01 — allopurinol SJS/TEN and DRESS	Screening in populations with high allele frequency
HLA-B*15:02 — carbamazepine SJS/TEN	Screening in people of South-East Asian ancestry
HLA-A*31:01 — carbamazepine DRESS	Considered in some guidelines
HLA-DQ2/DQ8 — coeliac disease	High negative predictive value: absence makes coeliac disease very unlikely
HLA-B27 — axial spondyloarthritis	Supportive, not diagnostic
HLA-DQB1*06:02 — narcolepsy type 1	Supportive

16.4 Chimerism after allogeneic stem cell transplantation

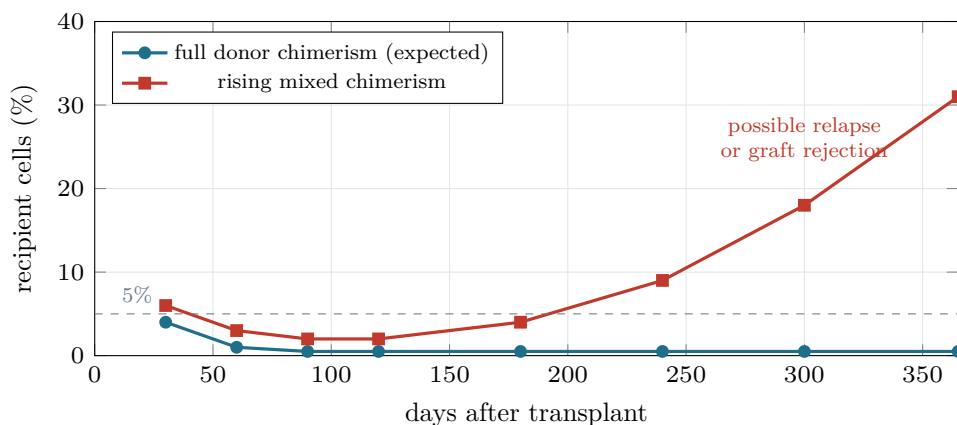


Figure 25: Chimerism monitoring after allogeneic stem cell transplantation. A rising proportion of recipient cells, especially in lineage-specific (T-cell or myeloid) fractions, prompts clinical review.

- **Purpose:** measure the proportion of donor-derived cells in blood or marrow, to confirm engraftment and detect graft failure or relapse.
- **Methods:** short tandem repeat (STR) PCR with capillary electrophoresis (sensitivity about 1–5%); qPCR or ddPCR of indels/SNPs that differ between donor and recipient (about 0.1%); NGS-based methods.
- **Lineage-specific chimerism:** testing sorted T cells (CD3) and myeloid cells (CD33/CD15) separately is more sensitive for early rejection or relapse.
- **Interpretation:** full donor chimerism (> 95–99% donor) is expected; increasing recipient cells (mixed chimerism) may herald relapse or rejection and prompts clinical action (e.g. immunosuppression withdrawal, donor lymphocyte infusion).

16.5 TREC/KREC newborn screening

- **TRECs** (T-cell receptor excision circles) are small DNA circles produced during T-cell receptor gene rearrangement in the thymus; they do not replicate, so they mark recently made (naïve) T cells. **KRECs** do the same for B cells.
- Measured by quantitative PCR on the newborn dried blood spot.
- **Low or absent TRECs** suggest severe combined immunodeficiency (SCID) or other T-cell lymphopenia (e.g. 22q11.2 deletion, prematurity) → repeat, then lymphocyte flow cytometry and specialist referral (Case H). Early diagnosis allows stem cell transplant or gene therapy before infections and before live vaccines such as BCG or rotavirus are given.

16.6 Inborn errors of immunity

- The IUIS classification (2022 update — Part B) lists about 485 inborn errors of immunity, grouped into 10 categories.
- **Testing:** targeted gene panels, exome or genome sequencing, with copy-number analysis; interpretation follows ACMG/AMP variant classification (pathogenic, likely pathogenic, VUS, likely benign, benign).
- **Examples:** X-linked agammaglobulinaemia (*BTK*), X-linked chronic granulomatous disease (*CYBB*), SCID (*IL2RG*, *ADA*, *RAG1/2*), familial haemophagocytic lymphohistiocytosis (*PRF1*, *UNC13D*), activated PI3K-delta syndrome (*PIK3CD*), hereditary angioedema (*SERPING1*).
- **Functional confirmation** (flow cytometry, protein expression, neutrophil oxidative burst) is often needed to classify variants.

16.7 Host genetics and host-response testing in infection

- **CCR5 Δ 32** homozygosity protects against CCR5-tropic HIV; **HIV tropism testing** (V3 loop genotype with geno2pheno) is needed before using maraviroc.
- **Host-response signatures:** blood transcriptomic signatures (e.g. a two-gene signature distinguishing bacterial from viral infection in febrile children) and interferon-stimulated gene signatures are being developed to support antimicrobial stewardship.
- **Immunoglobulin and T-cell receptor clonality testing** (gene rearrangement PCR) distinguishes clonal from reactive lymphoid proliferations — overlapping with haematopathology.

Check your understanding

1. A patient is HLA-B*57:01 positive. What is the clinical action?
2. Recipient-derived cells in the T-cell fraction rise from 2% to 15% over two months after transplant. What might this mean?
3. Why do TRECs reflect recent thymic output?

Unit 17 — Quality, verification and validation

Molecular infection tests sit inside the same quality system as all laboratory tests — ISO 15189, IQC, EQA — with extra attention to contamination, target sequence variation and commutability of standards.

Learning outcomes

1. Distinguish verification from validation.
2. Plan the performance studies for qualitative and quantitative molecular assays.
3. Set up IQC and interpret Levey–Jennings charts.
4. Describe EQA schemes and accreditation requirements.

17.1 Verification vs validation

	Verification	Validation
Applies to	Unmodified CE-IVD/IVDR-marked or FDA-cleared assays used as intended	Laboratory-developed tests, modified kits, new specimen types, NGS and metagenomic tests
Question	Does it perform as claimed in our hands?	Does it meet our clinical needs? (we set the claims)
Extent	Accuracy, precision, LoD confirmation, carry-over	All performance characteristics

17.2 Performance characteristics

Characteristic	Qualitative (detection)	Quantitative (viral load)
Accuracy	Agreement with reference method on positive and negative samples (positive and negative percent agreement)	Bias against reference; Bland–Altman on $\log_{10}$ values
Precision	Repeatability of results across runs, days, operators	SD of $\log_{10}$ values at low, mid, high levels
Limit of detection	Lowest concentration detected in $\geq 95\%$ of replicates (probit analysis)	LoD plus lower limit of quantification
Inclusivity	Detects all relevant genotypes, subtypes, lineages; in silico check of primer/probe sites	Quantifies all genotypes equivalently
Exclusivity (cross-reactivity)	No detection of related organisms and normal flora	Same
Interference	Blood, mucus, medications (e.g. nasal sprays), transport media	Same
Linearity / reportable range	—	Dilution series across the claimed range
Carry-over	High positives next to negatives	Same
Specimen stability	Time and temperature studies	Same

The validation guide by Burd (*Clin Microbiol Rev* 2010 — Part B) covers each step in detail.

17.3 Internal quality control

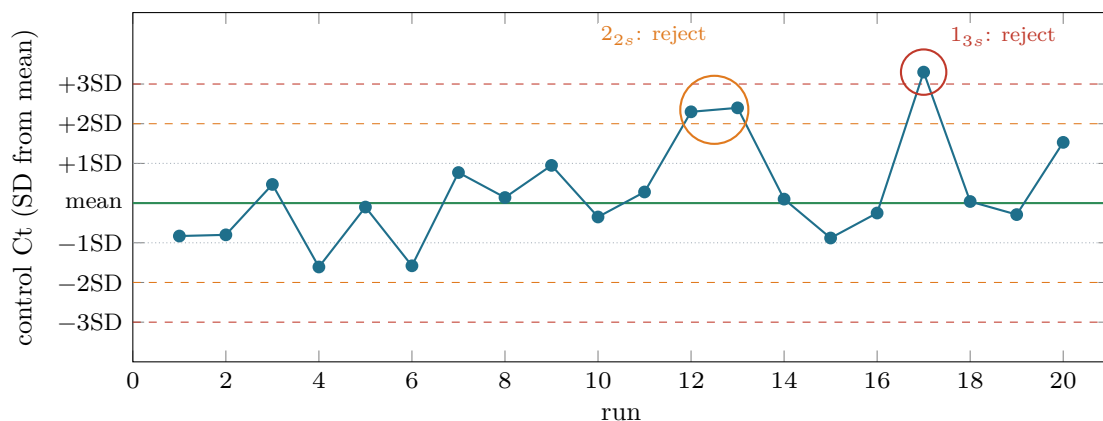


Figure 26: Levey–Jennings chart of a positive control's Ct with Westgard rule violations, used exactly as for chemistry IQC.

- **Per run:** positive control near the clinical decision point, negative control, NTC, internal control per sample.
- **Third-party controls** (independent of the kit) detect lot-to-lot problems.
- **Quantitative assays:** chart $\log_{10}$ control values or Ct on Levey–Jennings charts and apply Westgard rules (1-3s reject, 2-2s reject, 1-2s warning).
- **NGS:** positive control organism or library per run; negative controls; coverage thresholds per sample.
- **Target failure surveillance:** monitor for patterns such as single-target dropout in dual-target assays — it may reveal a new variant (Case G).

17.4 External quality assessment

Provider	Examples of molecular schemes
QCMD (Quality Control for Molecular Diagnostics)	Viral loads, respiratory and STI panels, TB, pathogen NGS
UK NEQAS Microbiology	Molecular detection of viruses, bacteria, fungi, parasites
INSTAND	Virology and bacteriology nucleic acid tests
CAP	Molecular microbiology surveys
WHO networks	TB, polio, measles/rubella, influenza laboratory networks
UK NEQAS for H&I / EFI-linked schemes	HLA typing and chimerism

17.5 Accreditation and regulation

- **ISO 15189:2022** — examination validation/verification, measurement uncertainty, IQC, EQA, risk management, competence of staff; bioinformatics pipelines are part of the examination.
- **EU IVDR** (Regulation 2017/746) — conditions for in-house tests in health institutions; other countries have their own frameworks.
- **Measurement uncertainty** for viral loads is usually derived from long-term IQC imprecision in $\log_{10}$ units.

Unit 18 — Reporting and interpretation

A molecular microbiology report must state clearly what was detected, how much, what was not tested, and what the result means for the patient.

Learning outcomes

1. Use correct wording for detected, not detected, invalid and quantitative results.
2. Write interpretive comments for common situations.
3. Apply notification, confidentiality and data-governance rules.

18.1 Report elements

Element	Notes
Patient and specimen	Two identifiers; specimen type and site; collection date and time
Test	Name, method, targets, and limitations (e.g. “does not detect HIV-2”)
Result	Detected / Not detected / Invalid / Indeterminate; quantitative value with units ($\log_{10}$ and IU/mL or copies/mL)
Interpretation	Clinical meaning, change from previous result
Recommendations	Repeat testing, further tests, specialist advice
Authorisation	Name and role; accreditation status

18.2 Wording examples

Not detected (qualitative): “HSV-1 and HSV-2 DNA not detected. A negative result early in the illness does not exclude HSV encephalitis; if clinical suspicion remains, repeat CSF testing in 3–7 days is recommended.”

Invalid: “Result invalid due to inhibition (internal control failure). Please send a repeat specimen.”

Viral load below quantification: “HIV-1 RNA detected, < 50 copies/mL (below the lower limit of quantification).”

Change in viral load: “CMV DNA 9,800 IU/mL ($3.99 \log_{10}$), increased from 2,400 IU/mL ($3.38 \log_{10}$) on [date] — a significant rise ($> 0.5 \log_{10}$).”

Resistance: “HIV-1 reverse transcriptase mutations M184V and K103N detected, conferring high-level resistance to lamivudine/emtricitabine and efavirenz/nevirapine (Stanford HIVdb version [x]). Discuss with the HIV team.”

Syndromic panel: “Rhinovirus/enterovirus RNA detected. Prolonged shedding can occur after resolved infection; interpret with clinical findings.”

18.3 Governance

- **Notifiable infections** must be reported to public health authorities as required by national law.
- **Confidentiality:** HIV, STI and genetic results require particular care in access and communication.
- **Pathogen genome data:** remove human reads before sharing; follow national rules for linking genomes to patient data.
- **Result communication:** urgent results (e.g. meningococcal or HSV detection in CSF, carbapenemase producers, multidrug-resistant TB) are telephoned according to local policy.

Practical exercises

Exercise 1 — Standard curve and quantification

A standard curve has slope -3.45 , intercept 39.8 and R^2 0.997 . A sample has Ct 27.1 . Calculate the efficiency and the $\log_{10}$ concentration.

Answer. $E = 10^{1/3.45} - 1 = 0.95 \rightarrow 95\%$ (acceptable). $\log_{10}$ concentration = $(27.1 - 39.8) / -3.45 = 3.68$ (about 4,800 IU/mL).

Exercise 2 — Is the change significant?

HBV DNA results: 18,000 IU/mL then 32,000 IU/mL. Is this a real increase?

Answer. $\log_{10}$ 18,000 = 4.26; $\log_{10}$ 32,000 = 4.51; difference 0.25 log — below the 0.5 log threshold, so within assay variation.

Exercise 3 — Digital PCR

20,000 partitions, 5,000 positive, partition volume 0.85 nL. Calculate copies/ μ L.

Answer. $p = 0.25$; $\lambda = -\ln(0.75) = 0.288$ copies per partition; $0.288 / 0.00085 \mu\text{L} \approx 338$ copies/ μ L.

Exercise 4 — Pooling

A laboratory pools 8 samples. A single positive sample has Ct 34 when tested alone, and the assay's limit is Ct 37. Will the pool be detected?

Answer. Dilution $1:8 \approx 3$ Ct $\rightarrow$ expected Ct about 37 — at the limit; the pool may be missed. Pooling reduces sensitivity for weak positives.

Exercise 5 — SNP distances

Five MRSA isolates from a neonatal unit have pairwise SNP distances: A–B 2, A–C 4, B–C 3, A–D 87, A–E 112. Which isolates may be part of an outbreak?

Answer. A, B and C are closely related and consistent with transmission (check timing and ward location); D and E are unrelated introductions.

Exercise 6 — Verification plan

Outline a verification for a new CE-IVD multiplex respiratory panel.

Answer outline. Accuracy: 50–100 retrospective samples covering each target (including low positives) and negatives, compared with the current method or reference; precision: low positives in replicate over several days; LoD confirmation for key targets; carry-over check; specimen types and transport media used locally; IQC and EQA plan; staff training; sign-off.

Case studies

Case A — CMV after kidney transplant

A recipient (donor CMV-positive, recipient negative) has plasma CMV DNA of 800, 2,400 and 9,800 IU/mL on weekly testing. After 3 weeks of valganciclovir at the correct dose the load is 15,000 IU/mL.

Discussion. Each rise exceeds 0.5 log — significant replication; start pre-emptive treatment per local threshold. A rising load after 2 or more weeks of adequate therapy suggests resistance: send for UL97 and UL54 genotyping (and UL56 if letermovir was used); review immunosuppression and drug levels.

Case B — HIV treatment failure

On tenofovir/emtricitabine/efavirenz, HIV RNA is 4,500 copies/mL on two occasions. Genotype: M184V, K103N.

Discussion. Confirmed virological failure. M184V → high-level lamivudine/emtricitabine resistance (but reduced viral fitness); K103N → efavirenz and nevirapine resistance. Address adherence; construct a new regimen with expert advice, guided by HIVdb interpretation.

Case C — Suspected HSV encephalitis

Day 1 CSF HSV PCR is negative; MRI shows temporal lobe changes.

Discussion. HSV PCR can be negative early. Continue aciclovir and repeat CSF PCR at 3–7 days; consider VZV, enterovirus, autoimmune encephalitis.

Case D — Encephalitis of unknown cause

Extensive testing is negative in an immunocompromised patient; CSF mNGS detects *Leptospira* reads above threshold, absent from negative controls.

Discussion. Plausible clinically and epidemiologically; confirm with targeted PCR or serology; treat. Shows the value of mNGS when targeted tests do not include the pathogen.

Case E — Carbapenemase outbreak

Four ICU *K. pneumoniae* isolates carry *blaKPC*. WGS: three are ST258 within 0–3 SNPs; one is ST15, more than 400 SNPs away.

Discussion. Clonal transmission of the ST258 strain; the ST15 isolate may have acquired the KPC plasmid by horizontal transfer — use long-read sequencing to compare plasmids. Infection control should address both clonal and plasmid spread.

Case F — Rifampicin resistance in a low-prevalence setting

Xpert MTB/RIF Ultra: “MTB detected low; rifampicin resistance detected” in a patient with no risk factors.

Discussion. The positive predictive value of resistance detection is lower where resistance is rare; silent mutations can give false resistance. Repeat Xpert on a second specimen and confirm with sequencing (targeted NGS or *rpoB* Sanger) and phenotypic testing before changing to an MDR regimen.

Case G — Target dropout

A dual-target SARS-CoV-2 assay suddenly shows many positives with one target negative.

Discussion. Likely a deletion in a new variant under the primer or probe site (as with S-gene target failure in the Alpha variant). Report positive based on the other target, inform the manufacturer and public health, sequence a selection, and review assay inclusivity.

Case H — SCID newborn screen

TRECs are undetectable on a newborn dried blood spot; repeat confirms.

Discussion. Urgent referral: lymphocyte subsets by flow cytometry, isolation precautions, no live vaccines (BCG, rotavirus), irradiated CMV-negative blood products, genetic testing; plan for definitive therapy.

Case I — Abacavir

A patient starting antiretroviral therapy is HLA-B*57:01 positive.

Discussion. Abacavir must not be prescribed; record as an allergy/contraindication in the health record.

Case J — Contamination event

Six unrelated patients on one run show low-level positives for the same target (Ct 36–38); the NTC is negative.

Discussion. Suspect contamination (e.g. positive control or a high-titre sample handled earlier). Hold results, repeat from the primary specimens on a new run, review plate layout, check environmental swabs, record a non-conformity.

Case K — Influenza in a stem cell transplant recipient

Influenza A (H1N1) RNA persists after 10 days of oseltamivir.

Discussion. Test for NA H275Y; if present, oseltamivir resistance — consider alternatives (e.g. zanamivir or baloxavir, depending on availability and guidance) and infection control.

Case L — Very high HHV-6 DNA

A well child has HHV-6 DNA of 6.5 log₁₀ copies/mL in whole blood on repeated testing.

Discussion. Suspect inherited chromosomally integrated HHV-6 (about one viral copy per cell). Confirm with hair follicle testing or ddPCR ratio; record the finding so future results are not misinterpreted as active infection. Antiviral treatment is not indicated.

Case M — *Pneumocystis* PCR in two patients

Patient 1 has HIV (CD4 30 cells/ μ L), hypoxia and diffuse infiltrates; BAL *Pneumocystis* PCR is strongly positive (Ct 24). Patient 2 has rheumatoid arthritis on methotrexate, a mild cough and a normal CT; BAL PCR is weakly positive (Ct 37).

Discussion. Patient 1: consistent with pneumocystis pneumonia — treat. Patient 2: low load with low clinical probability may represent colonisation; interpret with laboratory thresholds, β -D-glucan, imaging and clinical course. A negative BAL PCR would have made pneumocystis pneumonia very unlikely.

Case N — Malaria in a returning traveller

A traveller returning from Malaysian Borneo has fever and a parasitaemia of 2%; microscopy suggests *P. malariae*, but the patient is severely ill.

Discussion. *P. knowlesi* resembles *P. malariae* on microscopy but can cause severe, rapidly rising parasitaemia. Species-specific PCR confirms the diagnosis; manage as potentially severe malaria.

Case O — Amoebae in stool

Microscopy of a traveller's stool shows *Entamoeba* cysts; the patient is asymptomatic.

Discussion. Microscopy cannot separate pathogenic *E. histolytica* from non-pathogenic *E. dispar*. Species-specific PCR guides whether treatment is needed and avoids unnecessary therapy.

Self-assessment questions

1. Name the Baltimore group of HIV-1 and of hepatitis B virus.
2. Why can a single-target assay fail for a rapidly evolving virus?
3. Which tube additive must be avoided for PCR, and why?
4. What is the role of an internal control?
5. A 10-fold dilution changes Ct by about how much?
6. What efficiency corresponds to a slope of -3.32 ?
7. What is the minimum change in viral load usually regarded as significant?
8. Why are CMV loads from two laboratories not directly comparable?
9. What is ciHHV-6 and how is it confirmed?
10. Give two pitfalls of syndromic gastrointestinal panels.
11. What does NEAR stand for, and which platform uses it?
12. Classify bDNA and HC2 by amplification type.
13. How many primers does LAMP use, and what do the loop primers do?
14. What is collateral cleavage in CRISPR diagnostics?
15. Which gene region is targeted to detect rifampicin resistance in TB?
16. Why is genotypic absence of a carbapenemase not proof of susceptibility?
17. What HIV mutation causes high-level lamivudine resistance?
18. Which CMV gene is most often mutated in ganciclovir resistance?
19. What frequency of minority variant can Sanger reliably detect?
20. What is the ARTIC protocol used for?
21. Distinguish reference mapping from de novo assembly.
22. What is MLST?
23. What does a bootstrap value on a tree indicate?
24. Why are negative controls essential in metagenomic sequencing?
25. Which region is sequenced for broad-range fungal identification?
26. What SNP threshold suggests recent TB transmission?
27. Define two-field HLA resolution.
28. Which HLA allele must be screened before abacavir?
29. What does a low TREC result on newborn screening suggest?
30. Define LoD for a qualitative molecular assay.
31. Which rDNA regions are used for fungal species identification?
32. Why can a weakly positive *Pneumocystis* PCR be difficult to interpret?
33. Which *Plasmodium* species is often misidentified as *P. malariae* by microscopy?
34. What gene deletions cause false-negative HRP2 malaria rapid tests?
35. Name a molecular marker of artemisinin partial resistance.

Answers

Q	Answer
1	HIV-1: group VI; HBV: group VII
2	Mutations or deletions under primers/probes cause target failure or under-quantification
3	Heparin — inhibits Taq polymerase
4	Shows extraction and amplification worked; a failed internal control makes a negative result invalid
5	About 3.3 Ct
6	100%
7	$0.5 \log_{10}$ (about 3-fold)
8	Different assays, calibrators, specimen types; incomplete commutability of the WHO standard
9	Inherited chromosomally integrated HHV-6; hair/nail testing or ddPCR viral:human gene ratio
10	Detection of carriage; <i>C. difficile</i> colonisation; multiple detections of uncertain significance
11	Nicking enzyme amplification reaction; ID NOW

Q	Answer
12	Both signal amplification
13	4–6 primers; loop primers accelerate the reaction
14	Activated Cas12/Cas13 cleaves nearby non-target reporter molecules, generating signal
15	The 81-bp rifampicin resistance-determining region of <i>rpoB</i>
16	Other mechanisms (porin loss, efflux, uncommon enzymes) are not detected
17	M184V (or M184I)
18	UL97
19	About 15–20% or more
20	Tiled multiplex amplicon sequencing of viral genomes directly from specimens
21	Mapping aligns reads to a known reference; de novo assembly builds contigs without one
22	Multilocus sequence typing — alleles of 7 housekeeping genes define a sequence type
23	Statistical support for a grouping (clade)
24	Reagent and environmental contaminants (“kitome”) are common; detections must exceed controls
25	Internal transcribed spacer (ITS) region
26	≤ 5 SNPs (with > 12 making recent transmission unlikely)
27	Identifies the specific HLA protein (e.g. A*02:01)
28	HLA-B*57:01
29	Severe combined immunodeficiency or other T-cell lymphopenia
30	Lowest concentration detected in $\geq 95\%$ of replicates
31	ITS1 and ITS2 (the fungal barcode) and the D1/D2 region of 28S rDNA
32	Low loads may reflect colonisation rather than pneumonia, especially in non-HIV patients
33	<i>P. knowlesi</i>
34	<i>pfhrp2</i> and <i>pfhrp3</i>
35	<i>kelch13</i> propeller mutations (e.g. C580Y)

Glossary and key formulas

Formulas

$$E = 10^{-1/\text{slope}} - 1 \quad \text{Fold change} = 2^{-\Delta\Delta C_t} \quad \lambda = -\ln(1 - p) \quad Q = -10 \log_{10} P$$

$$\Delta \log = \log_{10}(\text{VL}_2) - \log_{10}(\text{VL}_1) \quad (\text{significant if } > 0.5)$$

Glossary

Term	Meaning
Amplicon	Product of a nucleic acid amplification reaction
Carrier RNA	Poly-A RNA added to improve recovery of low-level nucleic acid
cgMLST	Core-genome multilocus sequence typing
Commutability	Whether a reference material behaves like patient samples across assays
Consensus genome	The majority base at each position from sequencing reads
Ct (Cq)	Cycle at which fluorescence crosses the threshold
De novo assembly	Building a genome from reads without a reference
Heteroresistance	Coexistence of susceptible and resistant subpopulations
HLA	Human leukocyte antigen genes of the major histocompatibility complex
ITS	Internal transcribed spacer regions of ribosomal DNA; the fungal DNA barcode
rDNA	Ribosomal RNA genes; multi-copy targets for fungi and parasites
Index (barcode)	Sequence identifying a sample in pooled sequencing
IU	International unit, defined by a WHO International Standard
Kitome	Contaminating DNA in reagents and kits
LoD / LLoQ	Limit of detection / lower limit of quantification
Metagenomics	Sequencing all nucleic acid in a sample
Minority variant	A variant present in a fraction of a pathogen population
MLST	Multilocus sequence typing
NTC	No-template control
Phylogeny	Tree showing evolutionary relationships
Quasispecies	A population of related viral variants within a host
RRDR	Rifampicin resistance-determining region of <i>rpoB</i>
SNP distance	Number of single nucleotide differences between two genomes
STR	Short tandem repeat (used in chimerism and identity testing)
TREC / KREC	T-cell / kappa-deleting recombination excision circles
VAF	Variant allele frequency (fraction of reads with a variant)
WGS	Whole-genome sequencing

All figures in this resource are original schematics drawn for teaching. Thresholds, mutation lists and guideline details change: check the current version of each guideline before clinical use.

Part B

Self-study reading list

Free, open-access resources mapped to every unit

Part B — Self-study reading list

How to use this reading list

Every item is free to read: open-access papers in PubMed Central (PMC), free guidelines, textbooks, courses, databases and tools. Paper links go to the free full text; each PMC identifier was checked on 23 October 2026. **Core** items support the unit directly; **Extra** items are for deeper study.

Not listed because they are not free: standard textbooks such as the *Manual of Clinical Microbiology* and *Clinical Virology*, the MIQE guidelines paper, and ISO 15189. Use them through a library where available.

B1. Foundations (Units 1–4)

Resource	Type	What it covers	Use	Time
OpenStax <i>Biology 2e</i>	Free textbook	Ch. 14–17: DNA, genes, gene expression, biotechnology; Ch. 21–22: viruses, prokaryotes	Core	4 h
Baron (ed.), <i>Medical Microbiology</i> (4th ed.), NCBI Bookshelf	Free textbook (older edition)	Bacterial, viral, fungal and parasite biology	Core	as needed
ViralZone (SIB)	Free website	One-page summaries and genome diagrams for every virus family	Core	ongoing
ICTV	Free website	Current virus taxonomy	Extra	as needed
Domingo E et al. Viral quasispecies evolution. <i>Microbiol Mol Biol Rev</i> 2012	Review (PMC3372249)	Mutation rates, quasispecies, implications for resistance	Core	1 h
Lauring AS, Andino R. Quasispecies theory and the behavior of RNA viruses. <i>PLoS Pathog</i> 2010	Review (PMC2908548)	Short, clear introduction to quasispecies	Extra	30 min
WHO <i>Laboratory Biosafety Manual</i> , 4th edition	Free PDF	Risk-based biosafety	Core	1 h
UK Standards for Microbiology Investigations (RCPath)	Free standards	Specimen handling and investigation algorithms by syndrome	Core	as needed
Tan SC, Yiap BC. DNA, RNA, and protein extraction: the past and the present. <i>J Biomed Biotechnol</i> 2009	Review (PMC2789530)	Extraction chemistries	Core	45 min
Ali N et al. Current nucleic acid extraction methods and their implications to point-of-care diagnostics. <i>Biomed Res Int</i> 2017	Review (PMC5529626)	Lysis, solid-phase, automated and point-of-care extraction	Core	45 min

B2. PCR, real-time PCR and viral load (Units 5–7)

Resource	Type	What it covers	Use	Time
DNA Learning Center: PCR animation	Animation	The PCR cycle	Core	15 min

Resource	Type	What it covers	Use	Time
Kralik P, Ricchi M. A basic guide to real time PCR in microbial diagnostics. <i>Front Microbiol</i> 2017	Review (PMC5288344)	Chemistries, Ct, quantification, controls, validation	Core	1 h
Thermo Fisher <i>Real-time PCR Handbook</i>	Vendor handbook (free, registration)	Design, analysis, troubleshooting	Extra	2 h
Primer-BLAST and NCBI Virus	Free tools	Design primers; check conservation across viral sequences	Core (practical)	1 h
Hayden RT et al. Commutability of the first WHO International Standard for human cytomegalovirus. <i>J Clin Microbiol</i> 2015	Paper (PMC4572535)	Why CMV loads differ between laboratories	Core	45 min
Hayden RT et al. Comparative evaluation of three commercial quantitative CMV standards. <i>J Clin Microbiol</i> 2015	Paper (PMC4400765)	Secondary standards and bias	Extra	30 min
WHO consolidated HIV guidelines (2021)	Free guideline	Viral load monitoring, treatment failure, infant diagnosis	Core	2 h (selected chapters)
US HIV clinical guidelines (clinicalinfo.hiv.gov)	Free guideline	Viral load, resistance testing, HLA-B*57:01	Extra	as needed
AASLD–IDSA HCV guidance	Free guideline	HCV RNA testing and cure assessment	Extra	1 h

B3. Syndromic panels, digital PCR and other amplification methods (Units 8–9)

Resource	Type	What it covers	Use	Time
Ramanan P et al. Syndromic panel-based testing in clinical microbiology. <i>Clin Microbiol Rev</i> 2018	Review (PMC5740973)	Panel design, performance, interpretation, stewardship	Core	1.5 h
Quan PL et al. dPCR: a technology review. <i>Sensors</i> 2018	Review (PMC5948698)	Digital PCR principles and applications	Core	1 h
Fakruddin M et al. Nucleic acid amplification: alternative methods of PCR. <i>J Pharm Bioallied Sci</i> 2013	Review (PMC3831736)	LAMP, NASBA, TMA, SDA, RCA, HDA, LCR, bDNA	Core	1 h
Zanoli LM, Spoto G. Isothermal amplification methods for the detection of nucleic acids. <i>Biosensors</i> 2012	Review (PMC4263587)	Mechanisms with diagrams	Core	1 h
Notomi T et al. Loop-mediated isothermal amplification of DNA. <i>Nucleic Acids Res</i> 2000	Original LAMP paper (PMC102748)	Primer design and mechanism	Core	30 min

Resource	Type	What it covers	Use	Time
Wong YP et al. LAMP: a versatile technique for detection of micro-organisms. <i>J Appl Microbiol</i> 2018	Review (PMC7167136)	Readouts, uses, limitations	Core	45 min
Soroka M et al. LAMP: the better sibling of PCR? <i>Cells</i> 2021	Review (PMC8393631)	LAMP vs PCR	Extra	45 min
Gootenberg JS et al. Nucleic acid detection with CRISPR-Cas13a/C2c2. <i>Science</i> 2017	SHERLOCK paper (PMC5526198)	Cas13a diagnostics	Core	45 min
Chen JS et al. CRISPR-Cas12a target binding unleashes indiscriminate ssDNase activity. <i>Science</i> 2018	DETECTR paper (PMC6628903)	Cas12a diagnostics (HPV)	Core	45 min
Broughton JP et al. CRISPR-Cas12-based detection of SARS-CoV-2. <i>Nat Biotechnol</i> 2020	Paper (PMC9107629)	Clinical CRISPR assay vs RT-PCR	Core	45 min
Kellner MJ et al. SHERLOCK: nucleic acid detection with CRISPR nucleases. <i>Nat Protoc</i> 2019	Protocol (PMC6956564)	Practical workflow, lateral flow	Extra	1 h

B4. Antimicrobial resistance (Unit 10)

Resource	Type	What it covers	Use	Time
Lawn SD et al. Advances in tuberculosis diagnostics: the Xpert MTB/RIF assay. <i>Lancet Infect Dis</i> 2013	Review (PMC4844338)	How Xpert detects TB and rifampicin resistance	Core	1 h
Walker TM et al. Whole-genome sequencing for prediction of <i>M. tuberculosis</i> drug susceptibility. <i>Lancet Infect Dis</i> 2015	Paper (PMC4579482)	Genotypic prediction of TB resistance	Core	45 min
WHO catalogue of mutations in <i>M. tuberculosis</i> complex, 2nd edition (2023)	Free WHO document	Graded resistance mutations	Core (reference)	as needed
Tang MW, Shafer RW. HIV-1 antiretroviral resistance: scientific principles and clinical applications. <i>Drugs</i> 2012	Review (PMC3689909)	HIV resistance mechanisms and testing	Core	1 h
Stanford HIV Drug Resistance Database	Free tool	HIV (and other) genotype interpretation; mutation notes	Core (practical)	1 h
geno2pheno	Free tool	HIV tropism and resistance	Extra	30 min
Alcock BP et al. CARD 2023. <i>Nucleic Acids Res</i> 2023 and CARD website	Paper (PMC9825576) and tool	Bacterial resistance gene database	Core	45 min

Resource	Type	What it covers	Use	Time
ResFinder (Center for Genomic Epidemiology)	Free tool	Resistance genes from bacterial genomes	Core (practical)	30 min
TB-Profiler	Free tool	TB lineage and resistance from WGS	Extra	30 min

B5. Sequencing and bioinformatics (Units 11–12)

Resource	Type	What it covers	Use	Time
Behjati S, Tarpey PS. What is next generation sequencing? <i>Arch Dis Child Educ Pract Ed</i> 2013	Short introduction (PMC3841808)	NGS for clinicians	Core (start here)	20 min
Heather JM, Chain B. The sequence of sequencers. <i>Genomics</i> 2016	Review (PMC4727787)	Sanger to third generation	Core	45 min
Goodwin S et al. Coming of age: ten years of NGS technologies. <i>Nat Rev Genet</i> 2016	Review (PMC10373632)	Platform chemistries and error profiles	Core	1 h
Slatko BE et al. Overview of next-generation sequencing technologies. <i>Curr Protoc Mol Biol</i> 2018	Review (PMC6020069)	Library preparation and platforms	Extra	1 h
Didelot X et al. Transforming clinical microbiology with bacterial genome sequencing. <i>Nat Rev Genet</i> 2012	Review (PMC5049685)	Bacterial WGS in the clinic	Core	1 h
Balloux F et al. From theory to practice: translating whole-genome sequencing into the clinic. <i>Trends Microbiol</i> 2018	Review (PMC6249990)	Practical barriers and uses	Core	45 min
Quick J et al. Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples. <i>Nat Protoc</i> 2017	Protocol (PMC5902022)	Amplicon tiling (basis of ARTIC)	Core	1 h
Grubaugh ND et al. An amplicon-based sequencing framework for accurately measuring intrahost virus diversity (PrimalSeq and iVar). <i>Genome Biol</i> 2019	Paper (PMC6325816)	Minority variants and their accuracy	Extra	45 min
Rambaut A et al. A dynamic nomenclature proposal for SARS-CoV-2 lineages. <i>Nat Microbiol</i> 2020	Paper (PMC7610519)	Pango lineage system	Extra	30 min
ARTIC Network	Free protocols	Viral amplicon sequencing protocols and software	Extra	as needed
Galaxy Training: SARS-CoV-2 variant analysis	Free hands-on tutorial	Mapping, variant calling, consensus	Core (practical)	2–3 h

Resource	Type	What it covers	Use	Time
Galaxy Training: genome annotation and assembly topics	Free hands-on tutorials	Bacterial genome assembly and annotation	Extra (practical)	3–4 h
Nextclade	Free web tool	Clade assignment, QC, mutations	Core (practical)	30 min
PubMLST	Free database	MLST and cgMLST schemes	Core (practical)	30 min

B6. Metagenomics, 16S/ITS and genomic epidemiology (Units 13–14)

Resource	Type	What it covers	Use	Time
Gu W, Miller S, Chiu CY. Clinical metagenomic NGS for pathogen detection. <i>Annu Rev Pathol</i> 2019	Review (PMC6345613)	mNGS workflow and interpretation	Core	1 h
Chiu CY, Miller SA. Clinical metagenomics. <i>Nat Rev Genet</i> 2019	Review (PMC6858796)	Applications and challenges	Core	1 h
Wilson MR et al. Clinical metagenomic sequencing for diagnosis of meningitis and encephalitis. <i>N Engl J Med</i> 2019	Study (PMC6764751)	Prospective clinical evaluation	Core	45 min
Church DL et al. Performance and application of 16S rRNA gene cycle sequencing in the clinical microbiology laboratory. <i>Clin Microbiol Rev</i> 2020	Review (PMC7484979)	16S sequencing for identification	Core	1 h
Gardy JL, Loman NJ. Towards a genomics-informed, real-time, global pathogen surveillance system. <i>Nat Rev Genet</i> 2018	Review (PMC7097748)	Genomic surveillance	Core	1 h
Quick J et al. Real-time, portable genome sequencing for Ebola surveillance. <i>Nature</i> 2016	Paper (PMC4817224)	Nanopore sequencing in an outbreak	Extra	45 min
Walker TM et al. Whole-genome sequencing to delineate <i>M. tuberculosis</i> outbreaks. <i>Lancet Infect Dis</i> 2013	Paper (PMC3556524)	Origin of TB SNP thresholds	Core	45 min
Hadfield J et al. Nextstrain: real-time tracking of pathogen evolution. <i>Bioinformatics</i> 2018	Paper (PMC6247931) and Nextstrain	Phylogenetic tracking	Core	30 min
Microreact and Pathogenwatch	Free tools	Linking trees, maps, timelines; bacterial surveillance	Extra (practical)	1 h
Galaxy Training: microbiome topic	Free hands-on tutorials	Metagenomic classification, 16S analysis	Extra (practical)	3 h

B7. Mycology and parasitology (Unit 15)

Resource	Type	What it covers	Use	Time
Wickes BL, Wiederhold NP. Molecular diagnostics in medical mycology. <i>Nat Commun</i> 2018	Review (PMC6277409)	Fungal PCR, sequencing, identification	Core	1 h
Donnelly JP et al. Revision and update of the consensus definitions of invasive fungal disease (EORTC/MSGERC). <i>Clin Infect Dis</i> 2020	Consensus (PMC7486838)	Where PCR fits in diagnostic criteria	Core	1 h
White PL et al. <i>Aspergillus</i> PCR: one step closer to standardization. <i>J Clin Microbiol</i> 2010	Paper (PMC2849576)	EAPCRI standardisation	Extra	45 min
Schoch CL et al. Nuclear ribosomal ITS region as a universal DNA barcode marker for Fungi. <i>PNAS</i> 2012	Paper (PMC3341068)	ITS barcode	Extra	30 min
Verweij JJ, Stensvold CR. Molecular testing for clinical diagnosis and epidemiological investigations of intestinal parasitic infections. <i>Clin Microbiol Rev</i> 2014	Review (PMC3993103)	Stool parasite PCR	Core	1.5 h
Ariey F et al. A molecular marker of artemisinin-resistant <i>P. falciparum</i> malaria. <i>Nature</i> 2014	Paper (PMC5007947)	Discovery of <i>kelch13</i>	Core	45 min
Imwong M et al. Spread of a single multidrug resistant malaria parasite lineage. <i>Lancet Infect Dis</i> 2017	Paper (PMC5406483)	Genomic surveillance of resistance	Extra	45 min
CDC DPDx — laboratory identification of parasites	Free website	Parasite biology, diagnostics including molecular methods	Core	as needed
MalariaGEN	Free resource	<i>Plasmodium</i> genomic data and resistance markers	Extra	as needed

B8. Molecular immunology (Unit 16)

Resource	Type	What it covers	Use	Time
Marsh SGE et al. Nomenclature for factors of the HLA system, 2010. <i>Tissue Antigens</i> 2010	Nomenclature report (PMC2848993)	HLA naming rules	Core	45 min
HLA Nomenclature (hla.alleles.org)	Free website	Current nomenclature and allele lists	Core	as needed
Martin MA et al. CPIC guidelines for HLA-B genotype and abacavir dosing: 2014 update. <i>Clin Pharmacol Ther</i> 2014 and CPIC page	Guideline (PMC3994233)	HLA-B*57:01 screening	Core	30 min

Resource	Type	What it covers	Use	Time
Kwan A et al. Newborn screening for severe combined immunodeficiency in 11 screening programs in the United States. <i>JAMA</i> 2014	Study (PMC4492158)	TREC screening outcomes	Core	45 min
Tangye SG et al. Human inborn errors of immunity: 2022 update on the classification from the IUIS Expert Committee. <i>J Clin Immunol</i> 2022	Classification (PMC9244088)	Inborn errors of immunity and genes	Core (reference)	as needed
Richards S et al. ACMG/AMP standards for the interpretation of sequence variants. <i>Genet Med</i> 2015	Guideline (PMC4544753)	Classifying variants in immunodeficiency genes	Core	1.5 h
Herberg JA et al. Diagnostic test accuracy of a 2-transcript host RNA signature for discriminating bacterial vs viral infection in febrile children. <i>JAMA</i> 2016	Study (PMC5997174)	Host-response diagnostics	Extra	45 min

B9. Quality, validation and reporting (Units 17–18)

Resource	Type	What it covers	Use	Time
Burd EM. Validation of laboratory-developed molecular assays for infectious diseases. <i>Clin Microbiol Rev</i> 2010	Review (PMC2901657)	Step-by-step validation of infection assays	Core	1.5 h
Koboldt DC. Best practices for variant calling in clinical sequencing. <i>Genome Med</i> 2020	Review (PMC7586657)	Variant calling QC and artefacts	Extra	1 h
UK Standards for Microbiology Investigations (RCPATH)	Free standards	Reporting and quality guidance	Core	as needed

B10. Free courses and tools to bookmark

Resource	Use
EMBL-EBI online training	Genetic variation and bioinformatics courses (free)
Galaxy Training Network	Hands-on, browser-based NGS analysis
NCBI BLAST	Identify sequences (16S, ITS, viral genes)
NCBI Pathogen Detection	Public bacterial isolate genomes and resistance genes
NCBI Virus	Viral sequence data
IGV web app	Viewing read alignments
Stanford HIVdb	HIV resistance interpretation
Nextclade and Nextstrain	Viral clades and phylogenetic tracking
CARD, ResFinder, TB-Profler	Resistance prediction from genomes
PubMLST	Bacterial typing

Resource	Use
ViralZone	Virus family summaries
HLA Nomenclature	HLA alleles

B11. Reading log

Date	Item read	Three key points	Question for supervisor
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