AmPORE-TB

# Protocol

Research Use Only

| | |
|---|---|
| REF | OND-S-ATB01-RGL |
| For use with | OND-FM001-RGL \| Q-GRD-Mk1-ATB \| OND-E-FRK01-RGL |
| Σ | 96 tests including controls |

For Research Use Only. Not for use in diagnostic procedures.

The AmPORE-TB™ product has not undergone any regulatory agency review and is not certified under EU or UK medical device regulations.

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# Contents

# Version history

| Version | Date | Description of change |
|---|---|---|
| 1 | 01-OCT-2024 | Initial release |
| 2 | 19-MAR-2025 | Update to Section 3.3 |
| 3 | 30-SEP-2025 | • Detail added to Product description<br>• Detail added to reagents list<br>• Detail added to PCR set up guidance<br>• Detail added to AXP bead settling guidance<br>• Formatting and minor amends |

# Abbreviations

| Term | Description |
|---|---|
| AMK | Amikacin |
| AMR | Antimicrobial resistance |
| AXP | Ampure XP Beads |
| BCG | Bacillus Calmette-Guérin |
| BDQ | Bedaquiline |
| BR | Broad range |
| BSL | Biosafety Level |
| CAP | Capreomycin |
| CFU | Colony forming unit |
| CFZ | Clofazimine |
| CMX | Calibration mix |
| CPC | Cetylpydridinium chloride |
| DNA | Deoxyribonucleic acid |
| DLM | Delaminid |
| EB | Elution Buffer |
| EMB | Ethambutol |
| ETO | Ethionamide |
| FB | Flush Buffer |
| FCS | Flow Cell Storage Buffer |
| FLT | Flush Tether |
| FRK | Flow Cell Recalibration Kit |
| GC | GC Enhancer |
| HG | Hazard group |
| IC | Internal Control |
| INDELS | Insertions/deletions |
| INH | Isoniazid |
| KAN | Kanamycin |
| LBII | Loading Beads II |
| LFX | Levofloxacin |
| LoD | Limit of detection |
| LZD | Linezolid |
| MNPs | Multi-nucleotide polymorphisms |
| MOX | Moxifloxacin |
| MTBC | *Mycobacterium tuberculosis* complex |
| NALC | N-acetyl-l-cysteine |
| NaOH | Sodium hydroxide |
| NTC | No Template Control |
| NTM | Non-tuberculous mycobacteria |
| OND | Oxford Nanopore Diagnostics |
| PBS | Phosphate buffered saline |
| PC | Positive Control |
| PCR | Polymerase chain reaction |
| pDST | Phenotypic drug susceptibility testing |
| PMM | Platinum II Master Mix |
| PZA | Pyrazinamide |
| RAP F | Rapid Adapter F |
| RB | Recalibration Buffer |
| RB01-96 | Rapid barcode 01-96 |
| RIF | Rifampicin |
| SBII | Sequencing Buffer II |
| SNPs | Single nucleotide polymorphisms |
| SPRI | Solid phase reversible immobilisation |
| STM | Streptomycin |
| TB | Tuberculosis |
| TBPM | TB Primer Mix |
| WGS | Whole genome sequencing |
| WHO | World Health Organisation |

# 1 Product description

The AmPORE-TB™ test is intended for qualitative detection of *Mycobacterium tuberculosis* complex (MTBC) DNA extracted and sequenced from decontaminated sputum samples for the detection of mutations conditionally associated with drug resistance.

The assay consists of reagents for multiplex polymerase chain reaction (PCR) amplification of specific MTBC genomic sequences, barcoding and sequencing of the amplified products, and software for sequence analysis and interpretation to identify specific mutations as potential markers of resistance to specific antibiotics. Provided that sputum samples are extracted using a validated method, the product enables up to 88 samples in total to be assessed using a single flow cell across four sequencing runs.

This product is for Research Use Only. Not for use in diagnostic procedures.

AmPORE-TB Kits are intended for use by laboratory personnel with molecular biology and sequencing experience.

# 2 Principle of the examination method

The AmPORE-TB Kit has been developed by Oxford Nanopore Diagnostics to enable the simple and rapid analysis of genetic mutations thought to be associated with drug resistance in MTBC. This is based on multiple target regions of the bacterial genome and single nucleotide polymorphisms (SNPs), multi-nucleotide polymorphisms (MNPs), insertions/deletion (INDELS) and whole gene deletions associated with drug resistance in a highly multiplexed manner.

## 2.1 Workflow description

This workflow combines PCR amplification of the target regions of genes present in MTBC, rapid barcoded library preparation, and real-time nanopore sequencing. This enables the analysis of up to 22 individual samples of MTBC with a positive and negative (no template) control across one flow cell up to 4 times.

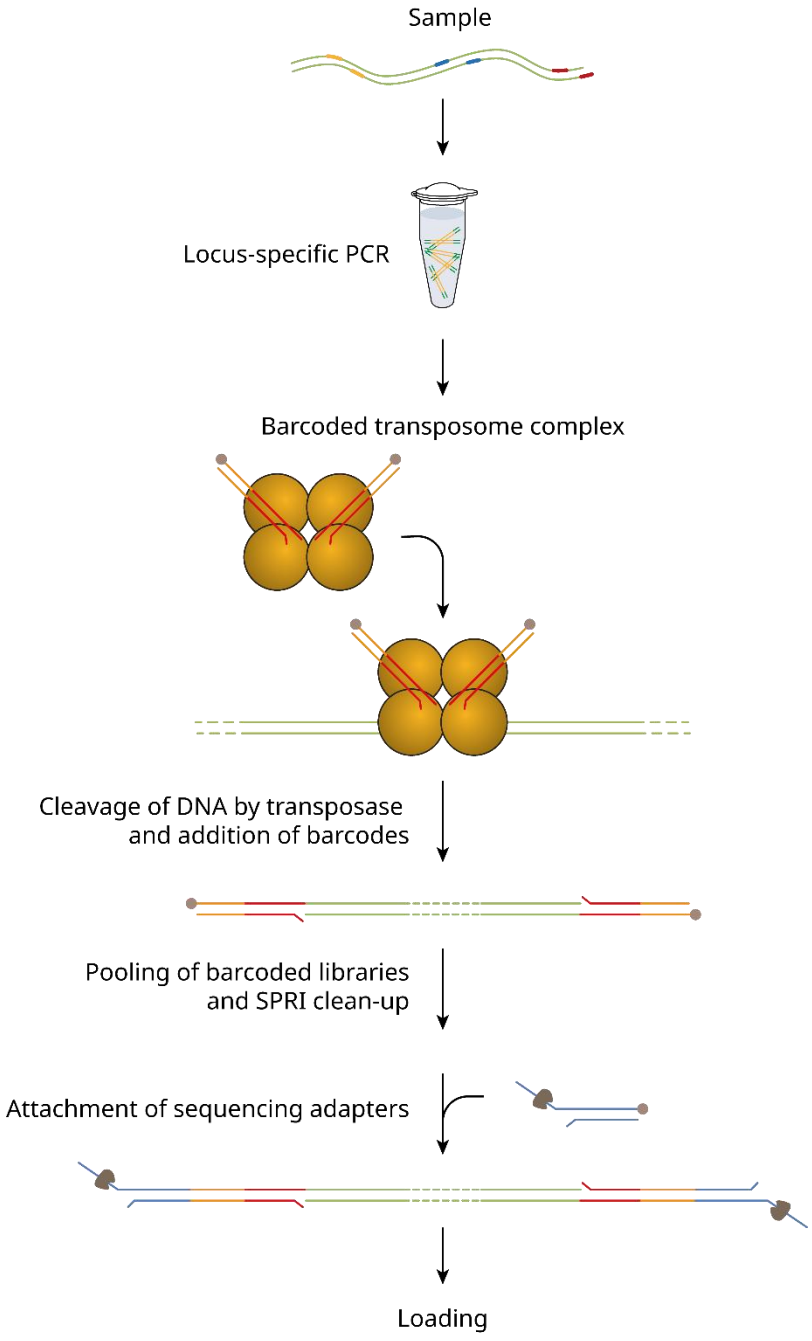
The workflow analyses the amplicon sequence data for genetic mutations associated with drug resistance using the catalogue of MTBC mutations curated by the World Health Organisation (WHO) (ISBN 9789240082410; https://www.who.int/publications/i/item/9789240082410). The mutations used to define resistance in the AmPORE-TB test are from groups 1 and 2 in the catalogue, i.e. mutations defined as 'associated with resistance' and 'associated with resistance interim' respectively. The workflow also classifies mutations as related to resistance because they fulfil a WHO expert rule. Please note that genetic mutations not part of the catalogue (group 1 and 2 and expert rules) are not analysed in the workflow.

At each of the pre-determined positions, we take a pileup of the sequencing reads and determine if the alternative allele in the resistance database is present at a threshold for which we have determined optimal performance. The workflow produces an analysis run report, designed as a full overview of the success or failure of the run based on control samples, and a one-page per-sample report.

These kits use PCR to amplify regions of 24 target genes found in MTBC and sequences them to identify specific resistance associated mutations. Additional targets amplified in this workflow are used for spoligotyping, non-tuberculous mycobacteria (NTM) detection and a plasmid-borne internal control (IC). Spoligotyping is a method for genotyping MTBC to identify potentially related strains. The IC is used for validating the procedure and any negative results, and *hsp65* is used for speciation of NTMs which can cause similar symptoms to tuberculosis (TB). Valid samples will have resistance associated genes and/or *hsp65* present, preventing amplification of the IC. If the target genes or *hsp65* do not amplify, the IC must be present (at ≥20x coverage) for the sample to be valid. Failure to detect either the target genes or IC in a sample identifies the sample as invalid and requires repeat testing. A positive control (PC) and a no template control (NTC) are included with up to 22 test samples (all spiked with IC). Further information about assay validity and the controls is included in Section 14 Quality Control and 2.3 Assay Validity.

| Drug resistance targets | |
|---|---|
| **Drug** | **Genes conferring resistance** |
| Ethambutol | *embA, embB* |
| Isoniazid | *fabG1, inhA, katG* |
| Pyrazinamide | *pncA* |
| Rifampicin | *rpoB* |
| Streptomycin | *gid,rpsL, rrs* |
| Amikacin | *eis, rrs* |
| Bedaquiline | *atpE, rv0678* |
| Capreomycin | *rrl, rrs, tlyA* |
| Clofazimine | *fbiA ,fbiB, fbiC, fgd1, rv0678* |
| Delamanid | *ddn, fbiA, fbiB, fbiC, fgd1,* |
| Ethionamide | *ethA, fabG1, inhA* |
| Kanamycin | *eis, rrs* |
| Levofloxacin | *gyrA, gyrB* |
| Linezolid | *rplC, rrl* |
| Moxifloxacin | *gyrA, gyrB* |
| Pretomanid | *ddn, fbiA, fbiB, fbiC, fgd1* |
| **Additional targets** | |
| **Application** | **Target** |
| NTM detection | *hsp65* |
| Typing *M. tuberculosis* complex | Spoligotyping (direct repeat locus) |
| Internal control | Unique DNA fragment |

To enable pooling of up to 22 samples and 2 controls into a single sequencing run after amplification, the rapid barcoding chemistry is used to barcode each individual sample with a unique barcode from the plate of Rapid Barcodes (RB01-96). These barcodes are on a transposome complex which simultaneously cleaves the PCR amplicons and attaches unique barcodes to the cleaved ends. After barcoding, the samples are pooled, cleaned by solid-phase reversible immobilization (SPRI) and sequencing adapters attached (to barcoded amplicons) for flow cell loading and sequencing.



## 2.2 Analysis pipeline

The AmPORE-TB analysis pipeline:

- Aligns the reads against the 27 genomic targets, 24 drug resistance-associated targets, the internal control, spoligotype CRISPR regions and hsp65.
- Reads are mapped to select for MTBC sequences.
- Identifies mutations from the WHO mutation catalogue associated with drug resistance.
- Determines the lineage of MTBC present based on spoligotyping.
- Generates files, including a HTML report and a CSV file detailing the success or failure of the assay. Information includes the barcode ID, control statuses and the test result including assay validity and drug resistance, with the specific mutations detected.

**Note:** Only reads that have had barcodes identified will be aligned to the target genes.

## 2.3 Assay validity

The AmPORE-TB analysis performs several quality control checks to ensure that the assay has been successful. The workflow determines the coverage (number of reads) of each amplicon in both the controls and test samples, and also assesses these relative to the positive control coverage.

An amplicon is considered "covered" when its median coverage is ≥20x. Neither *hsp65*, spoligotyping, or the internal control are used as amplicons for these calculations. The following criteria describe thresholds which are applied by default to each of the sample types included in the assay.

| Input | Description |
|---|---|
| Test sample | • For the drug resistance amplicon to pass and the sample to be considered valid, the following criteria must be met:<br>  • ≥10 amplicons are covered<br>  • *hsp65* coverage is ≥20x<br>• Internal control (QC) coverage is ≥20x<br>• If the sample meets the above criteria and the internal control has <20x median coverage, while sample coverage is significantly lower than the positive control, then MTBC is likely present in the sample at a level close to the limit of detection of the assay<br>• If the sample does not meet the criteria and the internal control has ≥20x median coverage, then MTBC is likely absent or below the limit of detection of the assay<br>• If the sample doesn't meet the above criteria and the internal control has <20x median coverage, then the analysis of this sample is considered failed |
| No template control (NTC) | For the control to pass and the **run** to be considered valid, the no template control must not have >2 drug resistance amplicons with a median coverage of >20x |
| Positive control (PC) | For the control to pass and the **run** to be considered valid, the positive control must have >20x median coverage in at least 22 amplicons |

For more information on quality control metrics please refer to Section 14.

# 3 Recommended equipment and materials

The following equipment and reagents have been used to verify this workflow. Alternative third-party reagents are available but have not been tested by Oxford Nanopore Diagnostics, therefore their performance with the assay is unknown.

A GridION Q sequencing device (Q-GRD-Mk1-ATB) is required to run the assay. Please refer to the GridION Q installation and configuration guide for instructions on how to install and configure your sequencing device for use.

## 3.1 Materials included

**Reagents**

- AmPORE-TB Kit (Oxford Nanopore Diagnostics OND-S-ATB01-RGL)
    - Rapid Barcode Plate (RB01-96); 8 µl per barcode
    - AMPure XP Beads (AXP); 2 x 1.2 ml
    - Sequencing Buffer II (SBII); 500 µl
    - Rapid Adapter F (RAP F); 10 µl
    - Elution Buffer (EB); 200 µl
    - Loading Beads II (LBII); 360 µl
    - Flush Tether (FLT); 200 µl
    - Flush Buffer (FB); 7.2 ml
    - Internal Control (IC); 120 µl
    - Positive Control (PC); 4 x 10 µl
    - No Template Control (NTC); 4 x 800 µl
    - Primer Mix (TBPM); 600 µl
    - Platinum II Master Mix (PMM); 2 x 1.4 ml
    - GC Enhancer (GC); 1.2 ml

- Flow Cell Recalibration Kit (Oxford Nanopore Diagnostics OND-E-FRK01-RGL)
    - Calibration Mix (CMX)
    - Recalibration Buffer (RB)
    - Flow Cell Storage Buffer (FCS)

**Consumables**

- Flow Cell R9 Version (Oxford Nanopore Diagnostics OND-FM001-RGL)

## 3.2 Materials required but not included

### Equipment

- GridION Q sequencer (Oxford Nanopore Technologies Q-GRD-Mk1-ATB)
    - Additional requirements, including a monitor, mouse, keyboard, power supply and network connection are described in the **Q GridION IT requirements**
- Benchtop centrifuge capable of speed up to 15,000 RPM (e.g. Eppendorf Centrifuge 5425/5425 R; Fisher Scientific 15881635)
- Plate centrifuge (e.g. Star Lab N2631-0008)
- Thermal Cycler (e.g. BioRad 1861096)
- Magnetic rack (e.g. Invitrogen DynaMag-2 Magnet; Fisher Scientific 10723874)
- Single channel pipettes (P1000, P200, P100, P20, P2; e.g Eppendorf, Gilson)
- Multi-channel pipettes (P10, P20; e.g. Eppendorf, Star Lab, Rainin)
- Fluorometer (e.g. Invitrogen Qubit 4; Fisher Scientific 16223001)
- FastPrep 5G bead beater* (MP Biomedicals 15260488)
- Maxwell RSC Instrument* (Promega AS4500)
- Class II microbiological safety cabinet
- Vortex mixer
- Fridge
- Freezer
- Weighing scales
- Heat block
- (Optional) PCR hoods for template and no template areas

### Consumables

- Eppendorf DNA 1.5 ml LoBind tubes (Sigma Aldrich EP0030108051-250EA)
- Eppendorf DNA 5 ml LoBind tubes (Sigma Aldrich EP0030108310-200EA)
- 0.2 ml thin-walled PCR tubes (Thermo Scientific AB0620)
- Qubit Assay tubes (Fisher Scientific Q32856)
- 96-well PCR Plate (Semi-skirted, straight edges; Star Lab I1402-9800)
- Clear Polyolefin StarSeal PCR (Star Lab E2796-9793)
- Lysing matrix E tubes* (MP Biomedicals 116914500)
- Pathogen Lysis Tubes L[†] (Qiagen 19092)

Reagents

- Qubit 1X dsDNA Broad Range (BR) Assay Kit (Fisher Scientific Q33265)
    - Qubit dsDNA BR Working Solution
    - Qubit dsDNA BR Lambda Standards
- Ethanol (Fisher Scientific BP2818100)
- Sodium hydroxide (NaOH; Sigma Aldrich S5881)
- Sodium citrate dihydrate (MP Biomedicals 219486880)
- N-Acetyl-L-cysteine (NALC; MP Biomedicals 210009825)
- Disodium phosphate anhydrous (Sigma Aldrich 1065860500)
- Monopotassium phosphate (Sigma Aldrich P0662)
- Phosphate buffered saline (PBS; Fisher Scientific 15374875)
- Maxwell RSC PureFood pathogen Kit* (Promega AS1660)
- Proteinase K (Qiagen 19133)
- Qiagen DNeasy Blood and Tissue Kit† (Qiagen 69504 or 69506)
- Buffer AL† (Qiagen 19075)
- Buffer ATL†‡ (Qiagen 19076)
- Tween-80† (Sigma Aldrich P4780-100ml)

*Only required for automated DNA extraction (with heat-kill)
†Only required for manual DNA extraction (with heat-kill)
‡Only required if using Qiagen DNeasy Blood and Tissue Kit 69506

Always read 3rd party materials/equipment supplier's documentation prior to use and follow the instruction for correct operation and any required maintenance.

# 4 Performance characteristics

## 4.1 Analytical limit of detection

Analytical limit of detection (LoD) for the AmPORE-TB test using both recommended extraction methods (manual and automated) with *M. tuberculosis* variant *bovis* Bacillus Calmette-Guérin (BCG) strain spiked sputum is 350 cfu/mL (based on molecular quantification) and 11.5 cfu/mL (based on colony plate counts).

The LoD using genome copies is 5 copies per reaction.

## 4.2 Repeatability

Repeatability was determined by using the same experimental conditions (reagent batch, instrument, samples) over a period of 12 days with two runs per day. Two MTBC control strains were used at two different concentrations (low and moderate). Overall, 100 % of both low and moderate concentration samples tested were positive across all operators.

## 4.3 Reproducibility

Reproducibility was determined at two testing sites (in-house and externally) using the same experimental conditions (reagent batch, samples) with a minimum of two operators per site over a period of five days, using the same samples as repeatability. Overall, 100 % of both low and moderate concentration samples tested were positive across all sites and operators.

## 4.4 Analytical specificity (cross-reactivity)

The AmPORE-TB test was evaluated using DNA extracted from a panel of common oral and respiratory tract commensals and pathogens along with closely related and clinically relevant NTMs that may cross-react with certain MTBC targets, plus selected relevant fungi and viruses. The panel (outlined in the tables below) consisted of 22 NTMs, 56 bacteria, 9 fungi and 13 viruses. In silico analysis was also used in place of any hazard group 3 or hard-to-culture organisms (e.g. *Mycobacterium leprae*). No cross-reactivity was observed with any of the microorganisms tested in the wet lab testing or in silico analysis.

| Non-tuberculous mycobacteria tested | |
|---|---|
| *Mycobacterium abscessus* | *Mycobacterium kumamotonense* |
| *Mycobacterium asiaticum* | *Mycobacterium marinum* |
| *Mycobacterium avium* | *Mycobacterium mucogenicum* |
| *Mycobacterium celatum* | *Mycobacterium scrofulaceum* |
| *Mycobacterium chelonae* | *Mycobacterium simiae* |
| *Mycobacterium flavescens* | *Mycobacterium smegmatis* |
| *Mycobacterium fortuitum* | *Mycobacterium terrae complex* |
| *Mycobacterium gastri* | *Mycobacterium thermoresistibile* |
| *Mycobacterium gordonae* | *Mycobacterium xenopi* |
| *Mycobacterium intracellulare* | *Mycobacterium malmoense* |
| *Mycobacterium kansasii* | *Mycobacterium szulgai* |

| Non-mycobacterial species tested | |
|---|---|
| *Actinomyces israelii* | *Neisseria sicca* |
| *Acinetobacter baumannii* | *Peptostreptococcus anaerobius* |
| *Acinetobacter calcoaceticus* | *Proteus mirabilis* |
| *Bacteroides fragilis* | *Proteus vulgaris* |
| *Bacillus cereus* | *Pseudomonas aeruginosa* |
| *Bacillus subtilis* | *Serratia marcescens* |
| *Burkholderia cepacia* | *Staphylococcus aureus* |
| *Citrobacter freundii* | *Staphylococcus epidermidis* |
| *Clostridium perfringens* | *Staphylococcus haemolyticus* |
| *Corynebacterium jeikeium* | *Staphylococcus lugdunensis* |
| *Eikenella corrodens* | *Streptococcus equi* |
| *Escherichia coli* (ESBL TEM-10) | *Streptococcus pneumoniae* |
| *Klebsiella pneumoniae* (KPC) | *Streptococcus pyogenes* |
| *Enterobacter aerogenes* | *Streptococcus agalactiae* |
| *Enterobacter cloacae* | *Streptococcus salivarius* |
| *Enterococcus faecalis* | *Stenotrophomonas maltophilia* |
| *Enterococcus faecium* | *Streptomyces anulatus* |
| *Escherichia coli* | *Veillonella parvula* |
| *Fusobacterium nucleatum* | *Streptococcus constellatus* |
| *Haemophilus influenzae* | *Streptococcus mitis* |
| *Haemophilus parainfluenzae* | *Streptococcus mutans* |
| *Haemophilus parahemolyticus* | *Streptococcus parasanguinis* |
| *Kingella kingae* | *Streptococcus sanguinis* |
| *Klebsiella pneumoniae* | *Yersinia enterocolitica* |
| *Klebsiella oxytoca* | *Nocardia brasiliensis* |
| *Legionella pneumophila* | *Nocardia otitidiscaviarum* |
| *Legionella micdadei* | *Klebsiella pneumoniae* (KPC) |
| *Leuconostoc mesenteroides* | *Klebsiella quasipneumoniae* (ESBL SHV-18) |

| Fungal species tested | |
|---|---|
| *Candida albicans* | *Cryptococcus neoformans* |
| *Candida glabrata* | *Penicillium rubens* |
| *Candida krusei* | *Rhizopus microsporus* |
| *Candida parapsilosis* | *Aspergillus fumigatus* |
| *Candida tropicalis* | |

| Viruses tested | |
|---|---|
| HIV | Parainfluenza 3 |
| Adenovirus | Parainfluenza 4 |
| Influenza A | Respiratory Syncytial Virus Type A |
| Influenza B | Respiratory Syncytial Virus Type B |
| Human metapneumovirus | Rhinovirus type 1A |
| Parainfluenza 1 | Varicella Zoster Virus |
| Parainfluenza 2 | |

| Organisms tested *in silico* | |
|---|---|
| *Blastomyces dermatitidis* | *Mycobacterium leprae* |
| *Histoplasma capsulatum* | *Mycoplasma pneumoniae* |
| Mumps | *Neisseria gonorrheae* |
| Rubella | *Neisseria lactamica* |
| Rubeola | *Neisseria meningitidis* |
| *Scedosporium spp* | *Nocardia farcinica* |
| *Chlamydophila pneumoniae* | *Pediococcus pentosaceus* |
| *Corynebacterium diptheriae* | *Rhodococcus equi* |
| *Corynebacterium pseudodiptheriticum* | *Streptococcus pneumoniae* |
| *Lactobacillus rhamnosus* | *Tsukamurella spp* |
| *Listeria monocytogenes* | |

## 4.5 Interfering substances

The AmPORE-TB test was evaluated against a panel of endogenous and exogenous potential interfering substances as detailed in the table below. Only one substance, cetylpydridinium chloride (CPC) resulted in interference with the AmPORE-TB test (when CPC used above 0.05 % (w/v)). There was no interference observed with any other substance tested.

| Interfering substances tested (highest concentration tested) | |
|---|---|
| Tobramycin (5 mg/mL) | Synex soother nasal spray (containing 0.05% w/v Oxymetazoline hydrochloride: (100%) |
| Mupirocin (0.62% w/v) | Resp ease 7% solution (7% NaCl solution: 5% v/v) |
| Nystatin oral suspension (20% v/v of 100,000 units/mL) | Benzocaine throat spray (5% w/v) |
| Isoniazid (50 µg/mL) | Guaifenesin (0.5 % w/v) |
| Acyclovir (50 µg/mL) | Physiologic saline (0.9 % NaCl: 100%) |
| Human blood (5% v/v) | Pentamidine isethionate (300 ng/mL) |
| Epinephrine (0.5% w/v) | Cetylpyridinium chloride (CPC; 0.5% w/v) |
| Tea tree oil (0.5% v/v) | Nicotine (0.03 mg/mL) |
| Human genomic DNA (100 ng per 0.7 mL sputum) | Blood plasma (100%) |
| Albuterol sulfate (100 µg/mL) | Caffeine (1.08E+01 mg/dL) |
| Corsodyl mouthwash (containing 0.2% w/v chlorhexidine digluconate: 20% v/v) | Paracetamol (1.56E+01 mg/dL) |
| Mucin – bovine (5% w/v) | Ibuprofen (2.19E+01 mg/dL) |
| Beclomethasone (5 µg/mL) | Loratadine (8.70E−03 mg/dL) |
| Zicam nasal gel (50% w/v) | |

## 4.6 Inclusivity

All the MTBC members and *M. tuberculosis* strains tested (from culture, DNA or *in silico*) were detected using the AmPORE-TB analysis workflow with all targets covered at ≥20x median coverage; hence the assay is considered 100 % inclusive of the MTBC.

| Sample ID | Sample type |
|---|---|
| *Mycobacterium tuberculosis* variant *bovis* BCG Strain Pasteur | Culture |
| *Mycobacterium tuberculosis* variant *bovis* BCG Strain Tice | |
| *Mycobacterium tuberculosis* H37Ra | DNA |
| *Mycobacterium tuberculosis* H37Rv | |
| *Mycobacterium tuberculosis* Strain X004439 | |
| *Mycobacterium tuberculosis* Strain TMC 331 | |
| *Mycobacterium tuberculosis* Strain TMC 303 | |
| *Mycobacterium caprae* | |
| *Mycobacterium microti* | |
| *Mycobacterium pinnipedii* | *In silico* |
| *Mycobacterium canettii* | |
| *Mycobacterium africanum* | |
| *Mycobacterium oryx* | |
| *Mycobacterium suricattae* | |
| *Mycobacterium mungi* | |
| *Mycobacterium dassie* | |

## 4.7 Variant detection

A limited panel of 104 highly characterised isolates was used to generate sensitivity and specificity values for the detection of variants and resistance by comparison to Illumina whole genome sequencing (WGS) data, and comparison to a composite reference (phenotypic drug susceptibility testing [pDST] + WGS data), respectively.

**Note:** These values are only indicative due to low sample numbers for some targets - performance will likely differ when using real clinical samples.

**Note:** Within the analysis workflow, when a sample is identified as close to the analytical LoD, the allele frequency threshold is raised for variant detection calling (from 15 % up to 40 %).

**Comparison to WGS data only.**

In total, WGS data was available for 15 drugs (CAP, EMB, RID, INH, MOX, LFX, AMK, PZA, KAN, BDQ, CFZ, DLM, ETO, LZD and STM).

| Drug | Sensitivity (%) | Specificity (%) | Number of known positives | Number of known negatives |
|---|---|---|---|---|
| Capreomycin (CAP) | 100 | 100 | 35 | 62 |
| Ethambutol (EMB) | 100 | 100 | 78 | 21 |
| Rifampicin (RIF) | 100 | 100 | 97 | 1 |
| Isoniazid (INH) | 100 | 100 | 96 | 2 |
| Moxifloxacin (MOX) | 100 | 100 | 54 | 41 |
| Levofloxacin (LFX) | 100 | 100 | 54 | 41 |
| Amikacin (AMK) | 100 | 100 | 36 | 57 |
| Pyrazinamide (PZA) | 97 | 100 | 63 | 32 |
| Kanamycin (KAN) | 100 | 100 | 53 | 45 |
| Bedaquiline (BDQ) | 50 | 100 | 2 | 97 |
| Clofazimine (CFZ) | 50 | 100 | 2 | 95 |
| Delamanid (DLM) | N/A | 98 | 0 | 97 |
| Ethionamide (ETO) | 100 | 98 | 54 | 41 |
| Linezolid (LZD) | N/A | 100 | 0 | 64 |
| Streptomycin (STM) | 100 | 100 | 55 | 42 |

**Comparison to pDST/WGS data**

In total, pDST and WGS data was available for nine drugs (INH, RIF, AMK, KAN, MOX, PZA, BDQ, DLM and LZD).

| Drug | Sensitivity (%) | Specificity (%) | Number of known positives | Number of known negatives |
|---|---|---|---|---|
| Rifampicin (RIF) | 100 | 100 | 87 | 1 |
| Isoniazid (INH) | 99 | 100 | 87 | 1 |
| Moxifloxacin (MOX) | 96 | 100 | 52 | 35 |
| Amikacin (AMK) | 97 | 100 | 37 | 47 |
| Pyrazinamide (PZA) | 95 | 100 | 63 | 26 |
| Kanamycin (KAN) | 95 | 100 | 55 | 33 |
| Bedaquiline (BDQ) | 50 | 100 | 2 | 87 |
| Delamanid (DLM) | N/A | 98 | 0 | 87 |
| Linezolid (LZD) | N/A | 100 | 0 | 53 |

# 5 Storage and stability

Please store third-party reagents following the manufacturer's recommendations.

The AmPORE-TB Kit (OND-S-ATB01-RGL) is shipped with ice packs at −20°C to −30°C and should be stored at −20°C to −30°C.

The Flow Cell Recalibration Kit (OND-E-FRK01-RGL) is shipped with ice packs at −20°C to −30°C and should be stored at −20°C to −30°C.

If the package is damaged or reagent tubes are leaking upon receipt, do not use the test reagents. Reagents may be compromised, leading to an incorrect result. Please contact the supplier. When dealing with multi-reaction volumes, users should not re-seal opened barcode plates.

The Flow Cell R9 Version (OND-FM001-RGL) is shipped at 2–8°C and stored at 2–8°C for up to 9 months. The expiry date is stated on the label of the product. Flow cells can be recalibrated up to 3 times. Following each recalibration, the flow cell should be stored at 2–8°C and used within 7 days. A flow cell check is performed at the start of each experiment to verify the integrity and correct functioning of the flow cell.

Flow cells must not be frozen. Freezing the flow cell causes irreversible damage to the product and will cause it to fail the flow cell check.

# 6 Considerations for using the AmPORE-TB test

## 6.1 Warnings

- Follow Good Laboratory Practice in relation to personal safety, including but not limited to the use of personal protective equipment when handling the samples and test reagents.
- Take care to prevent the reagents from contacting your skin, eyes, mucous membranes, etc.
- If contact occurs, immediately wash the affected area with soap and water. Thoroughly wash your hands after handling samples and test reagents.
- In the event of a serious incident, users should contact the Initial Importer or Manufacturer of the device and may also wish to contact the competent authority of the Member State where the device was used.

## 6.2 Precautions

**Personal protection**

- Refer to local or WHO health and safety guidance for handling diagnostic samples containing hazard group (HG)/BioSafety Level (BSL) 3 organisms.
- Decontaminate surfaces with 0.5% sodium hypochlorite (bleach) diluted with deionized or purified water.

**Reagent and consumable use**

- Prevent contamination of reagents by following standard contamination prevention procedures including but not limited to the use of personal protective equipment, separation of sample preparation pre-PCR and post-PCR, using filter pipette tips for handling DNA and using dedicated pipettes for pre-amplification areas and post-amplification areas.
- Controls must be kept separate from the samples to prevent contamination. The NTC must be kept separate from the PC.
- Reagents must be stored under the indicated storage conditions before and after use.
- Waste should be disposed of in accordance with local infectious waste disposal regulations.
- Do not mix different lots of reagents.
- We recommend using DNase-free pipette tips.
- Never reuse the consumables intended for single use (for example, tips, gloves, and test tubes).
- When using a pipette, care must be taken to use correct volumes and adhere to manufacturer's instructions. The use of multichannel pipettes is recommended where possible. Mouth pipettes must not be used.
- Do not use the test reagent if the package is damaged or reagent tubes are leaking (reagents may be compromised, leading to an incorrect result). Please contact technical support. Users should not re-seal opened plates.
- Do not use test reagents after their expiration date.

- Following recalibration, flow cells should be used within 7 days.
- The GridION Q device is designed to operate at 18–25°C.

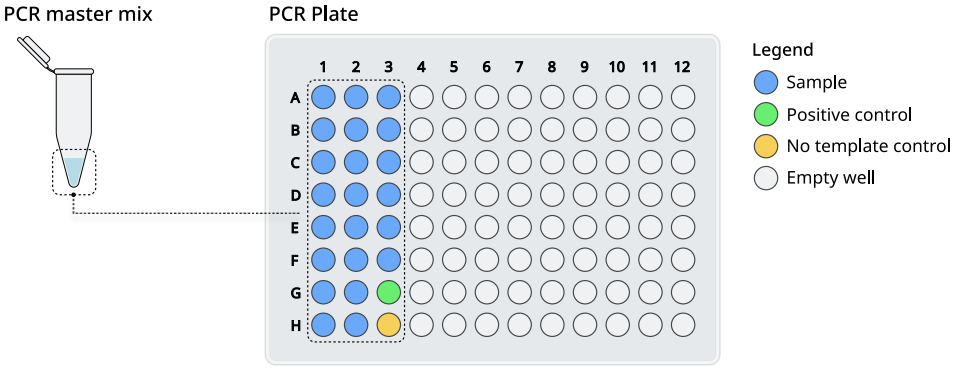
## 6.3 Limitations of the experimental procedure

- Twenty-two samples with two controls can be pooled together in a single run. While fewer samples per run could be processed using this protocol, this has not been validated by Oxford Nanopore as part of the product offering. The volumes of reagents provided has been optimised for use with 22 samples, fewer samples per run would reduce the total number of samples which can be tested per kit.
- False-negative results for the presence of MTBC may arise from:
    - Improper specimen collection
    - MTBC present in the sample but below the limit of detection
    - Degradation of the DNA during shipping/storage
    - Using inappropriate extraction or assay reagents
    - Failure to follow instructions for use
    - Using expired reagents
- False-positive results for the presence of MTBC may arise from:
    - Cross-contamination from incorrect handling of the positive control
    - Contamination during specimen handling or preparation
    - Cross-contamination between samples
    - Specimen mix-up
    - DNA contamination during product handling
- As with any molecular test, mutations within the primer binding sites of the AmPORE-TB test could affect primer binding, resulting in failure to detect the presence of MTBC and/or the presence of the target gene/s.
- The effect of vaccines, antibiotics, chemotherapeutic, immunosuppressant drugs or other inhibitory substances from human specimens have not been evaluated for their impact on assay performance.
- Negative results for antimicrobial resistance related mutations does not exclude the possibility of drug resistance as a result of mutations not detected or mutations in genes not targeted in this workflow.
- No mutation detected does not exclude the possibility of resistance.

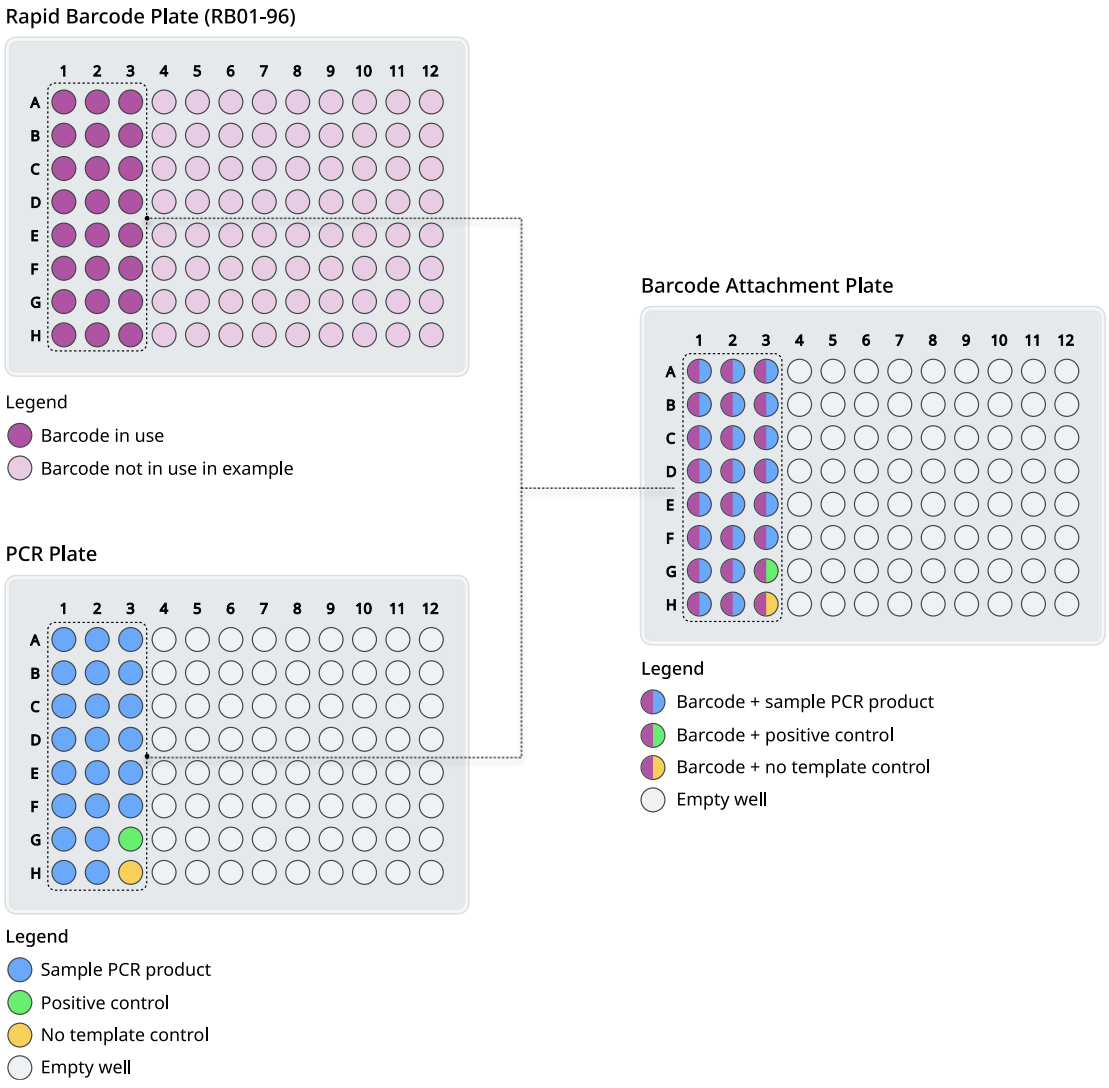
# 7 Overview of procedure from extracted DNA

## 7.1 Diagram of experimental procedure

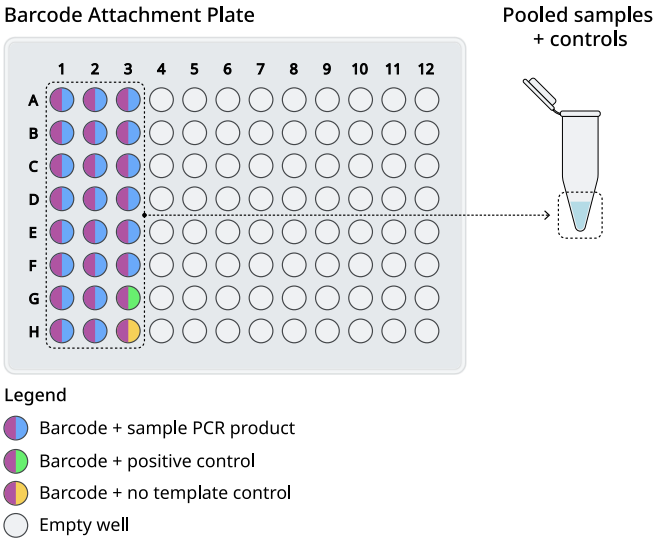
1. PCR master mix containing IC is transferred to all sample and control wells. Sample extracted DNA, extracted NTC, and PC are added as shown. PCR is used to amplify regions of target genes.




2. Unique barcodes are attached to PCR products to create a barcoded library.



3. Barcoded samples and controls are pooled, cleaned by solid-phase reversible immobilization (SPRI), and sequencing adapters are attached before loading onto the flow cell.




## 7.2 Recommendations for the experimental procedure

- Specimens must be collected, stored, and transported using procedures and conditions determined by the user to preserve the integrity of specimens for use in molecular assays for determining drug resistance. Inadequate or inappropriate specimen collection, storage and transport may yield invalid or incorrect test results. For correct procedures and conditions for specimen collection, storage, and transport, refer to local guidelines and device manufacturer instructions.
- Extraction of nucleic acid from specimens should be performed using the verified manual or automated extraction methods described in this document. The performance of other extraction methods has not been assessed and cannot be guaranteed. Use of other methods must be validated by the end user and is at their own risk.
- Nucleic acid contamination from the positive controls or specimens must be controlled by good laboratory practices and careful adherence to the procedures specified in this document.

# 8 Creating sample sheets

Before starting a sequencing run, a sample sheet must be uploaded at the time of sequencing. It must include an assay (indicating the analysis workflow version and assay, as well as library ID and number of samples in the library=sample count) and a sample (including barcode used, sample type and sample ID) information sections (as in the example below).

Sample IDs should not contain sensitive information and should be anonymised or pseudo-anonymised in accordance with local requirements

## 8.1 Locating the sample sheet template

1. Open LibreOfficeCalc using the shortcut in the Ubuntu favourites bar
2. Click File>New>Templates.
3. Select the AmPORE-TB assay template
4. Fill out the template with your sample details. If you have a barcode scanner, you can use this to enter sample and library IDs. Ensure you select the correct sample types as this cannot be changed later. If you want, you can optionally delete barcodes that have a sample type of no_sample.
5. Save the sample sheet. In the Save dialog, use the drop-down menu in the bottom-right to change the file type to be Text CSV (.csv) and then click Save in the top-right of the dialog.

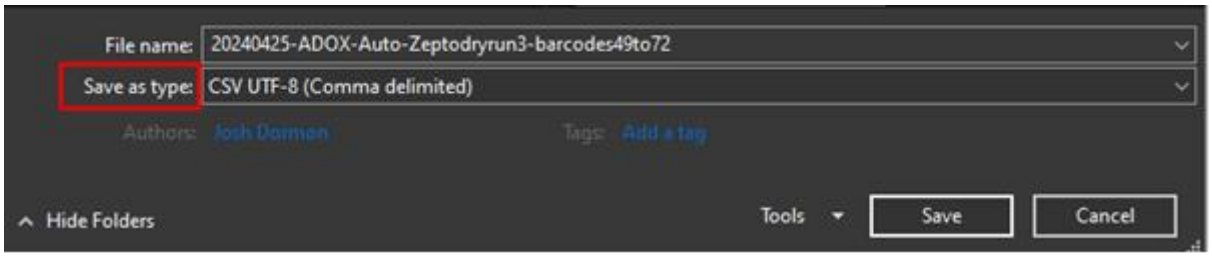
## 8.2 Example of a sample sheet format

- Version: The sample sheet format version must be included, e.g. `v1`
- `assay` is the specific assay to be used: e.g. `ampore-tb:v1.0`
- `library_id` is the identifier for the sequencing run. It can contain optional information about the extraction type, lab performing the run, date, barcodes used, etc.
- `sample_count` must match the number of rows in the sample information section and number of barcodes assigned a `sample_type`
- `barcode`: This column contains the individual barcodes assigned to each sample with the number written as 2 digits, e.g. “barcode01” … “barcode24”.
- `sample_type` specifies if the input is a sample or control. Two barcodes must be assigned as `positive_control` and `no_template_control`. The rest of the samples are designated as `sample`.
- `sample_id` must specify a unique identifier for each entry. Accepted characters: uppercase or lowercase letters, number, hyphen, full stop and underscore.

Example sample sheet:

| v1 | | | |
|---|---|---|---|
| | | | |
| assay | ampore-tb:v1.0 | | |
| library_id | lims-system-id-12345 | | |
| created_by | jsmith2 | | |
| created_at | 2023-03-04T14:07:14+01:00 | | |
| sample_count | 7 | | |
| lot | rbk | rbk-udi-123721348237 | |
| lot | tbdr | tbdr-udi-235273952348 | |
| | | | |
| well_position | barcode | sample_type | sample_id |
| A1 | barcode01 | sample | lims-sampleid-4234623 |
| A2 | barcode02 | sample | lims-sampleid-4234135 |
| A3 | barcode03 | positive_control | lims-sampleid-1234565 |
| A4 | barcode04 | sample | lims-sampleid-4235345 |
| A5 | barcode05 | sample | lims-sampleid-4234511 |
| A6 | barcode06 | no_template_control | lims-sampleid-1234578 |
| A7 | barcode07 | sample | lims-sampleid-4234567 |

Sample sheets must be saved as **.csv** (comma delimited) files. The easiest way to do this is to create an Excel file (or using LibreExcel on the GridION Q device) with the "save as type" option set to **csv** as below:



# 9 Experimental procedure from sample

## 9.1 Decontamination method

It is recommended to start this protocol with samples previously identified as TB positive using a rapid molecular diagnostic test (e.g. Xpert MTB/rif assay).

Prepare the working solutions below. These solutions can be prepared in advance and stored at room temperature (unless stated otherwise) for up to 4 weeks.

- **Sodium citrate solution**
  - Prepare 2% NaOH and 1.45% sodium citrate solution by weighing out 20.0 g NaOH and 14.5 g sodium citrate dihydrate using a fine balance. Add 1 L distilled water and shake until dissolved.
- **N-Acetyl-L-Cysteine (NALC) powder aliquots**
  - Weigh out aliquots of 0.25 g NALC powder using a fine balance. This is enough for 50 ml final solution. Store aliquots in the fridge and prepare final solution immediately before use.
- **Phosphate buffer (0.067 M)**
  - Weigh out 4.74 g disodium phosphate anhydrous and 4.54 g monopotassium phosphate using a fine balance. Add 1 L distilled water and shake until dissolved.

1. Just before starting, prepare a NALC-NaOH-sodium citrate solution by supplementing 50 ml of NaOH-Sodium citrate solution with the weighed aliquot of 0.25 g NALC powder and allow it to dissolve fully. (This can be scaled up or down dependent on sample size being processed).
2. Aliquot 700 µl of each sputum sample for processing. In addition, include the extraction NTC (700 µl NTC from AmPORE-TB Kit) at this stage.
3. Add 700 µl of NALC-NaOH-sodium citrate solution to each sample and control (extraction NTC).
4. Vortex the tubes and incubate them at room temperature for 10 minutes.
5. After the incubation, centrifuge at 15,000 × g for 3 minutes. Carefully discard the supernatant from all samples first, leaving the pellet intact.
6. Resuspend each pellet in 1 ml of Phosphate buffer.
7. Centrifuge at 15,000 × g for 3 minutes. Carefully discard the supernatant, leaving the pellet intact.
8. Proceed to either automated (section 9.2) or manual (section 9.3) extraction with heat kill.

## 9.2 Automated extraction (with heat-kill) method

To be performed after decontamination.

1. Resuspend each decontaminated pellet in 750 µl PBS and proceed to heat kill including extraction NTC.
2. Heat kill step: incubate the samples at 95°C for 15 minutes.
3. Transfer 750 µl to an MPBio Matrix E tube (for automated bead beating).
4. Bead beat at 6.0 m/s for 2 cycles of 45 seconds (90 seconds total).
5. Centrifuge the Matrix E tubes at 20,000 × g for 1 minute.
6. Transfer the liquid in 3 steps of 200 µl to a 1.5 ml Eppendorf tube, add 20 µl of Proteinase K to each tube and mix by quick vortexing.
7. Incubate tubes at 65°C for 5 minutes on a shaking incubator at 1000 RPM.
8. Progress to automated extraction on the Promega Maxwell. Follow the Maxwell Purefood Pathogen kit as per the protocol:
    a. Place cartridges in the Maxwell RSC and remove seals.
    b. Add 300 µl of Lysis Buffer (green lid - NOT Lysis Buffer A) to Well 1.
    c. Add the Proteinase K-treated sample to Well 1 (from step 8).
    d. Insert a plunger into Well 8.
    e. Place new elution tubes into tube slot with lid open.
    f. Add 50 µl of Elution buffer to each elution tube.
    g. Start extraction.
    h. The 50 µl eluate in the tube is ready to be used for PCR (N.B. if resin is present in the eluate, centrifuge at 10,000 × g prior to downstream processing for 1–2 minutes).

If not used on the same day, the eluate can be stored at 4°C for short term (1 week), or −20°C for longer term storage.

## 9.3 Manual extraction (with heat-kill) method

To be performed after decontamination. This method uses components from the Qiagen DNeasy Blood and Tissue Kit. Reagent DX is included with Qiagen Pathogen Lysis Tubes.

Prepare **phosphate-buffered saline (PBS) + 0.1% Tween** by adding 1 ml Tween-80 to 9 ml PBS (mix well). Add 0.5 ml 10% Tween-80 in 50 ml PBS and mix well. Upscale accordingly if necessary.

1. Resuspend each decontaminated pellet in 200 µl PBS + 0.1 % Tween-80 + 20 µl Proteinase K and incubate at 56°C for 10 minutes, including extraction NTC.
2. Heat kill step**:** incubate the tubes at 95°C for 15 minutes.

3. Invert mix Reagent DX before use, then add 1 µl to each tube, mix by pipetting and transfer the solution by pipetting to the Qiagen Pathogen Lysis Tubes L bead tube.

4. Vortex the tubes for 1 minute.

5. To each tube, add 250 µl of Buffer ATL and 20 µl of Proteinase K and mix by vortexing for 2 seconds.

6. Incubate at 56°C for 10 minutes.

7. While the samples are being incubated set up 350 µl of Buffer AL and 350 µl of 100 % Ethanol in a fresh 1.5 ml Eppendorf tube per sample.

8. After incubation (step 6) vortex the sample and transfer 350 µl from the bead tube (avoiding transfer of beads) to the tube containing buffer AL and 100% ethanol (step 7). Vortex for 15 seconds.

9. Transfer 600 µl to a DNeasy spin column and centrifuge at 6,000 × g for 1 minute. Discard the flowthrough from the collection tube (by pouring) and return the insert into the same collection tube.

10. Transfer the remainder of the sample to the DNeasy spin column and centrifuge at 6,000 × g for 1 minute. Transfer the column to a new collection tube.

11. Add 500 µl Buffer AW1 to the column and centrifuge at 10,000 × g for 1 minute. Transfer the column to a new collection tube.

12. Add 500 µl Buffer AW2 to the column and centrifuge at 20,000 × g for 3 minutes.

13. Transfer the column to a fresh 1.5 ml Eppendorf tube and add 50 µl of Buffer AE to the column membrane. Incubate at room temperature for 1 minute.

14. Centrifuge the sample at 10,000 × g for 1 minute.

15. Lift the column, collect the 50 µl eluate and add it back to the same column for a re-elution.

16. Centrifuge the sample at 10,000 × g for 1 minute. Discard the column and keep the eluate in the Eppendorf tube.

The 50 µl eluate is ready to be used for the multiplex PCR or can be stored at 4°C for 1 week, or at −20°C for longer.

Any excess eluate should be stored until all results have been reported in case there is a need to repeat the assay.

# 10 Experimental procedure from extracted DNA

## 10.1 Multiplex PCR amplification

1. Thaw the reagents listed below at room temperature and store on ice. Before using the reagents, vortex and briefly spin down.

| Reagents required |
|---|
| Platinum II MM (PMM) |
| GC Enhancer (GC) |
| Primer Mix (TBPM) |
| Internal Control (IC) |
| Positive Control (PC) |

We recommend performing steps 2 to 4 **on ice** in a pre-PCR no-template area. This may be a separate room or bench where no samples or extracted DNA will be handled during the library preparation steps.

2. Prepare the PCR master mix in a single sterile 1.5 ml Eppendorf DNA LoBind tube on ice in the order below.

| Reagent | Volume per sample (µl) | Volume (µl) for 22 samples and 2 controls with excess |
|---|---|---|
| GC enhancer (GC) | 10 | 260 |
| Primer Mix (TBPM) | 5 | 130 |
| Platinum II MM (PMM) | 25 | 650 |
| Total volume | 40 | 1040 |

All master mix preparation and PCR plate preparation steps must be performed on ice to ensure reagent stability and uniform amplification. Keep reagents, plasticware, and master mix on ice until transferring to the thermocycler.

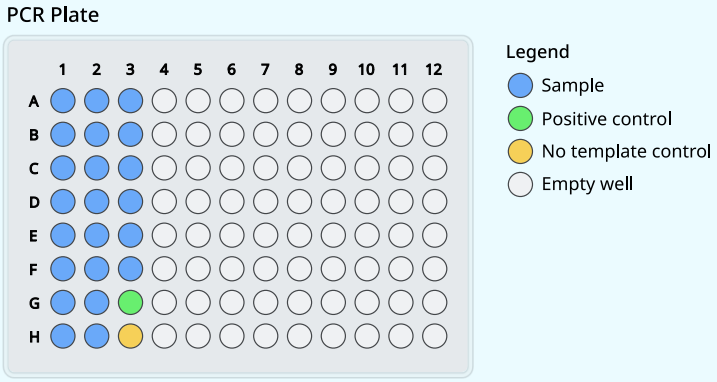
3. Vortex thoroughly.

Thorough vortexing is required to ensure sufficient mixing of the mastermix including the highly viscous GC enhancer.

In the next steps, we will set up the controls on a new 96-well plate that includes the IC, PC and NTC.

The following plate layout is recommended:

- IC is added to the PCR master mix and transferred to all sample and control wells (1A-3H).
- Sample extracted DNA is added to each sample well (1A-3F).
- PC and nuclease-free water are added to a single well (3G).
- NTC extract is added to a single well (3H).




In the next steps, we recommend the following:

- Use the NTC that has been processed using the chosen extraction protocol.
- Perform these steps **on ice** in a separate pre-PCR template addition area. This area can be a separate room or bench used to handle the samples for testing.

4. Keeping the master mix **on ice**, move to a template addition area.

5. Vortex the IC thoroughly, and briefly spin down. Add 26 µl of IC to the master mix, vortex thoroughly, and briefly spin down.

6. Keeping the PCR plate **on ice**, aliquot 41 µl of the master mix with IC into each PCR well for each sample plus PC and NTC.

7. Vortex the extracted DNA sample thoroughly, and briefly spin down. Add 9 µl of sample to each sample well.

8. Vortex the PC thoroughly, and briefly spin down. Add 5 µl of PC to the positive control well, plus 4 µl high-purity water.

9. Vortex the NTC extract thoroughly, and briefly spin down. Add 9 µl of NTC extract to the no template control well.

10. Seal the plate, vortex vigorously and briefly spin down.

Thorough vortexing is required to ensure sufficient mixing of the template in the viscous PCR mastermix.

11. Incubate the plate in a thermocycler with a heated lid set to 105°C using the following programme. If available, use a ramp rate of 4°C/sec (or maximum ramp rate available).

| Step | Temperature (°C) | Time (mm:ss) | Cycles |
|---|---|---|---|
| Heat activation | 94 | 05:00 | 1 |
| Denaturation | 94 | 00:20 | 35 |
| Annealing | 63 | 00:10 | |
| Extension | 66 | 01:20 | |
| Final extension | 68 | 04:00 | 1 |
| Hold | 10 | ∞ | 1 |

If necessary, the protocol can be paused at this point. The samples should be kept at 4°C and can be stored overnight.

The PCR plate and the remaining PCR products should be stored until all results have been reported in case there is a need to repeat the assay. Samples can be stored at 4°C overnight or at −20°C for longer periods.

We recommend performing the remainder of the library preparation in a separate post-PCR area. This area can be a separate room or bench used to only handle amplified samples.

12. After completion of the amplification programme, quantify the PC and NTC using the Qubit 1x dsDNA BR assay.

The PC is expected to be at least 5–10 ng/µl higher than the NTC. Further investigation is required if the NTC is >15 ng/µl.

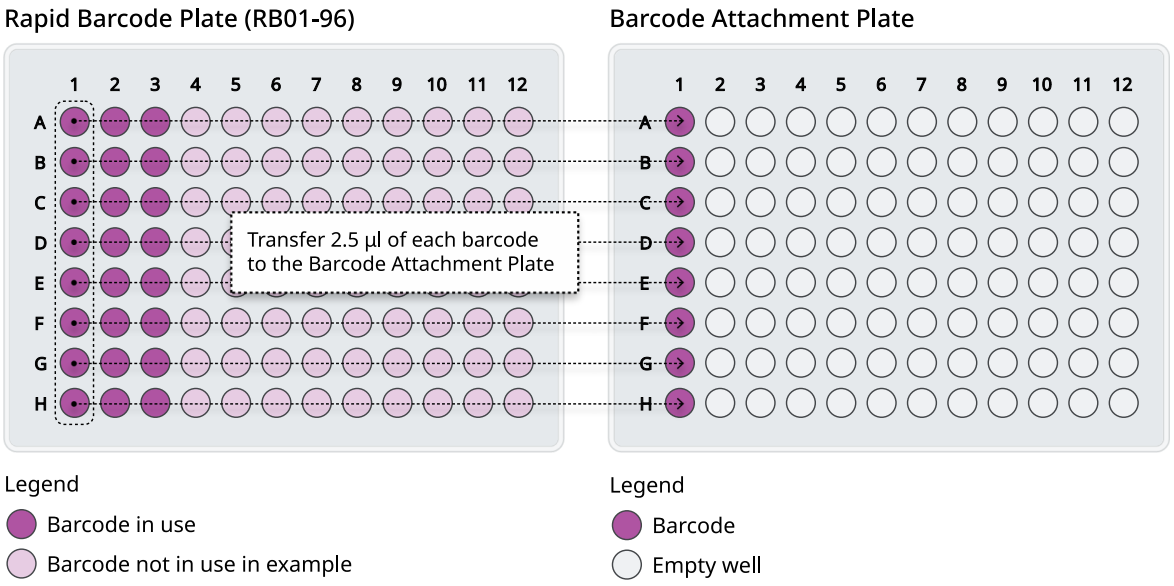
## 10.2 Addition of rapid barcodes

A unique barcode must be used for each sample and control that will be run on the same flow cell. A different set of barcodes must be used after each recalibration of a flow cell.

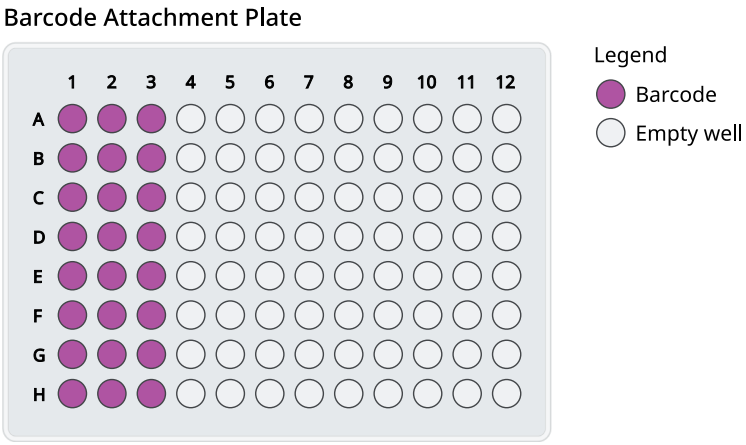
1. Thaw the reagents listed above at room temperature (18–23°C).

| Individual reagents |
|---|
| Rapid Barcode plate (RB01-96) |
| Rapid Adapter F (RAP F) |
| AMPure XP Beads (AXP) |
| Elution Buffer (EB) |
| Flush Buffer (FB; in preparation for flow cell loading) |

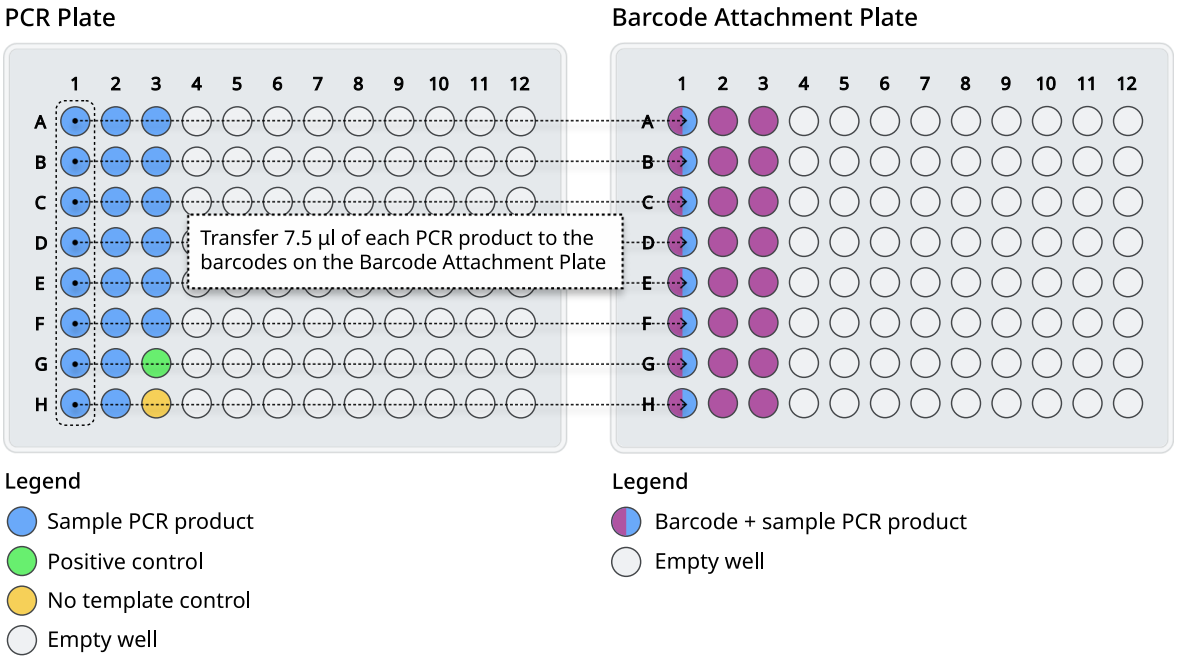
2. Spin down Rapid Barcode plate, Rapid Adapter F and Flush Buffer and then store on ice until use.
3. Store AMPure XP Beads and Elution Buffer at room temperature until use.
4. Briefly vortex and spin down the PCR plate.
5. Put the PCR plate containing the samples, the foil-sealed Rapid Barcode Plate, and a new 96-well plate (known as the Barcode Attachment Plate) on a flat surface.
6. Make sure that all plates are in the same orientation, with 1A in the top left corner.
7. Without removing the foil seal, use a multichannel pipette to pierce the foil seal of the first column of barcodes you wish to use from the Rapid Barcode Plate, pipette mix, and transfer 2.5 µl of each barcode to equivalent plate wells in the Barcode Attachment Plate (see example diagram below)



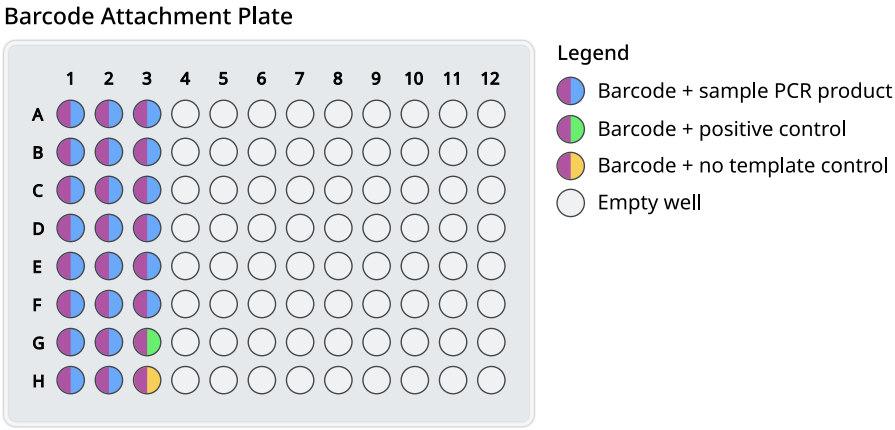
8. Repeat step 7 for the second and third columns of the barcodes to be used, so that all required barcodes are transferred to the Barcode Attachment Plate (see example diagram below).




9. Using a multichannel pipette, transfer 7.5 µl of the PCR product from the first column in the PCR Plate to the equivalent column in the Barcode Attachment Plate (see diagram below). Pipette mix the Barcode Attachment Plate 10 times using a multichannel pipette set at 7.5 µl.



10. Repeat step 9 for the remaining columns of the PCR Plate, so that all PCR products and controls are transferred to Barcode Attachment Plate.




11. Seal the Barcode Attachment Plate well to prevent evaporation and spin down briefly.

12. Set up the following programme in the thermocycler and incubate the Barcode Attachment Plate.

| Temperature (°C) | Time (mm:ss) | Cycles |
|---|---|---|
| 30 | 02:00 | 1 |
| 80 | 02:00 | |
| 10 | ∞ | |

13. Cut a 96 well plate seal to size and cover the pierced wells of the Rapid Barcode Plate.

## 10.3 AMPure XP bead wash

1. Equilibrate the AMPure XP beads at room temperature for 30 minutes.

2. Remove Barcode Attachment plate from thermocycler and spin down.

3. Pool all barcoded samples into a fresh 1.5 ml Eppendorf DNA LoBind tube. Mix by pipetting and briefly spin down.

4. Resuspend the AMPure XP Beads (AXP) by vortexing for 30 seconds.

5. Add an equal volume (1:1 ratio) of AMPure XP Beads (AXP) to your barcoded samples, mix by pipetting 10 times.

| Reagent | Volume per sample (µl) | Volume for 22 samples and 2 controls (µl) |
|---|---|---|
| AMPure XP Beads (AXP) | 10 | 240 |

6. Incubate at room temperature (18–23°C) for 5 minutes with gentle mixing by flicking.

7. In the meantime, freshly prepare 2.5 ml of 80% ethanol in nuclease-free water.

In the next steps, if the pellet on the magnet is disturbed at any point in the clean-up process, wait for the beads to pellet again and continue. If any beads are aspirated, dispense the beads back into the tube on the magnet and wait for the beads to pellet before continuing.

Bead settle times may vary – a higher initial sample volume or weaker magnets will require a longer settle time.

8. Briefly spin down the sample and place the tube on a magnet until the supernatant is clear and colourless (2 minutes).
9. Keeping the tube on the magnet, carefully remove and discard the supernatant without disturbing the pellet.
10. With the tube on the magnet, wash the beads by adding 1 ml of the freshly prepared 80% Ethanol to the tube without disturbing the beads. Incubate for up to 30 seconds. Remove and discard the ethanol.
11. Repeat the previous step for a second wash.

In the next steps, do not leave the pellet for an extended period to dry as this can reduce library yield. The beads may also be difficult to resuspend and will need to be pipetted well until the solution is uniform.

12. Spin down the sample tube and place back onto magnet (for 30 seconds).
13. Remove any residual ethanol and air-dry the pellet for 30 seconds.
14. With the tube off the magnet, resuspend the pellet in 15 µl of Elution Buffer (EB) and mix well by pipetting.

**Note:** Resuspend the beads well enough so that the solution is uniform.

15. Spin down the tube briefly, avoiding pelleting the beads.
16. Incubate for 10 minutes at room temperature (18–23°C) with occasional gentle flicking (ensure the tube is fully closed).
17. Place the tube on the magnet to pellet the beads until the eluate is clear and colourless for a minimum of 2 minutes.
18. Remove 14 µl of the eluate and collect in a new tube, taking care not to transfer any beads.

19. Quantify 1 µl of your final eluate using the Qubit 1X ds DNA BR Assay.

If the library pool concentration is <20 ng/µl, it is recommended to quantify a random selection of PCR products and compare to the negative control.

If the PCR was successful, the concentration of the majority of samples will be higher than the control, suggesting an issue with library preparation.

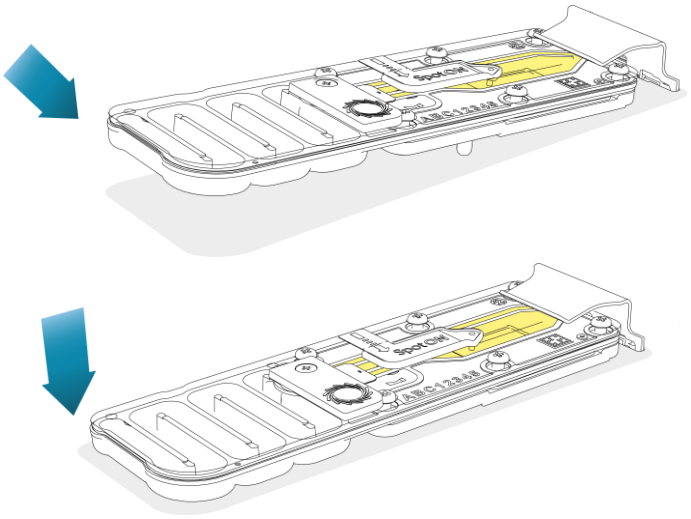
If the PCR concentrations are similar to the negative control, that suggests an issue with PCR.

# 11 Flow cell check and sample sheet selection

## 11.1 Flow cell check

Complete a flow cell check to assess the number of pores available before loading the library.

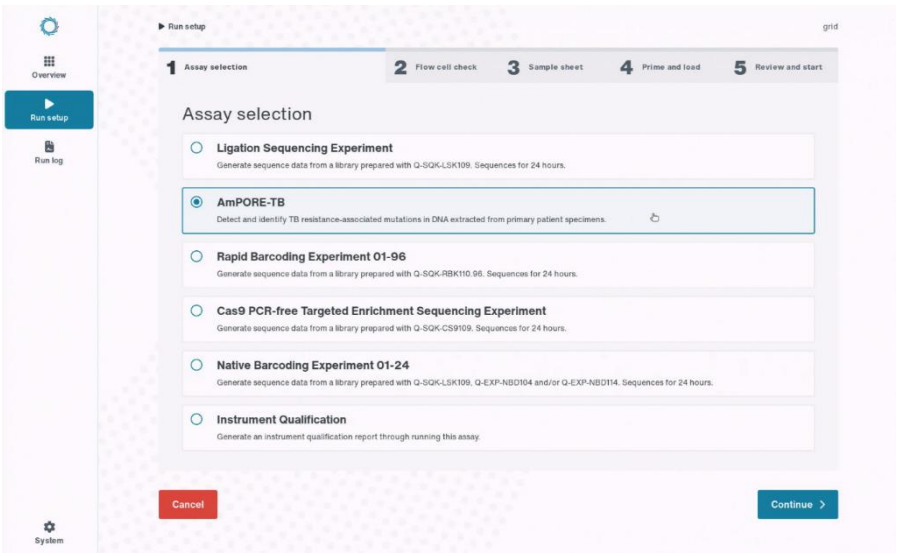
1. Open the device lid and slide the flow cell under the clip. Press down firmly on the flow cell to ensure correct thermal and electrical contact.



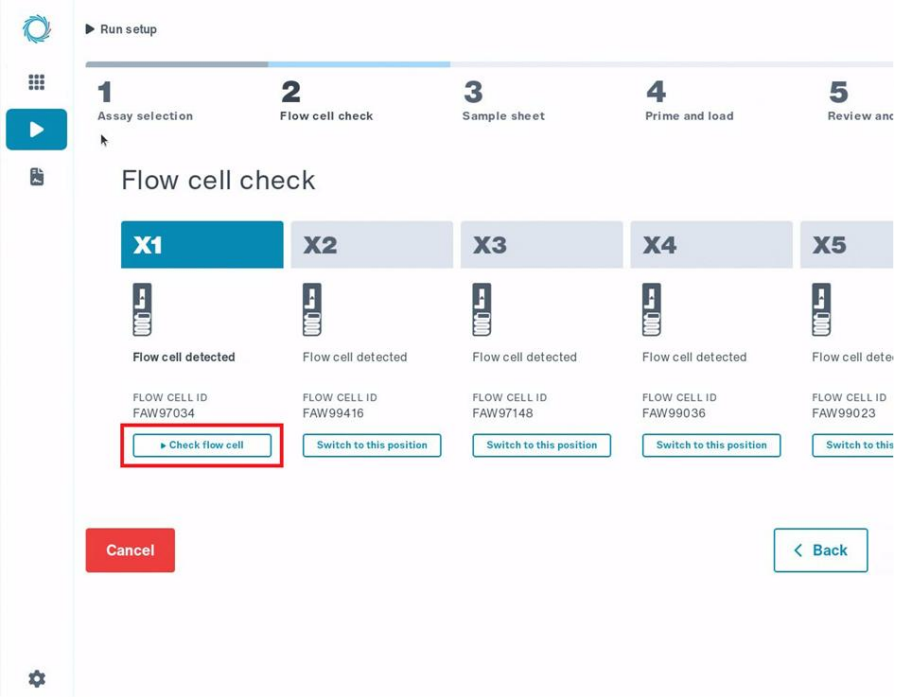
2. On the GridION Q home screen click on the wheel icon to start the sequencing software:

3. Once the software has opened, click **Run setup** underneath the relevant flow cell position.

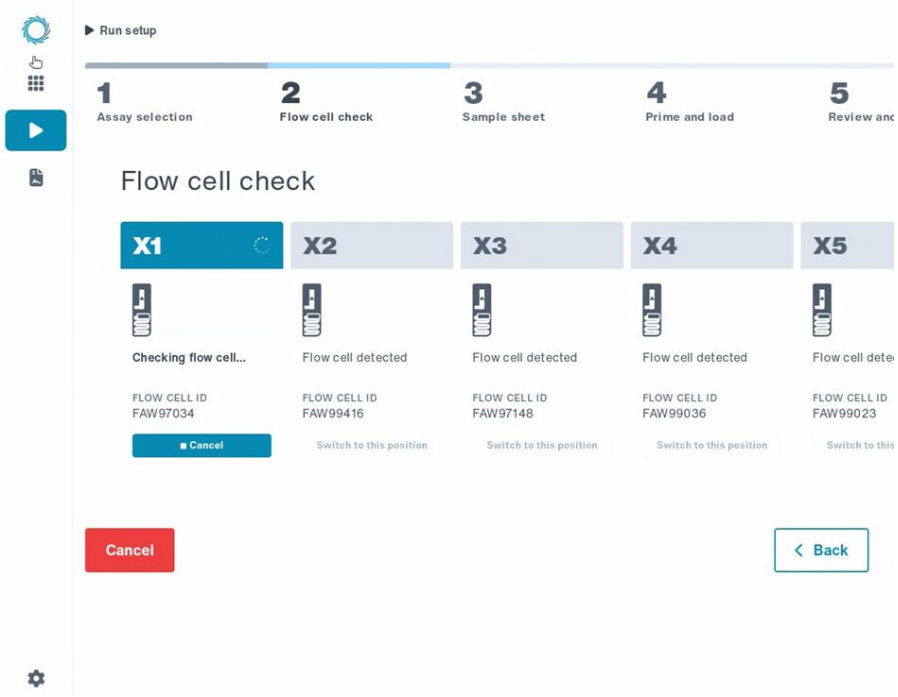
4. Select the **AmPORE-TB assay** then click **Continue.**



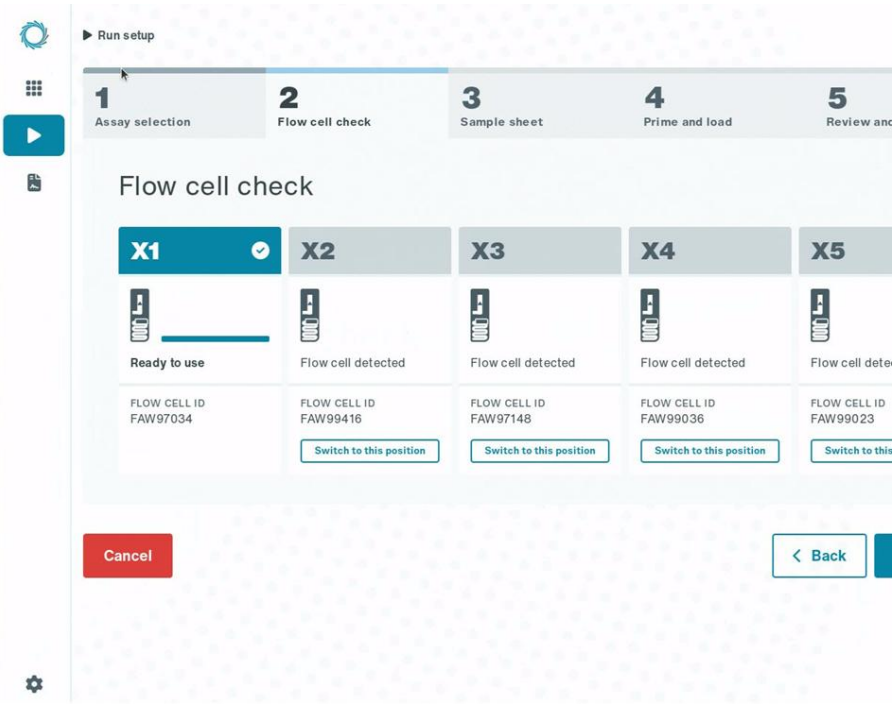
5. On the **Flow cell check** tab, click **Check flow cell** for the GridION Q position that contains the inserted flow cell.




6. Wait for the flow cell check to complete.



7. When a successful flow cell check has completed with the status **Ready to use**, click **Continue**.

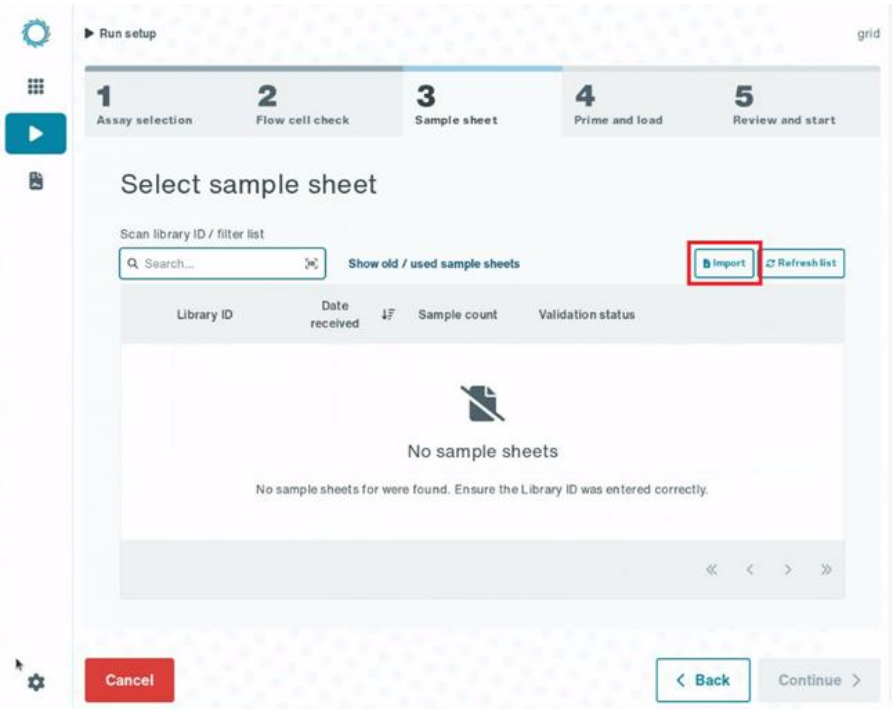



If the check fails, cancel the set up and repeat the Flow cell check. If a flow cell continues to fail, use a new flow cell and repeat the Flow cell check.

8. Follow the on-screen instructions.

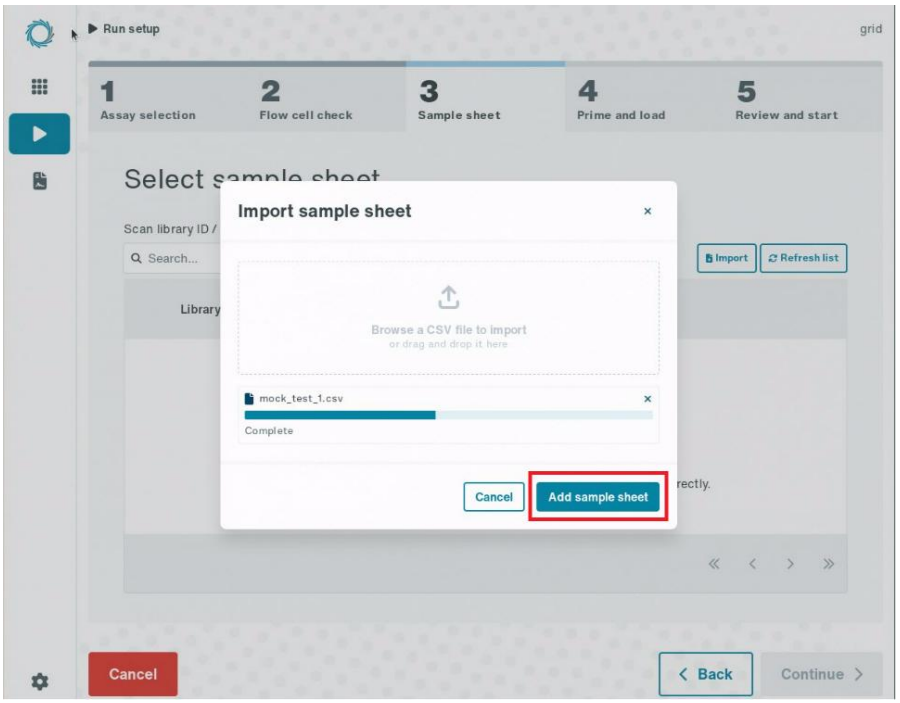
## 11.2 Sample sheet

1. On the **Sample sheet** tab, select your sample sheet from the list or click **Import.**

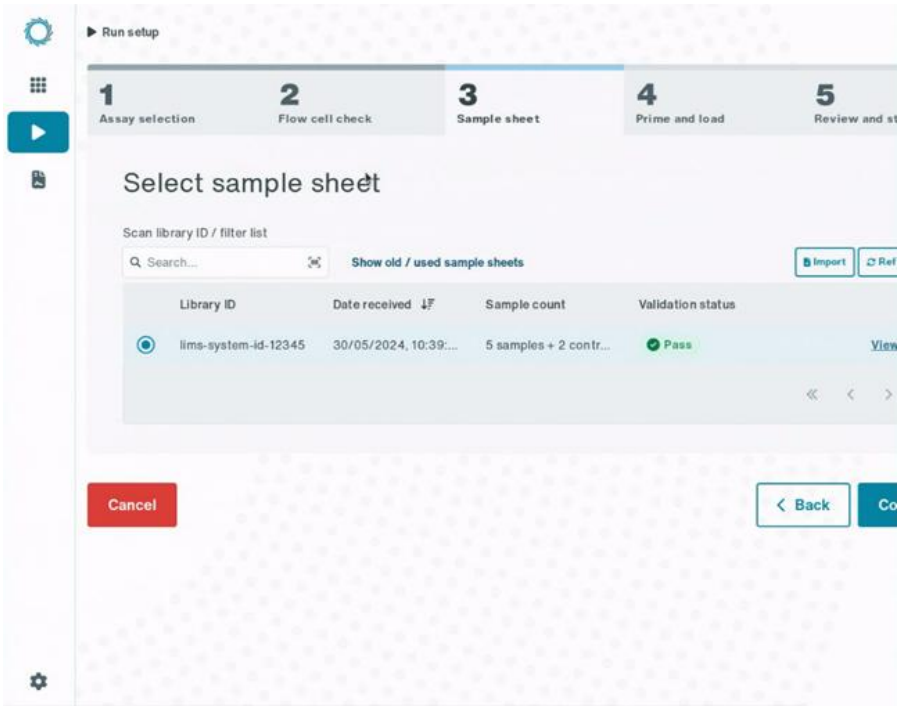



Ensure the correct sample sheet is selected. We recommend adding libraries to the sequencing device one at a time to avoid accidentally selecting an incorrect sample sheet.

2. If importing a sample sheet, locate the file and click **Add sample sheet**. If the sample sheet is rejected, check for barcode clashes between the sample sheet and the previous usage of the flow cell, and correct any issues.




3. Once the sample sheet has been approved, click **Continue**.



# 12 Flow cell loading

## 12.1 Preparing the library for loading

1. Thaw the reagents listed below at room temperature and store on ice. Before using the reagents, briefly spin down and pipette mix **three times**, unless stated otherwise.

| Individual reagents | Kit |
|---|---|
| Loading Beads II (LBII)<br>**Note:** Do not spin down; vortex to resuspend the beads. | AmPORE-TB Kit (OND-S-ATB01-RGL) |
| Flush Tether (FLT; pipette mix **10 times**) | |
| Flush Buffer (FB) | |
| Sequencing Buffer II (SBII) | |

2. Briefly spin down the Rapid Adapter F (RAP F) and mix by pipetting.

3. In a new 1.5 ml Eppendorf DNA LoBind tube, combine the following reagents with your library:

| Reagent | Volume per sample (µl) |
|---|---|
| Library | 11 |
| Rapid Adapter F (RAP F) | 1 |
| Total volume | 12 |

4. Gently mix by flicking the tube and spin down.

5. Incubate the reaction for 5 minutes at room temperature (18–23°C).

6. Store your prepared library on ice until ready to load onto the flow cell.

## 12.2 Flow cell preparation

We recommend all new users watch the Priming and loading your flow cell video before your first run.

1. Prepare the priming mix.

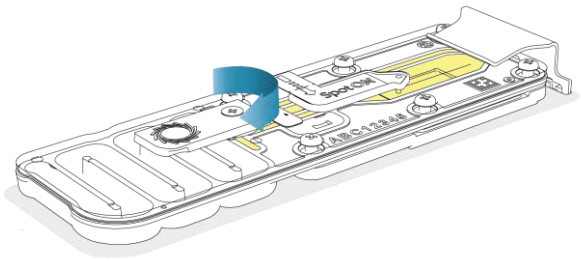
| Reagent | Volume per flow cell (µl) |
|---|---|
| Flush Tether (FLT) | 30 |
| Flush Buffer (FB) | 1,170 |
| Total volume | 1,200 |

2. Mix thoroughly.

We recommend using a P1000 pipette to prime your flow cell to create a seal with the ports in the flow cell.

3. Use the labelled image below to help correctly identify the different ports of the flow cell.


4. Locate the priming port next to the waste channel and slide the priming port cover clockwise to open.



Take care when drawing back buffer from the flow cell. Do not remove more than 20–30 µl, and make sure that the array of pores is always covered by buffer. Introducing air bubbles into the array can irreversibly damage pores.

5. After opening the priming port, remove any potential air bubbles under the cover by performing the following steps:

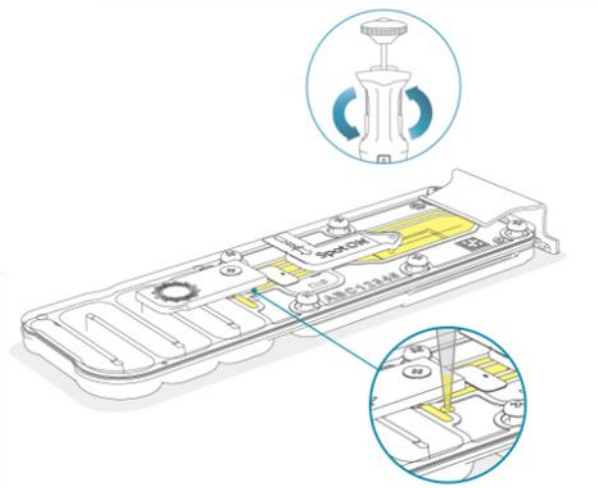
    a. Set a P1000 pipette to 200 µl.

    b. Carefully insert the tip into the priming port.

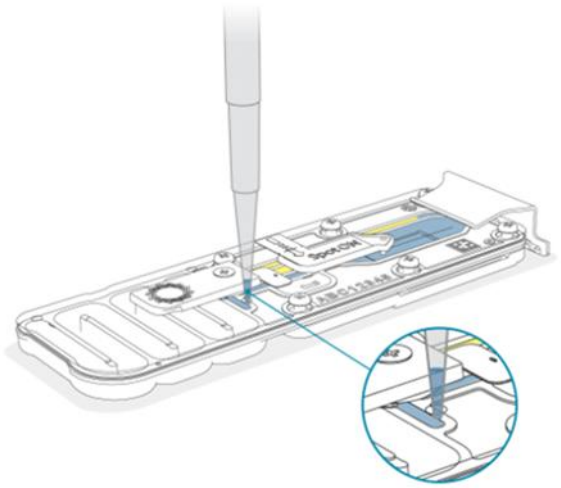
    c. Turn the wheel of the pipette until the dial shows 220–230 µl, to draw back 20–30 µl, or until you can see a small volume of buffer entering the pipette tip.

**Note:** Visually check that there is continuous buffer from the priming port across the sensor array.

Avoid the introduction of air bubbles by ensuring there are no bubbles or air gaps in the pipette tip before loading reagents.



6. Carefully load **800 µl** of the prepared priming mix into the flow cell via the **priming port**. Ensure you leave a small volume of fluid in the pipette tip to avoid the introduction of air bubbles.



7. **Wait for 5 minutes** after loading the priming mix. During this time, prepare the library for loading by following the next steps.

8. Vortex the thawed Sequencing Buffer (SBII) for 10 seconds and spin down.

9. Vortex Loading Beads II (LBII) immediately before using for 10 seconds, or until the beads are fully resuspended

The Loading Beads II (LBII) are a suspension of beads which settle very quickly. This reagent should be mixed immediately before every use to ensure the beads are resuspended before pipetting.

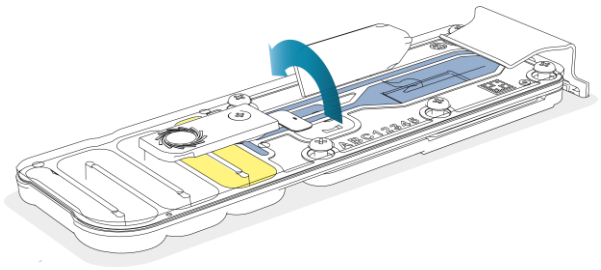
10. In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows:

| Reagent | Volume per flow cell (µl) |
|---|---|
| Adapted DNA Library | 12 |
| Sequencing Buffer II (SBII) | 37.5 |
| Loading Beads II (LBII), mixed immediately before every use | 25.5 |
| Total | 75 |

11. Pipette mix the entire contents of the tube 10 times.

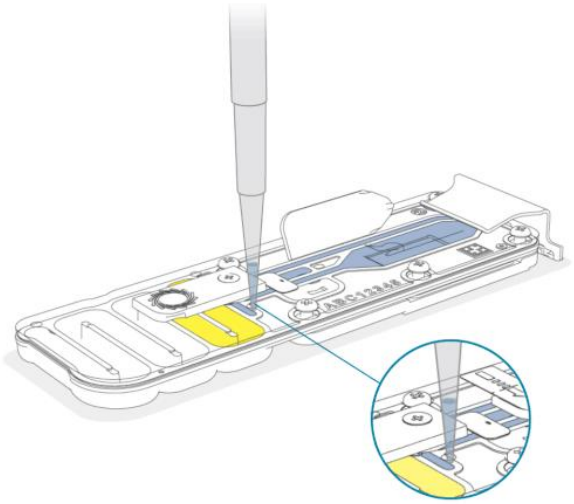
12. Locate the SpotON sample port cover that is on the sensor array. This cover is a plastic tab that is attached to the flow cell with a hinge at one end and a bung sealing the port on the sensor array.

13. Open the SpotON sample port cover by gently lifting the cover upwards to remove the bung from the port, whilst using the hinge to gently flip the cover over, as indicated below.



Avoid the introduction of air bubbles by ensuring there are no bubbles or air gaps in the pipette tip.

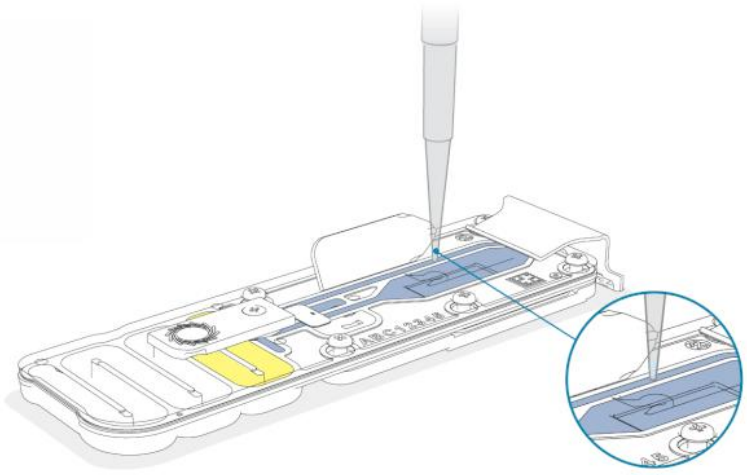
14. Via the **priming port**, carefully load 200 µl of the priming mix into the flow cell (**not** the SpotON sample port). Ensure you leave a small volume of fluid in the pipette tip to not introduce air bubbles. During this step, you will see buffer in the SpotON sample port.



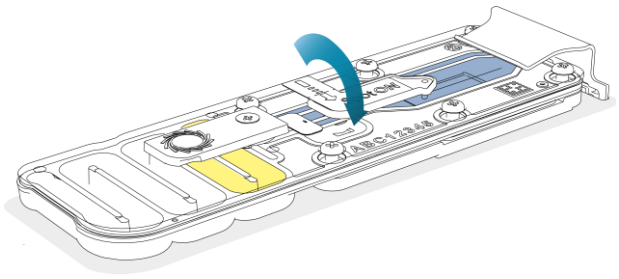
## 12.3 Flow cell loading

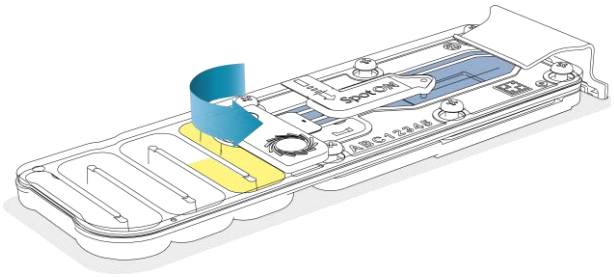
1. Mix the prepared library gently by pipetting up and down prior to loading the library to ensure the Loading Beads II (LBII) have not settled.

2. Via the **SpotON sample port**, load **75 µl** of the prepared library by hovering the pipette above the sample port and slowly dropping the prepared library into the port. Ensure each drop flows into the port.

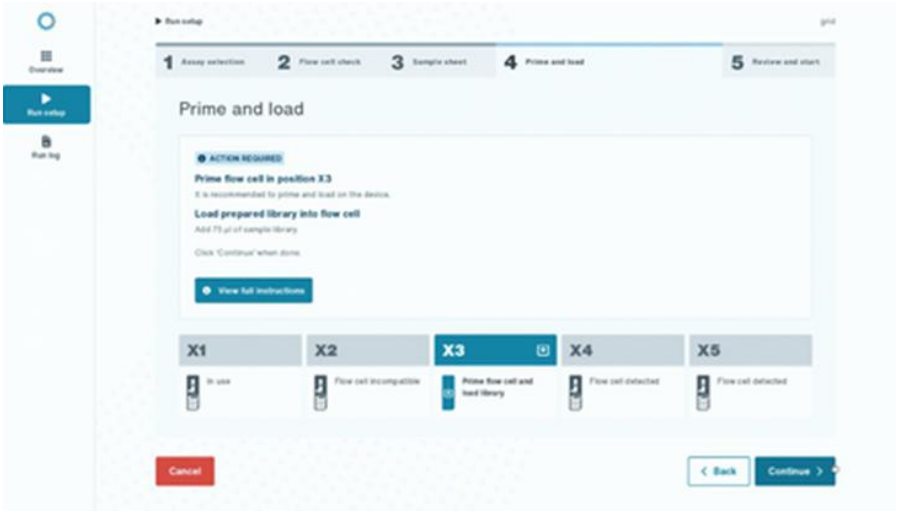


3. Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port. Slide the priming port closed and close the device lid.

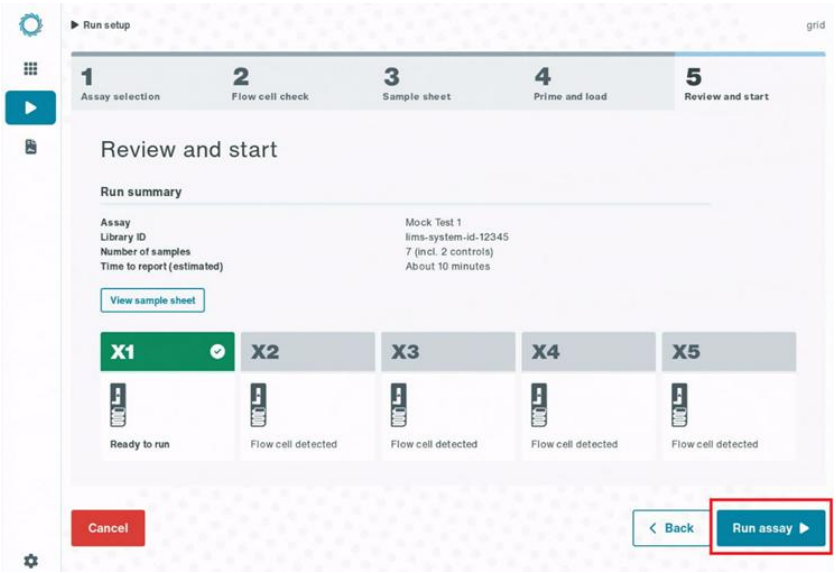




4. On the “Prime and Load” tab click **Continue** on the user interface once your library is loaded onto the flow cell.




5. Click **Run assay** to start your sequencing run.



6. After 30 seconds, the run will start. The sequencing overview page will display the run during sequencing.

7. At the end of the run, the workflow report can be accessed via the user interface, or you can download the file.

Do not remove a flow cell when a run is in progress. If a run is cancelled, the data will be lost and cannot be analysed.

# 13 Recalibrating and reusing a flow cell

This step aims to remove the initial library and prepare the flow cell for loading a subsequent library using the Flow Cell Recalibration Kit (OND-E-FRK01-RGL). After the flow cell has been recalibrated, a new library can be loaded, or the flow cell can be stored at 4°C. Each flow cell can be used up to 4 times, with recalibration after each run.

We recommend using a P1000 pipette to prime your flow cell to create a seal with the ports in the flow cell.

1. Place the tube of Calibration Mix (CMX) on ice. Do not vortex the tube.
2. Thaw a tube of Recalibration Buffer (RB) at room temperature and vortex to mix before spinning down. Then place on ice.
3. In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the following Recalibration Mix for the flow cell:

| Component | Volume per flow cell (µl) |
|---|---|
| Calibration Mix (CMX) | 2 |
| Recalibration Buffer (RB) | 398 |
| Total | 400 |

4. Mix well by pipetting, and place on ice. Do not vortex the tube.

It is vital the priming port and SpotON sample port cover are both closed to prevent air from being drawn into the flow cell which would cause a significant loss of pores.

5. Leave the flow cell inserted into the device and ensure the priming port cover and SpotON sample port cover are closed, as illustrated in the image below.


6. Insert a P1000 pipette into **waste port 1**, located near the priming port, and remove all fluid from the waste channel.

**Note:** As both the priming port and SpotON sample port are closed, no fluid should leave the sensor array area.

7. Slide the priming port cover clockwise to open the port.

Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

8. After opening the priming port, remove any potential air bubbles under the cover by performing the following steps:

   a. Set a P1000 pipette to 200 µl.

   b. Insert the tip into the priming port.

   c. Turn the wheel until the dial shows 220–230 µl, or until you can see a small volume of buffer entering the pipette tip.

**Note:** Visually check that there is continuous buffer from the priming port across the sensor array.

Avoid the introduction of air bubbles by ensuring there are no bubbles or air gaps in the pipette tip.

9. Carefully load 200 µl of the prepared Recalibration Mix into the flow cell via the **priming port** and start a timer for 5 minutes.

10. Once the 5-minute incubation is up, carefully load the remaining 200 µl of the prepared Recalibration Mix into the flow cell via the priming port.

11. Close the **priming port** and wait for **1 hour**.

It is vital the priming port and SpotON sample port cover are both closed to prevent air from being drawn into the flow cell, which would cause a significant loss of pores.

12. Insert a P1000 pipette into **waste port 1**, located near the priming port, and remove all fluid from the waste channel.


**Note:** As both the priming port and SpotON sample port are closed, no fluid should leave the sensor array area.

13. Thaw a tube of Flow Cell Storage Buffer (FCS) at room temperature and mix thoroughly by pipetting and spin down briefly.

14. Slide the flow cell priming port cover clockwise to open the port.

**Note:** The priming port cover is located next to the waste channel.

Take care when drawing back buffer from the flow cell. Do not remove more than 20-30 µl, and make sure that the array of pores are covered by buffer at all times. Introducing air bubbles into the array can irreversibly damage pores.

15. After opening the priming port, remove any potential air bubbles under the cover by performing the following steps:

    a. Set a P1000 pipette to 200 µl.

    b. Insert the tip into the **priming port.**

    c. Turn the wheel until the dial shows 220–230 µl, or until you can see a small volume of buffer entering the pipette tip.

**Note:** Visually check there is continuous buffer from the priming port across the sensor array.

Avoid the introduction of air bubbles by ensuring there are no bubbles or air gaps in the pipette tip.

16. Slowly add **500 µl** of Flow Cell Storage Buffer (FCS) via the **priming port** of the flow cell.

17. Close the **priming port.**

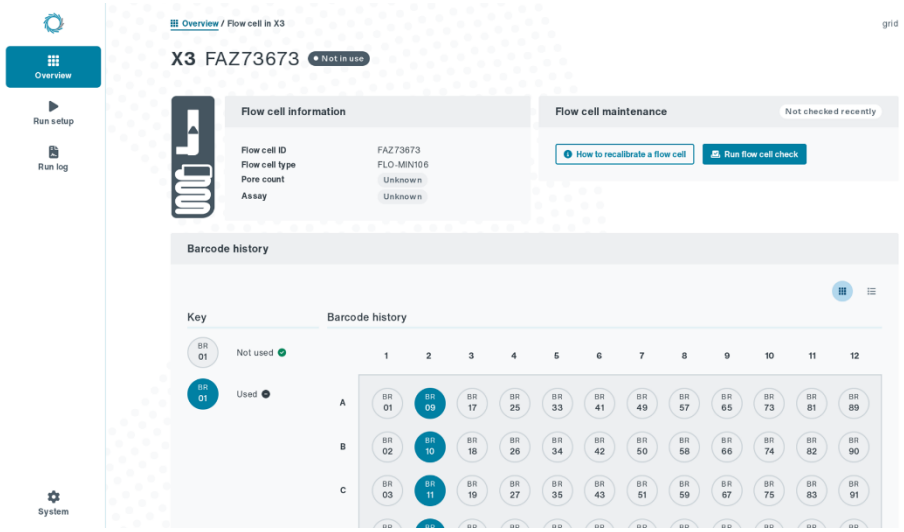
It is vital the priming port and SpotON sample port cover are both closed to prevent air from being drawn into the flow cell, which would cause a significant loss of pores.

18. Insert a P1000 pipette into **waste port 1**, located near the priming port, and remove all fluid from the waste channel.

**Note:** As both the priming port and SpotON sample port are closed, no fluid should leave the sensor array area.


19. The flow cell can now be stored in the provided blister pack at 4–8°C or used immediately.

20. When reusing a flow cell, it should be removed from storage and allowed to warm to room temperature for approximately 5 minutes. Check the barcode history, on the “Flow cell information” page (as shown below), before loading a library and sequencing to ensure different barcodes are used for subsequent sequencing runs.



# 14 Quality control

It is necessary to include at least one well for the no template control, one well for the positive control, and to include the internal control for all samples on the run.

- **Internal Control (IC):** The IC is provided and is added to every sample and control.
- **No Template Control (NTC):** The NTC is a nuclease-free water blank and is treated as a sample input having been processed from decontamination through the rest of the procedure as a sample.
- **Positive Control (PC):** The positive control used is ATCC Genomic DNA from the *Mycobacterium tuberculosis* strain H37Ra. PC is processed exactly like a sample, including the addition of the IC.

A run is considered invalid if any of the controls do not meet thresholds outlined in Section 2.3 **Assay validity.**

# 15 Analysis

## 15.1 Outputs

### Primary outputs

The primary output from the AmPORE-TB analysis software are reports which are presented in the sequencing software user interface:

- The Analytical Run Report in HTML format
- Per-sample reports in PDF format

### Secondary outputs

Secondary outputs are provided for troubleshooting or to enable LIMS compatibility only and are located on the file system of the sequencing device. Descriptions of the secondary outputs generated by AmPORE-TB analysis are given below.

| File | Description |
|---|---|
| <SAMPLE_NAME>.bam | Mapped reads for the sample. |
| <SAMPLE_NAME>.bam.bai | Index file for the mapped reads. |
| wf-tb-amr-report.csv | Tabular information for samples in the run for non-human processing/LIMS import. |

## 15.2 Analytical run report

The analytical run report contains the outcome of quality control checks at the run level, and results for all test samples on the sequencing run.

Analytical run reports are accessed from the **Run log** in the sequencing software. To display the report, locate the run in the list and click **View run**. In the Run reports section, locate the Analytical run report row and click **View**. The report can also be accessed via LIMS.

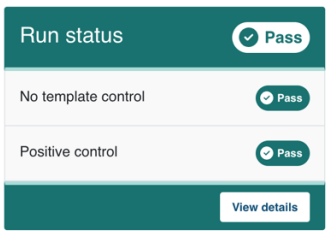
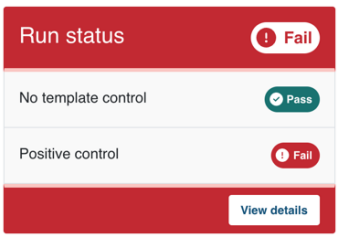
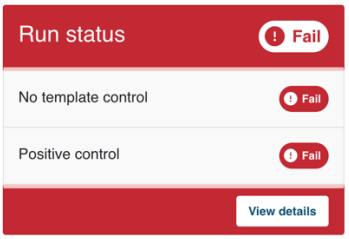
### Run summary

The run summary contains useful information about the assay and device used to generate the data used as input for the AmPORE-TB analysis. Descriptions of the metadata provided on the report are given in the table below.

| Field | Description |
|---|---|
| Report generated on | The date and time the report was created. |
| Analysis version | The version of the AmPORE-TB analysis used. |
| Started at | The date and time the sequencing run was started. |
| Started by | The user who started the sequencing run. |
| Device serial number | The serial number of the Oxford Nanopore Technologies' device used for sequencing. |
| Device type | The type of Oxford Nanopore Technologies device used for sequencing. |
| Library ID | The provided identifier of the library. |
| Sequencing software version | The version of the sequencing software used. |
| Flow cell ID | The identifier of the flow cell used. |
| Workflow broker | The version of EPI2ME installed on the device used to control analysis execution. |

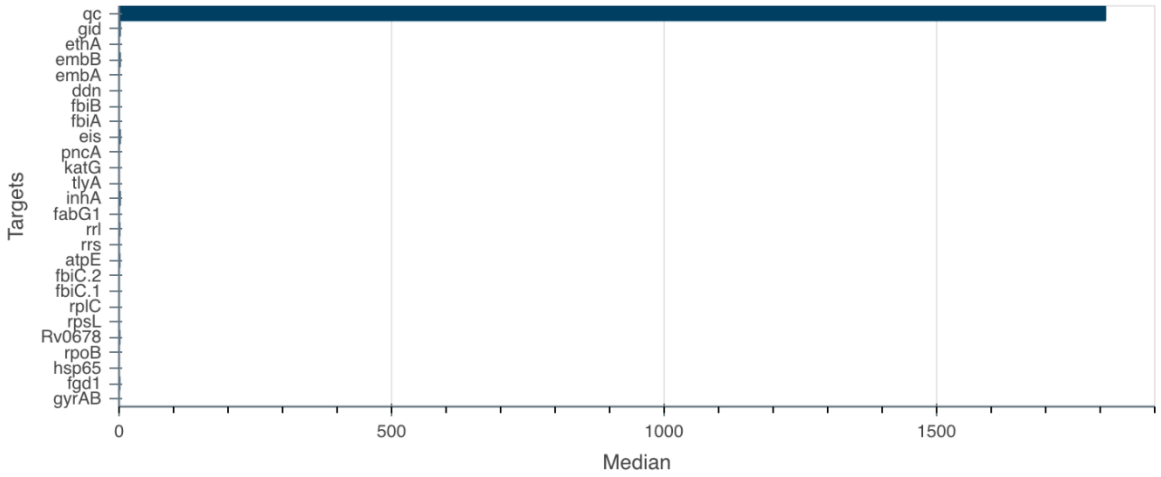
### Run status

In AmPORE-TB, a sequencing run failure occurs if either the positive or no template control fail quality control checks as described in Section 2.3. In the event of a run QC failure all samples on the run will be also set to fail and antimicrobial resistance evidence is not shown. Click **View details** to view more information on these controls.

| Examples of the run quality control status | | |
|---|---|---|
|  |  |  |
| A successful AmPORE-TB sequencing run. | A sequencing run where one of the process controls fails sequencing (e.g., either the no template control or the positive control). | A sequencing run where both process controls fail sequencing (failure of both the no template control and the positive control). |

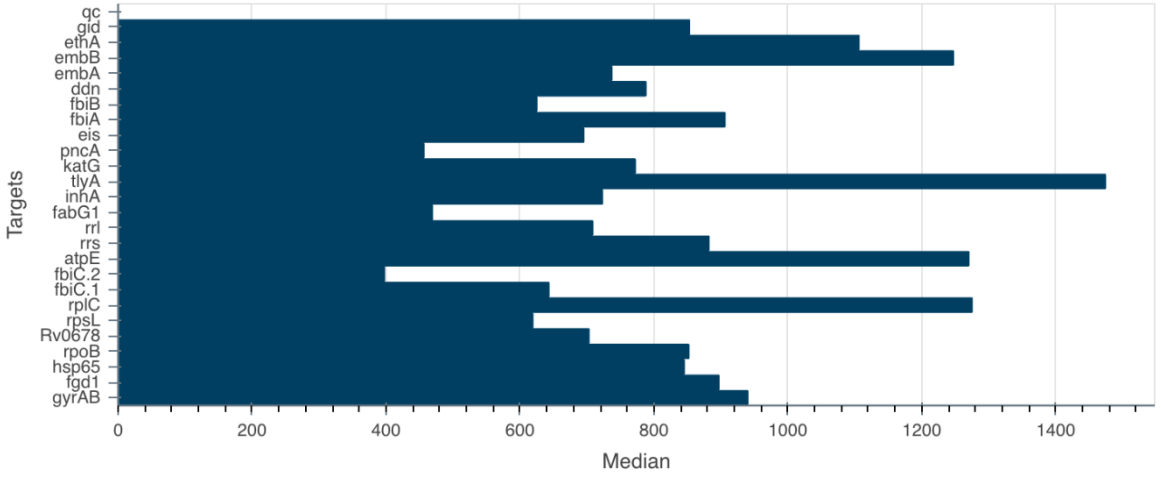
### No template control

An example coverage bar chart for the no template control is shown below for comparison to the criteria in Section 2.3.




### Positive control

An example coverage bar chart for the positive control is shown below for comparison to the criteria in Section 2.3.




You will notice that the “qc” target in the positive control does not have coverage. This is expected, as the internal quality control is competitive and is unlikely to amplify when DNA from the MTBC genome is present.

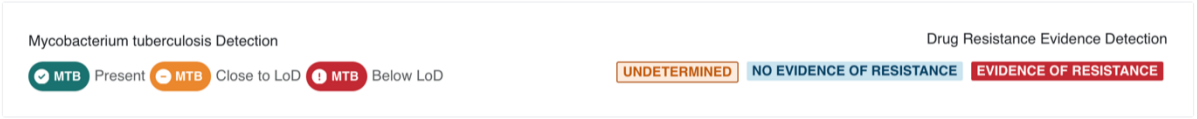
### Sample cards

Each sample is represented by a sample card, containing the sample name and associated barcode. This card contains at-a-glance results for the sample. Each sample card shows a label indicating the level of MTBC detection (green with tick, present; yellow with hyphen, close to limit-of-detection; red with exclamation, below the limit of detection). More information on the limit of detection is given below. Additionally, each sample card contains a label stating whether the sample has passed or failed, and shows the list of drugs, with those in blue indicating that the sample has no evidence of resistance to the drug, and those in red indicating that we have detected a mutation associated with resistance to

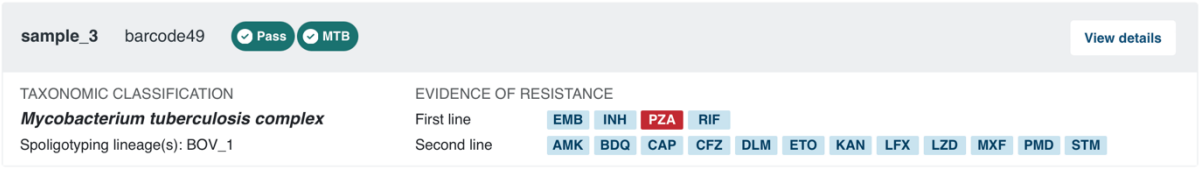
that drug. If the resistance status for a drug is unable to be determined due to lack of coverage of a target in the assay, this is flagged in orange as ‘undetermined’.

The taxonomic classification and the spoligotyping lineage are also displayed.

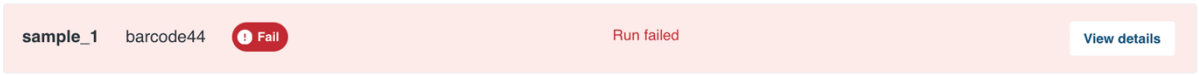
The key for the sample cards:




Sample cards provide at-a-glance information about multiple attributes of the sequenced sample:



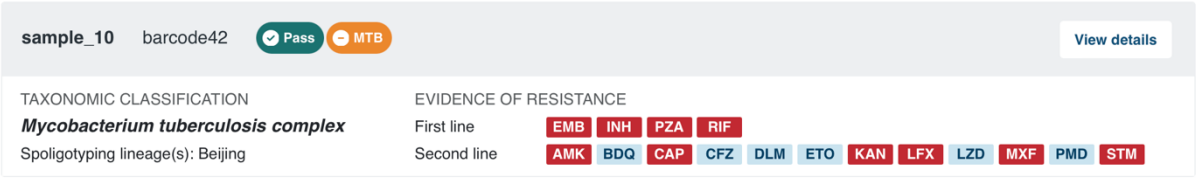

Example of a sample card when the run is deemed to have failed:



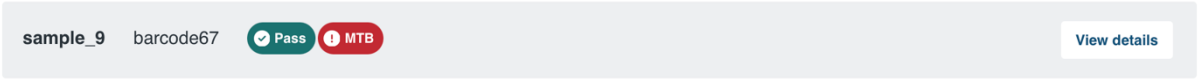

### Limit of detection

To ensure we are providing reliable calls, samples with uneven or low coverage will be subject to an increased allele frequency cut-off of 0.4 (or 40%). In some cases, these samples may be deemed to be “Close to LoD”. If insufficient coverage of the drug resistance targets is detected (<20x) then the sample will be deemed to be below LoD and no drug resistance evidence will be displayed.

Example of a sample card where MTBC is close to the limit of detection:



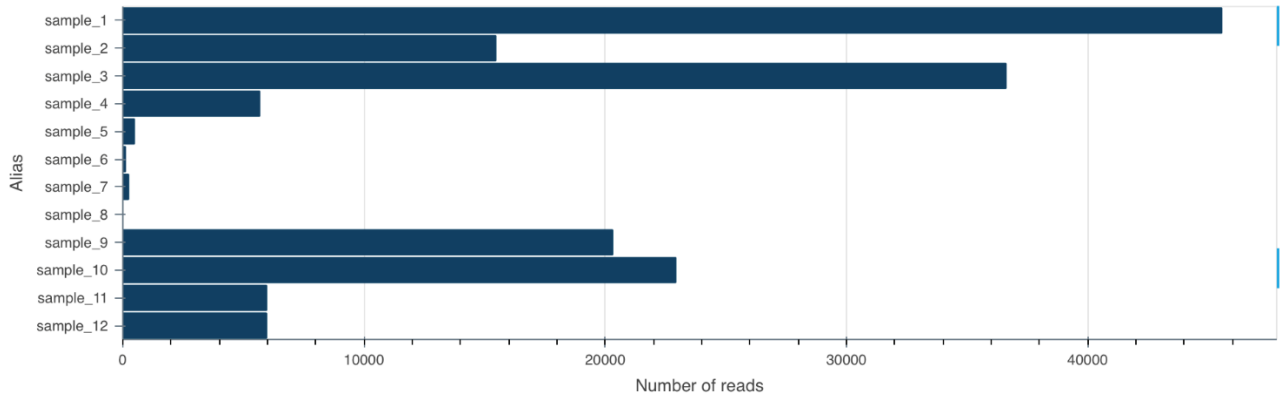

Example of a sample card where no MTBC has been detected:



### Sequencing summary

The bar plot displays the total number of reads generated for each sample in the run, after the samples have been screened for non-specific amplification.

Example of sequencing summary plot:

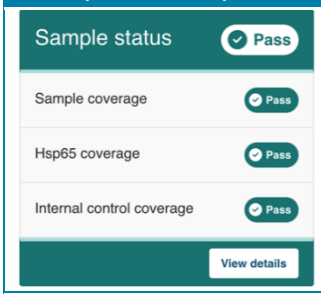
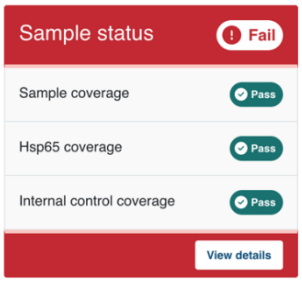
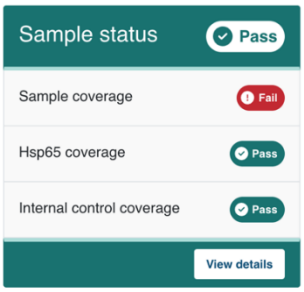
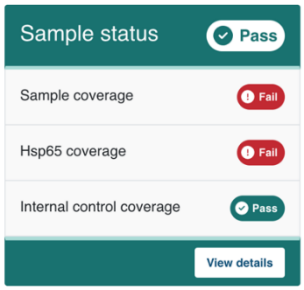
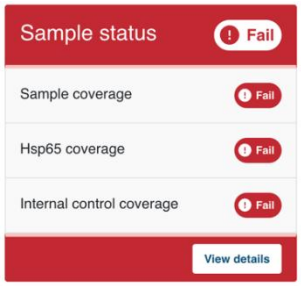



### Sample details

The **Sample details** section displays the barcode and read count for the sample, along with other information related to the assay run.

### Sample status

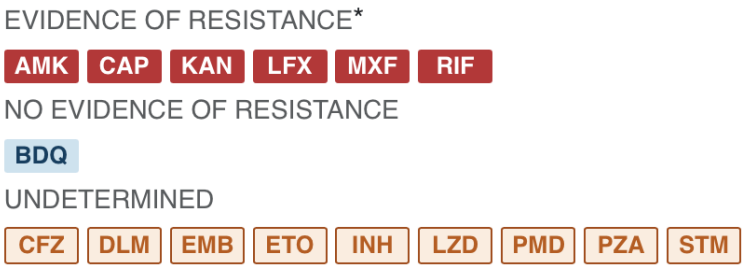
The **Sample status** panel provides a summary of quality control checks (Quality Control Checks, pages 1 and 2), including: sample coverage, hsp65 coverage, and internal control coverage. Clicking View details will display a panel with further detail on each coverage check, including the threshold required to pass, as well as coverage of individual targets within each check.

| Examples of sample status | |
|---|---|
|  | The sample has passed all quality control checks. |
|  | The AmPORE-TB run has failed due to one or both process controls failing, therefore no drug resistance information can be displayed. |
|  | An NTM has been detected (hsp65 is sufficiently covered but there are insufficient reads for the assay drug resistance targets in MTBC). |
|  | No MTBC or NTM present but the sample PCR was successful as the internal control is covered. |
|  | The sample has failed all quality control checks: hsp65 coverage is too low, sample coverage is too low, and the internal coverage is too low. |

### Resistance summary

A summary panel shows the status of the evidence of resistance for each drug. Drugs in blue indicate that the sample has no evidence of resistance to the drug, and those in red indicate that we have detected a mutation which has evidence of association with resistance to that drug. If the resistance status for a drug is unable to be determined due to lack of coverage of a target (one or more) for that drug in the assay, this is flagged as orange and "undetermined".

Example of the drug resistance summary for an individual sample:




This section also contains a table of all the mutations detected by the AmPORE-TB analysis that are associated with drug resistance, and has the following columns:

| Column | Description |
|---|---|
| Gene | The gene or locus in which the mutation has been found. |
| Position | The genomic position of the mutation, aligned against the MTBC reference genome (NC_000962.3). |
| REF | The reference base at the listed genomic position. |
| ALT | The mutation base, or indel, at the listed genomic position. |
| Detected Frequency | The proportion of reads the mutation has been found in compared to the total reads. |
| HGVS Protein | The HGVS nomenclature for the mutation at the protein level, if applicable (not all mutations will affect protein coding regions). |
| HGVS Nucleotide | The HGVS nomenclature for the mutation at the cDNA level. Applicable for all mutations. |
| Drug (Source) | The drug that this mutation causes resistance to and the source of that association. |

The list of mutations detected which are associated with resistance:

| Gene | Position | REF | ALT | Detected Frequency** | HGVS Protein | HGVS Nucleotide | Drug (Source***) |
|---|---|---|---|---|---|---|---|
| rpoB | 761155 | C | T | 0.92 | p.Ser450Leu | c.1349C>T | RIF (WHO G1) |
| rrs | 1473246 | A | G | 0.99 | Not applicable | n.1401A>G | AMK, CAP, KAN (WHO G1) |
| inhA | 1673425 | C | T | 0.92 | Not applicable | c.-777C>T | ETO, INH (WHO G1) |
| pncA | 2289214 | G | A | 0.89 | p.Gln10* | c.28C>T | PZA (WHO G1) |
| embB | 4247429 | A | G | 0.97 | p.Met306Val | c.916A>G | EMB (WHO G1) |

### Drug resistance association

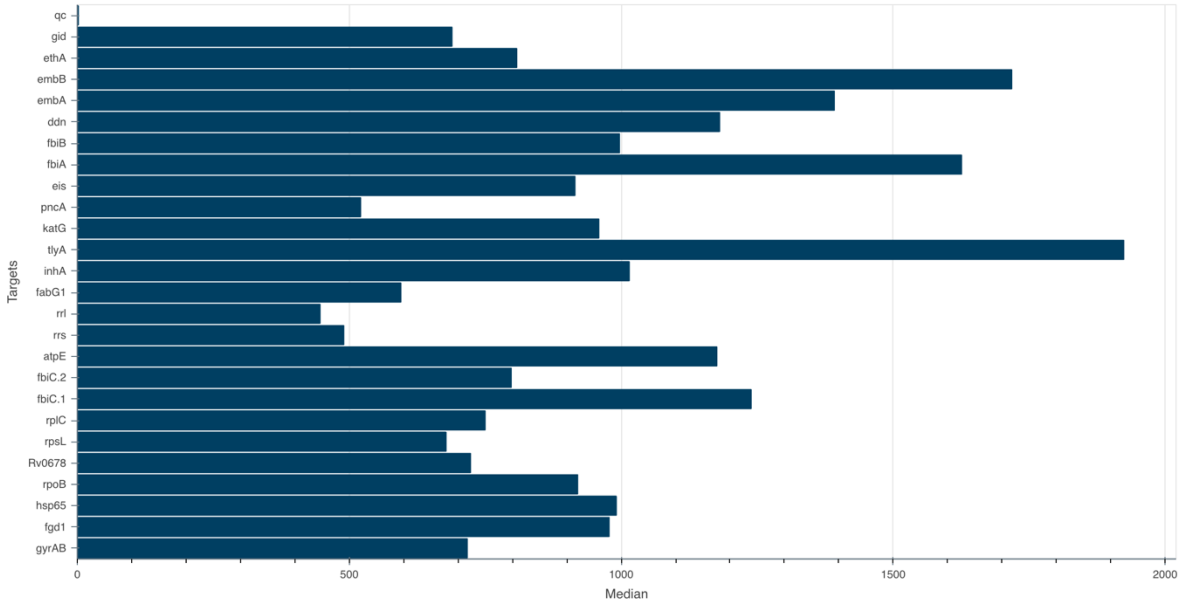
A mutation could be deemed to have evidence of association with resistance in three scenarios:

- The mutation matches a Group 1 or Group 2 mutation in the WHO 'Catalogue of mutations in *Mycobacterium tuberculosis* complex and their association with drug resistance' version 2 (WHO, 2023)
- The mutation fulfils an expert rule detailed in the WHO catalogue
- The whole target is deemed to be deleted in a gene known to have loss of function as a mode of resistance.

### Coverage

The bar plot in this section shows the median coverage of all the targets in the test for the sample. As noted above, the internal control ('qc') is competitive and therefore will only amplify in the absence of assay targets.

Example of the coverage plot for a test sample:

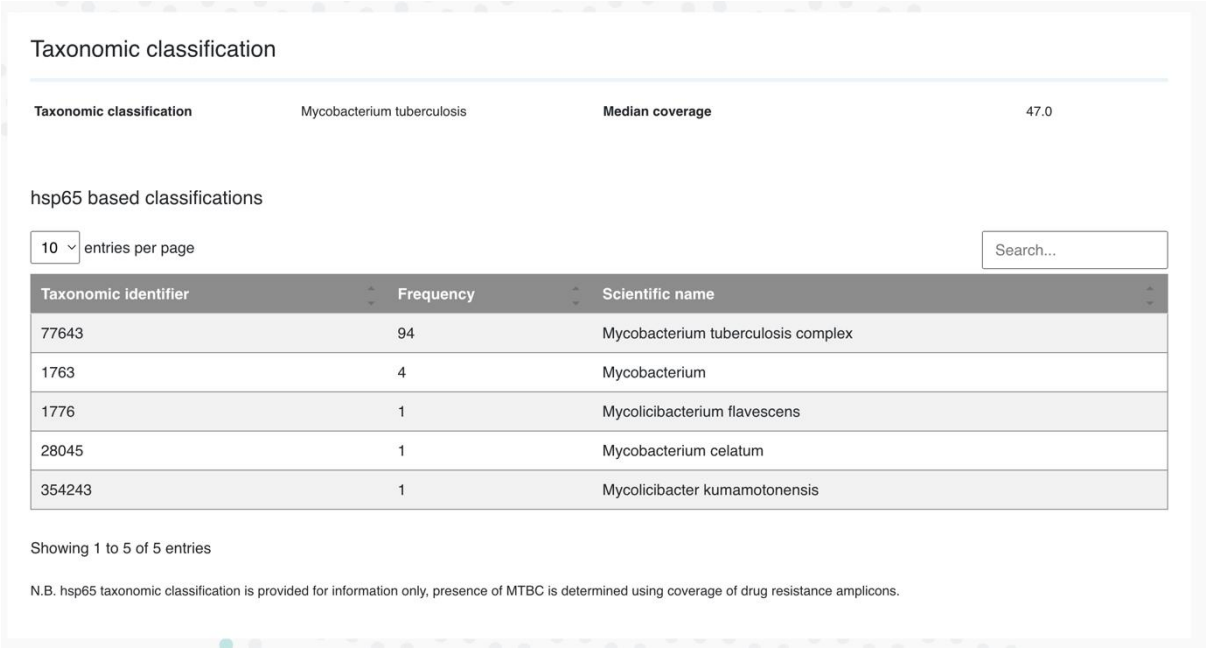


### Taxonomic classification

This section of the report provides an overview of the specific taxonomic classification of reads produced from *hsp65* amplification. The presence of MTBC is detected using drug resistance targets and taxonomic classification is provided for information only. The median coverage of the hsp65 locus is indicated, and a table is provided following hsp65-based classifications, which contains the following columns:

| Column | Description |
|---|---|
| Taxonomic ID | The taxonomic ID assigned to this record by Kraken2 |
| Frequency | The number of reads assigned to the taxonomic ID |
| Scientific name | The scientific name of the Genus or Species assigned to the taxonomic ID |

Example of the species identification section of the sample detail:



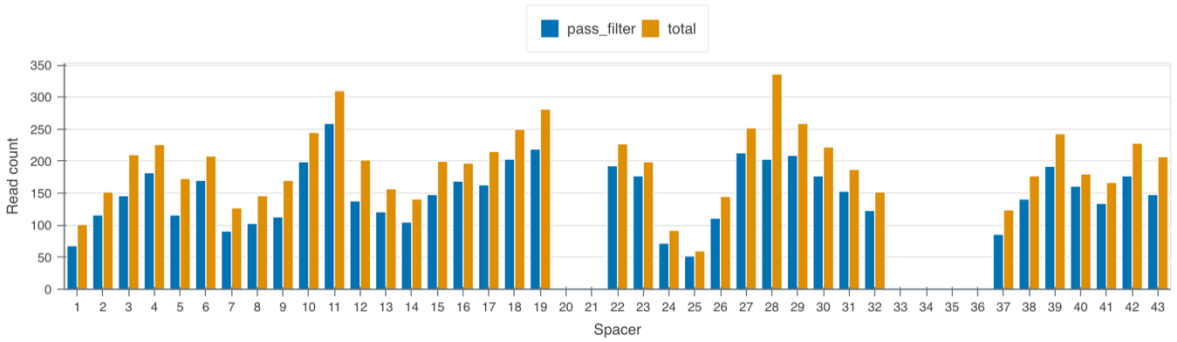

### Spoligotyping

In AmPORE-TB, spoligotyping is performed, which involves identifying the lineage of the MTBC present. This is for information only. In the MTBC genome, there is a region of direct repeat sequences. Between each of these repeat sequences are multiple unique spacer sequences. The presence of these spacer sequences enables 'typing' of the MTBC present in the sample. Previously, this was carried out by hybridisation, but sequencing data can detect the spacer through bioinformatic sequence analysis. For spoligotyping, the following results are displayed.

Description of the information provided in the spoligotyping section of the analytical run report:

| Key | Description |
|---|---|
| Sitvit2 lineage | The lineage assigned to the test sample by comparing the spoligotype with those in the Sitvit2 database. |
| Spoligotype binary | The presence (1) or absence (0) of each of the 43 spacers used for spoligotyping. |
| Octal | A 15-digit code which represents the spoligotype (base 8 number system with digits 0-7). |
| Total reads | The number of total reads mapping to the spoligotyping region. |
| Pass reads | The number of QC pass reads mapping to the spoligotyping region. |

Example of the spoligotyping bar plot:




## 15.3 Per-sample report

Alongside generating an overall report for all targets and all samples analysed, Am-PORE-TB also produces individual reports per sample in PDF format, with each section described below.

### Result summary

The results summary gives an overview of the individual sample, whether MTBC was detected or not and, if MTBC was detected, which drugs there is evidence for resistance.

Per sample report result summary example:

Mycobacterium tuberculosis was detected in sample_1

**sample_1 has evidence of resistance to amikacin, capreomycin, ethambutol, ethionamide, isoniazid, kanamycin, pyrazinamide and rifampicin**

Example of a failed sample run:

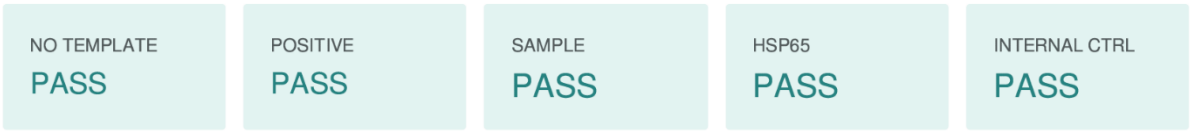
Mycobacterium tuberculosis was detected in sample_2

**Resistance information unavailable**

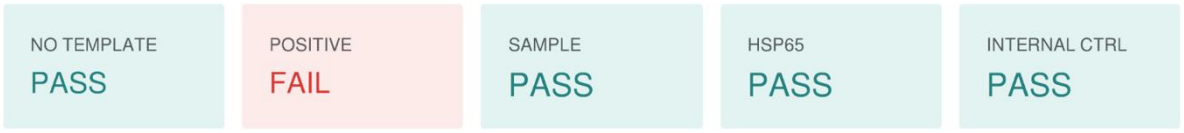
### Assay quality control checks

Like the analytical run report, the individual sample report shows whether the sample has passed or failed for each of the following criteria: no-template control, positive control, sample, *hsp65* and the internal control. A run will fail if either (a) the positive control has insufficient amplification and detection to pass and/ or (b) the no-template control has significant amplification outside of the internal control.

Per sample quality control checks, all pass:




Per sample quality control checks, example of failure:




### Evidence of drug resistance

This panel lists the first- and second-line drugs used to treat MTBC, and whether there has been resistance detected against each of these drugs in the sample. If resistance against a drug is detected, this is highlighted in red, and if no evidence of resistance against a drug is detected, this is highlighted in blue. If the resistance status for a drug is unable to be determined due to lack of coverage of a target in the assay, this is highlighted in orange.

The results are displayed in the per-sample report drug resistance table with three columns:

| Column | Description |
|---|---|
| Drug | The specific AMR target used for MTBC treatment, which is separated into first line and second line drugs. |
| Status | The status of that drug in terms of whether evidence of resistance has been detected. |
| Gene (Mutation, Frequency) | This column is only populated if evidence of resistance against the drug has been detected.<br><br>This column contains the associated resistance gene of that drug, the specific HGVS protein or nucleotide mutation (whichever is most appropriate) detected within that gene, and the proportion of the sequencing reads in which it has been found. |

Example of the drug resistance table:

First line drugs

| Drug | Status | Gene (Mutation, Frequency) |
|---|---|---|
| Ethambutol | Evidence of resistance | embB (p.Met306Val, 0.97) |
| Isoniazid | Evidence of resistance | inhA (c.-777C>T, 0.92) |
| Pyrazinamide | Evidence of resistance | pncA (p.Gln10*, 0.89) |
| Rifampicin | Evidence of resistance | rpoB (p.Ser450Leu, 0.92) |

Second line drugs

| Drug | Status | Gene (Mutation, Frequency) |
|---|---|---|
| Amikacin | Evidence of resistance | rrs (n.1401A>G, 0.99) |
| Bedaquiline | No evidence of resistance | |
| Capreomycin | Evidence of resistance | rrs (n.1401A>G, 0.99) |
| Clofazimine | No evidence of resistance | |
| Delamanid | No evidence of resistance | |
| Ethionamide | Evidence of resistance | inhA (c.-777C>T, 0.92) |
| Kanamycin | Evidence of resistance | rrs (n.1401A>G, 0.99) |
| Levofloxacin | No evidence of resistance | |
| Linezolid | No evidence of resistance | |
| Moxifloxacin | No evidence of resistance | |
| Pretomanid | No evidence of resistance | |
| Streptomycin | No evidence of resistance | |

## 15.4 CSV run report

The CSV report, wf-tb-amr-report.csv, provides a machine-readable tabular format of most of the results available in the HTML file described above and is intended for LIMS or for import into other downstream systems.

The table below details the first 12 columns contained in the report. The remaining columns are a list of drugs along with a semi-colon separated list of variants detected which have evidence of conferring resistance to that drug. If a sample call is 'negative', 'fail', or 'ntm', then there will be no variants listed.

| Field name | Description |
|---|---|
| sample | The sample name given in the sample sheet |
| barcode | The sample barcode given in the sample sheet |
| library_id | Library identifier |
| flowcell_id | Flow cell identifier |
| qc_status | Quality control status of the sample, either **pass** or **fail** |
| call | The final overall result for the sample:<br>• evidence_of_resistance evidence of resistance detected<br>• no_evidence_of_resistance no evidence of resistance detected<br>• negative sample deemed to be below LoD<br>• ntm non-tuberculous mycobacteria detected<br>• fail sample has failed quality control checks |
| n_reads | The number of reads generated for the sample, after the samples have been screened, and reads derived from non-specific amplification have been filtered out |
| passed_targets | A semi-colon separated list of amplicons which have passed the coverage threshold (*amplicon:median_coverage*) |
| failed_targets | A semi-colon separated list of amplicons which have failed the coverage threshold (*amplicon:median_coverage*) |
| hsp65_species | The top species hit resulting from taxonomic classification of reads produced from *hsp65* amplification |
| spoligotype_binary | The presence (1) or absence (0) of each of the 43 spacers used for spoligotyping, if available |
| spoligotype_lineage | The lineage assigned to the test sample by comparing the spoligotype with those in the Sitvit2 database, if available |
| AMK....STM | A column per drug containing any variants that have evidence of causing resistance, in the format *gene:variant:frequency.* Multiple mutations are separated by a semi-colon. |

# 16 Cleaning, decontamination, and waste disposal

## 16.1 General decontamination

1. Safely discard unused reagents, waste material, samples in accordance with local regulations, as these are potentially infectious.

2. Decontaminate surfaces with 0.5% sodium hypochlorite (bleach) diluted with deionized or purified water.

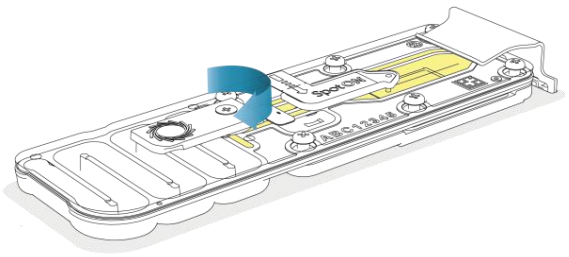
## 16.2 Flow cell flushing and returns

1. Request a flow cell returns box of the required size at: https://nanoporetech.com/upsflowcellreturn. Please note that the flow cell returns policy might vary by location. Contact your support representative for guidance.

Any liquid overflow from the flow cell may be hazardous and should be disposed of appropriately in accordance with local regulations and not disposed of directly into the domestic water supply. Avoid contact with strong acids or alkalis.

2. Remove the flow cell and place it on top of sufficient absorbent material on the bench to take up approximately 4 ml of flush waste per flow cell.

3. Close the SpotON port cover, ensuring the bung enters the SpotON port. Then slide open the priming port cover.



4. Place the flow cell on the absorbent material at a 45° angle. The reservoir end of the flow cell should be in contact with the absorbent material, which will capture any overflow of buffer/sample leaving the flow cell.

5. Using a P1000 pipette, slowly load a total of 4 ml water in 1 ml aliquots via the priming port so that the liquid fills the reservoir all the way to the waste channel port.

6. Once flushing is complete, close the priming port cover.


7. Using the pipette, remove the liquid from the waste reservoir via the **waste port 2**. Take care to leave sufficient liquid in the sensor chip area and neighbouring channels to ensure the sensor array remains submerged during transit.

8. Dispose of the absorbent material as biological waste, as detailed in local guidelines

9. Wipe the bench surface with a suitable disinfectant. Do not use any strong acids or alkalis.

10. Follow the rest of the returns process and book your flow cell collection as described: https://nanoporetech.com/upsflowcellreturn

# 17 Product use restriction/warranty

This product is for Research Use Only. Not for use in diagnostic procedures.

Oxford Nanopore Diagnostics products are shipped with documentation stating specifications, performances, and other technical information.

The software licence and device warranty contract will ensure your instrument is performing optimally by providing the latest up-to-date hardware and software.

# 18 Symbols

The device has the following information in the form of symbols:

| Symbol used | Meaning |
|---|---|
|  | Caution |
|  | Manufacturer |
|  | Distributor |
|  | 'Use by' date |
|  | Temperature limit |
|  | Contains sufficient for *n* tests |
|  | Consult product manual |
|   | Catalogue number |
|   | Lot number |
|  | Do not use if package has been damaged |

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