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The use of next-generation sequencing technologies for the detection of mutations associated with drug resistance in *Mycobacterium tuberculosis* complex: technical guide

World Health Organization

FIND
Because diagnosis matters
WHO collaborating centre for the evaluation of new diagnostic technologies

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Target Product Profile (TPP): Detection of resistance associated mutations in *Mycobacterium tuberculosis* complex utilizing Next Generation Sequencing

The TPP was drafted by FIND (Rebecca Colman, Timothy Rodwell, David Dolinger and Claudia Denkinger) with comments incorporated from CPTR assay development working group, CPTR surveillance working group, New Diagnostic Working Group, and a web-based survey sent out to GLI listserve and GHDonline. The final consensus TPP is presented in Appendix 3.

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Abbreviations

AMK	amikacin
BDQ	bedaquiline
CAP	capreomycin
CB	clinical breakpoint
CC	critical concentration
CFZ	clofazimine
CPTR	Critical Path to TB Drug Regimens
DCS	D-cycloserine
DLM	delamanid
DR-TB	drug-resistant tuberculosis
DST	drug susceptibility testing
FQs	fluoroquinolones
GFX	gatifloxacin
INH	isoniazid
IT	information technology
KAN	kanamycin
LFX	levofloxacin
LJ	Löwenstein-Jensen
LR	Likelihood ratio
LZD	linezolid
MDR-TB	multidrug-resistant tuberculosis
MFX	moxifloxacin
MGIT	BACTEC™ Mycobacterial Growth Indicator Tube™
MICs	minimum inhibitory concentrations
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
NGS	next-generation sequencing
OFX	ofloxacin
ONT	Oxford Nanopore Sequencing Technologies
OR	odds ratio
PCR	polymerase chain reaction
PGM	(Ion Torrent) Personal Genome Machine

PZA	pyrazinamide
QRDR	quinolone resistance-determining region
ReSeqTB	Relational Sequencing TB Data Platform
RIF	rifampicin
RRDR	rifampicin resistance-determining region
SLIs	second-line injectables
SMRT	Single Molecule Real-time Sequencing
SNP	single nucleotide polymorphism
SRL	supranational reference laboratory
TB	tuberculosis
TZD	terizidone
WGS	whole genome sequencing
WHO	World Health Organization
XDR-TB	extensively drug-resistant tuberculosis

Glossary of terms

Amplicon – A specific fragment of DNA from a target organism that is copied millions of times by polymerase chain reaction (PCR). Sequencing of these amplified fragments provides detailed information regarding variants at "targeted" loci of interest.

Breadth of coverage – Also known as 'genome coverage.' The percentage of nucleotide bases of a reference genome that are covered to a certain depth by sequencing. The breadth of coverage represents how much of a genome is covered by the sequenced fragments.

Clinical breakpoint –the concentration or concentrations of an antimicrobial agent which defines a minimum inhibitory concentration (MIC) above the critical concentration that separates strains that will likely respond to treatment from those which will likely not respond to treatment. This concentration is determined by correlation with available clinical outcome data, MIC distributions, genetic markers, and PK/PD data including drug dose. A dose increase can be used to overcome resistance observed at lower dosing, up until the maximum tolerated dose, and therefore a higher clinical breakpoint above which the particular drug is not recommended for use. The clinical breakpoint is used to guide individual clinical decisions in patient treatment. The clinical breakpoint is not applicable for drug resistance surveillance purposes.

Critical concentration – The lowest concentration of an anti-tuberculous agent *in vitro* that will inhibit the growth of 99% of phenotypically wild type strains of *Mycobacterium tuberculosis* complex.

***de novo* assembly** – During bioinformatics analyses, individual sequence reads are assembled into longer contiguous sequences in order to reconstruct the original sequence in the absence of a reference sequence or genome.

Depth of coverage – The number of sequencing reads at a given position in the sequenced genome. The depth of coverage represents how well a genome is covered by the sequenced fragments. The more reads there are, there more confidence there is that the reported sequence is accurate.

DNA fragment – A small piece of DNA produced by fragmentation or shearing of larger DNA molecules.

DNA library – The processed sample material that serves as the input material for NGS applications. A DNA library is obtained by fragmenting and sorting DNA to obtain fragments of a predefined length, attaching oligonucleotide adaptors to the ends of the fragments to enable sequencing and quantifying the final product for either targeted NGS or WGS.

FASTQ format – A text-based, raw data format for storing nucleotide sequence information and its corresponding quality scores (i.e. Phred scores).

GC content – The portion of nucleotide bases in a sequence that are either guanine (G) or cytosine (C). In general, the higher the GC content in a genome, the more difficult a genome is to sequence accurately.

Indels – An insertion or deletion of nucleotide bases in the genome of an organism.

Minimum inhibitory concentration (MIC) - the lowest concentration of an antimicrobial agent that prevents growth of more than 99% a microorganism in a solid medium or broth dilution susceptibility test.

Next-generation sequencing (NGS) – A "high-throughput, massively parallel" sequencing method used to determine the nucleotide sequence of a whole genome (i.e. whole genome

sequencing (WGS)) or part of a genome (i.e. targeted NGS) in a single biochemical reaction volume. NGS is performed by non-Sanger-based sequencing technologies that are capable of sequencing multiple DNA fragments in parallel, which are then pieced together and mapped to a reference genome using bioinformatics analyses.

Oligonucleotide adapters – Short nucleotide sequences that are ligated to DNA fragments to i) bind to a flow cell for next-generation sequencing; ii) allow for PCR-enrichment of adapter-ligated DNA fragments, only; or iii) allow for indexing or 'barcoding' of samples so multiple DNA libraries can be mixed together and run on one sequencing lane (known as 'multiplexing'), which is a means of increasing the speed and reducing costs associated with sequencing.

Phred score – A read editing program called Phred assigns a quality score to each base identified during sequencing, which is equivalent to the probability of error for that base. The Phred score is the negative log (base 10) of the error probability (e.g. a base with an accuracy of 99% receives a Phred score of 20). All NGS manufacturers use Phred scores as the measure of sequence quality reporting, although the estimation of error probability may be done in different ways.

Reference genome – The validated and published sequence of a known genome, gene, or artificial DNA construct. A reference sequence is usually stored in a public database and referred to by an accession number. A sequence produced by an NGS instrument may be aligned to a reference sequence or genome in order to assess NGS accuracy in targeted NGS or WGS applications and to find nucleotide changes (mutations) that are different from the reference genome.

Sanger sequencing – Technique for DNA sequencing based upon the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication, also known as 'the chain termination method.'

Sequence alignment – An algorithmic approach to best match consecutive nucleotide bases in one sequence with another sequence or genome (usually a reference genome) for the purposes of identifying genetic variation from the reference.

Sequence variants – Differences at specific positions between two aligned sequences. Variants include single-nucleotide polymorphisms (SNPs), insertions and deletions (indels), copy number variants, and structural rearrangements. In both targeted NGS and WGS applications, these variants are found after alignment of sequence reads to an accepted reference genome.

Sequencing by synthesis – Terminology used by Illumina (San Diego, California, USA) to describe the chemistry used in its NGS machines (e.g. MiSeq™ DX). The biochemistry involves a single-stranded template molecule, a sequencing primer, and DNA polymerase which adds nucleotides one by one to a DNA strand complementary to the template. Nucleotides are added to the templates in separate reaction mixes for each type of base. Each synthesis reaction is accompanied by the emission of light, which is detected by a camera. Each nucleotide is modified with a reversible terminator, so that only one nucleotide can be added to each template. After a cycle of four reactions adding just one G, A, T, or C base to each template, the terminators are removed so that another base can be added to all templates. This cycle of synthesis with each of the four bases and removal of terminators is repeated to achieve the desired read length.

Targeted next-generation sequencing (NGS) – NGS of specific genomic regions in a genome. Generally, targeted NGS is focused on sequencing a select set of genes or gene regions that have known or suspected associations with a specific pathogen (e.g. *Mycobacterium tuberculosis*) or a specific phenotype (e.g. drug resistance).

Whole genome sequencing (WGS) – The process of determining the complete genome sequence for a given organism at one time through NGS. This method can determine the order of all nucleotides in a given genome and detect any variations relative to a reference genome using bioinformatics analyses.

Executive summary

Next-generation sequencing (NGS) has great potential as a method for rapidly diagnosing drug-resistant tuberculosis (DR-TB) in diverse clinical reference laboratory settings worldwide. The NGS approach overcomes many of the significant challenges associated with conventional phenotypic testing as well as the limitations of other less comprehensive molecular tests by providing rapid, detailed sequence information for multiple gene regions or whole genomes of interest. However, the uptake of these technologies for DR-TB diagnosis has been hindered by concerns regarding costs, integration into existing laboratory workflows, technical training and skill requirements for utilization of the technology, and the need for expert guidance regarding the management and clinical interpretation of sequencing data.

Chapter 1 of this guide provides an overview of a general NGS workflow for both targeted NGS and WGS applications along with a description of the various instruments that are currently available for the sequencing of *Mycobacterium tuberculosis* complex (MTBC) pathogens. Detailed descriptions of commercially available NGS technologies are provided so that laboratories seeking to select and install an NGS workflow can objectively compare the strengths and weaknesses of each technology in order to identify a sequencer that best meets the needs of their laboratory.

Chapter 2 is a comprehensive review of the recent data supporting the characterization of MTBC resistance mutations as predictors of phenotypic drug resistance to the major anti-TB drug compounds isoniazid (INH), rifampicin (RIF), the fluoroquinolones (FQs), pyrazinamide (PZA) and the second-line injectables (SLIs), to help support NGS data analysis and results interpretation. The presented evidence and summary data for each mutation for different drugs of interest can ensure that targeted NGS and WGS applications are used to their fullest potential to describe the full resistance profiles of clinical strains and guide the appropriate treatment of TB and DR-TB infections.

In Chapter 3 we describe the implementation and accuracy of sequencing for DR-TB detection and characterization in a multi-country, population-based study. In the study of over 7,000 TB patients, investigators found sequencing to have very good accuracy for the prediction of INH, RIF, FQ and SLI resistance in comparison to phenotypic drug susceptibility testing (DST) results, with sufficient sensitivity to estimate the true prevalence of drug resistance for TB surveillance.

In Chapter 4 we outline various considerations for implementing NGS technologies, with a focus on implementing NGS in low- and middle-income countries. The goal of this chapter is to provide information to help laboratories recognize and address potential infrastructure limitations that could hinder or prevent the establishment of NGS workflows in different laboratory settings.

Although many NGS technologies will require additional optimization to further simplify workflows for the clinical diagnosis of DR-TB, the selected NGS technologies presented in this guide are all firmly on the path to early commercialization, and certain NGS platforms are already CE-IVD marked for *in vitro* diagnostic use and have been successfully integrated to reference laboratory workflows for routine DR-TB diagnosis and surveillance. Following additional optimization and commercialization, the next step for all NGS platforms is to achieve stringent regulatory approval, WHO endorsement, and local regulatory support for widespread implementation as *in vitro* diagnostic devices. Studies demonstrating the impact of these NGS technologies on improved DR-TB patient diagnosis and treatment outcomes will also be critical to further reduce obstacles to NGS implementation and promote technology uptake in intended use settings.

Background

In 2016, there were an estimated 10.4 million new cases of tuberculosis (TB) and over 1.6 million deaths attributed to TB [1]. Although global TB incidence and mortality rates have fallen in recent years, TB control efforts continue to fall short in reaching the 2030 targets of the End TB Strategy: to reduce TB deaths by 90% and TB incidence by 80%. One of the greatest threats to global TB care and prevention efforts is the persistence of drug-resistant TB (DR-TB), with 490,000 cases of multidrug-resistant TB (MDR-TB), or TB with resistance to at least the two first-line drugs, isoniazid and rifampicin, reported in 2016. The rise and spread of extensively drug-resistant TB (XDR-TB), or MDR-TB with resistance to at least one fluoroquinolone and a second-line injectable agent, has additionally complicated TB control efforts, with 6.2% of MDR-TB cases now classified as XDR-TB [1].

The successful diagnosis and treatment of M/XDR-TB depends upon universal access to accurate drug-susceptibility testing (DST). Conventionally, the diagnosis of drug resistance in *Mycobacterium tuberculosis* (MTB) strains has relied heavily upon slow culture and DST in liquid or solid media in biosafety level 3 laboratories. However, phenotypic results are only obtained after weeks to months of incubation, and many countries lack the resources to establish the stringent laboratory conditions required for these growth-based testing methods. Furthermore, phenotypic testing often lacks accuracy and reproducibility, especially for drugs such as pyrazinamide [2]. For these reasons, the use of rapid molecular tests is increasing worldwide. Rapid molecular diagnostic assays, such as the Cepheid Xpert MTB/RIF and Ultra assays (Cepheid, Sunnyvale, CA, USA), and the Hain GenoType MTBDR*plus* and MTBDRs*l* line probe assays (Hain Lifescience GmbH, Nehren, Germany), have been shown to lower the time to treatment initiation in TB and DR-TB patients [3]. Although the rollout of these technologies has led to a growing number of DR-TB cases detected and put on treatment, important diagnostic gaps remain. In 2016 only 41% of bacteriologically-confirmed and previously-treated TB patients were tested for rifampicin resistance, and only 39% of the notified DR-TB patients had additional DST results for the fluoroquinolones and second-line agents [1]. Altogether, only 22% of the estimated incident DR-TB cases were started on TB treatment [1]. Additional, rapid DR-TB diagnostic assays, including technologies that provide a comprehensive picture of the drug resistance profile of a TB clinical sample, are needed to fill these gaps.

Next-generation sequencing (NGS) presents an attractive option for DR-TB detection and characterization, and many NGS platform options now exist for DR-TB diagnosis. Unlike other DR-TB molecular assays, which rely upon the indirect identification of MTB and a limited set of resistance mutations through the hybridization of probes to specific genetic sequences, NGS assays can provide detailed sequence information for multiple gene regions or whole genomes of interest. All sequencing platforms rely upon a similar basic workflow to obtain sequencing reads from clinical samples; (1) DNA is first extracted from clinical samples or cultured isolates; (2) DNA goes through some enzymatic processing; (3) multiple fragments of DNA are sequenced in parallel, (4) and then bioinformatics analyses are used to map the individual reads to the reference genome. As each DNA base is sequenced multiple times, this assessment provides high depth to deliver accurate data and insight into occurrence of genetic polymorphisms. Specifically, NGS assays can confirm the presence or absence of indels and assess the occurrence of rare mutations and other sequence-level data that may evade detection by other molecular assays, such as the presence of heteroresistance, or a mix of multiple genetic populations, in a clinical sample [4]. Furthermore, NGS assays are flexible, in that they may be programed for a variety of applications, including the assessment of genetic information for additional organisms that may be present in a clinical sample.

Despite the advantages of NGS over other molecular methods for DR-TB identification and characterization, the uptake of these technologies has been hindered, especially in low- and middle-income countries, by cost limitations, the need for specialized and well-trained staff, a lack of readily-available data analysis and data storage solutions, and the lack of 'plug-and-play' solutions capable of obtaining sequencing information directly from primary clinical samples [5].

As NGS technologies progress through the diagnostic development pipeline and become more widely available for clinical use, reference laboratories should strive to fully understand the available platforms and associated workflows, as these NGS technologies are expected to be game changers for DR-TB diagnosis. This guide demonstrates that NGS can be successfully implemented as a high-performing DR-TB diagnostic, and performance is only expected to improve with our growing knowledge of the genetic basis of phenotypic drug resistance and the clinical relevance of TB genetic resistance markers.

Over time, NGS technologies that enable comprehensive, rapid DST are anticipated to reduce the need for phenotypic DST for DR-TB diagnosis, especially for drugs for which phenotypic methods are often unreliable, such as pyrazinamide. Importantly, even settings without access to phenotypic DST may successfully implement these technologies, accounting for important implementation considerations for targeted NGS applications such as sample referral mechanisms as well as data storage and connectivity needs and taking advantage of cloud-based interpretative software to remove the analytical barriers of NGS applications. Reference laboratories should also note that, once established, these tools would offer unmatched flexibility in regards to the options for various targeted NGS and WGS applications as well as possibilities for routine molecular epidemiological investigations, assessment of laboratory cross-contamination and the diagnosis of other infectious diseases, such as HIV or other drug-resistant, priority pathogens on the same platform [6].

Scope of the document

Recent advances, including the rise of high throughput NGS technologies and massively parallel sequencing, have reduced the time and costs associated with NGS, and have made these technologies reasonable options even for low- and middle-income countries. This guide summarizes the characteristics of available MTB NGS technologies along with the current knowledge of the genetic basis of MTB drug resistance and presents guidelines for NGS technology selection, procurement and implementation by TB reference laboratories in low- and middle-income countries for the diagnosis of drug resistance in TB clinical samples.

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1. Review of the current methods available for the sequencing of *Mycobacterium tuberculosis* complex

1.0 Introduction

Next-generation sequencing (NGS) is a powerful tool with the capacity to improve tuberculosis (TB) management and control through the rapid and accurate detection of all clinically relevant mutations, and thereby the rapid diagnosis of drug-resistant TB (DR-TB) in clinical specimens. This information is crucial for clinicians to make prompt decisions regarding the best therapy to adopt for treatment of multi- and extensively DR-TB (M/XDR-TB). The provision of rapid and accurate genetic sequencing information is especially important given the End TB strategy of universal drug susceptibility testing (DST) of all TB patients, as models have shown that MDR-TB incidence in many settings is the result of MDR-TB transmission, rather than treatment-related acquisition [1]. This effectively requires universal DST to identify DR-TB patients in endemic areas, rather than screening only previously-treated individuals. The genetic information obtained through either targeted NGS or whole genome sequencing (WGS) is invaluable to guide the diagnosis and treatment of TB populations in this context.

In addition to its application for the detection of drug resistance in clinical samples, technological advances in targeted NGS and WGS have raised the possibility for integration with routine molecular epidemiological investigations in different settings. In particular, the use of WGS in epidemiological investigations has allowed for in-depth resolution of transmission events, compared to traditional epidemiological investigations [2, 3]. This is important for the coordination and optimization of local and global TB control measures, especially in early detection of M/XDR-TB outbreaks. Thus, the main roles of WGS overlap with regards to public health: 1) the detection of genomic sequence variants to predict TB drug-resistance phenotypes and guide clinical decisions regarding treatment; 2) the identification of strain lineage and resistance mechanisms for TB surveillance; and 3) the recognition of genetically related strains for the resolution of transmission chains to direct TB control efforts [4]. This chapter will further explore the different NGS technologies currently available for clinical use for the purposes of DR-TB diagnosis and TB surveillance by reference laboratories.

1.1 Whole genome sequencing versus targeted next-generation sequencing

Advances in NGS technology have enabled the routine use of NGS for both targeted NGS and WGS of *Mycobacterium tuberculosis* complex (MTBC) samples, especially in high resource settings. WGS can provide the near complete genome of *Mycobacterium tuberculosis* (MTB) in a sample, while targeted NGS can generate MTB sequence data at specific genetic loci of interest. Although targeted NGS and WGS both rely upon the same basic NGS workflow, and both applications may be run on the same NGS instrument, the sample type input requirements and processing steps can vary widely according to the desired application.

Since drug resistance in MTB is mainly conferred through point mutations in specific gene targets, targeted NGS offers great promise for rapid diagnosis of DR-TB. Targeted NGS can be implemented through either an amplicon-based assay or a hybridization/capture-based assay to sequence large targets with a large depth of sequence coverage. The large depth of coverage offers high confidence for mutation detection and also enables detection of mixed populations, or heteroresistance, within a sample. Furthermore, this technology has been successfully implemented for the detection of TB drug resistance in clinical TB specimens. Although research is ongoing to better define DR-TB gene targets for targeted NGS, two amplicon-based technologies are currently in the TB diagnostic pipeline for DR-TB detection direct from sputum, including the Next Gen-RDST assay (Translational Genomics Research Institute, Phoenix, Arizona, USA) that can detect mutations in various MTBC gene regions associated with resistance to at least 7 drugs [5], as well as the Deeplex®-MycTB assay

(GenoScreen, Lille, France) for identification of mycobacterial species and prediction of MTBC drug resistance through targeted NGS of 18 resistance-associated gene targets in a 24-plexed amplicon mix (Appendix 1) [6, 7]. The ability to directly sequence TB clinical samples is invaluable to reference laboratories, as this reduces the time and cost associated with TB culture and further reduces the time to TB and DR-TB diagnosis, making these technologies an attractive option for DR-TB diagnosis in resource-limited settings, acknowledging that with the current technology the pooling of 24 to 48 specimens can save both hands-on-time and associated processing costs.

Currently, WGS is generally performed only on strains grown in culture due to the need for a relatively high quantity of good quality DNA to generate full WGS data for a given sample. Although there have also been attempts to perform WGS directly from sputum, results have been variable. One study, by Doughty et al., extracted MTB DNA from sputum and performed WGS using MiSeq (Illumina, San Diego, CA), but the assay could not predict drug resistance due to a high level of human DNA contamination in the samples [8]. Another study, by Brown et al., used a targeted enrichment approach (with oligonucleotide baits) to capture MTB DNA prior to performing WGS using MiSeq. The results showed good (>20x) depth of coverage and >98% genome coverage, but the associated costs of this approach would be prohibitive to application in low- and middle-income countries [9]. Finally, Votintseva et al. performed WGS on DNA extracted directly from MTB clinical samples via MiSeq using a low DNA concentration threshold (>0.05 ng/uL), below manufacturer recommendations [10]. Their results also showed a high level of human DNA contamination, though overall genotypic drug susceptibility predictions were comparable between direct and culture-based WGS in the study despite the poor sequencing quality, which inevitably impacts the reliability of variant calling. WGS direct from sputum will likely require further optimization, and even with improvements WGS may never obtain the desired sensitivity for applications such as heteroresistance detection. However, WGS offers other advantages over targeted NGS, including a higher genome coverage as required to combine genomic and epidemiological information to define transmission clusters during an outbreak, and the generated data contributes to our understanding of novel resistance mechanisms for both current and newer drugs as well as the identification of compensatory mutations. In this context, WGS remains invaluable for TB research and surveillance, though its clinical utility as a DR-TB diagnostic tool will require further investigation before routine WGS is more widely implemented for TB diagnosis.

In summary, laboratories may prefer to select instruments and optimize their sequencing workflows for targeted NGS applications if they plan to directly sequence TB sputum samples, while WGS workflows may be preferred for epidemiological investigations and various research applications. Regardless of whether targeted NGS or WGS applications are desired in a given setting, the point of entry of these technologies will likely be at national reference laboratories, as these laboratories oversee many different smaller laboratories and process a greater number of clinical specimens at any one time, making NGS applications more cost effective than phenotypic methods, even for resource-limited settings. It should also be noted that the cost effectiveness of these applications would only increase if additionally incorporated into workflows for the diagnosis of other multidrug-resistant organisms and diseases in the implementing laboratories.

1.2 The next-generation sequencing workflow

To realize the full potential of targeted NGS and WGS in improving the diagnosis, treatment and management of TB in any clinical setting, the general NGS workflow must be fully understood and considered so that it can be successfully integrated into existing laboratory workflows. Although different NGS platforms may have different sequencing chemistries, the general NGS workflow includes DNA extraction from a TB sample, library preparation, sequencing and data analysis (Figure 1). These steps are further detailed in the following sub-sections.

Figure 1: Conceptual NGS workflow

1.2.1 DNA extraction and quality control

The first step in any NGS workflow is TB DNA extraction and purification. This step can be crucial to the success of NGS applications, as the extracted genomic material for any sample must be of sufficient yield, purity and integrity to generate high quality sequencing data [11]. However, it is important to note that DNA quantity and quality requirements are dependent on the library preparation kit to be used and the desired sequencing application [12]. For example, the success of most WGS applications depends upon both a high quality and high quantity of input DNA to ensure that the effects of DNA contaminants, such as human DNA in a clinical sample [8], are minimized and that enough DNA is available to generate sequencing reads with high depth and coverage for downstream WGS analyses [9]. Considering that MTB has a complex cell wall that is difficult to lyse using conventional bacteriology methods, efficient extraction methods and DNA purification are crucial to ensure high quality DNA for NGS applications, especially WGS [11]. The DNA extraction method should sufficiently weaken the cell wall, releasing the DNA, and allowing for effective DNA purification in following steps. Many WGS applications rely upon an enrichment step to further promote sequencing success, either through MTB culture prior to DNA extraction or through additional processing, such as targeted enrichment steps, following extraction to ensure high DNA yield. Targeted NGS, in contrast, is designed to amplify specific MTBC markers and resistance genes, therefore avoiding some of these issues by design. For this reason, targeted NGS applications may generate valid results even for DNA inputs of minimal quantity and quality.

Generally, the choice of extraction methods will depend on the following:

- Starting material (i.e. direct specimen or culture)
- Duration of method and number of steps, including whether the time is dependent on the number of samples to be processed and options for automation of steps
- Number of necessary reagents/consumables
- Cost of reagents
- Integration into established workflow of the laboratory
- Desired NGS application (i.e. WGS and/or targeted NGS)
- NGS technology being used

Available, commonly used DNA extraction and purification methods include the following:

- Thermal methods (i.e. thermolysis)
- Organic/enzymatic-based methods (e.g. phenol-chloroform, proteinase K)
- Mechanical-based methods (e.g. sonication, bead beating, magnetic beads)
- Chemical-based methods (e.g. centrimonium bromide, sodium chloride)

Robotic machinery, such as automated pipettes, can also be used when performing MTB DNA extractions to save time during extraction steps. DNA extraction procedures require biosafety level 3 containment level when starting from direct specimens or cultured isolates, including appropriate safety equipment and work practices [13].

Whichever extraction method is chosen, as long as the extracted DNA meets minimal quality and quantity standards as defined for a given NGS instrument (Table 1), it can be used for either targeted NGS or WGS applications. Following extraction, the DNA is typically quantified using a spectrophotometer, a fluorescent detection system (e.g. a Qubit® fluorometer) or qPCR, in line with routine practice and molecular biology workflows. DNA size and integrity can also be examined by agarose gel electrophoresis or by microfluidic instruments, including Bioanalyzer (Agilent, Santa Clara,

California, USA), TapeStation (Agilent, Santa Clara, California, USA), LabChip (Perkinelmer, Waltham, Massachusetts, USA) and Fragment Analyzer (Advanced Analytical Technologies, Inc., Ankeny, Iowa, USA). These microfluidic instruments can produce an electropherogram that plots DNA concentration, yield and size.

1.2.2 DNA library preparation

Following DNA extraction, a DNA library, or a collection of specifically fragmented DNA to which oligonucleotide adaptors have been attached, must be prepared and quantified for both targeted NGS and WGS applications. The particular steps involved in library preparation will vary according to the NGS technology and desired application (Table 1). Generally, laboratories may choose between DNA library kits for targeted NGS and WGS. Kits meant for targeted NGS produce libraries that include specific, resistance-conferring genomic regions or lineage markers, while kits intended for WGS produce libraries comprised of fragments of the entire MTB genome. Library preparation for targeted NGS either involves a probe hybridization step, which is essential for library enrichment, or uses targeted PCR amplicons to create NGS libraries [14]. It should be noted that, regardless of the application (i.e. targeted NGS or WGS), the library preparation kits offered by a given manufacturer can often be used across several different NGS platforms from that manufacturer (e.g. the Nextera XT kit is compatible with all Illumina series instruments). Most kits allow for the multiplexing of samples during library preparation, and these kits can produce libraries given microgram to picogram quantities of starting material [14, 15].

Table 1: Examples of commercially available library preparation kits for different NGS instruments

Library preparation kit	System compatibility	NGS application	Input quantity [ng]
Nextera XT	All Illumina	WGS or Targeted	1
Nextera DNA Flex	All Illumina	WGS or Targeted	1-500
AmpliSeq	All Illumina	Targeted	1-100
Ion Xpress Plus Fragment	PGM and S5 Ion Torrent	WGS or Targeted	100
MuSeek	PGM and Proton	WGS or Targeted	100
Rapid Sequencing Kit	All ONT devices	WGS or Targeted	400
Ligation Sequencing Kit 1D	All ONT devices	WGS or Targeted	1000
Low Input by PCR Sequencing	All ONT devices	WGS or Targeted	< 100
1D^2 Sequencing	All ONT devices	WGS or Targeted	1000

The general steps of library preparation include DNA fragmentation, end-repair, phosphorylation of the 5′ prime ends, A-tailing of the 3′ ends to facilitate ligation to sequencing adapters, ligation of adapters, PCR, library purification and quantification [16]. DNA fragmentation can be done through physical (i.e. acoustic shearing or sonication), enzymatic (i.e. endonuclease or transposase activity) or chemical (i.e. hydroxyl radicals, such as iron-EDTA) methods. Most library preparation kits rely upon enzyme-based methods for DNA fragmentation. Laboratory technicians must critically follow the manufacturers' directions for all methods, with special attention paid to both temperatures and reaction times, as fragment size is dependent on the reaction efficiency [14]. It is also important to note the library insert size ranges and limitations (i.e. the length of the DNA sequence between the adapter sequences) for a given instrument to ensure best data yield. Fragment sizing may be evaluated during the library quantification steps using Agilent Bioanalyzer or TapeStation, which provide an electropherogram.

The following are some general factors to consider when selecting a library preparation kit:

- NGS instrument being used
- Quality and quantity of DNA required
- Application (i.e. targeted NGS and/or WGS)
- Cost
- Automation
- Duration of library preparation
- Compatibility with other NGS instruments (if desired)

It is critical to check the quality and quantity of the DNA before and after library preparation. This assessment helps to avoid the waste of reagents and consumables during library preparation and sequencing when starting from a poor-quality DNA. Most library preparation workflows have this functionality built in, though it should be noted that some workflows have been simplified to remove these quality assurance steps.

1.2.3 Next-generation sequencing: Platforms and considerations

A number of commercially available NGS technologies suitable for clinical use for DR-TB diagnosis and deployment in national reference laboratories are available [17], including the following:

Illumina MiSeq™

Initially produced and commercialized by Solexa, a company purchased by Illumina in 2007, this technology employs the 'sequencing by synthesis' approach, where a DNA polymerase synthesizes complementary strands to single-stranded DNA targets with synthesis halted by terminator nucleotides. This sequencing system currently dominates the global NGS market [18]. The platform series includes multiple instruments (Table 2) with varying throughput and read length.

One Illumina instrument currently available for the sequencing of microbial samples is the MiSeq™ DX module, which is the first FDA-regulated, CE-IVD marked NGS instrument for *in vitro* diagnostic use [19]. The instrument has a small footprint (0.3m^2), is capable of processing 1-96 samples per run, and can perform both targeted NGS and WGS applications [8-10, 20, 21], making this technology suitable for a range of reference laboratories, as well as for various applications from epidemiological surveillance to DR-TB diagnosis. The short-read technology has also been noted to have an overall lower error rate for TB applications compared to other sequencers [21], including superior performance compared to Ion Torrent Personal Genome Machine™ (PGM™) for GC rich regions, which is a benefit for MTB sequencing [22, 23]. A full GenoScreen (Lille, France) Deeplex®-MycTB targeted NGS workflow for the Illumina MiSeq instrument is available in Appendix 1.

ThermoFisher Scientific Ion Personal Genome Machine™

Manufactured by ThermoFisher Scientific (Waltham, Massachusetts, USA), this technology is based on 'sequencing by synthesis,' using semiconductor sequencing technology to detect pH changes caused by hydrogen ions released during DNA synthesis [24]. The technology uses emulsion PCR library amplification prior to sequencing, which can be a long and complicated process. However, there is the option of a separate, automated library preparation system that can be performed using the Ion Chef™ system. Use of the Ion Chef™ system for library preparation makes the speed of the process comparable to Illumina library preparation.

There is currently one CE-IVD marked Ion Torrent sequencer commercially available: the Ion PGM™ System [25]. The PGM instrument is roughly equivalent to the Illumina MiSeq instrument in terms of price, but the cost per sample is higher for the Ion Torrent platform. When selecting an Ion Torrent platform, there are four sequencing chips (Ion PI, Ion 314, Ion 316 and Ion 318) to choose from, with varying sequence data outputs. Compared to Illumina sequencing, an Ion Torrent sequencing run is generally shorter (hours versus days) and the reads are longer [26], though typically there is less data output available for bioinformatics analyses. Additionally, the reads obtained from Ion Torrent are only single-stranded, with a higher error rate due to the increased occurrence of long homopolymer regions [26].

Furthermore, read coverage in AT- or GC-rich (as is the case for MTB) regions has been noted to be poor with this technology, compared to MiSeq [22]. Nonetheless, this technology remains an option for TB reference laboratories seeking to perform either targeted NGS or WGS applications on a benchtop sequencer.

Oxford Nanopore MinION

MinION is a commercialized nanopore sequencing platform that was developed by Oxford Nanopore Sequencing Technologies (ONT) (Oxford, United Kingdom) in 2012. Although it is not yet CE-IVD marked for diagnostic use, the company is currently seeking regulatory approval [28]. MinION is a small (90g, hand-held) device that may be connected to a computer through a USB port cable [29, 30]. It identifies DNA bases by measuring changes in electric conductivity generated as DNA strands pass through a biological nanopore [30]. Since different nucleotides have different shapes, each distinct nucleotide is identified by its effect on the change of the ionic current as the bases pass through the nanopore [31]. A full WGS workflow for this technology is outlined in Appendix 2. For this technology, sample and library preparation steps are slightly shorter and the infrastructure demands are generally less demanding compared to Illumina technologies. Library preparation kits are available to multiplex up to 96 samples for targeted NGS and 12 samples for WGS. However, the portability of the device (MinION), combined with real-time detection of signals as the sequencing takes place, makes this technology ideal for real-time diagnostics especially in endemic settings, if adequate computing resources can be assured to process the 10-20GB of output data generated [30]. Compared to Illumina, this technology may have a higher cost per sample, but it offers longer reads (when using different extraction protocols that don't shear DNA) and therefore easier assembly during bioinformatics analyses. One of the main drawbacks to MinION is the high error rate (20-35%) although advances have been made in the assay chemistry to address this issue [29, 32]. With the ongoing improvements, ONT may become suitable for routine use in the near future.

QIAGEN: GeneReader NGS System

The GeneReader NGS System was developed by QIAGEN (Hilden, Germany) with the aim of addressing challenges to the widespread adoption of NGS applications in clinical diagnostic laboratories through a reduction in the current hands-on time required for the NGS workflow and a fully integrated bioinformatics pipeline [33]. The company intends to CE-IVD mark the NGS solution in the coming years. The GeneReader integrates highly parallel fluorescence-based sequencing chemistry with detection of corresponding fluorescent signal templates. Following DNA library construction, DNA amplified using a GeneRead QIAcube® is immobilized via direct bead-slide interaction and exposed to a DNA sequencing primer to produce a high-density array on a GeneReader Flow Cell, which is then read by the sequencer [34]. The platform's turntable design allows for the independent sequencing of up to 42 samples at a time. Available GeneReader software provides a FASTQ file of sequencing information generated by the technology. Although the QIAGEN GeneReader NGS System workflow is still being improved for resistance detection in TB clinical samples, it is likely that a true end-to-end workflow will soon be a reality for DR-TB diagnosis using this NGS technology.

While other NGS technologies, including PacBio instruments [35, 36], offer additional options for targeted NGS and WGS applications (Table 2), their high price points and computational resource requirements make them unlikely candidates for TB reference laboratory use in LMICs in the near future.

Table 2: List of all commercially available NGS instruments and characteristics

Instrument	Manufacturer	Chemistry	Detection	Data output [Gb]	Maximum read length [bp]	Sequencing time*	Estimated instrument cost [USD]	Strengths	Weaknesses
iSeq	Illumina	Sequencing by Synthesis (bridge PCR)	Fluorescence	0.3-1.2	2 x 150	9-17.5 hours	19,900	Low initial investment, short run time	Read length, low throughput
MiniSeq	Illumina	Sequencing by Synthesis (bridge PCR)	Fluorescence	1.7-7.5	2 x 150	4-24 hours	50,000	Low initial investment, short run time	Read length, low throughput
MiSeq	Illumina	Sequencing by Synthesis (bridge PCR)	Fluorescence	0.3-15	2 x 300	4-55 hours	100,000	Read length	Long run time
NextSeq	Illumina	Sequencing by Synthesis (bridge PCR)	Fluorescence	10-120	2 x 150	12-30 hours	250,000	Throughput	Long run time
HiSeq (2 500)	Illumina	Sequencing by Synthesis (bridge PCR)	Fluorescence	10-1000	2 x 150	< 3 days	650,000	Throughput, read accuracy	Long run time, high initial cost
Nova Seq (5000/6000)	Illumina	Sequencing by Synthesis (bridge PCR)	Fluorescence	2000-6000	2 x 150	16-44 hours	850,000-950,000	Throughput, read accuracy	Long run time, high initial cost
PGM	Thermo fisher	Sequencing by Synthesis (emulsion PCR)	Proton/Semi-conductor	0.08-2	400	3-10 hours	80,000	Short run time, read length	Low throughput, homopolymers
S5	Thermo fisher	Sequencing by Synthesis (emulsion PCR)	Proton/Semi-conductor	0.6-15	400	up to 19 hours	60,000	Read length	Homopolymers
Proton	Thermo fisher	Sequencing by Synthesis (emulsion PCR)	Proton/Semi-conductor	10-15	200	4-24 hours	149,000	Short run time, read length	Homopolymers

Instrument	Manufacturer	Chemistry	Detection	Data output [Gb]	Maximum read length [bp]	Sequencing time*	Estimated instrument cost [USD]	Strengths	Weaknesses
PacBio RSII	Pacific Biosciences	Single-molecule real-time	Fluorescence	0.5-1	60,000	up to 6 hours	750,000	Short run time, read length	High initial cost, high error rate
Sequel	Pacific Biosciences	Single-molecule real-time	Fluorescence	5-10	60,000	up to 20 hours	350,000	Short run time, read length	High initial cost, high error rate
MinION	ONT	Single-molecule real-time	Ion current change	10-20	100,000+	30 minutes-48 hours	1,000	Short run time, portable, read length, cost	High error rate
GridION	ONT	Single-molecule real-time	Ion current change	50-100	100,000+	30 minutes-48 hours	2,400	Short run time, read length	High error rate
PromethION	ONT	Single-molecule real-time	Ion current change	480-960	100,000+	30 minutes-48 hours	25,000	Short run time, read length	High error rate

* It should be noted that the ability to multiplex several samples within one run can help to compensate for long run times.
Qiagen was not included in the above table due to a lack of publicity available information regarding the assay. Described characteristics are current as of July 2018.

Despite the various differences among the NGS platforms, all of these platforms are suitable for DR-TB diagnosis in most settings given that infrastructure requirements are met and that experienced staff are available to perform the various NGS procedures. For this reason, NGS instruments should be selected according to what best suits a given laboratory workflow without stretching the laboratory's resources (Table 3). Whether a laboratory is deciding to set-up an in-house platform or out-source NGS services, the following should also be considered when comparing NGS technologies:

1. Cost. The costs associated with equipment and workflow set-up, the instrument, reagents, consumables, bioinformatics resources and equipment maintenance and support should all be considered when selecting an NGS instrument (please refer to Chapter 5 for additional information regarding NGS implementation). Cost can be evaluated as cost per sequencing run, cost per genome sequenced, or cost per megabase of output data when comparing NGS options.

2. Duration. Considerations should be made for the turn-around time of a targeted NGS or WGS run using a given instrument, from sample processing to data analysis, especially with regards to implications for improving patient management and disease control in a given setting. It should be noted that there is ongoing research to assess the use of MinION sequencing as a point-of-care diagnostic tool in this context [37].

3. Data Quality. The quality score limits for base calls from a particular platform are important indicators of assay reliability and robustness [38]. Generally, short-read sequencing platforms have associated better quality data than long-read sequencing platforms. A Phred score scale is typically used for quality assessment; a quality score of 20 (Q20) equates to 1 error for every 100 bases (1% error rate) while a Q30 score demonstrates 1 error for 1000 bases (0.1%), etc.

4. Data output. When considering the number of samples to be processed for targeted NGS or WGS in a given setting, it should be noted that some technologies might take a longer time to sequence a larger number of samples. This factor may have financial implications on the cost per sample and the amount of data output. For example, sample batching could result in reduction of cost per sample for any sequencing platform, but the amount of data generated per sample might diminish in turn, which needs to be taken into account for downstream bioinformatics analyses and in cost calculations, should there be a need for repeat sequencing of many samples to accomplish goals (i.e. epidemiological investigations).

Table 3: Comparison of commercially available NGS instruments

Sequencing platform	Sequencing kit	Number of samples per run	Sequencing cost* per sample (USD)	Typical run output	Sequencing run time	Type of analysis possible
Illumina MiSeq	MiSeq v2 (2x250bp)	54	$22.41	7.5-8.5 GB	39 hrs	SNP and INDEL analysis
Ion Torrent	Ion 318 Chip v2 (400 base)	13	$48.75	1.2-2 GB	7.3 hrs	SNP and INDEL analysis
Oxford Nanopore	Ligation Sequencing kit 1D	22 54	$42.86 $16.67	2-3 GB 4-8 GB	6 hrs 48 hrs	Genomic rearrangement, SNP, and INDEL analysis

Two run times are possible with the Oxford Nanopore system. Qiagen was not included in the above table due to a lack of publicly available information regarding the assay. All comparisons assume WGS is performed on MTB and average 30x coverage.
* Price for sequencing only, not including library preparation price, and not including bulk price discounts

Research is ongoing to improve the quality and quantity of data produced during both targeted NGS and WGS for DR-TB diagnosis, including various instrumentation improvements and reagent optimization. As the cost of sequencing, data transfer and analysis continue to decrease and the general NGS workflow becomes faster and more efficient, there will be the need for constant review of the NGS literature and manufacturer updates to ensure that the most recent information is considered for NGS instrument selection for a given setting.

1.2.4 Next-generation sequencing data analysis

The computational resources required for generating, analyzing, storing and managing sequencing output data, including the bandwidth for file transferring if such analyses are outsourced, is one of the main bottlenecks in the successful introduction and implementation of NGS technologies in a given setting [17]. This is especially true for WGS applications, as targeted NGS tends to generate smaller, more manageable datasets. Until such bottlenecks are overcome, the most immediate implementation strategy is for central-level reference laboratories to perform targeted NGS or WGS and data analysis, with peripheral centers referring samples and outsourcing sequencing and data management to reference laboratories as required. Of course, NGS outsourcing requires the existence of efficient sample referral networks, which may not be well established in certain settings. However, even if NGS is performed in-house, laboratories may still prefer to outsource NGS data analysis and data management, as NGS analysis pipelines can be extremely complex and data-intensive due to high data volumes and the need for sophisticated computational methods. The majority of available bioinformatics software requires some knowledge of the text-based command-line of the UNIX/Linux operating systems, allowing custom programming scripts and pipelines to automate data manipulation and analysis in a single step [17]. For those laboratories preferring to run their own NGS data analyses, Walker et al. provides an example of how an in-house bioinformatics pipeline can be applied to TB sequencing [39]. Some of the available NGS bioinformatics platforms are open-source while others are commercially available, though users often find graphical user interface software easier to use [40]. Some suitable products with a graphical user interface are offered through DNASTAR® (Madison, Wisconsin, USA), Applied Maths (Sint-Martens-Latem, Belgium), and QIAGEN (Hilden, Germany). Galaxy is a web-based platform that offers reproducible, data-intensive computational analysis functions [41]. The possibility of setting up analysis pipelines and accessing complex workflows through Galaxy makes this platform ideal for use in resource-limited settings, with automated analysis and interpretation pipelines that are easy to use.

Laboratories should note that the analysis and interpretation of both targeted NGS and WGS data to detect TB drug resistance directly from clinical samples requires a standardized, validated, coordinated, and collaborative analytical approach [11]. Under the Critical Path to TB Drug Regimens (CPTR) initiative, the Relational Sequencing TB Data Platform (ReSeqTB) was established to collect global TB patient data from multiple private and public databases and identify correlations between MTB mutations and clinically relevant resistance with the ultimate goal of guiding sequencing data interpretation for personalized TB patient care [42]. Currently, four web servers (CASTB, PhyResSE, TBProfiler, and GenTB) and two software solutions (KvarQ and Mykrobe Predictor TB) have been developed for facilitated interpretation of TB drug resistance from genomic data [43-48], with PhyResSE feeding information directly to ReSeqTB. Mykrobe Predictor and TBProfiler, in particular, have shown promising results for clinical applications, and might be valid options for a laboratory for DR-TB NGS bioinformatics analyses [49]. While there are several NGS analysis software options, the choice should ultimately depend on the ease of use, analysis speed, cost, accuracy (correlation with phenotypic results) and maintenance support. In addition to selecting and establishing a bioinformatics pipeline for the analysis of TB sequencing data, prospective users should also consider how they would validate a given analysis method in-house

and how version control would be provided, depending on local regulatory requirements. Finally, users should note that these analytical tools were developed based on the current knowledge of the molecular basis of TB drug resistance, and results will likely only improve with time given additional knowledge of TB drug resistance mechanisms and associations with phenotypic drug resistance, as discussed in the following chapter.

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2. Principles of using graded resistance-conferring mutations to detect resistance to anti-tuberculosis compounds

2.0 Introduction

The End TB Strategy calls for universal drug-susceptibility testing (DST) and treatment of all people with tuberculosis (TB), including drug-resistant TB (DR-TB). However, in 2016, only 22% of the estimated 600,000 people with MDR-TB received appropriate drug treatment [1]. Although the rapid and accurate detection of TB is a key factor in controlling the spread of disease, treatment success is largely reliant upon accurate information regarding TB drug susceptibility, especially for drug resistant cases [2, 3]. With the introduction of new drugs and regimens, it is crucial to provide a comprehensive drug susceptibility profile for TB patients in order to select effective therapies [4-8].

As discussed in the previous chapters, NGS technologies, in contrast to other molecular DST assays, have the potential to provide near-complete genetic data for clinical *Mycobacterium tuberculosis* complex (MTBC) strains. However, sequencing data interpretation is complex, and healthcare workers require clear rules and guidelines to determine the clinical relevance of genetic changes detected by sequencing technologies [9]. These guidelines require a high-quality, comprehensive catalogue of genetic markers of resistance, as well as a precise understanding of the level of resistance conferred by particular mutations, expressed as a range of minimum inhibitory concentrations (MICs) found in strains with these mutations, and insight into the degree of cross-resistance conferred for antibiotics with shared modes of action [10, 11]. Recent steps have been taken to grade MTBC mutations according to confidence as resistance markers as well as to better define the association between resistance mutations and conferred phenotypic drug resistance through a review of the association between strains with specific mutations and MIC data for drugs of interest [12, 13]. This chapter will summarize the existing evidence base for interpreting mutations when detected by targeted next-generation sequencing (NGS) or whole genome sequencing (WGS) for key first and second-line drugs. Specific rules can help researchers to best interpret the genetic data generated by sequencing to determine the full resistance profiles of clinical strains and guide appropriate treatment of infections.

2.1 Methods

2.1.1 Systematic review of mutations

All systematic review methods for the collation and analysis of MTBC mutation data are described further in Miotto et al. Briefly, a systematic literature review on the association of sequencing and phenotypic DST data for MTBC was undertaken for selected anti-TB drugs and resistance genes (loci considered are available as Supplementary 1 in [12]). Expert consensus from the global ReSeqTB Data Sharing Platform was used to define the loci with the highest likelihood of association with resistance [14], and the review was limited to those loci. A comprehensive search of the PubMed database for relevant citations was performed [12], and study quality was appraised using a modified Quality Assessment of Diagnostic Accuracy Studies-2 tool [15].

An expert, consensus-driven approach was used to develop a standardized procedure for grading drug resistance-associated mutations. Collated data were used to calculate the frequency of each mutation in resistant and susceptible MTBC isolates and to derive a likelihood ratio and odds ratio. In this approach, likelihood and odds ratios were used for objectively evaluating whether mutations were positively or negatively associated with phenotypic resistance. Using this rationale, the thresholds commonly adopted in evidence-based medicine were adapted to grade the MTBC mutations [16-18]. Further details are provided in online supplementary material [12]. Briefly, mutations were classified as having either high, moderate or minimal confidence for being associated with resistance, or as indeterminate or “not associated with resistance” (Table 1). Relevant data from this review for mutations associated with isoniazid (INH), rifampicin (RIF),

pyrazinamide (PZA), fluoroquinolones (FQs), and second-line injectables (SLIs) resistance are presented, below. Only those confidence levels established based upon combined liquid and solid media data for each resistance mutation were included in this analysis.

Table 1: Overview of confidence levels for grading mutations associated with phenotypic resistance

	LR* and OR	
	p-value	**value**
High confidence for association with resistance Strong association of the mutation with phenotypic drug resistance; sufficient evidence that the mutation confers or is strongly associated with drug resistance	< 0.05	>10
Moderate confidence for association with resistance Moderate association of the mutation with phenotypic drug resistance; additional data desirable for improved evidence that the mutation confers or is strongly associated with drug resistance	< 0.05	5 < … ≤ 10
Minimal confidence for association with resistance Weak association of the mutation with phenotypic drug resistance; inconclusive evidence that the mutation confers or is strongly associated with drug resistance. Substantial additional data required	< 0.05	1 < … ≤ 5
No association with resistance No evidence of association between the mutation and drug resistance	< 0.05	< 1
Indeterminate No statistically significant threshold reached; additional data required	≥ 0.05	

Only those confidence levels established based upon combined liquid and solid media data for each resistance mutation were included in this analysis.

The table shows the thresholds applied to likelihood ratios (LR) and odds ratios (OR) to grade the association of mutations with phenotypic drug resistance. LR*: positive likelihood ratio. "Additional data" is defined as a requirement for 1) more phenotypically drug resistant and susceptible isolates tested with the mutation in question; and/or 2) better understanding of the mechanism of drug resistance (e.g. to investigate epistasis, or the interactions between drug-resistance conferring mutations, lineage-specific genetic factors and compensatory mutations or synergistic factors when more than one mutation is required to confer resistance).

2.1.2 Systematic review of MIC data

Systematic review methods for the collection and analysis of MIC data are described further in the published Technical Expert Group Meeting Report and supplement [13, 19]. Briefly, in 2016, WHO commissioned FIND to perform a systematic review of available MIC data for phenotypically wild type and non-wild type strains, including associated sequencing data for relevant resistance genes. The medicines included in the review were the SLI agents [kanamycin (KAN), amikacin (AMK) and capreomycin (CAP)], clofazimine (CFZ) and bedaquiline (BDQ), cycloserine

(DCS) and terizidone (TRD), linezolid (LZD), delamanid (DLM), and the FQs [ofloxacin (OFX), levofloxacin (LFX), gatifloxacin (GFX) and moxifloxacin (MFX)]. The following media were considered: Löwenstein Jensen (LJ), Middlebrook 7H10/7H11 and BACTEC™ Mycobacterial Growth Indicator Tube™ (MGIT) 960. A comprehensive search of the PubMed database for relevant citations was performed and additional data were solicited from the WHO TB Supranational Reference Laboratory (SRL) Network. All studies with quantitative DST data for any of the listed compounds, testing at least three concentrations in relevant media to determine drug susceptibility for laboratory or clinical isolates, were included in the review as long as both the concentrations tested and MIC results were clearly defined.

MIC data were stratified by available genotypic DST results when available. For each study, the shape of the corresponding MIC distribution was assessed based on five main criteria: the inclusion of a control strain; whether the upper or lower end of the distribution was truncated; the consistency of the modes of the phenotypically wild type distribution; the overall number of phenotypically wild type isolates tested; and whether repeat MIC testing of individual isolates was done. In April 2017, the WHO Global TB programme convened a Technical Expert Group to review the evidence for different critical concentrations and clinical breakpoints used for DST for the above-mentioned drug-media combinations. The group assessed the presented MIC and sequencing data for each drug-medium combination. Depending on the quality and quantity of the data, critical concentrations (CCs) and/or clinical breakpoints (CBs) were established, maintained or revised. Owing to the lack of data, the CCs were also withdrawn in some cases. All results are available in the WHO revised table of CCs for TB DST [13], with all MIC files for each drug available through the FIND-hosted supplemental files [19]. Relevant data from this report for the FQs and SLIs are presented below.

Where review MIC data were not available (i.e. for INH, RIF and PZA), an abridged literature review was undertaken. Briefly, the PubMed database was queried for each drug and a full-text screen of all identified English-language publications was undertaken. The following search terms were used: (tuberculosis OR TB OR tuberculous) AND (isoniazid OR INH) AND (mutations OR katG OR inhA OR fabG1 OR ahpC OR mshA) AND (MIC OR MICs OR "minimum inhibitory concentration" OR "minimal inhibitory concentration" OR "minimum inhibitory concentrations" OR "minimal inhibitory concentrations") for INH studies; (tuberculosis OR TB OR tuberculous) AND (rifampicin OR rifampin OR RIF) AND (mutations OR rpoB) AND (MIC OR MICs OR "minimum inhibitory concentration" OR "minimal inhibitory concentration" OR "minimum inhibitory concentrations" OR "minimal inhibitory concentrations") for RIF studies; and (tuberculosis OR TB OR tuberculous) AND (pyrazinamide OR PZA) AND (mutations OR pncA) AND (MIC OR MICs OR "minimum inhibitory concentration" OR "minimal inhibitory concentration" OR "minimum inhibitory concentrations" OR "minimal inhibitory concentrations") for PZA studies. All identified studies and relevant, linked references underwent a full-text screening for MIC data. For this abridged review, only studies with clearly-defined phenotypic DST and MIC methodology and MIC results stratified by individual mutations in relevant gene regions were included in this chapter.

MIC data are summarized from the identified, relevant peer-reviewed publications in each drug table below. Each mutation (or double mutant) was classified as having either high, moderate or low associated MICs based upon the identified MIC distributions (Table 2).

Table 2: Overview of MIC data summations

MIC Data Summary*
High MICs None of the identified/available MIC distributions (in any media) span the current CC (either the 2012 WHO-recommended CCs [20], or, preferably, the most recent 2018 WHO-recommended CCs, if available [13]). For MFX, these distributions all have modes above the CB, otherwise they are considered to have moderate MICs.
Moderate MICs Some of the identified/available MIC distributions (in any media) span the current CC. For MFX, these distributions may be above the CC but span the CB.
Low MICs The majority of identified/available MIC distributions, across different media, include the current CC.
Need Additional Data Conflicting MIC results for any media or too few data points to establish conferred resistance (e.g. only 1 isolate tested in any media)

* For PZA, although MIC data is presented for both solid and liquid media, MIC data summaries are based only upon the availability of MGIT testing results, as WHO-recommended CCs have only been set for the MGIT960 system.

2.1.3 Summary data presentation

This chapter presents summary data from both the mutation and MIC data reviews, stratified by the following anti-tuberculosis drugs: INH, RIF, PZA, FQs and the SLIs. Collated data and mutation tables reflect both mutation confidence as markers of resistance given phenotypic DST results, as well as associated resistance levels for each mutation. Together, these data suggest the summary confidence in different MTBC resistance mutations as markers of phenotypic drug resistance.

2.2 Results

2.2.1 Isoniazid

Although the molecular mechanisms of INH resistance in MTBC have not been fully elucidated, the most frequent INH resistance-conferring mutations have been noted in the *katG* and the *fabG1/inhA* or *mabA/inhA* gene regions [21]. INH is a prodrug requiring activation by the catalase-peroxidase KatG enzyme prior to acting on cellular targets. Mutations in *katG* thereby disrupt INH activation to varying degrees. The *inhA* gene, in turn, encodes InhA, an enzyme involved in the synthesis of the bacterial cell wall mycolic acid. InhA is targeted by activated INH, which blocks the synthesis of mycolic acid, but mutations in *inhA* can prevent this interaction, whereas mutations in the *inhA/mabA* promoter can lead to drug titration and confer varying degrees of INH resistance. INH resistance has been associated with mutations in *kasA*, which also encodes an enzyme involved in mycolic acid synthesis, *furA*, a ferric acid upregulator, *ndh*, coding for NADH dehydrogenase, *oxyR-ahpC*, where mutations increasing expression of the encoded alkyl hydro peroxidase are believed to compensate for loss of *katG* activity, and *nat,* coding for arylamine N-acetyltransferase which inactivates INH, and *mshA*, the glycosyltransferase involved in mycothiol synthesis [22].

Relevant data for the *katG*, *inhA-mabA*, *furA*, and *mshA* INH resistance-associated genes were included in the review of INH mutation data. Data from 127 studies, with strains from 42 countries that were isolated from 1992-2014, were included in the review. The total number of isolates with available *katG* sequence data was 11,847, whereas 9,407 had *inhA-mabA* mutation data, 361 had *furA* mutation data and 288 had *mshA* mutation data. *ahpC* was not considered in the mutation data review, while

data on *furA* and *mabA* (*fabG1*) were largely lacking.

INH was not included in the MIC data review. In the abridged literature review, 122 studies were identified for full-text screening, of which 13 were found to have detailed MIC data for INH resistance-associated gene regions. These data are presented alongside mutation confidence data in Table 3 to suggest comparable INH resistance levels for these mutations in different media [23-35].

Miotto et al. found the *katG* S315I, S315N, S315T and combined frameshifts and premature stop codons to be high-confidence markers of INH resistance (Table 3). The abridged MIC data review largely supported these findings, as the *katG* S315N, S315T and pooled frameshifts and stop codons conferred high levels of phenotypic INH resistance in multiple different media. Additional MIC data is needed to confirm the level of INH resistance conferred by *katG* S315I mutants, though two of these mutants were found to have high MICs when tested in LJ medium. The *inhA* c-15t mutation was characterized as a moderate confidence resistance mutation by Miotto et al. The abridged MIC data review found these mutants to confer resistance at lower levels than the *katG* mutations, with some MIC distributions spanning the CC for certain media [26], and were therefore classified as moderate MIC mutations. The high-confidence *katG* S315 and *inhA* c-15t double mutants were found to have generally higher MICs than the individual mutants in all media tested. Mutations in *katG* coding region are also contributing to MIC increase when present. Additional data is necessary to establish confidence grading and conferred resistance levels for *mabA* (*fabG1*) and *ahpC-oxyR* mutants.

Using only the high, medium and low confidence-graded mutations identified in Miotto's review as predictors of phenotypic resistance (Table 3), the sensitivity and specificity for INH resistance detection were estimated to be 78% (95% CI 77.6-79.1%) and 100.0% (95% CI 99.7-100.0%), respectively. However, this estimate excludes mutations associated with phenotypic INH resistance that could not be confidence-graded due to insufficient data. If these mutations were also considered in addition to the confidence-graded mutations, the sensitivity and specificity for **INH resistance** detection would be **84%** (95% CI 82.9-84.3%) and **98%** (95% CI 96.8-98.4%), respectively.

Table 3: List of confidence-graded mutations associated with phenotypic INH resistance and associated MIC data

Gene	Mutations	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates)	INH Summary
inhA	c-15t	Moderate	10 (9-11%)	LJ	Lempens 2018 (n=43): 0.8 ≤ MIC ≤ 12.8 mg/L; **mode of 0.8 mg/L** Jagielski 2015 (n=5): 1 ≤ MIC ≤ 2.5 mg/L; **mode of 1 mg/L**	Moderate confidence, moderate MIC mutation.
				7H10	Gali 2006 (n=12): 0.25 ≤ MIC ≤ 2 mg/L; **mode of 0.5 mg/L**	
				7H11	Ennassiri 2017 (n=11): 0.5 ≤ MIC ≤ 1 mg/L; **mode of 1 mg/L** Guo 2006 (n=13): 0.2 ≤ MIC ≤ 0.4 mg/L; **mode of 0.2 mg/L**	
				MGIT	Kambli 2015 (n=16): 0.5 ≤ MIC ≤ 1 mg/L; **mode of 1 mg/L** Machado 2013 (n=16): 3 ≤ MIC > 10 mg/L; **mode of 3 mg/L** Machado 2016 (n=4): 3 ≤ MIC ≤ 20 mg/L; **mode of 3 mg/L**	
katG	S315I	High	0 (0-1%)	LJ	Jagielski 2015 (n=2): MIC = 10 mg/L	High confidence resistance mutation. Additional MIC data needed.
				7H10	–	
				7H11	–	
				MGIT	–	
	S315N	High	1 (1-1%)	LJ	Lempens 2018 (n=43): 0.8 ≤ MIC ≤ 12.8 mg/L Mei 2015 (n=9): 1 ≤ MIC ≤ 1.4 mg/L; **mode of 1 mg/L** Jagielski 2015 (n=1): MIC = 2.5 mg/L	High confidence, high MIC mutation.
				7H10	Gali 2006 (n=1): MIC = 8 mg/L	
				7H11	Guo 2006 (n=1): MIC =1 mg/L	
				MGIT	-	

Gene	Mutations	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates)	INH Summary
katG	**S315T**	High	54 (53-55%)	LJ	Lempens 2018 (n=78): 3.2 ≤ MIC ≥ 19.2 mg/L; **mode of 6.4 mg/L** Mei 2015 (n=18): 0.8 ≤ MIC ≤ 1.8 mg/L; **mode of 1 mg/L** Jagielski 2015 (n=50): 1 ≤ MIC ≤ 10 mg/L; **mode of 2.5 mg/L**	High confidence, high MIC mutation.
				7H10	Otto-Knapp 2016 (n=84): 0.25 ≤ MIC > 5 mg/L Gali 2006 (n=12): 4 ≤ MIC ≤ 16 mg/L; **mode of 8 mg/L** Cavusoglu 2006 (n=27): MIC ≥ 4 mg/L	
				7H11	Ennassiri 2017 (n=57): 2 ≤ MIC ≤ 10 mg/L; **mode of 10 mg/L** Cockerill 1995 (n=2): 8 ≤ MIC ≤ 16 mg/L Guo 2006 (n=8): 1 ≤ MIC ≤ 5 mg/L	
				MGIT	Kambli 2015 (n=50): 3 ≤ MIC ≥ 10 mg/L; **mode of 10 mg/L** Machado 2013 (n=1): MIC ≥ 3 mg/L Machado 2016 (n=2): 10 ≤ MIC ≤ 20 mg/L	
	Pooled frameshifts and premature stop codons	High	0 (0-1%)	LJ	Jagielski 2015 (n=2): 0.5 ≤ MIC ≤ 100 mg/L	High confidence, high MIC mutations.
				7H10	Gali 2006 (n=2): MIC > 32 mg/L	
				7H11	–	
				MGIT	Machado 2016 (n=1): MIC =128 mg/L	
katG + inhA	**S315** (T/G) and c-15t	High	N/A*	LJ	Lempens 2018 (n=13): MIC ≥ 19.2 mg/L; **mode of 25.6 mg/L** Jagielski 2015 (n=1): MIC = 5 mg/L	High confidence, high MIC mutations.
				7H10	–	
				7H11	Ennassiri 2017 (n=21): MIC ≥ 12 mg/L	
				MGIT	Kambli 2015 (n=20): MIC ≥ 10 mg/L	

Gene	Mutations	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates)	INH Summary
mabA (fabG1)	g609a	–	N/A	LJ	Lempens 2018 (n=1): MIC ≥ 19.2 mg/L	Need additional data.
				7H10	–	
				7H11	–	
				MGIT	Torres 2015 (n=1): MIC = 0.5 mg/L	
ahpC-oxyR	Genomic locus not considered		N/A 7H10 7H11 MGIT	LJ	Lempens 2018 (n=18): MIC ≤ 2 mg/L to ≥ 19.2 mg/L Jagielski 2015 (n=4): 0.5 ≤ MIC > 60 mg/L; **mode of 0.5 mg/L**	Need additional data.
				–		
				–		
				–		

The WHO-recommended INH CCs for phenotypic DST are 0.2mg/L in LJ, 0.2mg/L in 7H10, 0.2mg/L in 7H11 and 0.1mg/L in MGIT960 [20]. Phylogenetic SNPs and other mutations with no causative association with INH resistance (*inhA* **g-102a**, t-80g, g-47c, and **T4I**, *furA* **L68F** and *katG* A110V, **R463L** and L499M, *mshA* 187V and **N111S**), as well as silent mutations (*inhA-mabA* G3G, L44L, L88L, S142S, G205G, E261E, K239K, P241P, A281A, A291A, T308T, T326T, K327K, T344T and L611L, *mshA* G106G, D208D, A298A, C409C, L244L and L261L), are not shown in the above table. For those mutations with noted confidence grading, standard type represents associations based on nominal p-values (putative); **bold type** represents associations based on corrected p-values. Limitations: Ennassiri et al. did not sequence isolates (genotypic results were obtained through LPA testing), and Kambli only performed confirmatory sequencing for a subset of isolates for which genotype was determined by LPA.

* 11,082 total INH-resistant isolates were evaluated. Pooled sensitivity estimates from the systematic review include isolates with multiple mutations.

2.2.2 Rifampicin

RIF resistance in MTBC is most commonly associated with mutations in a well-defined, 81-base pair region of the *rpoB* gene [36]. Generally, upwards of 95% of RIF-resistant MTBC have mutations occurring in this Rifampicin Resistance-Determining Region, or RRDR [37, 38]. These mutations generally impede binding of RIF to the beta subunit of DNA-dependent RNA polymerase in MTBC [39, 40], resulting in RIF resistance.

Relevant data for the *rpoB* RIF resistance-associated gene were included in the review of RIF mutation data. Data from 95 studies, with strains from 37 countries that were isolated from 1992-2014, were included in the review. The total number of isolates with available *rpoB* sequence data was 13,424.

RIF was not included in the MIC data review. In abridged literature review, 120 studies were identified for full-text screening, of which 16 were found to have detailed MIC data for RIF resistance-associated gene regions. These data are presented alongside mutation confidence data in Table 4, to suggest comparative RIF resistance levels for these mutations in different media [27, 30, 41-53]. Mutations are presented using both *E. coli* and *M. tuberculosis* numbering to aid in data interpretation, as different molecular tools rely upon different TB mutation nomenclature for accurate test interpretation.

Using only the high, medium and low confidence-graded mutations identified in Miotto's review as predictors of phenotypic resistance (Table 4), the sensitivity and specificity for RIF resistance detection were estimated to be 91% (95% CI 89.8-91.1%) and 100% (95% CI 99.9-100.0%), respectively. However, this estimate excludes mutations associated with phenotypic RIF resistance that could not be confidence-graded due to insufficient data. If these mutations were also considered in addition to the confidence-graded mutations, the sensitivity and specificity for RIF resistance detection would be 96% (95% CI 95.2-96.1%) and 99% (95% CI 98.1-98.8%), respectively. RIF resistance is almost exclusively associated with mutations occurring in the *rpoB* gene RRDR.

Miotto et al. characterized the *rpoB* Q513K, Q513L, Q513P, F514dupI, D516A, D516F, D516G and L533P, D516V, delN518, H526C, H526D, H526G, H526L, H526R, H526Y, S531F, S531L and S531W as high confidence, while D516Y, S522L, H526P and L533P were characterized as moderate confidence and L511P, H526N and I572F were characterized as minimal confidence markers of RIF resistance (Table 4). The abridged MIC data review found data to be lacking for many of these mutations, especially in solid media, though the *rpoB* Q513K, Q513L, Q513P, D516V, H526D, H526R, H526Y and the S531 mutants were confirmed to have associated high MICs across media. For other mutations, D516Y and L533P were found to have associated moderate MICs across media, while the L511P and H526N mutations were found to have associated low MICs across media.

Using only the high, medium and low confidence-graded mutations identified in Miotto's review as predictors of phenotypic resistance (Table 4), the sensitivity and specificity for RIF resistance detection were estimated to be 91% (95% CI 89.8-91.1%) and 100% (95% CI 99.9-100.0%), respectively. However, this estimate excludes mutations associated with phenotypic RIF resistance that could not be confidence-graded due to insufficient data. If these mutations were also considered in addition to the confidence-graded mutations, the sensitivity and specificity for **RIF resistance** detection would be **96%** (95% CI 95.2-96.1%) and **99%** (95% CI 98.1-98.8%), respectively.

Table 4: List of confidence-graded mutations associated with phenotypic RIF resistance and associated MIC data

Gene	Mutations (MTB numbering)	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates and Laboratory Mutants)	RIF Summary
rpoB	Q513K (Q432K)	High	1 (0-1%)	LJ	Rigouts 2013 (n=2): MIC > 640 mg/L	High confidence, high MIC mutation.
				7H10	–	
				7H11	–	
				MGIT	Berrada 2016 (n=3): MIC ≥ 8 mg/L	
	Q513L (Q432L)	High	0 (0-0%)	LJ	–	High confidence, high MIC mutation.
				7H10	Miotto 2018 (n=3): MIC > 20 mg/L	
				7H11	Anthony 2005 (n=1): MIC = 256 mg/L	
				MGIT	Berrada 2016 (n=1): MIC ≥ 8 mg/L	
	Q513P (Q432P)	High	0 (0-1%)	LJ	Rigouts 2013 (n=3): MIC > 640 mg/L	High confidence, high MIC mutation.
				7H10	Cavusoglu 2006 (n=1): MIC ≥ 32 mg/L	
				7H11	–	
				MGIT	Berrada 2016 (n=2): MIC ≥ 8 mg/L Abanda 2017 (n=2): MIC > 8 mg/L	
	F514dupl (F433dupl)	High	0 (0-0%)	LJ	–	High confidence resistance mutation. Additional MIC data needed.
				7H10	–	
				7H11	–	
				MGIT	Jamieson 2014 (n=1): MIC = 20 mg/L	
	D516A (D435A)	High	0 (0-0%)	LJ	–	High confidence resistance mutation. Additional MIC data needed.
				7H10	–	
				7H11	–	
				MGIT	Kambli 2015 (n=1): MIC = 10 mg/L	

Gene	Mutations (MTB numbering)	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates and Laboratory Mutants)	RIF Summary
rpoB	D516F (D435F)	High	0 (0-1%)	LJ	Rigouts 2013 (n=4): MIC > 640 mg/L	High confidence resistance mutation. Additional MIC data needed.
				7H10	–	
				7H11	–	
				MGIT	Berrada 2016 (n=1): MIC = 2 mg/L Kambli 2015 (n=1): MIC = 10 mg/L	
	D516G + L533P	High	N/A*	LJ	–	High confidence resistance mutation. Additional MIC data needed.
				7H10	–	
				7H11	–	
				MGIT	Berrada 2016 (n=1): MIC > 8 mg/L	
	D516V (D435V)	High	6 (5-6%)	LJ	Rigouts 2013 (n=15): 320 ≤ MIC > 640 mg/L	High confidence, high MIC mutation.
				7H10	Cavusoglu 2006 (n=1): MIC = 8 mg/L	
				7H11	–	
				MGIT	Berrada 2016 (n=18): MIC ≥ 8 mg/L Kambli 2015 (n=3): MIC = 10 mg/L Jamieson 2014 (n=3): 20 ≤ MIC ≤ 50 mg/L; **mode of 50 mg/L** Sirgel 2013 (n=29): 10 ≤ MIC ≤ 15 mg/L	
	delN518 (delN437)	High	0 (0-0%)	LJ	–	High confidence resistance mutation. Additional MIC data needed.
				7H10	–	
				7H11	–	
				MGIT	Berrada 2016 (n=1): MIC = 4 mg/L	

Gene	Mutations (MTB numbering)	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates and Laboratory Mutants)	RIF Summary
	H526C (H445C)	High	0 (0-1%)	LJ	–	High confidence resistance mutation. Additional MIC data needed.
				7H10	Cavusoglu 2006 (n=1): MIC = 8 mg/L Miotto 2018 (n=2): MIC > 20 mg/L	
				7H11	–	
				MGIT	Berrada 2016 (n=3): 2 ≤ MIC ≤ 8 mg/L Kambli 2015 (n=1): MIC = 2 mg/L	
rpoB	H526D (H445D)	High	4 (3-4%)	LJ	Rigouts 2013 (n=11): MIC ≥ 640 mg/L	High confidence, high MIC mutation.
				7H10	Cavusoglu 2004 (n=1): MIC > 128 mg/L	
				7H11	Pozzi 1999 (n=9): 16 ≤ MIC ≥ 128 mg/L Anthony 2005 (n=4): 128 ≤ MIC ≤ 256 mg/L; **mode of 256 mg/L**	
				MGIT	Berrada 2016 (n=3): MIC ≥ 8 mg/L Kambli 2015 (n=7): MIC > 20 mg/L Jamieson 2014 (n=1): MIC = 160 mg/L	
	H526G (H445G)	High	0 (0-0%)	LJ	–	High confidence resistance mutation. Additional MIC data needed.
				7H10	–	
				7H11	–	
				MGIT	Berrada 2016 (n=1): MIC = 2 mg/L	
	H526L (H445L)	High	1 (1-2%)	LJ	Rigouts 2013 (n=10): MIC ≥ 640 mg/L	High confidence resistance mutation. Additional MIC data needed.
				7H10	–	
				7H11	–	
				MGIT	Berrada 2016 (n=4): 2 ≤ MIC ≤ 8; **mode of 2 mg/L** Abanda 2017 (n=2): 0.25 ≤ MIC < 4 mg/L Jamieson 2014 (n=2): 0.5 ≤ MIC ≤ 2 mg/L	

Gene	Mutations (MTB numbering)	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates and Laboratory Mutants)	RIF Summary
	H526R (H445R)	High	2 (1-2%)	LJ	Rigouts 2013 (n=5): MIC > 640 mg/L	High confidence, high MIC mutation.
				7H10	Cavusoglu 2006 (n=4): MIC ≥ 32 mg/L	
				7H11	Pozzi 1999 (n=1): MIC ≥ 128 mg/L Anthony 2005 (n=4): 128 ≤ MIC ≤ 512 mg/L; **mode of 256 mg/L**	
				MGIT	Berrada 2016 (n=2): MIC ≥ 8 mg/L Abanda 2017 (n=3): MIC > 8 mg/L Kambli 2015 (n=3): MIC > 20 mg/L	
rpoB	H526Y (H445Y)	High	6 (5-6%)	LJ	Rigouts 2013 (n=14): MIC ≥ 640 mg/L	High confidence, high MIC mutation.
				7H10	Cavusoglu 2006 (n=2): MIC ≥ 32 mg/L	
				7H11	Pozzi 1999 (n=1): MIC ≥ 128 mg/L Anthony 2005 (n=4): 128 ≤ MIC ≤ 256 mg/L; **mode of 256 mg/L**	
				MGIT	Berrada 2016 (n=1): MIC ≥ 8 mg/L Kambli 2015 (n=3): MIC > 20 mg/L Jamieson 2014 (n=2): 100 ≤ MIC ≥ 160 mg/L	
	S531F (S450F)	High	0 (0-0%)	LJ	–	High confidence, high MIC mutation.
				7H10	Miotto 2018 (n=2): MIC > 20 mg/L	
				7H11	–	
				MGIT	Berrada 2016 (n=1): MIC ≥ 8 mg/L	

Gene	Mutations (MTB numbering)	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates and Laboratory Mutants)	RIF Summary
	S531L (S450L)	High	52 (51-53%)	LJ	Rigouts 2013 (n=10): MIC ≥ 640 mg/L	High confidence, high MIC mutation.
				7H10	Schön 2013 (n=17): 16 ≤ MIC ≤ 128 mg/L Cavusoglu 2006 (n=19): MIC ≥ 32 mg/L Miotto 2018 (n=1): MIC > 20 mg/L	
				7H11	Pozzi 1999 (n=21): 32 ≤ MIC ≥ 128 mg/L Anthony 2005 (n=4): MIC = 256 mg/L	
				MGIT	Berrada 2016 (n=5): MIC ≥ 8 mg/L Kambli 2015 (n=57): MIC > 20 mg/L Jamieson 2014 (n=21): 100 ≤ MIC ≥ 160 mg/L; **mode of 160 mg/L** Sirgel 2013 (n=1): MIC > 10 mg/L Machado 2016 (n=5): 80 ≤ MIC ≤ 320mg/L; **mode of 320 mg/L**	
rpoB	S531W (S450W)	High	2 (1-2%)	LJ	Rigouts 2013 (n=4): MIC ≥ 640 mg/L	High confidence, high MIC mutation.
				7H10	Schön 2013 (n=1): MIC = 256 mg/L Cavusoglu 2006 (n=4): MIC ≥ 32 mg/L	
				7H11	Anthony 2005 (n=1): MIC = 256 mg/L	
				MGIT	Berrada 2016 (n=3): MIC ≥ 8 mg/L Abanda 2017 (n=1): MIC > 8 mg/L Jamieson 2014 (n=3): MIC ≥ 160 mg/L	

Gene	Mutations (MTB numbering)	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates and Laboratory Mutants)	RIF Summary
	D516Y (D435Y)	Moderate	2 (2-2%)	LJ	Rigouts 2013 (n=15): 320 ≤ MIC > 640 mg/L	Moderate confidence, moderate MIC mutation.
				7H10	Schön 2013 (n=1): MIC = 0.5 mg/L van Ingen 2011 (n=7): 0.5 ≤ MIC ≤ 2 mg/L; **mode of 2 mg/L** Cavusoglu 2006 (n=2): MIC = 8 mg/L Miotto 2018 (n=3): MIC = 9 mg/L	
				7H11	–	
				MGIT	Berrada 2016 (n=4): 0.25 ≤ MIC ≤ 0.5 mg/L Abanda 2017 (n=4): MIC > 8 mg/L Kambli 2015 (n=3): 0.25 ≤ MIC ≤ 10 mg/L	
	S522L (S441L)	Moderate	0 (0-1%)	LJ	Rigouts 2013 (n=2): MIC > 640 mg/L	Moderate confidence resistance mutation. Additional MIC data needed.
				7H10	–	
				7H11	–	
				MGIT	Berrada 2016 (n=1): MIC = 2 mg/L Jamieson 2014 (n=2): MIC = 20 mg/L	
	H526P (H445P)	Moderate	0 (0-0%)	LJ	–	Moderate confidence resistance mutation. Additional MIC data needed.
				7H10	–	
				7H11	Anthony 2005 (n=1): MIC = 256 mg/L	
				MGIT	–	

Gene	Mutations (MTB numbering)	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates and Laboratory Mutants)	RIF Summary
rpoB	L533P (L452P)	Moderate	3 (2-3%)	LJ	Rigouts 2013 (n=14): 160 ≤ MIC > 640 mg/L Andres 2014 (n=2): 5 ≤ MIC ≤ 10 mg/L	Moderate confidence, moderate MIC mutation.
				7H10	Cavusoglu 2006 (n=1): MIC = 4 mg/L Miotto 2018 (n=1): MIC = 3 mg/L	
				7H11	Nguyen 2015 (n=4): 0.248 ≤ MIC ≤ 0.9 mg/L	
				MGIT	Berrada 2016 (n=2): MIC= 0.5 mg/L Abanda 2017 (n=1): MIC > 8 mg/L Kambli 2015 (n=4): 2 ≤ MIC ≤ 4 mg/L; **mode of 4 mg/L** Ho 2013 (n=1): MIC = 1.0 mg/L Andres 2014 (n=2): MIC = 0.5 mg/L	
	L511P (L430P)	Minimal	1 (1-2%)	LJ	Rigouts 2013 (n=6): 80 ≤ MIC ≤ 640 mg/L	Minimal confidence, low MIC mutation.
				7H10	Schön 2013 (n=2): 0.125 ≤ MIC ≤ 1 mg/L	
				7H11	Nguyen 2015 (n=2): 0.248 ≤ MIC ≤ 0.7 mg/L	
				MGIT	Berrada 2016 (n=4): MIC ≤ 0.25 mg/L Abanda 2017 (n=2): MIC ≤ 0.25 mg/L Jamieson 2014 (n=2): MIC ≤ 0.125 mg/L Ho 2013 (n=3): 0.25 ≤ MIC ≤ 1 mg/L	
	H526N (H445N)	Minimal	1 (1-1%)	LJ	Rigouts 2013 (n=5): 80 ≤ MIC > 640 mg/L	Minimal confidence, low MIC mutation.
				7H10	Miotto 2018 (n=1): MIC = 3 mg/L	
				7H11	-	
				MGIT	Berrada 2016 (n=3): 0.125 ≤ MIC ≤ 0.25 mg/L; **mode of 0.125 mg/L** Abanda 2017 (n=1): MIC > 8 mg/L Kambli 2015 (n=1): MIC = 0.25 mg/L Jamieson 2014 (n=1): MIC = 0.25 mg/L	

Gene	Mutations (MTB numbering)	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates and Laboratory Mutants)	RIF Summary
rpoB	I572F (I491F)	Minimal	0 (0-1%)	LJ	Rigouts 2013 (n=7): 40 ≤ MIC > 640 mg/L	Minimal confidence resistance mutation. Additional MIC data needed.
				7H10	–	
				7H11	–	
				MGIT	–	

The WHO-recommended RIF CCs for phenotypic DST are 40.0mg/L in LJ, 1.0mg/L in 7H10, 1.0mg/L in 7H11 and 1.0mg/L in MGIT960 [20]. The high-confidence mutations *rpoB* **D516G**, D516ins, D516N, S522Q, H526F, S531Q, S531Y, D626E, M515I and D516Y, F505V and D516Y, S512T, Q513H and L533P, and Q513-514ins (*E. coli* numbering system) were not included in the table above, as no MIC data were identified for isolates with these unique mutations for any medium of interest. For those mutations with noted confidence grading, standard type represents associations based on nominal p-values (putative); **bold type** represents associations based on corrected p-values. Silent mutations (MTB numbering system, *rpoB* D184D, F433F, L440L, T444T, R448R, L452L, G453G, G455G, S458S, R461R, G463G, R467R, V469V, P471P, E481E, G485G, S495S, L778L, G876G, and A1075A) are also not shown in the above table. Limitations: Anthony et al. only determined MICs for *M. tuberculosis* MTB72 (ATCC35801) laboratory mutant, Cavusoglu et al. and Machado et al. 2016 did not sequence isolates (genotypic results were obtained through LPA testing), and Kambli only performed confirmatory sequencing for a subset of isolates for which genotype was determined by LPA.

* 8,549 total RIF-resistant isolates were evaluated. Pooled sensitivity estimates from the systematic review include isolates with multiple mutations.

2.2.3 Fluoroquinolones

The FQs are among the most important anti-tuberculosis drugs, including the most effective second-line drugs [54], with the later generation FQs (levofloxacin and moxifloxacin) becoming vital components in regimens for the successful treatment of DR-TB. The MTBC cellular target of the FQs is DNA gyrase, a type II topoisomerase consisting of two subunits encoded by *gyrA* and *gyrB* [55]. FQ resistance in MTBC is conferred through genetic mutations in one of these two subunits, with most mutations occurring between the *gyrA* or *gyrB* quinolone resistance-determining regions (QRDRs), from codons 74-113 and codons 500 to 538, respectively [56, 57]. The majority of FQ resistance has been associated with mutations occurring in the *gyrA* QRDR, at codons 88, 90, 91 and 94 [58]. *gyrB* QRDR mutations, in contrast, typically occur with lower sensitivity and specificity [59].

Focusing solely on isolates with phenotypic MFX DST data in the mutation review, relevant data for the *gyrA* and *gyrB* FQ resistance-associated genes were included in the review of FQ mutation data. Data from 75 studies, with strains from 36 countries that were isolated from 1991-2013, were included in the review. The total number of isolates with available *gyrA* sequence data was 1,019, whereas 735 had *gyrB* mutation data.

The FQs were included in the MIC data review. A full-text screen was performed for 2,225 total records, and 65 studies with relevant FQ MIC data for different media were included in the final review. Summary MFX MIC data is presented for each mutation, stratified by media, in Table 5. *gyrB* mutations are presented using two different numbering systems to aid in data interpretation, as different molecular tools rely upon different TB mutation nomenclature for accurate test interpretation.

Only the *gyrA* mutations G88C, A90V, S91P, D94A, D94G, D94N and D94Y and the *gyrB* mutation A504V were determined to be high-confidence MFX resistance markers by Miotto et al. (Table 5). The full MIC data review confirmed that the G88C, D94G and D94N mutations were associated with high MFX MICs across different media, along with the D94H mutation, a *gyrA* mutation for which no confidence grading was available. Although there was also no confidence grading available for *gyrA* D89N, this mutation was confirmed to have moderate MICs, along with the high confidence S91P and D94Y mutations, while the high confidence *gyrA* A90V and D94A mutations were found to have low MICs. Additional MIC data is needed to confirm the MIC distributions for all *gyrB* mutations.

The sensitivity and specificity of gyrA mutations for predicting phenotypic resistance to FQs in general, and MFX specifically, has been underestimated in past reports due to phenotypic reference standard critical concentrations that were set too high. These have recently been corrected [13], and will likely increase the accuracy of these mutations as predictors of phenotypic resistance. Using only the high, medium and low confidence-graded mutations identified in Miotto's review as predictors of phenotypic resistance (Table 5) and considering this limitation, the sensitivity and specificity for **MFX resistance** detection were estimated to be **89%** (95% CI 86.4-91.7%) and **100%** (95% CI 99.2-100.0%), respectively. Sensitivity for detecting phenotypic MFX resistance was increased to 94% (95% CI 91.5-95.5%) when all resistance-associated mutations were considered, including those mutations that could not be confidence-graded due to insufficient data.

Table 5: List of confidence-graded mutations associated with phenotypic MFX resistance and associated MIC data

Gene	Mutations	Confidence Grading	Pooled Sensitivity (95% CI)**	Media	MIC Evidence (Mouse*** and Clinical Isolates)	MFX Summary
gyrA	G88C	High	1 (0-2%)	LJ	–	High confidence, high MIC mutation.
				7H10	1 study (n=1); MIC 8	
				7H11	–	
				MGIT	3 studies (n=3); MIC 4->4; **mode of 4 mg/L**	
	D89N	–	N/A	LJ	1 study (n=3); MIC >8	Moderate MIC mutation.
				7H10	2 studies (n=4); MIC 1-4; **mode of 4 mg/L**	
				7H11	1 study (n=6); MIC 4-8	
				MGIT	3 studies (n=3); MIC 0.5-2.5; **mode of 0.5 mg/L**	
	A90V	High	17 (14-20%)	LJ	3 studies (n=21); MIC 0.5->4; **mode of 2 mg/L**	High confidence, low MIC mutation.
				7H10	5 studies (n=52); MIC ≤0.06-8; **mode of 1 mg/L**	
				7H11	2 studies (n=11); MIC 1-4; **mode of 1 mg/L**	
				MGIT	12 studies (n=111); MIC 0.18-2.5 mg/L; **mode of 1 mg/L**	
	S91P	High	3 (2-5%)	LJ	2 studies (n=6); MIC 2-4; **mode of 2 mg/L**	High confidence, moderate MIC mutation.
				7H10	3 studies (n=6); MIC 1-4; **mode of 2 mg/L**	
				7H11	2 studies (n=4); MIC 1-4; **mode of 4mg/L**	
				MGIT	11 studies (n=37); MIC 0.5->7.5; **mode of 1 mg/L**	
	D94A	High	7 (5-10%)	LJ	2 studies (n=11); MIC 0.5->4	High confidence, low MIC mutation.
				7H10	3 studies (n=25); MIC 0.25-8; **mode of 1 mg/L**	
				7H11	2 studies (n=2); MIC 1-2	
				MGIT	9 studies (n=45); MIC 0.25-2.5; **mode of 1 mg/L**	

Gene	Mutations	Confidence Grading	Pooled Sensitivity (95% CI)**	Media	MIC Evidence (Mouse*** and Clinical Isolates)	MFX Summary
gyrA	**D94G**	High	29 (26-33%)	LJ	3 studies (n=21); MIC 1->8	High confidence, high MIC mutation.
				7H10	6 studies (n=60); MIC 0.25-16; **mode of 4 mg/L**	
				7H11	2 studies (n=11); MIC 1-8; **mode of 4 mg/L**	
				MGIT	12 studies (n=151); MIC 0.5-8; **mode of 2 mg/L**	
	D94H	–	N/A	LJ	1 study (n=1); MIC >4	High MIC mutation.
				7H10	–	
				7H11	1 study (n=2); MIC 4-8	
				MGIT	6 studies (n=11); MIC 1-2.5; **mode of 2 mg/L**	
	D94N	High	6 (4-8%)	LJ	2 studies (n=2); MIC 4->4	High confidence, high MIC mutation.
				7H10	2 studies (n=12); MIC 4-16; **mode of 4 mg/L**	
				7H11	2 studies (n=3); MIC 1-8; **mode of 8 mg/L**	
				MGIT	7 studies (n=18); MIC 0.5->4; **mode of 2mg/L**	
	D94Y	High	3 (2-5%)	LJ	–	High confidence, moderate MIC mutation.
				7H10	4 studies (n=9); MIC 0.25-8; **mode of 1 mg/L**	
				7H11	1 study (n=3); MIC 2-8; **mode of 8 mg/L**	
				MGIT	6 studies (n=7); MIC 1-4; **mode of 2 mg/L**	
gyrB	**D461H (D500H)**	–	N/A	LJ	–	Additional data needed.
				7H10	2 studies (n=2); MIC 1	
				7H11	1 study (n=4); MIC 2	
				MGIT	1 study (n=1); MIC 0.5	

Gene	Mutations	Confidence Grading	Pooled Sensitivity (95% CI)**	Media	MIC Evidence (Mouse*** and Clinical Isolates)	MFX Summary
gyrB	D461N (D500N)	–	N/A	LJ	–	Additional data needed.
				7H10	1 study (n=2); MIC 0.5	
				7H11	1 study (n=2); MIC 1	
				MGIT	–	
	N499D (N538D)	–	N/A	LJ	–	Additional data needed.
				7H10	3 studies (n=3); MIC 1-4	
				7H11	–	
				MGIT	1 study (n=1); MIC 0.5	
	N499S (N538S)	–	N/A	LJ	1 study (n=1): MIC >8	Additional data needed.
				7H10	–	
				7H11	–	
				MGIT	1 study (n=1); MIC 1.5	
	N499K (N538K)	–	N/A	LJ	–	Additional data needed.
				7H10	1 study (n=1); MIC 4	
				7H11	–	
				MGIT	–	
	A504V (A543V)	High	N/A****	LJ	–	High confidence resistance mutation. Additional MIC data needed.
				7H10	–	
				7H11	1 study (n=2); MIC 0.5-1	
				MGIT	2 studies (n=2); MIC 0.12-0.25	

The most recent WHO-recommended MFX CCs for phenotypic DST are 1.0mg/L in LJ, 0.5mg/L in 7H10, 0.5mg/L in 7H11 and 0.25mg/L in MGIT960 [13]. The WHO-recommended MFX CBs for phenotypic DST are 2.0mg/L in 7H10 and 1.0mg/L in MGIT960 [13]. The high-confidence mutation *gyrB* E459K was not included in the table above, as no MIC data was identified for isolates with this unique mutation for any medium of interest. Phylogenetic SNPs, and mutations with no causative association with moxifloxacin resistance (*gyrA* E21Q, **S95T**, G247S, G668D, and V712L), as well as silent mutations (*gyrA* A125A, L197L, L198L, T272T, A343A, A384A, E485E, L549L, I568I, A581A, V678V, and G809G, *gyrB* T625T), are also not shown in the above table. For those mutations with noted confidence grading, standard type represents associations based on nominal p-values (putative); **bold type** represents associations based on corrected p-values. Notable limitations of included datasets are noted in the published WHO report.

* *gyrB* 1998 numbering given in parentheses [60]. 2002 updated numbering given [61].

** 567 total MFX-resistant isolates were evaluated. Pooled sensitivity estimates from the systematic review include isolates with multiple mutations.

*** Mouse mutant data were only included in the 7H11 data summaries

**** Estimates not available for MFX-resistant isolates

2.2.4 Pyrazinamide

Knowledge of PZA susceptibility can inform decisions on the choice and design of effective DR-TB regimens. Culture-based PZA phenotypic DST is both difficult to perform and often produces unreliable results [62, 63]. Currently, Bactec MGIT 960 liquid culture method is the only WHO-recommended methodology for PZA susceptibility testing, though even this testing is associated with a high rate of false-positive resistance results [62, 64]. The lack of a rapid molecular test to diagnose PZA resistance rapidly ahead of start of treatment implies that even where PZA DST is available, the results usually only become available after treatment has been started.

PZA resistance is most commonly associated with mutations in the MTBC *pncA* gene and promoter, which lead to a reduction or loss of pyrazinamidase activity [65]. Unfortunately, the molecular detection of PZA resistance is complicated by the fact that a diversity of mutations confer PZA resistance, and no single gene region, or hot spot, holds the majority of PZA resistance mutations. Instead, these mutations are scattered throughout the *pncA* gene and promoter [66]. For these reasons, sequencing offers great promise for the rapid and accurate detection of PZA resistance, though there is a pressing need to correlate the many *pncA* mutations with conferred levels of phenotypic drug resistance to best guide the interpretation of *pncA* sequencing results.

Relevant data for the *pncA* PZA-resistance associated gene were included in the review of PZA mutation data. Data from 81 studies, with strains from 36 countries that were isolated from 1990-2014, were included in the review. The total number of isolates with available *pncA* sequence data was 4,949.

PZA was not included in the MIC data review. In abridged literature review, 42 studies were identified for full-text screening, of which 14 were found to have detailed MIC data for PZA resistance-associated gene regions. These data are presented alongside mutation confidence data in Table 6, to suggest comparative PZA resistance levels for these mutations in different media [48, 64, 67-79].

A wide variety of *pncA* mutations were determined to be high-confidence PZA resistance markers (Table 6). The full MIC data review confirmed that the majority of these mutations were associated with high MICs in MGIT, including *pncA* a-11g, t-7c, A3E, L4S, I6T, D8G, Q10P, C14R, G17D, Y34D, H51Q, H57D, H57Y, P62Q, W68C, T76P, F94L, G97D, R123P, V128G, G132A, G132S, T135P, H137P, V139G, T142K, V155G, L172P, M175T, M175V, combined frameshifts and premature stop codons, though additional mutations were identified in the abridged MIC data review that were confirmed to have high MICs in more than one isolate tested by MGIT or a combination of MGIT and other media (i.e. *pncA* V9G, F13L, A28T, K96Q, G97A and V163G). It should also be noted that although *pncA* D12G and F58L were considered minimal confidence resistance mutations and P54L was considered to be a moderate confidence resistance mutation in the mutation review, they were found to have a high MIC in the abridged MIC data review. Finally, although confidence grading was not available for *pncA* mutations E37V, S65A, D110G, A170V and V180I, these mutations were found to have low associated MICs.

Table 6: List of confidence-graded mutations associated with phenotypic PZA resistance and associated MIC data

Gene	Mutations	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates)	PZA Summary
pncA	a-11g	High	3 (2-4%)	LJ	Ghiraldi 2011 (n=1): MIC = 1600 mg/L	High confidence, high MIC mutation.
				MGIT	Morlock 2017 (n=1): MIC > 800 mg/L Li 2016 (n=9): 128 ≤ MIC ≤ 256 mg/L; **mode of 128 mg/L**	
	t-7c	High	0 (0-1%)	7H10	Morlock 2000 (n=1): MIC = 100 mg/L	High confidence, high MIC mutation.
				MGIT	Aono 2014 (n=2): MIC > 1600 mg/L Li 2016 (n=2): MIC = 128 mg/L	
	A3E	High	0 (0-1%)	MGIT	Aono 2014 (n=4): MIC ≥ 1600 mg/L; **mode of 1600 mg/L**	High confidence, high MIC mutation.
	A3P	–	N/A	LJ	Barco 2006 (n=1): MIC > 800 mg/L	Additional data needed.
	L4S	High	0 (0-1%)	7H10	Endoh 2002 (n=1): MIC > 100 mg/L Morlock 2000 (n=1): MIC = 800 mg/L	High confidence, high MIC mutation.
				MGIT	Li 2016 (n=1): MIC = 256 mg/L	
	I6T	High	0 (0-1%)	MGIT	Werngren 2012 (n=1): MIC = 256 mg/L Gonzalo 2014 (n=2): MIC = 400 mg/L	High confidence, high MIC mutation.
	V7A	–	N/A	LJ	Barco 2006 (n=1): MIC = 400 mg/L	Additional data needed.
	D8G	High	0 (0-1%)	MGIT	Li 2016 (n=2): MIC = 256 mg/L	High confidence, high MIC mutation.
	D8N	High	0 (0-0%)	MGIT	Li 2016 (n=1): MIC = 256 mg/L	High confidence resistance mutation. Need additional MIC data.
	D8A	–	N/A	MGIT	Li 2016 (n=1): MIC = 256 mg/L	Additional data needed.

Gene	Mutations	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates)	PZA Summary
pncA	V9G	–	N/A	MGIT	Aono 2014 (n=1): MIC = 1600 mg/L Li 2016 (n=1): MIC = 256 mg/L	High MIC mutation.
	Q10P	High	0 (1-2%)	7H10	Endoh 2002 (n=1): MIC > 100 mg/L Morlock 2000 (n=1): MIC = 800 mg/L	High confidence, high MIC mutation.
				7H11	Hirano 1998 (n=1): MIC > 800 mg/L	
				MGIT	Aono 2014 (n=2): MIC > 1600 mg/L Li 2016 (n=1): MIC = 512 mg/L	
	D12A	High	1 (0-1%)	7H10	Hu 2000 (n=1): MIC = 1500 mg/L	High confidence resistance mutation. Need additional MIC data.
				MGIT	Li 2016 (n=1): MIC = 512 mg/L	
	D12N	High	0 (0-1%)	MGIT	Li 2016 (n=1): MIC = 512 mg/L	High confidence resistance mutation. Need additional MIC data.
	F13L	–	N/A	MGIT	Li 2016 (n=1): MIC = 512 mg/L Morlock 2017 (n=1): MIC > 100 mg/L	High MIC mutation.
	F13S	–	N/A	MGIT	Li 2016 (n=1): MIC = 512 mg/L	Additional data needed.
	C14R	High	1 (0-1%)	7H11	Hirano 1998 (n=1): MIC > 800 mg/L	High confidence, high MIC mutation.
				MGIT	Li 2016 (n=2): MIC = 256 mg/L	
	G16S	–	N/A	MGIT	Li 2016 (n=1): MIC = 256 mg/L	Additional data needed.
	G17D	High	0 (0-0%)	7H11	Hirano 1998 (n=2): MIC > 800 mg/L	High confidence, high MIC mutation.
	L19P	High	0 (0-0%)	7H10	Hu 2000 (n=1): MIC = 1500 mg/L	High confidence resistance mutation. Need additional MIC data.

Gene	Mutations	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates)	PZA Summary
pncA	G24D	High	0 (0-1%)	MGIT	Li 2016 (n=1): MIC = 512 mg/L	High confidence resistance mutation. Need additional MIC data.
	A28T	–	N/A	MGIT	Morlock 2017 (n=2): MIC = 100 mg/L	High MIC mutation.
	D33A	–	N/A	MGIT	Li 2016 (n=1): MIC = 256 mg/L	Additional data needed.
	Y34D	High	0 (0-1%)	MGIT	Aono 2014 (n=4): 400 ≤ MIC ≥ 1600 mg/L	High confidence, high MIC mutation.
	Y34S	–	N/A	7H11	Hirano 1998 (n=2): MIC > 800 mg/L	Additional data needed.
	Y41H	–	N/A	7H10	Hu 2000 (n=1): MIC = 600 mg/L	Additional data needed.
	H43P	–	N/A	7H11	Hirano 1998 (n=1): MIC > 800 mg/L	Additional data needed.
	A46V	High	0 (0-1%)	7H11	Hirano 1998 (n=1): MIC = 400 mg/L	High confidence resistance mutation. Need additional MIC data.
	T47P	–	N/A	7H10	Hu 2000 (n=1): MIC = 900 mg/L	Additional data needed.
	H51Q	High	1 (1-1%)	MGIT	Aono 2014 (n=1): MIC = 1600 mg/L Li 2016 (n=1): MIC = 1024 mg/L Werngren 2012 (n=1): MIC > 1024 mg/L	High confidence, high MIC mutation.
	H51R	High	0 (0-1%)	LJ	Barco 2006 (n=1): MIC > 800 mg/L	High confidence resistance mutation. Need additional MIC data.
				7H11	Hirano 1998 (n=1): MIC > 800 mg/L	
				MGIT	Li 2016 (n=1): MIC = 1024 mg/L	
	H51P	–	N/A	7H10	Morlock 2000 (n=1): MIC = 800 mg/L	Additional data needed.
				7H11	Hirano 1998 (n=1): MIC > 800 mg/L	
	H51Y	–	N/A	MGIT	Li 2016 (n=1): MIC > 1024 mg/L	Additional data needed.

Gene	Mutations	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates)	PZA Summary
pncA	P54S	High	0 (0-0%)	MGIT	Werngren 2012 (n=1): MIC > 1024 mg/L	High confidence resistance mutation. Need additional MIC data.
	P54Q	–	N/A	LJ	Barco 2006 (n=1): MIC > 800 mg/L	Additional data needed.
				MGIT	Li 2016 (n=1): MIC = 512 mg/L	
	P54T	–	N/A	7H10	Hu 2000 (n=1): MIC = 800 mg/L	Additional data needed.
	H57D**	High	1 (1-2%)	7H11	Hirano 1998 (n=7): MIC > 800 mg/L	High confidence, high MIC mutation.
				MGIT	Li 2016 (n=1): MIC = 1024 mg/L Gonzalo 2014 (n=2): MIC > 800 mg/L	
	H57P	High	0 (0-0%)	MGIT	Li 2016 (n=1): MIC > 1024 mg/L	High confidence resistance mutation. Need additional MIC data.
	H57R	High	1 (1-1%)	LJ	Barco 2006 (n=1): MIC > 800 mg/L	High confidence resistance mutation. Need additional MIC data.
				MGIT	Li 2016 (n=1): MIC = 1024 mg/L	
	H57Y	High	0 (0-1%)	MGIT	Li 2016 (n=3): MIC > 1024 mg/L	High confidence, high MIC mutation.
	H57Q	–	N/A	MGIT	Li 2016 (n=1): MIC = 1024 mg/L	Additional data needed.
	H57N	–	N/A	LJ	Ghiraldi 2011 (n=1): MIC > 3200 mg/L	Additional data needed.
	F58S	–	N/A	MGIT	Li 2016 (n=1): MIC = 1024 mg/L	Additional data needed.
	S59P	High	0 (0-1%)	LJ	Barco 2006 (n=1): MIC > 800 mg/L Ghiraldi 2011 (n=1): MIC = 1600 mg/L	High confidence resistance mutation. Need additional MIC data.
				MGIT	Aono 2014 (n=1): MIC = 1600 mg/L	

Gene	Mutations	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates)	PZA Summary
pncA	P62Q	High	0 (0-0%)	MGIT	Aono 2014 (n=2): MIC = 800 mg/L	High confidence, high MIC mutation.
	P62T	–	N/A	MGIT	Li 2016 (n=1): MIC > 1024 mg/L	Additional data needed.
	P62R	–	N/A	7H10	Morlock 2000 (n=1): MIC > 800 mg/L	Additional data needed.
	D63G	High	0 (0-1%)	7H11	Hirano 1998 (n=1): MIC > 800 mg/L	High confidence resistance mutation. Need additional MIC data.
	D63A	–	N/A	MGIT	Li 2016 (n=1): MIC = 512 mg/L	Additional data needed.
	S65P	–	N/A	MGIT	Li 2016 (n=1): MIC = 32 mg/L	Additional data needed.
	S67P	High	0 (0-1%)	7H11	Hirano 1998 (n=1): MIC > 800 mg/L	High confidence resistance mutation. Need additional MIC data.
				MGIT	Li 2016 (n=1): MIC = 512 mg/L	
	W68C	High	0 (0-0%)	MGIT	Li 2016 (n=2): MIC ≥ 1024 mg/L	High confidence, high MIC mutation.
	W68R	High	1 (0-1%)	MGIT	Li 2016 (n=1): MIC = 1024 mg/L	High confidence resistance mutation. Need additional MIC data.
	W68L	–	N/A	LJ	Barco 2006 (n=2): MIC > 800 mg/L	Additional data needed.
				MGIT	Chan 2007 (n=1): MIC	
	P69R	–	N/A	7H10	Hu 2000 (n=1): MIC = 1200 mg/L	Additional data needed.
	H71Y	High	0 (0-1%)	MGIT	Aono 2014 (n=1): MIC = 1600 mg/L	High confidence resistance mutation. Need additional MIC data.

Gene	Mutations	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates)	PZA Summary
pncA	C72R	High	0 (0-1%)	7H10	Hu 2000 (n=1): MIC = 1500 mg/L	High confidence resistance mutation. Need additional MIC data.
				7H11	Hirano 1998 (n=1): MIC > 800 mg/L	
	C72P	–	N/A	7H11	Hirano 1998 (n=1): MIC > 800 mg/L	Additional data needed.
				MGIT	Li 2016 (n=1): MIC > 1024 mg/L	
	T76P	High	2 (1-2%)	7H10	Morlock 2000 (n=1): MIC = 400 mg/L	High confidence, high MIC mutation.
				MGIT	Li 2016 (n=2): MIC ≥ 1024 mg/L	
	H82R	High	0 (0-1%)	MGIT	Li 2016 (n=1): MIC = 512 mg/L	High confidence resistance mutation. Need additional MIC data.
	H82Y	–	N/A	MGIT	Morlock 2017 (n=1): MIC = 100 mg/L	Additional data needed.
	L85P	High	1 (1-2%)	7H10	Morlock 2000 (n=2): MIC > 800 mg/L Hu 2000 (n=1): MIC = 800 mg/L	High confidence resistance mutation. Need additional MIC data.
	F94L	High	1 (0-1%)	MGIT	Li 2016 (n=5): MIC = 512 mg/L	High confidence, high MIC mutation.
	F94C	–	N/A	MGIT	Li 2016 (n=1): MIC = 256 mg/L	Additional data needed.
	F94S	High	1 (0-1%)	MGIT	Li 2016 (n=1): MIC = 256 mg/L	High confidence resistance mutation. Need additional MIC data.
	K96N	High	0 (0-0%)	7H10	Hu 2000 (n=2): MIC = 900 mg/L	High confidence resistance mutation. Need additional MIC data.
	K96R	High	0 (0-0%)	MGIT	Li 2016 (n=1): MIC = 256 mg/L	High confidence resistance mutation. Need additional MIC data.

Gene	Mutations	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates)	PZA Summary
pncA	K96Q	–	N/A	MGIT	Aono 2014 (n=1): MIC > 1600 mg/L Li 2016 (n=1): MIC = 256 mg/L	High MIC mutation.
	K96T	–	N/A	LJ	Ghiraldi 2011 (n=1): MIC > 3200 mg/L	Additional data needed.
	G97D	High	0 (0-1%)	7H10	Morlock 2000 (n=1): MIC = 400 mg/L	High confidence, high MIC mutation.
				MGIT	Aono 2014 (n=1): MIC = 1600 mg/L Li 2016 (n=1): MIC = 512 mg/L Gonzalo 2014 (n=2): MIC > 800 mg/L	
	G97S	High	0 (0-1%)	7H10	Morlock 2000 (n=1): MIC = 400 mg/L	High confidence resistance mutation. Need additional MIC data.
				MGIT	Li 2016 (n=1): MIC = 1024 mg/L	
	G97A	–	N/A	MGIT	Hoffner 2013 (n=1): MIC > 800 mg/L Li 2016 (n=1): MIC = 512 mg/L	High MIC mutation.
	T100P	–	N/A	MGIT	Li 2016 (n=1): MIC = 512 mg/L	Additional data needed.
	S104R	High	0 (0-1%)	7H11	Hirano 1998 (n=1): MIC > 800 mg/L	High confidence resistance mutation. Need additional MIC data.
	G105D	–	N/A	LJ	Barco 2006 (n=2): MIC > 800 mg/L	Additional data needed.
	G108R	High	0 (0-1%)	LJ	Barco 2006 (n=1): MIC > 800 mg/L	High confidence resistance mutation. Need additional MIC data.
	T114P	–	N/A	7H11	Hirano 1998 (n=1): MIC > 800 mg/L	Additional data needed.
	N188T	–	N/A	7H10	Hu 2000 (n=1): MIC = 900 mg/L	Additional data needed.
	L120P	High	1 (1-1%)	MGIT	Hoffner 2013 (n=2): MIC > 800 mg/L Gonzalo 2014 (n=2): MIC > 800 mg/L	High confidence resistance mutation. Need additional MIC data.

Gene	Mutations	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates)	PZA Summary
pncA	L120R	–	N/A	LJ	Barco 2006 (n=1): MIC > 800 mg/L Ghiraldi 2011 (n=1): MIC = 800 mg/L	Additional data needed.
	R121P	–	N/A	7H10	Hu 2000 (n=1): MIC = 1000 mg/L	Additional data needed.
	R123P	High	0 (0-0%)	MGIT	Werngren 2012 (n=1): MIC > 1024 mg/L Gonzalo 2014 (n=2): MIC > 800 mg/L	High confidence, high MIC mutation.
	V125F	High	0 (0-0%)	MGIT	Werngren 2012 (n=1): MIC = 256 mg/L	High confidence resistance mutation. Need additional MIC data.
	V128G	High	1 (0-1%)	MGIT	Hoffner 2013 (n=1): MIC > 800 mg/L Gonzalo 2014 (n=2): MIC > 800 mg/L	High confidence, high MIC mutation.
	V130G	–	N/A	7H10	Morlock 2000 (n=1): MIC > 800 mg/L	Additional data needed.
				7H11	Hirano 1998 (n=2): MIC > 800 mg/L	
				MGIT	Li 2016 (n=1): MIC = 512 mg/L	
	V130A	–	N/A	MGIT	Li 2016 (n=1): 512 ≤ MIC ≤ 1024 mg/L Whitfield 2015 (n=2): 25 ≤ MIC < 100 mg/L	Additional data needed.
	G132A	High	0 (0-0%)	MGIT	Li 2016 (n=1): MIC = 1024 mg/L Werngren 2012 (n=1): MIC = 1024 mg/L Gonzalo 2014 (n=2): MIC > 800 mg/L	High confidence, high MIC mutation.
	G132D	High	0 (0-0%)	7H11	Hirano 1998 (n=1): MIC > 800 mg/L	High confidence resistance mutation. Need additional MIC data.
				MGIT	Li 2016 (n=1): MIC = 1024 mg/L	
	G132S	High	0 (0-1%)	MGIT	Li 2016 (n=1): MIC > 1024 mg/L Werngren 2012 (n=1): MIC > 1024 mg/L	High confidence, high MIC mutation.
	G132C	–	N/A	MGIT	Li 2016 (n=1): MIC = 1024 mg/L	Additional data needed.
	I133S	–	N/A	LJ	Barco 2006 (n=1): MIC > 800 mg/L	Additional data needed.

Gene	Mutations	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates)	PZA Summary
pncA	A134V	High	0 (0-1%)	7H10	Morlock 2000 (n=1): MIC > 800 mg/L	High confidence resistance mutation. Need additional MIC data.
	T135P	High	1 (1-1%)	7H10	Morlock 2000 (n=1): MIC > 800 mg/L	High confidence, high MIC mutation.
				MGIT	Li 2016 (n=2): 512 ≤ MIC ≤ 1024 mg/L	
	H137P	High	0 (0-0%)	MGIT	Aono 2014 (n=1): MIC > 1600 mg/L Li 2016 (n=1): MIC = 1024 mg/L	High confidence, high MIC mutation.
	H137R	–	N/A	7H10	Morlock 2000 (n=2): MIC ≥ 800 mg/L	Additional data needed.
				MGIT	Li 2016 (n=1): MIC = 1024 mg/L	
	H137D	–	N/A	MGIT	Li 2016 (n=1): MIC > 1024 mg/L	Additional data needed.
	C138Y	High	0 (0-1%)	7H10	Morlock 2000 (n=1): MIC > 800 mg/L Hu 2000 (n=1): MIC = 600 mg/L	High confidence resistance mutation. Need additional MIC data.
	C138R	–	N/A	MGIT	Li 2016 (n=1): MIC = 1024 mg/L	Additional data needed.
	C138W	–	N/A	MGIT	Li 2016 (n=1): MIC = 1024 mg/L	Additional data needed.
	V139G	High	1 (0-1%)	7H10	Morlock 2000 (n=1): MIC > 800 mg/L	High confidence, high MIC mutation.
				MGIT	Aono 2014 (n=1): MIC > 1600 mg/L Li 2016 (n=1): MIC = 1024 mg/L	
	V139L	High	0 (0-1%)	7H10	Morlock 2000 (n=1): MIC = 100 mg/L Hu 2000 (n=1): MIC = 900 mg/L	High confidence resistance mutation. Need additional MIC data.
				MGIT	Li 2016 (n=1): MIC = 512 mg/L	
	V139A	–	N/A	7H10	Hu 2000 (n=2): MIC = 1000 mg/L	Additional data needed.
				7H11	Hirano 1998 (n=1): MIC > 800 mg/L	
				MGIT	Li 2016 (n=1): MIC = 1024 mg/L	

Gene	Mutations	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates)	PZA Summary
pncA	Q141P	High	1 (1-1%)	MGIT	Aono 2014 (n=1): MIC > 1600 mg/L	High confidence resistance mutation. Need additional MIC data.
	T142A	High	0 (0-1%)	7H11	Hirano 1998 (n=1): MIC > 800 mg/L	High confidence resistance mutation. Need additional MIC data.
				MGIT	Li 2016 (n=1): MIC = 1024 mg/L	
	T142K	High	0 (0-1%)	7H10	Hu 2000 (n=3): 300 ≤ MIC ≤ 600 mg/L	High confidence, high MIC mutation.
				7H11	Hirano 1998 (n=2): MIC > 800 mg/L	
				MGIT	Li 2016 (n=1): MIC = 1024 mg/L Werngren 2012 (n=1): MIC > 1024 mg/L	
	T142M	High	0 (0-1%)	MGIT	Li 2016 (n=1): MIC = 1024 mg/L	High confidence resistance mutation. Need additional MIC data.
	T142P	–	N/A	MGIT	Li 2016 (n=1): MIC > 1024 mg/L	Additional data needed.
	A143G	–	N/A	MGIT	Li 2016 (n=1): MIC = 512 mg/L	Additional data needed.
	A146V	–	N/A	7H11	Hirano 1998 (n=1): MIC > 800 mg/L	Additional data needed.
	T153I	–	N/A	MGIT	Li 2016 (n=1): MIC = 512 mg/L	Additional data needed.
	T153N	–	N/A	7H10	Morlock 2000 (n=2): MIC = 800 mg/L	Additional data needed.
	V155G	High	1 (1-1%)	MGIT	Werngren 2012 (n=2): MIC ≥ 1024 mg/L Gonzalo 2014 (n=2): MIC > 800 mg/L	High confidence, high MIC mutation.
	V155E	–	N/A	LJ	Barco 2006 (n=1): MIC > 800 mg/L Ghiraldi 2011 (n=1): MIC = 1600 mg/L	Additional data needed.
	V163G	–	N/A	MGIT	Li 2016 (n=4): 512 ≤ MIC ≥ 1024 mg/L; **mode of 1024 mg/L**	High MIC mutation.

Gene	Mutations	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates)	PZA Summary
pncA	V163A	–	N/A	MGIT	Whitfield 2015 (n=1): MIC ≤ 100 mg/L	Additional data needed.
	S164P	–	N/A	LJ	Barco 2006 (n=1): MIC > 800 mg/L Ghiraldi 2011 (n=1): MIC = 1600 mg/L	Additional data needed.
	L172P	High	1 (1-1%)	7H10	Morlock 2000 (n=2): MIC = 200 mg/L	High confidence, high MIC mutation.
				7H11	Hirano 1998 (n=1): MIC > 800 mg/L	
				MGIT	Li 2016 (n=1): MIC = 1024 mg/L Werngren 2012 (n=1): MIC = 1024 mg/L	
	M175T	High	0 (0-0%)	MGIT	Li 2016 (n=1): MIC = 1024 mg/L Werngren 2012 (n=1): MIC > 1024 mg/L	High confidence, high MIC mutation.
	M175V	High	0 (0-1%)	MGIT	Li 2016 (n=3): MIC > 1024 mg/L	High confidence, high MIC mutation.
	V180F	High	0 (0-0%)	MGIT	Li 2016 (n=2): MIC = 512 mg/L	High confidence resistance mutation. Need additional MIC data.
	Pooled frameshifts and premature stop codons	High	15 (13-16%)	LJ	Barco 2006 (n=3): 400 ≤ MIC > 800 mg/L Ghiraldi 2011 (n=1): MIC > 3200 mg/L	High confidence, high MIC mutations.
				7H10	Endoh 2002 (n=1): MIC > 100 mg/L Morlock 2000 (n=6): 400 ≤ MIC ≥ 800 mg/L Hu 2000 (n=13): 400 ≤ MIC > 2000 mg/L	
				7H11	Hirano 1998 (n=5): MIC > 800 mg/L	
				MGIT	Aono 2014 (n=17): MIC ≥ 1600 mg/L Hoffner 2013 (n=2): 200 ≤ MIC > 800 mg/L Li 2016 (n=27): 256 ≤ MIC ≥ 1024 mg/L; **mode of 1024 mg/L** Werngren 2012 (n=3): 256 ≤ MIC > 1024 mg/L Gonzalo 2014 (n=2) 200 ≤ MIC > 800 mg/L	

Gene	Mutations	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates)	PZA Summary
pncA	V7G	Moderate	1 (0-1%)	7H11	Hirano 1998 (n=2): MIC > 800 mg/L	Moderate confidence resistance mutation. Need additional MIC data.
	Q10R	Moderate	0 (0-1%)	MGIT	Li 2016 (n=1): MIC = 512 mg/L	Moderate confidence resistance mutation. Need additional MIC data.
	P54L	Moderate	1 (0-1%)	MGIT	Aono 2014 (n=2): MIC > 1600 mg/L Li 2016 (n=1): MIC = 512 mg/L Werngren 2012 (n=1): MIC = 512 mg/L	Moderate confidence, high MIC mutation.
	W68G	Moderate	1 (0-1%)	MGIT	Li 2016 (n=1): MIC = 1024 mg/L	Moderate confidence resistance mutation. Need additional MIC data.
	K96E	Moderate	0 (0-1%)	MGIT	Li 2016 (n=1): MIC = 256 mg/L	Moderate confidence resistance mutation. Need additional MIC data.
	K96T	Moderate	0 (0-1%)	LJ	Barco 2006 (n=1): MIC > 800 mg/L	Moderate confidence resistance mutation. Need additional MIC data.
	A171T	–	N/A	7H10	Morlock 2000 (n=1): MIC > 800 mg/L	Additional data needed.
	M175I	Moderate	0 (0-1%)	MGIT	Li 2016 (n=1): MIC > 1024 mg/L	Moderate confidence resistance mutation. Need additional MIC data.
	D12G	Minimal	1 (1-1%)	MGIT	Li 2016 (n=1): MIC = 1024 mg/L Gonzalo 2014 (n=2): MIC > 800 mg/L	Minimal confidence, high MIC mutation.
	E37V	–	N/A	MGIT	Whitfield 2015 (n=2): MIC = 50 mg/L Morlock 2017 (n=1): MIC = 50 mg/L	Low MIC mutation.

Gene	Mutations	Confidence Grading	Pooled Sensitivity (95% CI)*	Media	MIC Evidence (Clinical Isolates)	PZA Summary
pncA	F58L	Minimal	1 (0-1%)	7H11	Hirano 1998 (n=1): MIC = 400 mg/L	Minimal confidence, high MIC mutation.
				MGIT	Werngren 2012 (n=2): 128 ≤ MIC ≤ 512 mg/L	
	S65A	–	N/A	MGIT	Li 2016 (n=2): MIC = 32 mg/L	Low MIC mutation.
	H71R	Minimal	1 (0-1%)	7H10	Morlock 2000 (n=1): MIC > 800 mg/L	Minimal confidence resistance mutation. Need additional MIC data.
				MGIT	Aono 2014 (n=1): MIC > 1600 mg/L	
	D110G	–	N/A	MGIT	Whitfield 2015 (n=13): MIC = 50 mg/L	Low MIC mutation.
	I133T	Minimal	1 (1-1%)	MGIT	Li 2016 (n=1): MIC = 512 mg/L	Minimal confidence resistance mutation. Need additional MIC data.
	I133N	–	N/A	7H10	Morlock 2000 (n=1): MIC = 800 mg/L	Additional data needed.
	A170V	–	N/A	MGIT	Whitfield 2015 (n=1): MIC = 75 mg/L Morlock 2017 (n=1): MIC = 75 mg/L	Low MIC mutation.
	V180I	–	N/A	MGIT	Whitfield 2015 (n=1): MIC = 75 mg/L Morlock 2017 (n=1): MIC = 75 mg/L	Low MIC mutation.

For brevity, MIC results were only shown for media that were tested. The WHO-recommended PZA CC for phenotypic DST is 100.0mg/L in MGIT960 [20]. The high-confidence mutations *pncA* t-12c, V7G, D8E, **K48T**, **D49G**, D49N, P62L, S66P, **H71D**, H71Q, **L85R**, G97C, **Y103H**, L116P, **L116R**, **V125G**, **T135N**, **inframe – R148ins**, L151S, L159P, T160P, G162D, T168P, and V180G, as well as the moderate-confidence mutation A171E and minimal-confidence mutation **V139A,** were not included in the table, above, as no MIC data was identified for isolates with these unique mutations for any medium of interest. Mutations with no association with PZA resistance (*pncA* **indel – c-125del, I31T, L35R, T47A, I6L**, K48T, and T114M), as well as silent mutations (F13F, A20A, A26A, L27L, A36A, A38A, A39A, A46A, G55G, K96K, S65S, S74S, G75G, T76T, G78G, G124G, D129D, C138C, G150G, E173E), are also not shown in the above table. For those mutations with noted confidence grading, standard type represents associations based on nominal p-values (putative); **bold type** represents associations based on corrected p-values. Limitations: it was assumed that Hu et al. generated MICs through testing on 7H10 media, though this was not clearly stated in the paper.

* 2,710 total PZA-resistant isolates were evaluated. Pooled sensitivity estimates from the systematic review include isolates with multiple mutations.

** Marker for *Mycobacterium bovis* [80]

The estimation of the accuracy of mutations as predictors of phenotypic PZA resistance poses unique challenges. The known resistance-conferring mutations are diverse and distributed over several hundred base pairs of the pncA gene and its promoter. This gene region also includes many mutations that do not confer PZA resistance. Furthermore, these mutations generally occur infrequently, even in very large strain collections, reducing our statistical power to classify their association with PZA resistance with any confidence. Based only on the confidence-graded mutations identified as markers of resistance in Miotto's review (Table 6), the sensitivity and specificity for PZA resistance detection were 53% (95% CI 51.2-55.0%) and 100% (95% CI 99.9-100.0%), respectively. When all the mutations associated with resistant phenotypes in the review were considered, however, the sensitivity and specificity for PZA resistance detection were 83% (95% CI 81.7-84.5%) and 94% (95% CI 92.9-94.7%), respectively.

Manually curating the mutation lists, together with additional, published genotype/phenotype associations further refined the accuracy of PZA resistance detection and yielded sensitivity and specificity for detection of phenotypic PZA resistance of 76% (95% CI 74.1-77.3%) and 100.0% (95% CI 99.9-100.0%), respectively. This estimate excludes mutations associated with phenotypic PZA resistance that could not be confidence-graded due to insufficient evidence. If these mutations were also considered, the sensitivity and specificity for **PZA resistance** detection would be **83%** (95% CI 81.9-84.7%) and **98%** (95% CI 96.9-98.1%).

The reduced sensitivity of sequencing of the pncA gene for the detection of PZA resistance compared with phenotypic DST is likely due to the limited reproducibility of the phenotypic test. This factor also makes it more difficult to establish resistance associations for the rarer pncA mutations.

2.2.5 Second-line injectable drugs

This section describes the correlation between the mutations associated with phenotypic resistance to the second-line injectable drugs (AMK, KAN and CAP). Currently AMK and STR are the only injectable agents recommended for use in the treatment of DR-TB. STR was not included in this review. Although there are differences in the mechanisms of action of the three compounds, it is generally understood that both AMK and KAN bind the 16S rRNA in the MTBC 30S ribosomal subunit, inhibiting protein synthesis [81], while CAP interferes with translation in MTBC, and has been shown to inhibit phenylalanine synthesis in mycobacterial ribosomes [82]. The primary gene regions associated with *M. tuberculosis* second-line injectable drug resistance include mutations in the *rrs* genes, which generally confer resistance to all three injectables, the *tlyA* gene mutations, most commonly associated with resistance to CAP, and the *eis* promoter mutations, which may be considered markers of KAN resistance [83].

Relevant data for the *rrs*, *tlyA*, *eis* and *whib7* SLI resistance-associated genes were included in the review of SLI mutation data. Data from 104 studies, with strains from 43 countries that were isolated from 1985-2013, were included in the review. The total number of isolates with available *rrs* sequence data was 2,105 for AMK, 1,727 for KAN and 2,533 for CAP. 2,029 isolates had available *eis* sequence data and 56 isolates had available *whiB7* sequence data for KAN. Another 1,854 isolates had available *tlyA* sequence data for CAP.

AMK, KAN and CAP were all included in the MIC data review. A full-text screen was performed for 3,815 total records, and 49 studies with relevant SLI MIC data for different media were included in the final review. Summary AMK, KAN and CAP MIC data are presented for associated resistance mutations, stratified by media, in Tables 7-9.

Amikacin

For mutations associated with AMK resistance (Table 7), only the *rrs* mutations a1401g and g1484t were determined to be high-confidence resistance markers by Miotto et al. The full MIC data review confirmed that these *rrs* mutations were associated with high MICs across different media. Although there was no confidence grading available for the *rrs* c1402t and *eis* c-14t mutations, these mutations were found

to have low associated MICs in the MIC data review.

Using only the high, medium and low confidence-graded mutations identified in Miotto's review as predictors of phenotypic resistance (Table 7: bolded mutations), the sensitivity and specificity for AMK resistance detection were estimated to be 80% (95% CI 76.7-82.3%) and 98% (95% CI 96.8-98.4%), respectively. However, this estimate excludes mutations associated with phenotypic AMK resistance that could not be confidence-graded due to insufficient data. If these mutations were also considered in addition to the confidence-graded mutations, the sensitivity and specificity for **AMK resistance** detection would be **79%** (95% CI 75.8-81.5%) and **100%** (95% CI 99.7-100.0%), respectively.

Table 7: List of confidence-graded mutations associated with phenotypic AMK resistance and associated MIC data

Gene	Mutations	Combined Media Confidence Grading	Pooled Sensitivity (95% CI)*	Media	AMK MIC Evidence (*in vitro* and Clinical Isolates)	AMK Summary
rrs	a1401g	High	74 (71-77%)	LJ	3 studies (n=74): 20 ≤ MIC > 160 mg/L	High confidence, high MIC mutation**.
				7H10	7 studies (n=168): 10 < MIC > 256 mg/L	
				7H11	–	
				MGIT	8 studies (n=143): MIC > 8	
	c1402t	–	N/A	LJ	1 study (n=4): 7.5 ³ MIC ≤ 15; **mode of 15 mg/L**	Low MIC mutation.
				7H10	4 studies (n=14): 1 ≤ MIC ≤ 32 mg/L	
				7H11	–	
				MGIT	–	
	g1484t	High	1 (1-2%)	LJ	–	High confidence, high MIC mutation.
				7H10	2 studies (n=25): 10 < MIC > 64 mg/L	
				7H11	–	
				MGIT	–	
eis	c-14t	–	N/A	LJ	–	Low MIC mutation.
				7H10	3 studies (n=22): 0.5 ≤ MIC ≤ 32; **mode of 2-4 mg/L**	
				7H11	–	
				MGIT	4 studies (n=14): 0.25 ³ MIC ≤ 2; **mode of 1 mg/L**	

The most recent WHO-recommended AMK CCs for phenotypic DST are 30.0mg/L in LJ, 2.0mg/L in 7H10 and 1.0mg/L in MGIT960 [13]. Silent mutations (*eis* S48S, P90P) are not shown in the above table. For those mutations with noted confidence grading, standard type represents associations based on nominal p-values (putative); **bold type** represents associations based on corrected p-values. Limitations: MIC data for *in vitro* (laboratory-generated) isolates were only included for isolates that were tested on 7H10.

*815 total AMK-resistant isolates were evaluated. Pooled sensitivity estimates from the systematic review include isolates with multiple mutations.

**Exception to MIC data summary rules (Table 2), since only 1 of the 74 isolates tested in LJ had an MIC below the CC.

Kanamycin

For mutations associated with KAN resistance (Table 8), the *rrs* mutations a1401g, c1402t and g1484t were categorized as high-confidence resistance markers, along with the *eis* promoter mutation c-14t and g-10a, by Miotto et al. Additional *eis* promoter mutations c-12t and g-37t were considered minimal confidence markers of KAN resistance. The full MIC data review confirmed that the a1401g and g1484t mutations were associated with generally high KAN MICs, though the c1402t mutation was only found to be associated with low MICs. For the *eis* promoter mutations, c-14t and g-37t were associated with moderate KAN MICs, while c-12t was associated with low KAN MICs. Additional MIC data was needed to confirm the MIC distribution for g-10a mutants.

Using only the high, medium and low confidence-graded mutations identified in Miotto's review as predictors of phenotypic resistance (Table 8), the sensitivity and specificity for KAN resistance detection were estimated to be 76% (95% CI 73.4-78.7%) and 97% (95% CI 96.2-97.9%), respectively. However, this estimate excludes mutations associated with phenotypic KAN resistance that could not be confidence-graded due to insufficient data. If these mutations were also considered in addition to the confidence-graded mutations, the sensitivity and specificity for **KAN resistance** detection would be **68%** (95% CI 65.3-71.2%) and **100%** (95% CI 99.8-100.0%), respectively.

Table 8: List of confidence-graded mutations associated with phenotypic KAN resistance and associated MIC data

Gene	Mutations	Combined Media Confidence Grading	Pooled Sensitivity (95% CI)*	Media	KAN MIC Evidence (*in vitro* and Clinical Isolates)	KAN Summary
rrs	a1401g	High	44 (41-47%)	LJ	3 studies (n=74): MIC ³ 120 mg/L	High confidence, high MIC resistance mutation.
				7H10	6 studies (n=163): 16 ≤ MIC > 256 mg/L	
				7H11	–	
				MGIT	5 studies (n=105): MIC > 20 mg/L	
	c1402t	High	1 (0-1%)	LJ	1 study (n=4): 15 ≤ MIC ≤ 30 mg/L	High confidence, low MIC resistance mutation
				7H10	3 studies (n=11): 4 ≤ MIC ≤ 20 mg/L	
				7H11	–	
				MGIT	–	
	g1484t	High	1 (0-1%)	LJ	–	High confidence, high MIC resistance mutation.
				7H10	2 studies (n=25); MIC > 10 mg/L	
				7H11	–	
				MGIT	–	
eis	g-10a	Moderate	11 (9-13%)	LJ	–	Moderate confidence resistance mutation. Additional MIC data needed.
				7H10	3 studies (n=47): 2 ≤ MIC ≤ 80; mode of 4 mg/L	
				7H11	–	
				MGIT	3 studies (n=16): 2.5 ≤ MIC > 80; mode of 10mg/L	

Gene	Mutations	Combined Media Confidence Grading	Pooled Sensitivity (95% CI)*	Media	KAN MIC Evidence (*in vitro* and Clinical Isolates)	KAN Summary
eis	c-12t	Minimal	2 (1-3%)	LJ	–	Minimal confidence, low MIC mutation.
				7H10	2 studies (n=4): 4 ≤ MIC ≤ 10; **mode of 4 mg/L**	
				7H11	–	
				MGIT	3 studies (n=5): 1.25 ≤ MIC ≤ 5; **mode of 5mg/L**	
	c-14t	High	4 (3-6%)	LJ	–	High confidence, moderate MIC mutation.
				7H10	4 studies (n=25): 4 ≤ MIC ≤ 40; **mode of 16 mg/L**	
				7H11	–	
				MGIT	3 studies (n=1): 0.62 [3] MIC ≤ 40; **mode of 20mg/L**	
	g-37t	Minimal	2 (1-3%)	LJ	–	Minimal confidence, moderate MIC mutation.
				7H10	2 studies (n=5): 8 ≤ MIC ≤ 20; **mode of 20 mg/L**	
				7H11	–	
				MGIT	3 studies (n=1): 2.5 ≤ MIC ≤ 20; **mode of 10mg/L**	

The most recent WHO-recommended KAN CCs for phenotypic DST are 30.0mg/L in LJ, 4.0mg/L in 7H10 and 2.5mg/L in MGIT960 [13]. Mutations with no causative association with KAN resistance (**a1338c**) and silent mutations (*eis* S48S, P90P) are not shown in the above table. For those mutations with noted confidence grading, standard type represents associations based on nominal p-values (putative); **bold type** represents associations based on corrected p-values. Limitations: MIC data for *in vitro* (laboratory-generated) isolates were only included for isolates that were tested on 7H10.

*979 total KAN-resistant isolates were evaluated. Pooled sensitivity estimates from the systematic review include isolates with multiple mutations.

Capreomycin

For mutations associated with CAP resistance (Table 9), the *rrs* mutations a1401g, c1402t and g1484t were categorized as high-confidence resistance markers, along with most *tlyA* mutations. The full MIC data review confirmed that the c1402t and g1484t mutations were associated with high CAP MICs across different media, though the a1401g mutant MICs were lower overall, and therefore characterized as having moderate MICs. For the *tlyA* mutations, these mutations when identified in the full MIC data review conferred high CAP MICs.

Using only the high, medium and low confidence-graded mutations identified in Miotto's review as predictors of phenotypic resistance (Table 9), the sensitivity and specificity for CAP resistance detection were estimated to be 73% (95% CI 69.7-75.5%) and 98% (95% CI 97.2-98.4%), respectively. However, this estimate excludes mutations associated with phenotypic CAP resistance that could not be confidence-graded due to insufficient data. If these mutations were also considered in addition to the confidence-graded mutations, the sensitivity and specificity for **CAP resistance** detection would be **71%** (95% CI 68.3-74.2%) and **100%** (95% CI 99.8-100.0%), respectively.

Table 9: List of confidence-graded mutations associated with phenotypic CAP resistance and associated MIC data

Gene	Mutations	Combined Media Confidence Grading	Pooled Sensitivity (95% CI)*	Media	CAP MIC Evidence (*in vitro* and Clinical Isolates)	CAP Summary
rrs	a1401g	High	59 (56-62%)	LJ	3 studies (n=74): 8 ≤ MIC > 160; **mode of 160 mg/L**	High confidence, moderate MIC mutation.
				7H10	9 studies (n=263): 4 ≤ MIC > 160; **mode of 8 mg/L**	
				7H11	–	
				MGIT	7 studies (n=153): 5 ≤ MIC ≤ 25; **mode of 10 mg/L**	
	c1402t	High	1 (1-2%)	LJ	1 study (n=4): MIC > 160 mg/L	High confidence, high MIC mutation.
				7H10	5 studies (n=15): 20 ≤ MIC > 160 mg/L	
				7H11	–	
				MGIT	–	
	g1484t	High	1 (0-2%)	LJ	–	High confidence, high MIC mutation.
				7H10	3 studies (n=26); 10 < MIC > 320 mg/L	
				7H11	–	
				MGIT	–	
tlyA	(Combined)	Generally High	4 (3-5%)	LJ	–	High confidence, high MIC mutations.
				7H10	3 studies (n=48): 0.5 ≤ MIC ≤ 160; **mode of 40 mg/L**	
				7H11	–	
				MGIT	1 study (n=5): 5 ≤ MIC ≤ 25; **mode of 25 mg/L**	

The most recent WHO-recommended CAP CCs for phenotypic DST are 40.0mg/L in LJ, 4.0mg/L in 7H10 and 2.5mg/L in MGIT960 [13]. Mutations with no causative association with CAP resistance (rrs **c517t**, *tlyA* D149H) and silent mutations (*tlyA* L11L) are not shown in the above table. For those mutations with noted confidence grading, standard type represents associations based on nominal p-values (putative); **bold type** represents associations based on corrected p-values. Limitations: MIC data for *in vitro* (laboratory-generated) isolates were only included for isolates that were tested on 7H10.

*919 total CAP-resistant isolates were evaluated. Pooled sensitivity estimates from the systematic review include isolates with multiple mutations.

2.3 Conclusions

Rapid evidence-based triage of patients with DR-TB infections to appropriate TB treatment regimens can only be achieved using genotypic DST methods. However, as recently shown in Heyckendorf et al., composing an effective treatment regimen remains a challenge even given information from the current WHO-endorsed genotypic methods (e.g. GeneXpert MTB/RIF and line probe assays) [87]. Whole genome sequencing can provide additional information to help design TB treatment regimens although our knowledge related to the molecular bais of resistance for the newer drugs is still limited at present. Together, the compiled mutation and MIC data for INH, RIF, MFX, PZA and AMK can help guide the interpretation of current genotypic methods and NGS results and ultimately direct the TB treatment. Although the stated confidence grading and MIC distributions must be considered provisional (especially for drugs such as INH, RIF and PZA, for which a full MIC data review has not been conducted to date), the knowledge base regarding the relationship between the genetic mutations in drug resistance genes and the levels of drug resistance conferred by these mutations for isolates tested on different media will only grow in the future, especially as WGS becomes more widely implemented, providing further evidence to strengthen conclusions and support certain mutations as markers of phenotypic drug resistance and, ultimately, clinical outcome. It is also important to note, considering the observed discrepancies in phenotypic DST results for certain *rpoB* or *gyrA* mutants in different media [41, 85], as well as the complications of diagnosing PZA drug resistance given both complex molecular mechanisms of resistance and the difficulties of phenotypic testing for PZA resistance, that NGS might be considered a more robust reference standard for defining certain drug resistance profiles.

While many of the diagnostic sensitivity estimates observed in the systematic review for the graded mutations were lower than WHO thresholds for phenotypic resistance detection (e.g. 84% for INH, as opposed to >90%) [86], this is an underestimate of the potential sensitivity of genotypic prediction of resistance, given that most systematic review data were generated using targeted, Sanger sequencing. WGS datasets are expected to provide new knowledge in the genetic bases of phenotypic drug resistance in MTB.

Importantly, as with any evidence-based approach, confidence levels and associated resistance levels can change in either direction given the accumulation of additional data for both infrequent mutations and mutations with little associated MIC data, as well as for those mutations with established confidence grading and MIC distributions. The limited knowledge of MTB genetic diversity in certain settings may present an obstacle to NGS implementation in these regions. Additionally, it is important to note that the current grading is mainly based on the assumption that *in vitro* resistance levels are characterized according to CCs, which may be used as a proxy for clinical resistance. The availability of additional clinical outcome data could help to better characterize CBs; thus, an updating of the current grading system could be needed in the future. Furthermore, not all studies included data on all known resistance genes for any drug, which limits the confidence in the resistance conferred by certain rare mutations, such as *ahpC* mutations and INH resistance, or *eis* promoter mutations and AMK resistance. Similarly, silent mutations were considered to be wild type for purposes of this analysis. Moreover, certain mutations might have had falsely high MIC distributions, given the presence of undetected compensatory mutations in other gene regions not sequenced in the identified studies in the MIC review. In general, the current grading approach is not able to accurately weigh combinations of mutations. It should also be noted that sequencing technologies are limited in their ability to detect low frequencies (<10-20%) of resistant strains in a mixture with susceptible strains, whereas phenotypic testing can detect resistant strains making up only 1% of the total population [86], leading to discordances between the detected genotype and the resistant phenotype for some drugs, particularly the FQs [87]. Finally, breakpoint artifacts (i.e. inappropriately high CCs) might have been a major source of misclassification of phenotypes, especially for

drugs such as RIF, for which a full MIC review has not been conducted to better define the CC, which appears to be set too high given the fact that resistance is inconsistently defined on a phenotypic level in different media [88]. Nonetheless, the presented evidence will be of particular value for the interpretation of sequencing diagnostics for DR-TB detection and characterization to guide individualized treatment regimens.

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3. Accuracy of sequencing in a multi-country, population-based study for determining drug resistance in *Mycobacterium tuberculosis* complex

3.0 Introduction

Drug resistance surveillance is a key component of tuberculosis (TB) control programmes. TB surveillance data can be used to estimate disease burden, support diagnostic and treatment services, design effective treatment regimens, monitor intervention effectiveness, and, if systematically undertaken, allow individually-tailored patient care. In most countries with a high TB burden, drug resistance is monitored through periodic epidemiological surveys that have largely relied on conventional, growth-based methods, including culture and phenotypic drug susceptibility testing (DST). However, phenotypic results for *Mycobacterium tuberculosis* complex (MTBC) are only obtained after weeks to months of incubation, and many developing countries face additional limitations that challenge phenotypic testing, such as the lack of resources to rapidly transport and process samples or to establish the stringent laboratory conditions needed for growth-based methods. These factors make it difficult to repeat epidemiological surveys at regular intervals and so, despite the many benefits of surveillance data for TB control, the implementation of routine diagnostic testing for drug susceptibility as recommended by World Health Organization (WHO) as part of the End TB Strategy remains insufficient [1].

Rapid molecular diagnostic tests that do not rely on slow-growing MTBC culture are essential to the management and surveillance of TB drug resistance. Although the roll-out of rapid molecular diagnostics such as Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) has started to increase access to susceptibility testing for rifampicin resistance, greater efforts are needed to meet the WHO target of universal DST [2]. In particular, there is a critical need for new, rapid and easy-to-perform molecular drug susceptibility tests for a wide range of anti-TB drugs [3], as well as a clear set of criteria for the interpretation of molecular test results.

Given the ability of sequencing technologies to generate a complete picture of the drug resistance profile of a clinical sample, and the flexibility of sequencing applications to accommodate a growing knowledge base surrounding the association between specific mutations and phenotypic drug resistance in MTBC, sequencing-based diagnostics for TB drug resistance present an attractive option for the surveillance of TB drug resistance in various settings. From 2010 to 2014, population-based survey studies were conducted in seven resource-limited, high TB burden countries [4]. This chapter presents the methods used for sequencing clinical isolates in this study, as well as the overall and country-specific performance of sequencing for the detection of resistance to the major first- and second-line anti-TB drugs: rifampicin (RIF), isoniazid (INH), ofloxacin (OFX), moxifloxacin (MFX), pyrazinamide (PZA), kanamycin (KAN), amikacin (AMK) and capreomycin (CAP).

3.1 Methods

3.1.1 Study design and participants

Population-based surveys [5] were conducted in Azerbaijan [6], Bangladesh [7], Belarus [8], Pakistan [9], Philippines [10], South Africa [11], and Ukraine [12] between 2010 and 2014. Sites were selected in each country either by cluster sampling or by including all diagnostic facilities in the country. All consecutive patients with pulmonary TB who presented at the study sites, both newly diagnosed and previously treated, were eligible for enrolment.

3.1.2 Phenotypic testing

Strains were isolated using Löwenstein Jensen (LJ) media or BACTEC™ Mycobacterial Growth Indicator Tube™ (MGIT) 960 (Becton Dickinson, Sparks, MD, USA) liquid culture system (Table 1). Identification of MTBC was performed using biochemical and immunochromatographic tests [13]. Susceptibility testing was conducted using the LJ proportion method or MGIT 960

with 1% as the critical proportion according to manufacturers' instructions, at the following critical concentrations recommended at that time by the WHO for LJ and MGIT 960, respectively [14]: RIF, 40.0 µg/mL and 1.0 µg/mL; INH, 0.2 µg/mL and 0.1 µg/mL; PZA, 100.0 µg/mL (MGIT 960); OFX, 4.0 µg/mL and 2.0 µg/mL; MFX, 0.5 µg/mL (MGIT 960); KAN, 30.0 µg/mL and 2.5 µg/mL; AMK, 30.0 µg/mL and 1.0 µg/mL; CAP, 40.0 µg/mL and 2.5 µg/mL. Strains showing discrepant results between *pncA* sequencing and phenotypic testing of PZA in MGIT 960 were also tested using the Wayne enzymatic pyrazinamidase assay, with slight modifications [15]. All laboratory methods were standardized and the national as well as subnational reference laboratories involved in the study successfully passed the external quality assessment for phenotypic testing conducted by the supranational reference laboratories (SRLs) linked to them prior to survey initiation, including proficiency testing on a panel of strains for selected drugs (RIF, INH, PZA, KAN, AMK, CAP, OFX and MFX) and retesting [5].

3.1.3 Sequencing methods

Genetic sequencing data were obtained for clinical isolates either through whole genome sequencing (WGS) or targeted Sanger sequencing, using one of three different technologies. The SRLs involved in the study and performing genotypic DST ensured high-quality results by participating in annual intra-network proficiency programmes focused on molecular tests and including sequencing.

1. Illumina next-generation sequencing (NGS) technology (Illumina Inc., San Diego, CA, USA).

a. DNA extraction. Commercially-available (para)magnetic- (Maxwell 16 Cell DNA Purification kit, Promega, Madison, WI, USA; NucliSENS easyMag, BioMérieux, Marcy l'Etoile, France) or CTAB (N-cetyl-N,N,N-trimethyl ammonium bromide)/NaCl protocol were used to extract DNA from strains, according to manufacturer's instructions and published procedures [16]. DNA quantity and quality were assessed using fluorometric- and UV-based methods (by means of a Qubit double-stranded DNA assay and spectrophotometer), respectively. The integrity of genomic DNA was confirmed by agarose gel electrophoresis.

b. Library preparation and sequencing. Paired-end libraries were prepared using the Nextera XT DNA Sample Preparation kit [17], requiring 1 ng of pure input genomic DNA only. After normalization and pooling, the libraries were sequenced on Hiseq 2500, NextSeq 500 or MiSeq platforms (reads length 101bp, 151bp and 251bp, respectively), according to the manufacturer's instructions. Batching of samples for WGS was performed according to the volume of data (in GigaBases) achievable by each Illumina platform per run and the targeted depth, permitting to run up to 192 samples simultaneously on the highest throughput instrument (HiSeq).

c. Bioinformatics analysis and results reporting. The reads generated were checked for quality and aligned to the *M. tuberculosis* H37Rv reference genome (GenBank AL123456.3). A published online analysis pipeline (PhyResSE) was applied to ensure quality of variant calls for sequences generated by Illumina technology [18] with the following checks: no unusual depth (>2× compared to average coverage); no additional variant calls within 12 base pairs of another SNP or indel; at least 5 reads calling variants; and consensus on >75% reads, including at least one read for each direction. In addition, a standardized analysis pipeline developed by the laboratories participating in the study was used to confirm and crosscheck all WGS results [19]. A package of bioinformatics software developed in-house was used for recalibration, indels realignment and variant calling, with the following filters: 4 reads covering each direction; a total of 4 reads with a Phred score greater or equal to 20; and allele frequency greater or equal to 75%.

2. Ion Personal Genome Machine (PGM) NGS technology (Thermo Fisher Scientific Inc., Waltham, MA, USA).

a. DNA extraction. A commercially-available column-based system (QIAamp DNA Mini Kit, Qiagen, Hilden, Germany) was used to extract DNA from strains, according to manufacturer's instructions and published procedures [20].

b. Library preparation and sequencing. Library and emulsion PCR were performed

following manufacturer's instructions and pooled libraries sequenced on the Ion PGM instrument (reads length: 400 bp). Batching of DRS samples for WGS was performed according to the volume of data (in GigaBases) achievable via the Thermo Fisher platform and the targeted depth.

c. Bioinformatics analysis and results reporting. The reads generated were checked for quality and aligned to the *M. tuberculosis* H37Rv reference genome (GenBank AL123456.3). Total variant calling was performed by using the CLC Genomics Workbench 7 tool (Qiagen, Hilden, Germany) with the following filters: minimum reads coverage: 10; minimum count of reads calling variants: 2; minimum frequency of reads calling variants: 10%; minimum frequency of reads calling variants in each direction: 5%. Pyro-error variants in homopolymer regions with a minimum length of 3 and a frequency below 0.8 were removed.

3. Targeted Sanger technology (Thermo Fisher Scientific Inc.).

a. DNA extraction. DNA was extracted from pure cultures by thermal lysis and sonication as previously described [21].

b. Sequencing. The relevant genomic regions of *rpoB*, *katG*, *fabG* promoter, *inhA*, *pncA*, *gyrA* and *gyrB* were targeted [22], using the primers as reported by Zignol et al. 2018. PCR products were sequenced using 3730xl platform (Thermo Fisher Scientific Inc.), according to manufacturer's instructions.

c. Bioinformatics analysis and results reporting. The output results were analyzed by ClustalW application (BioEdit software, Ibis Biosciences, Abbott Company, Carlsbad, CA, USA) and Sequencher software (Sequencher version 4.9 DNA sequence analysis software, Gene Codes Corporation, Ann Arbor, MI USA). Sequences were aligned to the *M. tuberculosis* H37Rv reference strain (GenBank AL123456.3) to identify nucleotide mutations. Any discordant results were re-analyzed.

In the event of doubtful calls or phenotypic-genotypic discrepancy, the following measures were taken: WGS reads aligning to the reference genome were visually re-checked and and a second pipeline was applied; Sanger sequences were individually re-analyzed to confirm variant calls. Sequencing was repeated or the sample excluded if reads coverage or quality were not sufficient for drug resistance interpretation for at least *rpoB*, *katG*, *inhA*, *fabG* promoter, *pncA*, *gyrA*, *gyrB*, *rrs* and *eis* genomic regions.

Nucleotide variants detected by the different sequencing technologies were annotated based on the *M. tuberculosis* H37Rv reference genome. Mutations in *gyrB* gene were reported in line with the annotation proposed by Camus et al, locating the Quinolone Resistance-Determining Region between codons 461 and 499 [23].

The role of variants detected within the targeted genomic regions was assigned according to the recently developed, standardized approach for the grading of mutations in *M. tuberculosis,* according to their association with drug resistance [24] and as discussed in the previous chapter. Systematic reviews and available databases were additionally used to infer the role of mutations not classified with the grading system and for substitutions not linked to resistance [18, 22, 25-29].

In accordance with the abovementioned approach to mutation grading [24], mutations classified as having high, moderate, and minimal confidence were assumed as true markers or resistance, overruling the phenotypic result whenever such mutations were observed.

3.1.4 Statistical analysis

The accuracy of sequencing compared with phenotypic testing results was assessed for the following genes: *rpoB* for rifampicin; *katG, inhA* and *fabG* promoter for isoniazid; *pncA* for pyrazinamide; *gyrA* and *gyrB* for ofloxacin and moxifloxacin; *rrs* and *eis* for kanamycin; and *rrs* for amikacin and capreomycin. Pooled distributions for the sensitivity (denoted *se*) and specificity (denoted *sp*) of the tests were obtained using random effects modeling after logit transform and a restricted maximum likelihood estimator [4].

Mutations classified in the "high confidence", "moderate confidence" or "minimal confidence" categories as discussed in the preceding chapter [24], were considered as conferring true resistance even if the phenotypic testing result showed susceptibility (i.e. phenotypic false negatives) as these categories did not account

for MIC-based evidence. The specificity of sequencing was thus set at 100%. The statistical approach to data analysis and summary statistics are further detailed in Zignol et al. 2018.

The average cost of performing genetic sequencing in the study was also calculated and compared to the average cost of phenotypic first- and second-line DST.

3.2 Results

3.2.1 Quality of sequencing results

A reads coverage of 69.2x relative to the reference genome was obtained on average from samples undergoing WGS, with a mean percentage of unambiguous coverage relative to the complete reference genome of 96%. Specifically, more than 95% of samples with WGS results achieved a reads coverage of at least 20x, as minimum threshold accepted to analyze results with high accuracy (and 86% of these achieved more than 30x). A mean reads coverage relative to the reference genome of at least 30x was obtained from both of the NGS technologies used (Illumina technology 70,8x vs Ion PGM Thermo Fisher Scientific 30,9x). This depth enabled the analysis of the drug resistance-associated genomic regions similarly. However, as expected, samples sequenced by Illumina technology achieved higher mean reads coverage than those sequenced by Ion PGM. Nevertheless, the accuracy of sequencing results compared to phenotypic testing for samples undergoing Ion PGM was in line with findings from Illumina sequencing.

3.2.2 Sequencing results

A total of 7,094 patients were enrolled in the study. The number of clinical isolates per site, and the phenotypic methods used for *M. tuberculosis* isolation and DST by site, are given in Table 1.

Table 1: Laboratory methods for isolation of M. tuberculosis, phenotypic susceptibility testing and sequencing by country

Country	Number of isolates	Isolation of *M. Tuberculosis* and phenotypic susceptibility testing		Sequencing method
		Löwenstein-Jensen	MGIT 960	
Azerbaijan	751	*M.tb* isolation, DST to: RIF, INH	DST to: OFX, MFX, PZA, AMK, CAP	WGS using Illumina
Bangladesh	949	*M.tb* isolation, DST to: RIF, INH	DST to: OFX, MFX, PZA, KAN	WGS using Illumina
Belarus	197		*M.tb* isolation, DST to: RIF, INH, OFX, MFX, PZA, KAN, AMK, CAP	WGS using Ion PGM
Pakistan	1,461	*M.tb* isolation, DST to: RIF, INH	DST to: OFX, MFX, PZA, KAN, CAP	Sanger or WGS using Illumina*
Philippines	1,017	*M.tb* isolation, DST to: RIF, INH		Sanger
South Africa	1,578		*M.tb* isolation, DST to: RIF, INH, OFX, MFX, PZA, KAN, AMK, CAP	WGS using Illumina
Ukraine	1,141	*M.tb* isolation, DST to: RIF, INH, OFX, KAN, AMK, CAP	DST to: MFX	WGS using Illumina

LJ: Lowenstein-Jensen; DST: drug susceptibility testing; RIF: rifampicin; INH: isoniazid; OFX: ofloxacin; MFX: moxifloxacin; PZA: pyrazinamide; KAN: kanamycin; AMK: amikacin; CAP: capreomycin; WGS: whole genome sequencing.
* of the 1,461 isolates from Pakistan, 1,254 underwent Sanger sequencing and the remaining 207 underwent WGS using Illumina

The number of patients with MTBC isolates for which both genotypic and phenotypic testing results were available varied between drugs: 7,010 for RIF, 7,018 for INH, 5,110 for OFX, 4,793 for MFX, 2,993 for PZA, 623 for KAN, 690 for AMK and 764 for CAP (Table 2). Data on injectable agents (KAN, AMK and CAP), and on PZA for Ukraine, were available only for patients with RIF-resistant tuberculosis.

Table 2: Number of clinical M. tuberculosis isolates included in the study and pooled sensitivity values of genetic sequencing compared to phenotypic testing stratified by RIF resistance status for each respective locus or loci

Drug	Locus	RIF-susceptible cases		RIF-resistant cases		All cases	
		no. of isolates	sensitivity % (95%CI)	no. of isolates	sensitivity % (95%CI)	no. of isolates	sensitivity % (95%CI)
RIF	*rpoB*	–	–	–	–	7,010	91 (87-94)
INH	*katG & inhA*	6,065	81 (66-90)	953	90 (81-95)	7,018	86 (74-93)
OFX	*gyrA & gyrB*	4,244	76 (51-90)	866	88 (83-92)	5,110	85 (77-91)
MFX	*gyrA & gyrB*	4,010	81 (53-94)	783	91 (85-95)	4,793	88 (81-92)
PZA	*pncA*	2,310	37 (22-54)	683	55 (40-70)	2,993	51 (35-66)
PZA*	*pncA*	2,310	50 (33-67)	683	54 (40-68)	2,993	54 (39-68)
KAN	*rrs & eis*	–	–	623	79 (58-91)	–	–
AMK	*rrs*	–	–	690	90 (82-95)	–	–
CAP	*rrs*	–	–	764	81 (56-93)	–	–
MDR-TB		–	–	–	–	6,986	85 (75-91)
XDR-TB		–	–	–	–	756	74 (53-87)

*adjusted with Wayne's test results

Table 3 presents the number of clinical *M. tuberculosis* isolates included in the study and the pooled sensitivity values of genetic sequencing compared to the adjusted phenotypic testing. The overall pooled sensitivity values for genetic sequencing among all TB cases were 91% (95% confidence interval - CI: 87-94%) for *rpoB* (RIF); 86% (95%CI: 74-93%) for *katG, inhA* and *fabG* promoter combined (INH); 85% (95%CI: 77-91%) for *gyrA* and *gyrB* combined (OFX); and 88% (95%CI: 81-92%) for *gyrA* and *gyrB* combined (MFX). The sensitivity for *pncA* (PZA), when compared with MGIT 960 testing adjusted for the results of the Wayne's test, was 54% (95%CI: 39-68%).

Table 3: Number of clinical M. tuberculosis isolates included in the study and the pooled sensitivity values stratified by RIF resistance status for each respective locus or loci

Drug	Locus	Country	RIF-susceptible cases		RIF-resistant cases		All cases	
			no. of isolates	sensitivity % (95%CI)	no. of isolates	sensitivity % (95%CI)	no. of isolates	sensitivity % (95%CI)
rifampicin	*rpoB*	Azerbaijan	–	–	–	–	745	95 (90-98)
		Bangladesh	–	–	–	–	941	86 (76-93)
		Belarus	–	–	–	–	191	93 (86-97)
		Pakistan	–	–	–	–	1,455	85 (76-91)
		Philippines	–	–	–	–	1,003	89 (81-95)
		South Africa	–	–	–	–	1,535	87 (79-94)
		Ukraine	–	–	–	–	1,140	94 (91-96)

Drug	Locus	Country	RIF-susceptible cases		RIF-resistant cases		All cases	
			no. of isolates	sensitivity % (95%CI)	no. of isolates	sensitivity % (95%CI)	no. of isolates	sensitivity % (95%CI)
isoniazid	*katG & inhA*	Azerbaijan	616	81 (72-88)	129	85 (78-90)	745	83 (78-88)
		Bangladesh	871	94 (82-99)	70	84 (73-92)	941	88 (81-93)
		Belarus	97	90 (70-99)	94	99 (94-100)	191	97 (93-99)
		Pakistan	1,353	65 (56-73)	99	75 (65-83)	1,452	69 (62-75)
		Philippines	937	80 (72-87)	76	90 (80-96)	1,013	84 (78-89)
		South Africa	1,464	48 (37-59)	76	84 (72-93)	1,540	62 (54-70)
		Ukraine	781	92 (86-96)	355	97 (95-98)	1,136	96 (93-97)
ofloxacin	*gyrA & gyrB*	Azerbaijan	615	67 (9-99)	130	85 (69-95)	745	84 (68-94)
		Bangladesh	860	95 (84-99)	68	100 (71-100)	928	96 (87-100)
		Belarus	97	0 (0-85)	93	93 (76-99)	190	87 (69-96)
		Pakistan	511	73 (65-81)	96	86 (65-97)	607	75 (68-82)
		South Africa	1,426	50 (1-99)	77	83 (52-98)	1,503	79 (49-95)
		Ukraine	782	83 (36-100)	355	90 (80-96)	1,137	89 (80-95)
moxi-floxacin	*gyrA & gyrB*	Azerbaijan	615	100 (15-100)	130	88 (72-97)	745	89 (73-97)
		Bangladesh	860	98 (87-100)	68	100 (71-100)	928	98 (90-100)
		Belarus	97	0 (0-85)	93	89 (71-96)	190	83 (64-94)
		Pakistan	511	80 (72-87)	96	95 (75-100)	607	82 (75-89)
		South Africa	1,416	50 (1-99)	77	91 (59-100)	1,493	85 (55-98)
		Ukraine	555	100 (48-100)	275	92 (82-98)	830	93 (83-98)
pyra-zinamide	*pncA*	Azerbaijan	616	58 (28-85)	129	54 (43-65)	745	55 (44-65)
		Bangladesh	49	31 (11-59)	24	40 (19-64)	73	36 (21-54)
		Belarus	99	75 (19-99)	98	83 (72-90)	197	82 (72-90)
		Pakistan	98	25 (3-65)	46	35 (20-54)	144	33 (20-50)
		South Africa	1,477	24 (11-41)	76	64 (45-80)	1,553	43 (31-56)
		Ukraine	–	–	281	48 (40-55)	–	–
pyra-zinamide*	*pncA*	Azerbaijan	616	70 (35-93)	129	54 (43-65)	745	56 (45-66)
		Bangladesh	49	33 (12-62)	24	38 (18-62)	73	36 (21-54)
		Belarus	99	75 (19-99)	98	81 (71-89)	197	81 (71-89)
		Pakistan	98	29 (4-71)	46	36 (20-55)	144	35 (21-52)
		South Africa	1,477	57 (29-82)	76	60 (42-76)	1,553	59 (44-73)
		Ukraine	–	–	281	48 (40-55)	–	–

Drug	Locus	Country	RIF-susceptible cases		RIF-resistant cases		All cases	
			no. of isolates	sensitivity % (95%CI)	no. of isolates	sensitivity % (95%CI)	no. of isolates	sensitivity % (95%CI)
kanamycin	*rrs & eis*	Belarus	–	–	97	89 (73-97)	–	–
		Pakistan	–	–	74	100 (15-100)	–	–
		South Africa	–	–	83	67 (58-75)	–	–
		Ukraine	–	–	369	86 (71-95)	–	–
amikacin	*rrs*	Azerbaijan	–	–	140	86 (71-95)	–	–
		Belarus	–	–	97	92 (74-99)	–	–
		South Africa	–	–	84	67 (9-99)	–	–
		Ukraine	–	–	369	95 (86-99)	–	–
capreo-mycin	*rrs*	Azerbaijan	–	–	141	80 (65-91)	–	–
		Belarus	–	–	97	96 (76-100)	–	–
		Pakistan	–	–	73	67 (9-99)	–	–
		South Africa	–	–	84	33 (4-78)	–	–
		Ukraine	–	–	369	90 (80-96)	–	–

*adjusted with Wayne's test results; CI - confidence interval

The average cost of genome sequencing was 150 USD per sample. Considering that DST was performed in two rounds, with first-line drugs tested initially and second-line drugs subsequently, the average cost of conventional DST to RIF, INH, OFX, MFX, PZA, KAN, AMK and CAP was 230 USD. These costs included the cost of kits and reagents, as well as staff time.

3.3 Conclusions

A multi-country surveillance project of over 7,000 patients found genetic sequencing to have very good accuracy for the prediction of resistance to RIF, INH, FQs and the injectable agents. These performance estimates were consistent with previously published results [24, 30], and implied that the sensitivity of sequencing compared to phenotypic testing can be used to estimate the true prevalence of drug resistance for TB surveillance.

Notably, the ability of genetic sequencing to predict phenotypic resistance was most problematic for PZA in this study. This finding was unsurprising due to our incomplete understanding of the role of mutations in conferring resistance to PZA; 42% of all *pncA* mutations were unclassified in the dataset. Although the majority of these *pncA* mutations have been previously reported to be associated with resistance, there was insufficient power to classify them by conferred resistance due to their infrequent occurrence. Additionally, the phenotypic test for PZA susceptibility has inadequate reproducibility [31, 32], making it a weak test with which to make comparisons between phenotypic and genotypic results.

The strengths of this study include that the tested isolates were representative of the entire TB patient population in 7 resource-limited countries with high TB or DR-TB burdens. Furthermore, the patient management varied between settings, and included those newly diagnosed with TB as well as those who were previously diagnosed. Although phenotypic results were considered the gold standard for this study, phenotypic results were overwritten by genetic results in the case of resistance mutation identification. This is supported by the fact that phenotypic tests are suboptimal for certain drugs [33, 34], and so clinical decisions are often taken using a combination of

phenotypic and genotypic testing results. The use of sequencing for resistance detection in this context will likely only improve given additional data regarding the association between genetic polymorphisms and phenotypic drug resistance, and the recent revisions to the WHO-recommended critical concentrations for DST, especially for the second-line drugs [35].

Although only laboratory methods recommended by WHO were used in this study, and all laboratories passed proficiency testing before beginning the project, some variability in phenotypic results between laboratories and between media types could have influenced outcomes. Different platforms were used for DNA sequencing, including Sanger sequencing, which may have slightly different coverage of some genomic regions [36]. However, despite these variations and considering the complications of performing genetic sequencing in certain environments, overall this study demonstrated the high accuracy of sequencing in diverse settings and clinical backgrounds. These results support sequencing as an *M. tuberculosis* diagnostic and surveillance tool, especially given the limitations of conventional phenotypic methods for this purpose, and considering that the available molecular diagnostic tools are only capable of detecting resistance to a limited number of drugs. Once properly standardized and made economically feasible, this technology would be a game-changer for countries with limited laboratory and sample referral capacity. However, there remain practical considerations for setting up sequencing in resource-limited settings, and countries should consider such factors as the potential to batch specimens, training requirements, harmonization of sample preparation methods, and external quality assurance systems to best implement these technologies [37-39].

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4. Implementation considerations for drug-resistant tuberculosis sequencing in low- and middle-income countries

4.0 Introduction

Although next-generation sequencing (NGS) has the potential to significantly accelerate diagnosis for the purposes of guiding individualized tuberculosis (TB) treatment compared to conventional phenotypic drug susceptibility testing (DST) methods, the uptake of these technologies by reference laboratories has been slow, especially in low and middle-income countries [1]. There are several reasons for this, including the cost associated with establishing a new technology, limited technical experience with NGS instruments, workflows and clinical interpretation of data, and lack of commercially available, standardized NGS workflows in the context of a rapidly evolving commercial and regulatory landscape. Considering these obstacles, many laboratories have avoided NGS or preferred to outsource clinical sequencing to centralized laboratories or specialist sequencing companies. However, outsourcing has its own complexities and added costs, and requires efficient sample referral systems and results transmission networks which can also result in sample-to-answer delays.

As both sequencing technologies and sequencing workflows continue to become cheaper and simpler, even low-throughput, low-capacity laboratories can now consider implementing these technologies in-house. Before these clinical laboratories can implement sequencing, however, several factors need to be considered, including: instrument selection, set-up, support and training requirements, standardization of NGS workflow and analysis pipelines, quality assurance and control, data and information technology (IT) requirements, and data interpretation and reporting formats. Fortunately, there are several global public-private partnerships working to help make this technology more accessible to those with the greatest drug-resistant TB (DR-TB) burden and most need, as well as ongoing global efforts to standardize clinical NGS for DR-TB diagnosis and accelerate national and global regulatory approval of NGS technology as an IVD solution. In this chapter we outline some important considerations for reference laboratories considering implementation of NGS technologies for DR-TB diagnosis and surveillance.

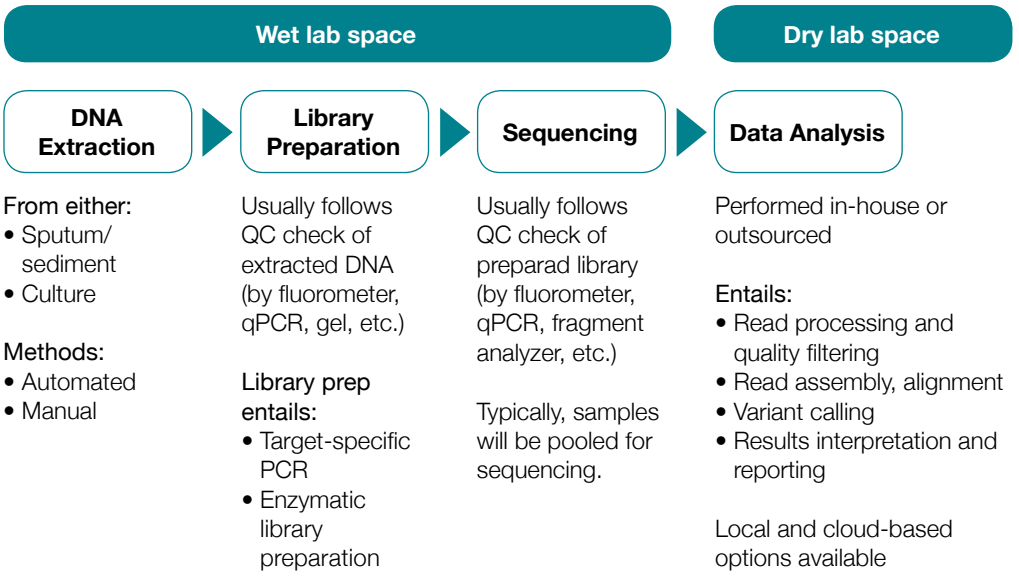
4.1 Setting up an NGS workflow

Although there are a wide variety of NGS technologies and application-specific workflows, a general sequencing workflow involves the use of at least three separated wet laboratory areas. One clean space should be reserved for all pre-amplification procedures (reagent preparation and pre-PCR steps), one space is required for sample preparation and DNA extraction, and a third space is needed for all amplification/post-amplification procedures. Laboratories already running molecular assays such as the line probe assays (LPAs) may be able to adopt existing lab spaces for the NGS workflow, though testing for contamination potential between samples and technologies should be conducted periodically and addressed, when encountered. Well-defined and separated spaces or, ideally, a single room, should be dedicated to each step of this workflow. If sequencing data analyses are to be conducted in the same laboratory, dry laboratory space should also be allocated for bioinformatics analyses (Fig. 1).

Sample preparation

Sample preparation for sequencing requires two steps: nucleic acid extraction and library preparation. These procedures involve processing direct clinical samples containing *M. tuberculosis* (MTB) for downstream targeted NGS (or MTB enrichment followed by WGS); or processing DNA extracted from MTB cultures for downstream targeted NGS or WGS, and so must be performed in laboratories complying with applicable biosafety level standards, with all steps implemented in accordance with appropriate biosafety standards. The processing of sputum specimens for DNA extraction, in particular, should be performed in a biological safety cabinet (i.e. Class II A2 cabinets) under at least biosafety level 2 standards [4]. It is essential

Figure 1: Conceptual NGS workflow contains both wet- and dry-laboratory workspace



to avoid any DNA cross-contamination when performing molecular biology protocols, and the use of different rooms and dedicated UV-C germicidal light-equipped, laminar flow cabinets for each task, together with a unidirectional workflow, can serve to reduce the risks of contamination of DNA and PCR product.

Sequencing

The NGS sequencing instruments should be placed within the wet lab space (ideally, a separate room) specifically dedicated to amplification/post-amplification procedures. These platforms should be located in rooms with a reliable heat and/or air conditioning system to regulate the room temperature (the optimal working temperature for most NGS technologies is generally in the range of 19-25°C) [5]. NGS instruments should be kept in a clean, dry space on stable bench tops and protected from vibrations as much as possible. Access to the instruments should be limited to well-trained staff that have demonstrated competency on the platforms. A continuous power supply should be guaranteed during instrument runs to avoid interruption and the consequent waste of samples, reagents and consumables.

4.2 Additional equipment requirements

The first step to successful implementation of NGS solutions for DR-TB diagnosis is the selection of an appropriate sequencing instrument to fit the needs of the clinical laboratory and to run the desired NGS applications. As discussed in Chapter 1, these instruments differ widely in chemistry, throughput, price, time to completion of a sequencing run and NGS read length, among other factors. It is important to choose a sequencing instrument that is available broadly, has good local supply distribution and support, and is relatively inexpensive. It should also have reliably low error rates and be capable of high or low throughput processing, depending on individual laboratory needs. Whichever NGS technology is selected, however, it is important to understand that most NGS instruments and workflows are still considered specialized equipment and require expert set up with all installation activities performed by the manufacturer's engineers. Potential setup complexities include taking into account power supply considerations, including back-up options, electrical connections, computing hardware and software, a maintenance plan (e.g. weekly, monthly, and/or pre-run checks),

equipment warranty, and necessary training. Another important consideration when setting up NGS workflows is the need for additional equipment. These auxiliary equipment needs are specific not only to the selected sequencer (e.g. the Ion Personal Genome Machine (PGM) requires both a compressed nitrogen cylinder and water purification system), but also to the DNA fragmentation method used (enzymatic or non-enzymatic) and to the intended applications (targeted NGS, WGS or both). Additionally, the local availability of NGS product suppliers, distributors and technical support should be considered when purchasing and installing both NGS technologies and accessory equipment in order to avoid any potential delays associated with the delivery of reagents and the provision of technical assistance accompanying or following installation.

While it is not possible to provide general guidance regarding the additional equipment required for any and all NGS workflows due to the specific needs of each sequencing instrument and application, we have provided an example of the most common elements of a broadly utilized NGS workflow to help illustrate the types of equipment that should be considered. In Table 1, below, we list the additional equipment that is most commonly used for sample preparation (prior to sequencing) in an Illumina MiSeq workflow.

Table 1: Additional types of equipment required for sample preparation for a "generic" NGS workflow based on an Illumina MiSeq workflow (enzymatic fragmentation)*

Workflow element	Type of equipment required	Description
DNA extraction	Automated DNA extraction instrument	There is a large variety of available commercial instruments for semi or fully automated extraction of DNA from patient samples.
	Manual extraction	Most manual extraction kits do not require additional equipment other than those items listed below.
Liquid handling	Molecular biology grade pipettors	Several steps in the NGS workflow require accurate measuring of microliter to milliliter volumes. Manual, single channel, axial pipettors are most commonly used, but automated options are also available for high throughput laboratories.
DNA quantification	Fluorometer	Quantification of extracted DNA is required at one or more stages of most NGS pipelines. Any one or more of the listed types of instruments can be used for DNA quantification. Some workflows might require a fragment analyzer AND one other DNA quantification instrument.
	Spectrophotometer	
	RT PCR instrument	
	Fragment analyzer	
Amplification & incubation	Thermocycler	Almost all NGS workflows (both targeted & WGS) require one or more steps of DNA amplification and incubation of reagents. There are many commercially available options.
Sample purification	Magnetic stand	Many reasonably-priced, commercially available options for separating different reagent components using magnetic beads.
	High-speed microplate shaker	For high-throughput reagent mixing.
	Centrifuge	Tube or plate centrifuges available, depending on workflow needs.
	Vortexer	For reagent mixing.
Sample & reagent storage	-20 Freezer	Any reliable, cost-effective freezer is suitable for storing samples and reagents.

* This list is intended to be used for illustration purposes only and is not intended to be exhaustive.

The listed equipment is relevant for a mostly manual workflow. There are also semi-automated and fully-automated workflows provided by select manufacturers that use liquid handling workstations to automate the DNA extraction and library preparation steps described below, but these solutions will most likely only be cost-effective for very high-throughput clinical laboratories.

Another consideration for NGS users is the consumables and reagents associated with NGS workflows. Depending on the selected sequencing instrument and workflow, the user must either acquire and maintain a variety of different supplies or work with commercial partners that supply "kits" of consolidated supplies. NGS lab managers will need to understand appropriate international and local regulatory and import procedures, as well as processes to monitor reagent quality and shelf life to ensure that only high-quality sequencing data is generated. Additionally, the laboratory must establish standard operating procedures for handling the reagents and chemicals used for targeted NGS and WGS to ensure both quality and safety.

Finally, when selecting and installing NGS solutions, laboratories should contemplate the following infrastructure considerations:

- Unidirectional work flow for the techniques involved in targeted NGS and/or WGS
- Space requirements: consider the instrument dimensions and weight to account for the necessary bench space and positioning of the instrument; dedicated spaces for general molecular biology techniques (ensuring physical separation between pre/post-PCR spaces); storage space for consumables/supplies, reagents and kits
- Electrical requirements: power specifications (voltage/current/frequency/power draw), safety measures (e.g. protective earth) and uninterruptible power supply systems, as appropriate for the selected technology
- Environmental requirements: controlled temperature (air conditioning), humidity, elevation, air quality, ventilation and vibration
- Network and internet connections
- Computing requirements: instrument computer hardware (e.g. processor, memory, GPU processor, storage and operating system), external storage, data back-ups and appropriate data security measures.

Additional details and considerations for TB laboratory strengthening and technology implementation at different levels of the TB laboratory network may be found in the Global Laboratory Initiative Practical Guide to TB Laboratory Strengthening [2]. Users may also refer to the Genoscreen targeted NGS and Oxford Nanopore Sequencing Technologies (ONT) WGS workflows outlined in the appendices for further direction in establishing specific workflows for different NGS applications (Appendix 1-2).

4.3 Support and training requirements

The successful integration of both targeted NGS and WGS into routine clinical reference laboratory workflows will depend, in no small part, on the experience of the laboratory personnel involved. Ideally, any staff performing sequencing should have a molecular biology skillset that lends itself to the performance of wet laboratory techniques and troubleshooting of any issues that may arise when performing NGS applications. Laboratory technicians should be properly trained on all assay operating procedures, from DNA extraction to library preparation and sequencing, and be continuously updated by assay manufacturers on the use of sequencing kits, instruments and reagents. If the laboratory has staff experienced in performing NGS applications, those persons should train any new staff until they are able to perform targeted NGS and/or WGS procedures independently, producing consistent and accurate results. Ultimately, the time dedicated to NGS training depends on the background and experience of the laboratory trainees (especially in regards to training and experience in molecular biology techniques and sequencing principles), and it may take several weeks of full-time training to ensure a single technician is adequately trained in the full NGS workflow. NGS training may be divided into different sessions: theory, overview and principles of the technology (starting from either principles

of molecular biology or directly to sequencing, depending on the trainee's experience); a detailed description of the overall laboratory workflow and review of targeted NGS or WGS standard operating procedures and protocols as they are incorporated into this workflow; multiple, practical demonstrations by trainers, including examples of encountered issues and troubleshooting, followed by targeted NGS or WGS procedures performed multiple times by trainees who are mentored by trainers, with the final procedures independently performed by trainees with support provided by trainers, if needed; and finally, external quality assurance. Ideally, all technicians performing NGS should independently perform successful sequencing of a sample test panel with known genotypic results after completing supervised training requirements. Competency for both targeted NGS and WGS applications should be evaluated and signed-off by the trainers and laboratory records of these training assessments should be kept by the laboratory for each technician performing NGS applications (Table 2).

Table 2: Performance activities for NGS competency training plan

Elements	Performance criteria
1. Preparation of equipment and reagents	1. Set-up of equipment/instrumentation required for the test 2. Performance of pre-use (calibrations), after use and safety checks 3. Selection, storage and use of appropriate reagents 4. Preparation of the correct volumes and appropriate labeling of reagents required for the test
2. Sample processing and DNA extraction	1. Preparation of worksheets and identification of samples 2. Use of appropriate extraction methods and adherence to standard operating procedures 3. Storage of samples/DNA in accordance with requirements 4. Maintenance of chain of custody traceable to all staff, for all samples
3. Library preparation and sequencing	1. DNA quality and quantity assessment 2. Use of the correct reagents and kits 3. Use of appropriate controls and reference standards 4. Following of standard operating procedures for library preparation and set up of the sequencing instrument 5. Prevention of cross-contamination
4. Data processing	1. Ensure results are consistent with expectations and reference standards 2. Reporting of atypical observations 3. Record and report results in accordance with test output 4. Interpretation of data trends/results and reporting 5. Troubleshooting procedures, including handling of problems related to reagents or equipment 6. Data storage requirements and data management and retention policies
5. Safe work environment	1. Use of safe work practices and appropriate personal protective equipment 2. Minimization of waste generation 3. Storage of equipment and reagents 4. Appropriate cleaning of equipment and safe use of reagents 5. Safe disposal of waste including hazardous waste and tested samples

Although close internal laboratory training and monitoring of technicians is necessary to ensure technician competence and confidence in performing targeted NGS and WGS procedures, sequencing companies and their distributing partners often offer support contracts to laboratories implementing new NGS platforms. NGS companies and distributors may provide technical specialists who can assist customers either remotely or in-person on instrument installation and operation, NGS workflows, experimental set-up and troubleshooting. The customers may select from different NGS support packages depending on their specific needs. In addition to these NGS provider services, public online NGS forums and communities can also help users in designing and performing targeted NGS and WGS experiments, as well as interpreting obtained results and troubleshooting. Some websites and online forums available for this purpose include: SEQanswers (http://seqanswers.com/), Biostars (https://www.biostars.org/) and LinkedIn's (LinkedIn Corporation, Sunnyvale, California, USA) Genomics forum (https://www.linkedin.com/groups/1907871/profile). If the selected NGS instrument is placed within a genomic facility at a research institute, the facility staff may also be able to guide users through different NGS applications and troubleshooting.

4.4 Bioinformatics and data interpretation

Sequencing data analyses are the last, and arguably the most important step, of any NGS workflow for DR-TB diagnosis. While the processing and interpretation of sequencing data requires relatively complicated and intensive, expert bioinformatics knowledge, in the past few years there has been an explosion of both commercial and publicly accessible semi and fully-automated bioinformatics tools and platforms to support both expert and non-expert analysis and interpretation of MTB NGS data. These tools have democratized NGS analysis and made even complex analyses and clinical interpretation accessible to non-experts. The majority of currently available tools are focused on providing cloud-based WGS pipelines for processing raw sequence data. These analyses compare generated sequencing data to a reference MTB genome to identify mutations and relate the identified mutations to associated drug resistance phenotypes. The long-term goal of these efforts is to facilitate prediction of clinically relevant MTB drug resistance phenotypes directly from NGS data.

Bioinformatics tools for non-experts

The largest efforts to establish and maintain large repositories of genomic and phenotypic data, and provide access to non-expert bioinformatics solutions for surveillance and diagnosis of DR-TB are led by two global consortiums of NGS experts: The ReSeqTB Consortium and CRyPTIC. These consortiums have accumulated genotypic and phenotypic data from many thousands of MTB strains collected worldwide. Both also provide access to cloud-based solutions for automatic processing of raw WGS data from MTB strains, with outputs including comprehensive mutation lists and drug resistance reports for users with minimal expertise. While these tools are still only suitable for surveillance and research use, the ReSeq platform is being transferred to WHO in Q1 of 2019 (ReSeqWHO) to support global DR-TB surveillance in the short term, and clinical diagnosis of DR-TB for patient care from both targeted NGS data and WGS in the long term, once the platform is fully operational and validated for clinical use [7, 8, 9]. Likewise, the CRyPTIC platform will provide similar WGS analysis services and ultimately the two platforms will be made interoperable.

Recent advances in WGS of MTB have led to the development of several other user-friendly tools that can characterize and predict drug resistance to various anti-TB drugs direct from WGS data. Some of these WGS tools are web-based, including PhyResSE [12], TBProfiler [13], ResFinder [14], and GenTB [15], while others can be run off-line, including TGS-TB and Mykrobe predictor [16, 17]. Of these tools, only Mykrobe predictor is currently compatible with both Illumina and Oxford Nanopore WGS data, while the others are optimized mostly for Illumina sequencing data.

Bioinformatics tools for experts

For those laboratories with some bioinformatics capabilities and expertise, or those wishing to develop their own NGS analytical pipelines

or conduct analyses beyond those offered by the existing pipelines, it is important to note that prior to applying conventional bioinformatics pipelines or analyses to generate results for a sequencing run, there is a need to evaluate instrument metrics during and after the sequencing run. Platforms, such as FastQC (Table 3), assess different NGS quality statistics such as quality score, intensity data by cycle, amount of data, and average read length. There may be additional processing needs for NGS data quality analysis, such as the trimming and combination of multiple sequencing files (e.g. a multi-file sequencing run would require concatenation and trimming of adapters prior to any secondary analyses). After sequencing quality metrics are met according to the control parameters (i.e. base score and quality score), special attention must be paid to post-sequencing data handling and analysis. Typically, secondary analysis involves steps such as the assessment of quality control metrics, read alignment to reference genome(s), or *de novo* assembly steps. Tertiary analysis then involves steps such as variant calling, necessary alignment refinement (e.g. around indels), genome functional annotation, phylogenetic analysis, and mutation analysis. At this point, clinically significant results are interpreted and reported.

There are many different types of commercially available software packages that contain almost complete bioinformatics software optimized for particular NGS applications. Easy-to-use web interfaces facilitate application use, even for users without knowledge of programming and scripting languages. These workflow management platforms are typically cloud-based, such as Galaxy Cloudman and Cloud Virtual Resource [10, 11], with various settings and parameters that can be customized by the user for specific use cases. Analyses on these platforms can be automated by the creation of workflows that ensure reproducible results. They also offer data storage space and computational resources necessary for the analysis. However, the dependence on a single, shared file system, such as with the Galaxy platform, means that the platform is only able to analyze one specimen at a time, limiting its use for large datasets.

For laboratories with strong bioinformatics support, targeted NGS and WGS data analysis is often performed using a laboratory tailored collection of open-source software programs programmed to function together in a customized solution. These software programs are run using text-based command-line of UNIX or Linux operating systems that allow developing scripts and application of automated bioinformatics pipelines (Table 3) [3]. The advantage of using open-source software programs is that they are free of charge and the user has full control over all of the versions installed. Accurate versioning of the software is essential, and when upgrading to the newest software versions users should perform a re-analysis test as part of the validation of the newer version of the software. Some drawbacks of these command-line software programs are that they tend to be difficult to install, they are prone to software bugs, there is no graphical interface and IT support is often limited.

In regards to NGS data reporting, most bioinformatics output results include sequence variants such as single nucleotide polymorphisms (SNPs), deletions, insertions and structural variants. However, the interpretation of these results also depends on the user having basic knowledge of the expected targeted NGS or WGS outputs, especially when there are errors. The relative impact of errors can vary based on the sample preparation method, sequencing platform and bioinformatics analysis tools used. For example, some of the rapid analysis tools that directly analyze sequencing data can miss conventional or novel mutations due to computational weaknesses. For this reason, the user is ultimately responsible for ensuring the integrity of targeted NGS or WGS data reporting. Users should critically evaluate the NGS results generated for every sample and assess whether the results meet analytical requirements. The incorporation of internal controls into the pipeline is also critical for identifying any potential sources of bias in this context. If the laboratory does not have a bioinformatician on staff, it is advisable to collaborate with other laboratories with bioinformatics experience for an additional data check.

Table 3: Software programs most commonly used in the analysis of targeted NGS and WGS data.

Application	Software	Link	Commercial/ Open-source
Quality checks	BaseSpace BioNumerics CLC Genomic Workbench FastQC nanoQC Picard Tools	https://basespace.illumina.com www.applied-maths.com www.clcbio.com http://www.bioinformatics.babraham.ac.uk https://github.com/wdecoster/nanoQC https://broadinstitute.github.io/picard/	Commercial Commercial Commercial Open-source Open-source Open-source
De novo assembly	BioNumerics SeqSphere CLC Genomic Workbench SPAdes Celera Assembler Velvet Canu minimap2 DNASTAR	www.applied-maths.com www.ridom.de www.clcbio.com http://bioinf.spbau.ru/spades https://sourceforge.net/projects/wgs-assembler https://www.ebi.ac.uk/~zerbino/velvet/ https://github.com/marbl/canu/releases https://github.com/lh3/minimap2 https://www.dnastar.com	Commercial Commercial Commercial Open-source Open-source Open-source Open-source Open-source Commercial
Reference-based alignment	BWA BioNumerics DNASTAR	http://bio-bwa.sourceforge.net/ www.applied-maths.com https://www.dnastar.com	Open-source Commercial Commercial
Consensus and variant calling	BioNumerics CLC Genomic Workbench DNASTAR Samtools GATK Freebayes GenomicConsensus Nanopolish	www.applied-maths.com www.clcbio.com https://www.dnastar.com http://samtools.sourceforge.net/ https://software.broadinstitute.org/gatk/ https://github.com/ekg/freebayes https://github.com/PacificBiosciences/GenomicConsensus https://github.com/jts/nanopolish	Commercial Commercial Commercial Open-source Open-source Open-source Open-source Open-source
Annotation	Prokka SnpEff RAST Patric	http://www.vicbioinformatics.com http://snpeff.sourceforge.net/ http://rast.theseed.org/FIG/rast.cgi https://www.patricbrc.org/	Open-source Open-source Open-source Open-source
Genome and variant browser	Integrated Genomics Viewer Artemis Tablet Varb	http://software.broadinstitute.org/software/igv/ http://www.sanger.ac.uk/science/tools/artemis https://ics.hutton.ac.uk/tablet http://www.markdpreston.com/software	Open-source Open-source Open-source Open-source

When selecting from among the many free or commercially available bioinformatics tools, users should be cautious to ensure they select the best tools for their analysis. Factors to be considered include: compatibility of the tool with the given reads generated by a sequencing platform; tool output files; whether the technology is constantly maintained or updated (which is especially relevant given the speed at which the NGS field is evolving); additional computational resources needed; downstream analysis workflow; and the time taken to complete the analysis.

4.4.1 Results reporting

Both targeted NGS and WGS applications produce data that enables clinicians and public health practitioners to retrieve several types of information from MTB genomes, ranging from bacterial species identification and strain relatedness to a comprehensive characterization of the strain's drug resistance profile. Additionally, targeted NGS and WGS data can be used to expose potentially clinically relevant information not routinely reported in phenotypic clinical DST reports, such as the proportion of mixed resistant and susceptible strains found in a clinical sample or the presence of specific MTB mutations that can predict not just resistance at the WHO critical concentration (which is most commonly reported in phenotypic DST reports) but also the likely level of drug resistance conferred by that mutation (see Chapter 3 for additional details). This emphasizes the need for common terminology and standardization in the reporting of genomic information to maximize its utility. It also highlights the need for training of health practitioners in the interpretation of these standardized genomic reports in order to translate this information into actionable information to improve treatment decisions, conduct contact tracing and understand national and international DR-TB surveillance patterns.

In order to have the most impact, an NGS clinical reporting template should include the following essential sequencing and DST details in an easy-to-read format for both targeted NGS and WGS applications:

- Sequencing type (WGS or targeted NGS)
- Sequencing platform utilized
- Bioinformatics software/pipeline and version used for analysis
- Drugs of interest included in resistance profile (first/second-line/new/re-purposed drugs)
- Known drug resistance-conferring target gene(s) and respective loci IDs
- Mutations detected, including:
  - % of resistance alleles at loci of interest (to identify mixed infections when present)
  - Coding effect (synonymous or non-synonymous)
  - Amino acid changes (when non-synonymous)
- Phenotypic resistance prediction
- Phylogenetic lineage/sub-lineage/local-strain information
- Detected genomic clustering (if any)
- Comments (for disputed or uncertain mutations).

Additional information could be reported either in the initial results code or as an annex to document regarding the quality of the generated sequencing outputs:

- Type of clinical ample collected and DNA extraction method
- Average depth of coverage across the amplicons (targeted NGS) or genome (WGS)
- Percentage of reads mapped to the reference genome
- Percentage of reference genome covered (i.e. sequencing breadth).

A standardized reporting format should also consider which gene numbering system to use for identifying the location of detected mutations in order to avoid misunderstandings due to different annotation systems. Historically, TB investigators have reported some common MTB mutations using genome numbering schemes from other bacteria (e.g. the *rpoB* gene is most often reported using the *E. coli* gene numbering scheme). Ideally, given that the H37Rv strain is the most widely used MTB reference genome worldwide, gene numbering nomenclature should be standardized according to the sequence of this strain. In 2017, a meeting of global experts was convened in London by FIND and Critical Path to TB Drug Regimens Initiative in order to standardize language for reporting of

NGS testing results. One of the outputs of this meeting was a proposed clinical sequencing report template for MTB (Fig. 2) that is currently under review for publication [18]. We include a draft of this template, here, for demonstration purposes.

Figure 2: Example of a standardized clinical WGS report for MTB*

Barcode XXXXXXXXXXXX

MYCOBACTERIUM TUBERCULOSIS SEQUENCING REPORT

Sample Details

Patient Name	JOHN DOE	Patient ID	12345678910
Birth Date	2000-JAN-01	Location	SOMEPLACE
Sample Type	SPUTUM	Sample Collection Date	2016-DEC-25
Sample Source	PULMONARY	Sequenced From	CULTURED ISOLATE (LJ)
Sample ID	A12345678	Sample Received Date/Time	2017-JAN-02, 12:22
Laboratory Technician	TECHNICIAN NAME	Report Date/Time	2017-JAN-05, 11:45
Requested By	REQUESTER NAME	Requester Contact	REQUESTER@EMAIL.COM

Assay Details

Sequencer	ILLUMINA HISEQ 2500	Method	WHOLE GENOME SEQUENCING
Pipeline	RESEQTBV.3.2C (https://platform.reseqtb.org)	Reference	H37RV (NC_000962.3)

Final Result

The sample was positive for ***Mycobacterium tuberculosis.***
It is **resistant to isoniazid, rifampin, capreomycin, kanamycin, ofloxacin, and moxifloxacin.**

Lineage

Mycobacterium tuberculosis, lineage 2.2.1 (**East-Asian Beijing**).

Drug Susceptibility

Resistance is reported when a high likelihood resistance-conferring mutation is detected in loci of interest.[1] **No mutation detected does not exclude the possibility of resistance.**

☐ No mutations detected
☐ Multi-drug resistance predicted
☑ **Extensive drug resistance predicted**

	Interpretation	Drug	Gene Target (Mutation, Allele %)	Comments
First Line	Resistant	Isoniazid	*katG* (Ser315Thr, 100%)	
		Rifampin	*rpoB* (Ser450Leu, 100%)	Rifabutin resistance likely
	Sensitive	Ethambutol		No mutation detected
		Pyrazinamide		***Expert consultation advised***
Second Line	Resistant	Capreomycin	*rrs* (C1402T, nucleotide 100%)	At least low-level resistance predicted
		Kanamycin	*rrs* (C1402T, nucleotide 100%)	
		Moxifloxacin	*gyrA* (Ala90Val, 14%)	
		Ofloxacin	*gyrA* (Ala90Val, 14%)	
	Sensitive	Amikacin		No mutation detected
		Ethionamide		No mutation detected
		Streptomycin		No mutation detected

Disclaimer

[1]Loci of interest derived from ReSeqTB Data Platform and from Miotto P, et al. Eur Respir J. 2017 PMID: 29284687
Low frequency hetero-resistance below the limit of detection by sequencing may affect typing results. The interpretation provided is based on the current understanding of genotype-phenotype relationships. All results reference the *M. tuberculosis* mutation numbering system which differs from the *E. coli* numbering system.

Authorized By

Name	AUTHORIZER NAME	Position	LAB SUPERVISOR
Signature		Date	2017-JAN-05
Reporting Laboratory	LAB NAME	LAB ADDRESS	LAB PHONE NUMBER

*For demonstration purposes, only.

4.5 Quality assurance and control

For both targeted NGS and WGS applications, rigorous, standardized sequencing protocols that rely upon strict analysis criteria, error reporting and version control are required to ensure reliable genomic variant calls [3, 19]. A laboratory implementing NGS applications should have detailed and documented procedures and protocols in place for optimal DNA extraction, target enrichment, library preparation and post-sequencing analysis. Starting with specimen preparation, the laboratory should assess the source of the specimen meant for targeted NGS or WGS testing since this can affect the DNA extraction method. MTB from liquid or solid culture media is handled differently from MTB direct from sputum, and these different sample types and preparation methods should all meet the DNA quality and quantity minimums required for either targeted NGS or WGS testing (Refer to the NGS Target Product Profile in Appendix 3). Quality control methods must be well developed to accurately assess the minimum amount of DNA required for sequencing and to adjust DNA inputs for different NGS instruments. Accurate library qualification and quantification can be critical for optimal sequencing (further details provided in Chapter 2). The inclusion of positive and negative controls is also essential for the detection of errors during the sample and library preparation processes. During sequencing, certain quality metrics must be met for particular platforms. For example, a good quality Illumina run would generate an amount of reads within the expected Q score range above Q20 or Q30 [20], a particular set of minimum depth and coverage criteria, and a low error rate based on the sequencer's positive control.

Given the inherent differences between NGS platforms and informatics tools, specific thresholds can be defined for each laboratory as a means of monitoring all optimal quality metrics for analytical performance. Currently, large research consortia are collaborating to process thousands of MTBC genomes with the final aim to generate consensus on standardized analysis pipelines and variant interpretation criteria as a top priority for large-scale TB sequencing efforts. Global consortia, including ReSeqTB and CRyPTIC, are currently validating their pipelines (i.e. establishing the diagnostic performance of specific sequencing approaches for drug resistance detection and epidemiological purposes) by comparing targeted NGS and WGS results with reference sequences. This analysis will consider sequencing assay parameters such as accuracy, reproducibility, repeatability, robustness and limit of detection in the post-sequencing analyses. The identification of appropriate quality control thresholds and acceptable minimum requisites through this collaborative effort will help to further promote the implementation of sequencing in accredited clinical practice. Well-characterized standards can be manipulated *in silico* to assess the impact that quality metrics such as read depth and error rates can have on prediction accuracy by the pipeline bioinformatic algorithm.

Although the definition of quality thresholds and the standardization of bioinformatics analysis pipelines are still evolving subjects for NGS of the MTB genome, certain considerations may be helpful for setting up both targeted NGS and WGS quality assurance and control protocols. The following are some of the quality metrics to be considered for quality control during sample preparation and sequencing steps:

- DNA quality and quantity (more details provided in Appendix 3)
- Library quantification and qualification
- Sequencing coverage
- Base call quality score.

Quality control metrics undertaken during data analysis to ensure accuracy and consistency:

- Number and length of sequencing reads
- Reads duplication
- Strand bias
- Reads quality score
- Mapping/assembly quality
- Average reference coverage breadth
- Percentage of mapped and unmapped reads
- Percentage of the reference genome covered
- Read breadth of single variant calls.

Internal and external quality control processes of the entire wet and dry laboratory workflows should also be established to ensure the

generated data meets international standards. Detailed standard operating procedures involved in targeted NGS and WGS applications should be documented to ensure reproducibility of methods. Table 4 outlines some of the key quality control considerations and quality indicators for the different steps of the NGS workflow to help ensure reproducibility. Any samples selected for internal validation testing should be representative of a wider pool of MTB strains with varying lineage and genotype. Performing external quality assessment (EQA) for WGS is often complicated due to the need to control for high-quality DNA extraction, library preparation steps, sequencing reactions, and the bioinformatics analysis. Currently, there are limited EQA programs for NGS of MTB. In such cases, it is advisable to take part in inter-laboratory comparison of processes as a way of monitoring laboratory performance. Another common practice is splitting samples and repeating the entire test process for the same samples within the individual laboratory. Results from such tests should be well documented since these reference practices are essential for accreditation and certification. Laboratories could also consider undergoing an on-site evaluation using a standardized EQA checklist, such as provided by a supranational reference laboratory, to ensure that workflows comply with quality standards.

4.6 Data and Information technology requirements

NGS technologies generate hundreds of gigabytes to terabytes of data per instrument and per run. This digital information must be stored, backed up and processed using dedicated secure systems to ensure data availability, security and traceability, in addition to the timely delivery of accurate results. As the laboratory makes plans to set up NGS for various applications, they should also make sure to consult an IT department regarding these crucial issues. IT may assist with setting up the instrument, selecting the optimal computer hardware, data storage and transfer options, and installation of all necessary software. This is crucial to avoid downstream problems and ensure the laboratory has sufficient computational resources prior to the introduction of NGS technologies. Installation of computational hardware will depend on the expected amount of data to be generated from a particular sequencing technology as well as the type and frequency of analyses to be performed [19]. Laboratories should carefully consider their workload, network speed/performance, data security and confidentiality concerns, existing infrastructure and all associated data management and IT costs to select the optimal data storage/computing solution for their NGS technology. As the field is maturing, many labs are adopting a cloud computing model for genomic analysis and storage due to its elasticity, reproducibility, and privacy features [21, 22].

4.6.1 Data storage

Based on cost and ease of use, different laboratories may opt for different types of long- or short-term data storage. Local data storage using external hard drives is an attractive option, especially for smaller laboratories, as it does not require much in terms of established bioinformatics infrastructure. External hard drives may also be programmed to undergo automatic back-ups at set time periods, ensuring efficient data management with little operator involvement. However, this option is generally suitable only for settings with smaller sequencing workloads, such as those performing targeted NGS or WGS for research or routine sequencing in low TB incidence settings, as the stored data may only be recovered directly from the source computer or hard drive (there is no option for remote access), and there is the risk of data loss if the device is damaged in any way. Another option for local storage includes the use of flash drives, although these can easily be infected with viruses. These local storage options are available through many retailers and come in a wide array of storage capacities, allowing users to select the options that would best fit the needs of their environment. A third local storage option is the use of a scalable, local computing cluster that can be custom-tailored for a particular laboratory. This strategy involves connecting multiple computers to enable high-

Table 4: Key quality indicators and quality control considerations for NGS workflows.

NGS workflow step	Standardization process	Quality metric
DNA extraction	Specimen/sample quality	Adequate sample purity (with consideration for other organism DNA, human DNA or inhibitors in the sample)
	Specimen/sample quantity	Sufficient starting material (with consideration for targeted NGS or WGS application, i.e. cfu/mL)
	DNA quality	Adequate DNA purity
	DNA quantity	Sufficient starting material – see Appendix 3
		Adequate volume
Library preparation	DNA quality	Correct fragment size
		Adequate DNA purity
	DNA quantity	Sufficient starting material
		Adequate volume
Sequencing	Quality of the run	Base quality score (e.g. Phred Q score)
		Median base quality by cycle and percentage of bases above quality threshold (with considerations for adapter trimming and GC content)
		Indexed sequence capture percentages
	Sequence base-calling	Number of reads
		Read length
		Percentage of unique reads
		Percentage of duplicate reads
Assembly	Reference-guided assembly	Appropriate reference
		Percentage of reads correctly mapped to reference genome or target
		Average coverage of genome or target region
		Average depth of coverage
		Read duplication
		Mapping quality scores (e.g. % MTBC by Kraken Taxonomic Sequence Classification System, or % mapping by Burrows-Wheeler Aligner)
	de novo assembly	Number of contigs
		Contig length, N50
		Number of scaffolds
		Complete or partial assembly (percent assembly size)

NGS workflow step	Standardization process	Quality metric
Variant calling	SNP calling/variant detection	Variant call quality score
		Number and percentage of reads with the variant detected
		Percentage of novel variants, concordance rates with reference target or sequence
		Strand bias
		Allelic read percentages (incl. different variant types and portions and ratios of base substitutions)
		Variant allele frequency (heterozygous and homozygous calls)

speed and intensive data calculations. However, the requirement of sophisticated network systems and maintenance costs may make this an unfavorable option for many reference laboratories.

Alternatively, sequencing data can be stored, accessed and queried using online cloud services like BaseSpace, Google Cloud, and Amazon Drive, as long as these systems protect the privacy of patient data and comply with any national guidelines for the storage and use of patient data. Likewise, data can be stored through online public databases such as the Sequence Read Archive or the European Nucleotide Archive. This approach enables high-level data computing, ensures secure storage and management of data as well as continuous support from software/hardware owners, and has the benefit of remote data access through any device connected to the network. Limitations to cloud-based data solutions include the need for a stable and high-speed internet connection for data upload and access. In selecting data storage options, both targeted NGS and WGS users should consider that sequence data from TB samples should ideally be stored by two different systems in order to avoid the loss of any raw data.

4.6.2 Data transfer/ upload

In addition to physical hardware that can be used for data transfer, a laboratory can set up secure network protocols for data transfers. In establishing these protocols, technicians should review some of the required regulations for transfer of data, including data encryption, applicable file formats, stringent access control, and firewalls. It is important to note that all transferred data for clinical samples is generally de-identified to maintain confidentiality. Technicians should also ensure that sufficient bandwidth is in place to allow for data transfers, especially if data analyses are done off-site or by a third party, and that network stability does not compromise the success of data transfers. The data should be in standard formats, such as FASTQ, VCF or BAM, which makes it easier for the intended user to transfer and access files. Most of the files may be in zipped folders for easier data transfer and to save space. In order to ensure that data was not corrupted during transfers, users may compare the checksums (e.g. md5sum) of both the original and copied file to confirm that the files are the same. Concerning targeted NGS and WGS results, all NGS results should be accompanied by a log file detailing the steps taken to get the result to ensure reproducibility between different NGS runs.

4.7 Ethical considerations for the use of information obtained from human samples

Given that both targeted NGS and WGS approaches yield an unprecedented amount of genetic data, researchers and clinicians should note that special considerations should be made for the collection and use of genetic information obtained from human clinical samples. In the

case of WGS, in particular, it is likely that human DNA present in the initial clinical samples will be amplified and sequenced to some extent, and so it is important that patients are informed of their rights to protection of this data and all plans for sequencing data use, and that these rights are safeguarded when it comes to the use of their personal, genetic data [23]. Relevant issues for genetic data collection and use include the adequacy of the patient's informed consent and the transparency of all research activities conducted in research settings, as well as the storage and follow-up use of clinical samples in this context. While it is essential to ensure that the benefits of genomic medicine are available to TB patients, researchers and clinicians should strive to act as stewards for all research participants and transparent research should be promoted to ensure researcher-participant trust in all contexts. Specific, local ethical and legal frameworks appropriate for the sequencing era of research are urgently required to direct sequencing research and protect human subjects providing clinical samples, especially in resource-limited settings. Until these guidelines are in place, Institutional Review Boards/Ethics Committees will have to analyze the eligibility of different studies and sequencing work on a case-by-case basis, and laboratories seeking to implement sequencing should ensure adequate data protections are in place to ensure the confidentiality of patient data.

4.8 References

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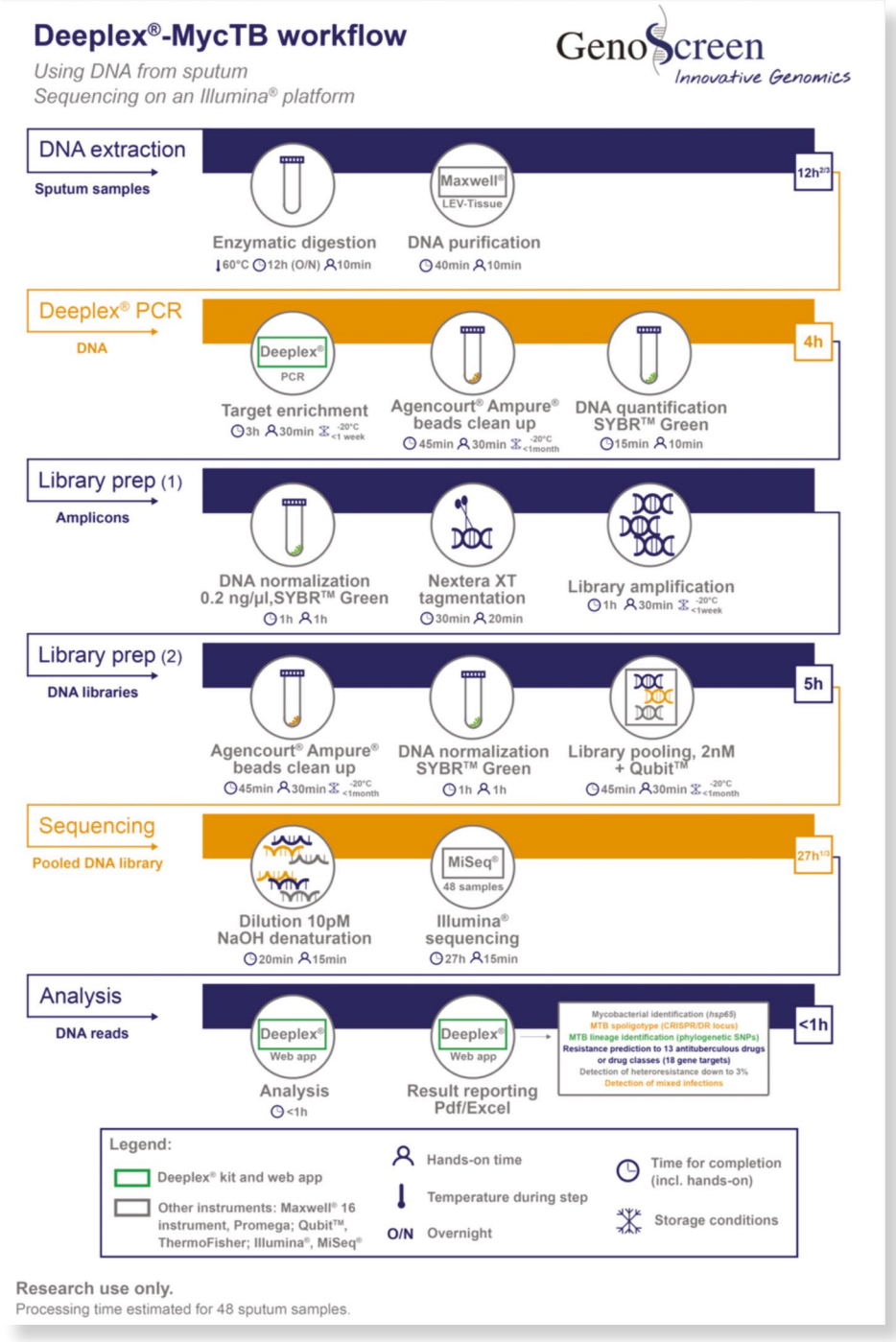
Appendix 1. Example of an End-to-End Sequencing Workflow for TB DST: The Genoscreen Deeplex®-MycTB Targeted NGS workflow for the Illumina MiSeq



The above workflow reflects the most recent Genoscreen Deeplex®-MycTB workflow for performing targeted NGS direct from TB sputum samples using the Illumina MiSeq instrument. The workflow is based on a MiSeq run for 48 tests, including controls. Following the clean-up of amplicon mixtures, DNA should be quantified, with minimal DNA concentration requirements of above 3-5ng/µl necessary to help ensure success of subsequent steps. DNA quantification is also recommended following the clean-up of DNA libraries, prior to DNA normalization, and at the library pool step. For the individual DNA libraries, the minimal target value is any concentration above 0 ng/µl. No DNA quality check is currently recommended at any point in the workflow. The assay contains an internal control to detect any PCR inhibition.

Appendix 2. Example of an End-to-End Sequencing Workflow for TB DST: The Oxford Nanopore Sequencing Technologies WGS workflow for MinION.

A complete nanopore-only assembly of an XDR *Mycobacterium tuberculosis* Beijing lineage strain identifies novel genetic variation in repetitive PE/PPE gene regions

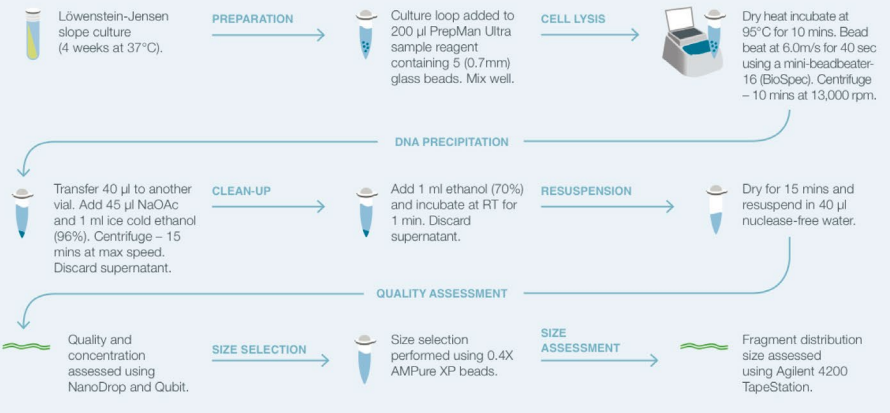
With over 10 million new cases reported each year and an increasing incidence of drug resistant infections, tuberculosis (TB) is one of the leading global threats to human health. To better understand the genomic changes that facilitate the emergence and spread of drug resistant *M. tuberculosis* strains (the predominant cause of TB), researchers at the University of Queensland, Australia, utilised nanopore sequencing to deliver a complete *de novo* genome assembly of a highly-transmissible XDR strain from Papua New Guinea[1].

The MinION allowed the full drug-resistance profile to be determined with complete phenotypic concordance. Novel insights were also gained on GC-rich and repetitive genes that are intractable to traditional short-read sequencing technologies.

Sample preparation

This sample preparation protocol, as described by Bainomugisa *et al*[1] for *M. tuberculosis*, can be applied to other bacterial species.

Sample preparation ~ 1.5 hours



Library preparation

As per 1D Genomic DNA sequencing by ligation for the MinION device using SQK-LSK108, with some amendments[1].

Library preparation ~ 1.5 hours (single sample) ~ 2 hours (multiplex)

Oxford Nanopore also provides transposase-based library preparation kits, which offer a fast, 10-minute workflow. Both ligation- and transposon-based kits are compatible with barcoding, enabling cost-effective analysis of multiple samples on a single flow cell. For more information, visit www.nanoporetech.com.

nanoporetech.com nanoporetech.com/publications

The above workflow reflects the most recently published Oxford Nanopore Sequencing Technologies (ONT) workflow for performing WGS from TB cultures from Bainomugisa et al. [1]. During sample preparation, the DNA purity should be measured by NanoDrop at an OD 260/280 of 1.8 and OD 260/230 of 2.0-2.2. The Qubit input mass was 1 µg. ONT recommends long-term storage of high molecular weight DNA in TE buffer. For DNA fragment sizing, the average size as measured by pulse-field or low percentage agarose gel analysis is > 1 kb, though > 8 kb is considered optimal for WGS. During library preparation, DNA recovery should be measured by Qubit both post end-prep, at ~700 ng, and post adapter ligation, at ~400 ng. All buffers used in the workflow should be free of specific contaminants and surfactants. MinION sequencing results may be fed into data analysis platforms such as Metrichor (ONT) or Mykrobe, as discussed further in Votintseva et al. [2]. It should be noted that DNA extraction may also start from MGIT960 cultures, following the method described by Votintseva et al. to extract and purify DNA [3].

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Appendix 3. Target Product Profile - Detection of resistance associated mutations in *Mycobacterium tuberculosis* complex utilizing Next Generation Sequencing

Definitions and clarifications

- Characteristic – refers to a specific requirement or attribute that is measurable
- Minimal – for a specific characteristic refers to the lowest acceptable output for that characteristic. For clarification, solutions must meet the 'Minimal' standard in order to be acceptable (CAVEAT: a test may still be acceptable if shortcomings pertain to the soft targets or if hard targets are only missed marginally)
- Optimal – for a specific characteristic, provides an output for that characteristic which is considered a 'stretch' from a performance characteristic. Meeting or exceeding the 'Optimal' characteristics provides the greatest differentiation and the greatest impact for the end users, clinicians and patients. Developers would ideally design and develop their solutions to meet 'Optimal' characteristic for as many cases as possible.

Note: The optimal and minimal requirements/ characteristics/attributes define a range for the performance output.

Abbreviations: DST - Drug susceptibility testing, RIF – Rifampin, FQ – Fluoroquinolones, MOX-Moxifloxacin, LVX-levofloxacin, PZA – Pyrazinamide, INH – Isoniazid, AMK-amikacin, EMB-ethambutol, BDQ-bedaquiline, DLD-delamanid, LZD-linezolid, CLF- Clofazimine, CYC – Cycloserine, ETH-ethionamide, XDR-extensively drug resistant TB (INH, RIF, FQ, AMK,), NGS – Next-Generation Sequencing, WGS – Whole Genome Sequencing, Xpert MTB/RIF-GeneXpert MTB & RIF, Mtb – *Mycobacterium tuberculosis*, NTM - non-tuberculous mycobacteria.

Characteristic	Optimal	Minimal	Explanations/Limitations	Ref
Scope				
Key assumptions	• This TPP is intended to provide guidance for development of assays which will utilize Next Generation Sequencing (NGS) as the platform for the detection of mutations in the *M. tuberculosis* complex (MTBC) genomes for drug resistance determination. MTBC as per reference: *M. tuberculosis, M. bovis, M. africanum, M. canetti, M. microti, M. caprae, M. pinnipedi, M. mungi* and *M. orygis*. • This TPP will only provide guidance on the sequencing assay component of far more reaching overall solutions. The assumed input into the assay is DNA/nucleic acid material extracted directly from primary patient samples (e.g. sputum), and output is data to be fed into an analysis backend solution. Separate TPPs for DNA extraction and back-end analysis and archiving will guide their design. • The minimal and optimal characteristics are broad ranges to encompass all types of next generation sequencing (e.g. WGS, targeted NGS) • Though the test for which this TPP is providing characteristics could be utilized for clinical management and other needs, the TPP will focus primarily on the characteristics required for a surveillance sequencing test for the determination of the presence of resistance associated mutations for specified anti-mycobacterial drugs • Timeline considered 5 years to implementation and impact analysis			(1)
Rationale	In the short-term, the assay will provide support for the identification of **optimal,** individualized TB regimen and /or drug selection for treatment at the reference center level for patient populations identified in intended use section. In the longer term, the assay will provide support for guiding **effective first-line TB therapy** in the context of the roll-out of new TB treatment regimens post 2018 The assay can also be used for culture-free surveillance of drug resistant TB direct from primary patient samples at a national level The assay in conjunction with follow up studies can examine clinical relevance of drug resistance allele and wild type allele mixtures			
Intended Use	The intended use of this assay and associated system is to test primary samples (i.e. sputum) from confirmed TB patients at risk for DR-TB for the presence of resistance associated mutations of high confidence in pre-identified loci of interest. The assay aids in the determination of the presence of resistance associated mutations and can add to epidemiologic investigations. This assay is not indicated for use as a stand-alone diagnostic for determining the presence of MTBC. Patient populations: Patients with bacteriologically confirmed rifampicin-resistant MTBC as determined by a validated molecular or phenotypic assay Patients in whom drug resistance is suspected Patients where first line therapy(ies) has failed Patients were second line therapy(ies) has failed			

Characteristic	Optimal	Minimal	Explanations/Limitations	Ref
	Patients where relapse of MTBC infection has occurred Patients who initiated first- line anti-mycobacterial therapy, therapy was discontinued and are now about to re-initiate anti-mycobacterial therapy Patients who have an underlying predisposition that makes treatment difficult (e.g. HIV, diabetes) Patients who have close contact with known M/XDR patient all clinical samples collected under a defined protocol for surveillance efforts			
Goal of Test	To detect and identify a predefined set of MTBC resistance-associated mutations in DNA extracted directly from primary patient specimens (e.g. sputum)		The solution will serve as a reflex test and will be performed only after a patient is confirmed to be infected with MTBC and to be at risk for drug-resistant TB (based on clinical, phenotypic, and/or genotypic evaluations)	
Drug resistance tested*	All anti-mycobacterial drugs for which there is evidence of association with resistance and the correlation of genotypic to phenotypic presentation is known (e.g. all drugs used for treating TB patients)	RIF, INH, FQ, AK, PZA	FQ, AK and PZA have well-documented, and moderately to highly sensitive and specific, known genomic targets. Currently, EMB, BDQ, DLD, LZD, ETH, CFZ, CYC do not yet have well-defined mechanism of resistance or well-documented genomic targets. As more information is gathered, genomic targets for additional resistance makers will need to be included. Considering the 5 year timeframe, we expect major improvements in the knowledge of drug resistance mechanisms.	(2-8)
Assay Design	The assay shall be designed in such a manner that the addition of or removal of genome targets or enhanced performance characteristics of the assay does not require extensive analytical and clinical re-verification and re-validation of the assay as a whole.		The assay should be designed in such a manner that it can be updated with new drug resistance genomic targets as needs arise with minimal re-development, re-validation and re-registration required. The knowledge of new genomic targets that serve as additional resistance markers is expanding and will need to be incorporated into the assay over the life of the assay.	
Target Population	Countries with medium to high TB prevalence by WHO categorization. See intended use for patient population. This test may have additional value for clinical monitoring of patients under treatment.		Children < 11 years of age have limited ability to produce large sputum volumes with sufficient bacterial loads. This makes testing with sequencing methods challenging. Therefore, initial validation studies and intended use claims will focus on patients >11 years old, with the caveat that optimal solutions should include evaluations of assay performance on primary samples from young children (< 11 years) with pulmonary and extra pulmonary TB and DR-TB.	(8, 9)

Characteristic	Optimal	Minimal	Explanations/Limitations	Ref
Target user of test	Laboratory worker able to operate a medium complexity device (as defined by FDA)	Laboratory worker able to operate a high complexity device (as defined by FDA)	Optimally, laboratory workers would only need to move samples from one component of the NGS solution to the next and be able to execute the steps in a manner designed to reduce cross-contamination. The different instruments (i.e. Library preparation and sequencing) involved should have pushbutton ease of use. Minimally acceptable requirements would include that laboratory worker's need to have the ability and training to run standard molecular techniques including PCR for library preparation, preparation and handling of samples (i.e. pipetting, accurate measurement) and loading and running of the sequencing platform.	(10, 11)
Lowest setting of implementation (health system level)	District level hospital (level 2, Primary-level hospital)	Central National TB Reference labs (level 3)	Limitations in setting are defined by: (i) training required to setup, run and maintain a sequencing assay and system; (ii) environmental requirements of sequencing instruments (e.g. minimum to moderate tolerance of extremes in temperature, humidity, electricity supply and dust); and (iii) requirement for sample batching (see section on batching).	(9, 12)
Pricing				
Price for individual test (only includes reagent and consumables; at scale; ex-works)	targeted NGS: < 15 USD/ sample for genotypic DST[1] WGS: < 30 USD/sample for genotypic DST[1]	targeted NGS: < 60 USD/ sample for genotypic DST[1] WGS: < 100 USD/sample for genotypic DST[1]	Cost per sample is defined as cost per complete set of mutation results from a single patient sample. Costs should include a characteristic that addresses cost per patient sample as relative to batch size required to achieve that cost, as cost is directly affected by batch size for most NGS approaches. This is a critical characteristic to help labs determine optimal sample flow for greatest cost efficiency. Modeling data suggests that an all-inclusive assay (i.e. DNA extraction, multiplexed second-line NGS DST + Xpert MTB/RIF, and analysis) would be more cost-effective than GX followed by culture-based DST if the cost was below 45 USD and thus help uptake of solution in LMIC. However, needs to be a balance between what is currently achievable in a cost of goods and what the market can stand.	(13)

[1] Referes to the combined cost of the library assay and sequencing when run at the highest efficiency throughput

Characteristic	Optimal	Minimal	Explanations/Limitations	Ref
Capital costs for instrumentation (library preparation and sequencing instrument)	≤ 50,000 USD	≤ 150,000 USD	Capital costs includes instrumentation for library preparation and sequencing. Current solutions potentially include the need for PCR machine (US $5,000), automated pipetor (US $50-60,000) RT-PCR machine (US $12,000) and NGS sequencing platform (at max US $125,000) Availability of rental options is necessary if price >US$10,000	
Instrument maintenance costs	8% of purchase price/year	15% of purchase price/year	Initial for 2 years, for the entire system (all required system platforms i.e. sample prep etc.)	
Batching (per sequencing run)	1-100 samples	1-20	Maximum flexibility is optimal for clinical care, even at the reference laboratory level, where the ability to handle small batch sizes cost-effectively will ensure optimal utility for a variety of patient load environments. For surveillance, the ability to batch large numbers together is more desirable. Currently, most NGS platform costs (primarily cartridges and reagent kits) are determined per sequencing run, which have been designed to maximize the number of base pairs sequenced per run as until now NGS has been primarily focused on capturing an entire human genome in a single run. As Mtb is a relatively small genome (~4M bp), the only way to make Mtb sequencing runs cost-effective or even "cost-possible" is to batch as many Mtb samples in the run as possible. This is especially true for targeted NGS where only a small fraction of the 4M bp are sequenced per Mtb sample. Batching this many samples, even in a reference laboratory, requires sufficient patient samples be collected before performing a sequencing run, which increases TTR for each individual patient.	

Characteristic	Optimal	Minimal	Explanations/Limitations	Ref
Batching (for library preparation)	1-96 samples	1-20	Maximum flexibility is optimal. For clinical care, even at the reference laboratory level, the ability to handle small batch sizes cost-effectively will ensure optimal utility for a variety of patient load environments. For surveillance, the ability to batch large numbers together is more desirable. Ideally, library preparation batching should be optimized to match desired sequencing batching in order to maximize efficiency and workflow.	
Performance				
Diagnostic sensitivity of sequencing against phenotypic reference standard	> 99% sensitivity for detection of phenotypic resistance to RIF > 90% sensitivity for detection of phenotypic resistance to INH, FQ, AK, PZA Estimates of optimal sensitivity of sequencing as a predictor of phenotypic resistance to BDQ, DLD, LZD, ETH, CYC and CFZ has not yet been determined	> 95% sensitivity for detection of phenotypic resistance to RIF > 90% sensitivity for detection of phenotypic resistance to INH, and FQ > 85% sensitivity for detection of phenotypic resistance to AK, and PZA	While the genetic mutations that confer phenotypic resistance to most first and second-line TB drugs have been identified and well-documented, detection of these mutations is still an indirect measurement of phenotypic resistance, given that expression of phenotypic resistance is affected by other unmeasured or unmeasurable variables, and that characterization of phenotypic resistance is itself imperfect for many drugs. The diagnostic sensitivity of even a 100% accurate sequencing assay against a phenotypic reference standard will therefore rarely reach 100%. Optimal and minimal thresholds for diagnostic sensitivity from any sequencing platform therefore need to exceed or match the best available estimates for the highest sensitivity likely achievable based on large, globally relevant data sets. The minimal threshold sensitivity set here for RIF, INH, FQs, AK and PZA as are consistent with estimates of the global sensitivity of sequencing as a predictor of phenotypic resistance as determined in a systematic review of the literature completed in March 2016. Optimal thresholds are estimates of what should be achievable with an optimal NGS solution with expanded genetic targets and better defined phenotypic reference standards.	(14-21)

Characteristic	Optimal	Minimal	Explanations/Limitations	Ref
			Estimates of sensitivity for prediction of phenotypic resistance to BDQ, DLD, LZD, ETH and CFZ are still being developed. It should be noted that the presence of heteroresistance or mixed populations may also affect sensitivity when comparing genotypic data to phenotypic resistance. Special care will be needed in setting thresholds of resistance identification in low level subpopulations. (see section on mixed population /heteroresistance below)	
Diagnostic specificity of sequencing against phenotypic reference standard	The specificity of sequencing as a predictor of phenotypic reference standards for all drugs shall exceed 98%	The specificity of sequencing as a predictor of phenotypic reference standards for all drugs shall exceed 95%	While the genetic mutations that confer phenotypic resistance to many first and second-line TB drugs have been identified and well-documented, detection of these mutations is still an indirect measure of phenotypic resistance, given that expression of phenotypic resistance is affected by other unmeasured variables, and that characterization of phenotypic resistance is itself imperfect for many drugs. The diagnostic specificity of even a 100% accurate sequencing assay against a phenotypic reference standard will therefore rarely reach 100%. Optimal and minimal thresholds for diagnostic specificity from any sequencing platform therefore need to exceed or match the best available estimates for the highest specificity likely achievable based on large, globally relevant data sets. Additionally, interpretation of sequencing data should only include genetic mutations for which the documented specificity exceeds 95%. The thresholds set here are consistent with estimates of the global specificity of well-documented genetic mutations associated with phenotypic resistance as determined in a systematic review of the literature completed in March 2016.	(14-17, 20, 21)

Characteristic	Optimal	Minimal	Explanations/Limitations	Ref
Diagnostic sensitivity of sequencing against genetic reference standard	>98% sensitivity for detection of targeted SNPs for resistance to RIF, INH, FQ, AK, PZA when compared to a well characterized genetic sequencing reference standard			
Diagnostic specificity of sequencing against genetic reference standard	The specificity of sequencing as a predictor of genetic sequencing reference standards for all drugs shall exceed 98%			
Limit of Detection – mutation detection (detection of majority population – mutation or WT for a specific position)	Targeted NGS: 100 genomes in a reaction (0.5 pg of Mtb DNA) WGS: 2.03 x 10^3 genomes in a reaction (equivalent to 10 pg of Mtb DNA)	Targeted NGS: 5,000 genomes in a reaction (0.025 ng of Mtb) WGS: 2.03 x 10^5 genomes in a reaction (equivalent to 1 ng of Mtb DNA)	Limit based on genomic copies needs to be determined from DNA input into assay. (for context scanty sputum smear positive = 5,000 Mtb genomic copies/mL) The number of organisms is refereeing to the amount of purified DNA after extraction, and independent for the efficiency of the DNA extraction performed.	
Limit of Detection – heteroresistance (detection of minority population - mutation or WT for a specific position)	Targeted NGS: 1 resistant organism in with 99 resistant organisms in a reaction (1%) WGS: 20 resistant organisms with 2010 susceptible in a reaction (1%)	Targeted NGS: 500 resistant organisms in with 4500 resistant organisms in a reaction (10%) WGS: 2.03 x 10^4 resistant organisms with 1.83 x 10^5 susceptible in a reaction (10%)	The number of organisms is referring to the amount of purified DNA after extraction, and independent for the efficiency of the DNA extraction performed.	

Characteristic	Optimal	Minimal	Explanations/Limitations	Ref
Analytical specificity (WGS)	No cross reactivity of drug resistance gene regions with other non-MTBC organisms including NTM. Identification of MTBC species	Minimal cross reactivity of drug resistance gene regions with other non-MTBC organisms including NTM	Almost none of the drug resistance gene targets utilized to date for predicting phenotypic drug resistance in MTBC appear to cross react with NTMs with the exception of targets in the *rrs* and *eis* genes which are used for prediction of AK resistance and can theoretically cross-react with NTMs based on sequence homology observed. Optimal NGS solutions will be able to identify MTBC species detected in sample (as per FDA reference: *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. canetti*, *M. microti*, *M. caprae*, *M. pinnipedi*, *M. mungi* and *M. orygis*) but minimal solutions would not include that ability.	
Analytical specificity (targeted NGS)	No cross reactivity of drug resistance gene targets with other non-MTBC organisms including NTM. Identification of MTBC species optimal	Minimal cross reactivity of drug resistance gene targets with other non- MTBC organisms including NTM	Almost none of the drug resistance gene targets utilized to date for predicting phenotypic drug resistance in MTBC appear to cross react with NTMs with the exception of targets in the *rrs* and *eis* genes which are used for prediction of AK resistance and can theoretically cross-react with NTMs based on sequence homology observed. All sequencing assays should include an evaluation of target-specific cross reactivity with NTMs to demonstrate no cross-reactivity with a selection of reference standard NTMs. Optimal NGS solutions will be able to identify MTBC species detected in sample	(1)
Indeterminate results detection	<2% within samples that meet the LOD criteria	< 5% within samples that meet the LOD criteria	Not more than 2% (optimal) and 5% (minimal) of samples sequenced should have indeterminate results. Indeterminate results are defined as outputs from the NGS sequencer that fail to identify either wildtype or mutated sequence at the target gene regions due to error or lack of coverage. All sequencing platforms need to include a validation analysis of error rates for the sequencing chemistry and assay performance with diverse clinical specimens (i.e. specimens that contain a representative diversity of resistance mutations and combinations of mutations).	

Characteristic	Optimal	Minimal	Explanations/Limitations	Ref
Mixed population/ heteroresistance detection	Be able to identify known resistant alleles down to ≤ 1% of an ad-mixture (ex. 10 resistant reads in 1,000 reads)	Be able to identify known resistant alleles down to <10% of an ad-mixture (ex. 10 resistant reads in 100 reads)	Mixed populations or heteroresistance refers to the presence of more than one allele at a particular genomic position (presence of both wild type and resistant allele) in a single clinical sample. Can be due to spontaneous mutation in a clonal infection or an infection with multiple strains. While the clinical relevance of the presence of low-level mixed populations (<10%) are still unproven, a mix of ~10% resistant alleles are likely to result in a resistant phenotype when submitted for phenotypic DST by growth-based methods. Therefore, NGS platforms with the ability to accurately detect ~10% resistant alleles are more likely to be concordant with phenotypic testing than platforms unable to detect those low-level proportion of resistant alleles. While the clinical relevance of resistant alleles making up <10% of the population in a clinical sample has yet to be clearly documented, NGS platforms aiming to monitor the presence and change of low-level resistant populations should have the ability to detect at least 1% resistant alleles among 99% wildtype genomes in order to detect minor changes in population proportions accurately, which will increase knowledge on the effects of low level populations and patient outcomes.	(22-26)
Characterization of population mixtures	Identification of portion of alternate/wildtype alleles	Identification of mutations with no quantification	Optimally, NGS platforms should have sufficient read quality and reproducibility to quantify the proportion of resistance/wildtype alleles in all targeted gene regions. NGS platforms claiming this capability should be able to demonstrate analytical reproducibility and consistency of the ratio of alleles from known mixture standards.	
Interfering substances	No interference by those substances known to occur in human respiratory and pulmonary tracts, including blood and anti-mycobacterial drugs that could potentially inhibit a PCR reaction. Substances used to treat and / or alleviate respiratory disease or symptoms.		Relevant interfering substances include, but are not limited to, endogenous substances such as blood and mucus, and exogenous substances such as topical nasal and throat medications and oral medications that may be secreted into respiratory secretions.	(1)

Characteristic	Optimal	Minimal	Explanations/Limitations	Ref
			(Refer to Table 3. Substances and Classes for Interference Testing in the Class II Special Controls Guideline: Nucleic Acid-Based In Vitro Diagnostic Devices for the Detection of Mycobacterium tuberculosis Complex in Respiratory Specimens - Guideline for Industry and Food and Drug Administration Staff)	
Operational				
Sample type	MTBC DNA extracted directly from primary patient samples and primary cultures		It is expected that NGS platforms will require a DNA extraction step prior to library preparation and sequencing. Optimal solutions will successfully sequence MTBC DNA extracted directly from patient samples such as raw sputum, sediment, or other EPTB samples, and will not require any MTBC culture step before DNA extraction. This will enable broad use of NGS technology in facilities with no MTBC culture facilities, and to accelerate use of "culture-free" surveillance methods globally. Minimal criteria include DNA from MTBC culture as it is not currently feasible to reliably perform WGS on MTBC DNA extracted directly from clinical samples.	
Manual sample prep (total hands-on steps after obtaining DNA)	Maximum 2 steps	Maximum 10 steps	Optimal is PCR/library preparation in 1 step, sequencing as step 2. Steps include DNA amplification, multistep library preparation and QA/QC of prepared libraries Once extracted DNA has entered the library preparation pipeline for sequencing, instrument operator intervention should be kept to a minimum, optimally only requiring intervention to progress the sample through the different instruments.	(27, 28)
Reagent integration	Maximum 2 external reagents	Maximum 4 external reagents	The existing sequencing kits include 1 prep kit and 1 seq kit, but the prep kit has several reagents in it.	

Characteristic	Optimal	Minimal	Explanations/Limitations	Ref
Time-to-result (library prep and sequencing)	≤ 24 hrs	≤ 72 hrs	While NGS solutions are not currently envisioned to be point-of-care assays, the time-to-result (TTR) is still an important parameter for determining optimal use of the assay as a clinical diagnostic since optimal patient care requires rapid decision making. NGS TTR is influenced primarily by NGS run parameters (sequence read length, sequencing strategy, depth of reads etc.) and needs to be balanced against the value of each of these parameters to the accuracy/utility of the NGS assay. While batching requirements will set the minimum number of samples that need to be collected before a run can start, it is still optimal to have library prep and seq runs take <=24 hours overall when used as a diagnostic assay. Time-to-result for NGS-based surveillance is less critical.	(29, 30)
Maximum daily throughput	> 100	No minimal for max daily throughput due to the minimal time being greater than 24 hrs.	Optimal maximum daily throughput is based on one run of one instrument deployed at a reference laboratory.	(31)
Biosafety	Universal precautions		Extracted MTBC DNA is the starting sample for sequencing DST solutions, there should be minimal biosafety concerns. DNA extraction front end solutions will need to demonstrate that extracted DNA does not contain remnant infectious material.	(32, 33)
Waste disposal solid	Normal disposal with care of amplified material so as to not contaminate PCR workspace and sequencing runs.		No infectious waste disposal required.	
Waste disposal Infectious material	No infectious waste disposal		Extracted MTBC DNA is the starting sample for sequencing DST solutions, there should be no infectious waste material waste. DNA extraction front end solutions will need to demonstrate that extracted DNA does not contain remnant infectious material.	

Characteristic	Optimal	Minimal	Explanations/Limitations	Ref
Multi-use platform	Yes (ideally at least HIV, HCV, HBV on the same instrument)		NGS platforms should be open platforms, capable of sequencing whatever amplicon or whole genome DNA is used as the template for sequencing. While it is recognized that amplicon design for targeted NGS, library preparation and downstream analysis of NGS data might need to be tailored for specific pathogens, optimal platforms will require minimal to no reagent, process or hardware adaptation to accommodate a wide variety of pathogen DNA as source material. Multi-use platforms will increase utility, improve cost-effectiveness and accelerate uptake of NGS technology.	
Instrumentation	Single integrated system ideally modular to allow module expansion of throughput	Up to 3 instruments that are independent of one another	Ideally a single device is preferred for efficiency and speed, but up to three modules are acceptable (e.g., PCR for library prep, PCR or RT-PCR for quality control and sample standardization, and a sequencing instrument).	
Power requirements	Capable of running off standard electrical outlet as supplied currently worldwide plus UPS (to complete current cycle); circuit protector. UPS and circuit protector must be integrated within the instrument or instruments.	Capable of running off standard electrical outlet as supplied currently worldwide plus UPS (to complete current cycle); circuit protector.	As NGS solutions intended deployment environments are reference laboratories, no special power requirements beyond those stated are required.	(27, 28)
Maintenance/ calibration	Preventative Maintenance 2 years; include maintenance alert; remote or no calibration	Preventative Maintenance 1 year; include maintenance alert; remote or no calibration	A maintenance alert is necessary to ensure proper functionality in settings where it is unlikely that the same person will always handle the device and records will be kept on duration of use. Furthermore, it will be essential that only simple tools/ minimal expertise is necessary to do the maintenance given the quantity of devices that is likely going to be in use and service visits are unlikely to be feasible outside of urban settings.	(34-36)

Characteristic	Optimal	Minimal	Explanations/Limitations	Ref
Data analysis	Integrated data analysis to generate a standard FASTQ file		A separate TPP will address the backend analysis and interpretation of FASTQ files Minimum standards for data to be included in "standardized" FASTQ fie. The FASTQ file output should be able to be easily converted in a FASTQ file format which is defined in the backed analysis TPP	
Regulatory requirements	Assay and platform manufacturing in compliance with ISO EN 13485 - 2010 (Medical Device Management Standard) or higher standards and/or regulations and in compliance with ISO IEC 62304 Medical Device Data Systems. Manufacturing facility certified and authorized for use by a stringent regulatory authority that is a member of the of the International Medical Device Regulators Forum (IMDRF) formerly known as Global Harmonization Task Force (GHTF); registered for in vitro diagnostic manufacturing. Assay and platform development in compliance with ISO EN 13485 and ISO EN 14971 - 2007 (Medical Device Risk Management)			
Instrument performance data export (connectivity and interoper-ability)	Full data export (on usage of device, error/invalid rates, and personalized, protected results data) over USB port and network. Network connectivity through Ethernet, WiFi, and/or GSM/UMTS mobile broadband modem. Results should be encoded using a documented standard (such as HL7) and be formatted as JSON text.	Integrated ability for full data export from the device in a user-friendly format (on usage of device, error/invalid rates, and non-personalized results data) over USB port.	We expect LIMS connectivity capacity to be available at reference laboratory level of deployment.	(27, 28, 37)

Characteristic	Optimal	Minimal	Explanations/Limitations	Ref
	JSON data should be transmitted through HTTP(S) to a local or remote server as results are generated. Results should be locally stored and queued during network interruptions and sent as a batch when connectivity is restored.			
Electronics and software	Integrated		Separate PC or user interface hardware (e.g. tablet) for running the sequencing platform not ideal due to added complexity of interoperability requirements, maintenance and updating.	
Operating temperature/ humidity	Between +5 to +40°C 90% non-condensing humidity	Between +19 to +25°C 75% non-condensing humidity	While high environmental temperatures and high humidity are often a problem in countries where TB is endemic, optimal and minimal thresholds were based on expectation of some environmental control in reference laboratories and district hospitals where NGS is expected to be deployed. Current minimums are based on Illumina specifications	(27, 34, 38)
Reagent Kit transport	No cold chain required; Tolerance of transport stress for a minimum of 72 hrs. at –20 to +50°C	Tolerance of transport stress for a minimum of 72 hrs. at 0 to + 40°C	Refrigerated transport is costly and often cannot be guaranteed during the entire transportation process. Frequent delays in transport are commonplace. Optimal kits will therefore not require refrigeration.	(27, 28, 38)
Reagent Kit storage/ stability	2 years	6 months	High environmental temperatures and high humidity is often a problem in many countries where TB is endemic especially in transport of reagents/systems.	(27, 28, 34)

Characteristic	Optimal	Minimal	Explanations/Limitations	Ref
Reagent Kit temperature storage/ stability	+15°C to +30°C, 80% non-condensing humidity	-10°C to 40°C, 80% non-condensing humidity	High environmental temperatures and high humidity is often a problem in many countries where TB is endemic especially in transport of reagents/systems. Current systems require refrigeration or freezing of reagents to enable stable storage.	(27, 28, 34, 38)
Additional supplies (not included in kit)	Pipettes, Pipette tips. Standard supplies for PCR		As intended deployment is a reference laboratory and minimal instrumentation and training include PCR use, standard PCR supplies will be needed. However, minimal performance requirements and product specifications will be determined and provided as part of the overall procedure	
Internal Process Quality Control	Full process control, controlling for sample processing, amplification and detection. Positive control to ensure assay is functioning and negative controls for examination of cross contamination. This includes examining cross-contamination of samples during the sample processing, during sequencing, and minimize false positive results due to contamination or carryover of samples on the sequencer.		NGS assay should include at least one MTBC DNA target to confirm the presence of MTBC DNA in a patient sample. This MTBC target (or set of targets) should be present in every sample, if they are not present it signals a sample failure. Every sequencing run should have an overall sequencing run positive and negative (no template) controls to ensure the sequencing portion is working appropriately and to examine error rates for each run. The positive control DNA should be a well characterized MTBC strain, and thus if the positive control produces a different result than expected than a possible error in sample prep or sequencing has occurred. If the negative control generates a call of >10% than contamination has occurred, and the samples will need to be reevaluated. Other control processes maybe developed and used.	(34-36, 39)
Training & education needs	3 days (or 24 work hrs.) at the level for a laboratory technician	1 week (or 40 work hrs.) at the level for a laboratory technician	Optimally, training should be minimal and include instruction on simple sample handling, contamination control and instrument use and be completed in three days. Acceptable training would be longer to accommodate more complex sample handing, contamination control and multiple instrument use and instruction.	

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