The use of next-generation sequencing for the surveillance of drug-resistant tuberculosis

An implementation manual

World Health Organization

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Diagnosis for all

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Contents

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Abbreviations and acronyms

BSL	biosafety level
DNA	deoxyribonucleic acid
DR-TB	drug-resistant TB
DST	drug susceptibility testing
EQA	external quality assessment
FIND	Foundation for Innovative New Diagnostics
FQ	fluoroquinolone
IT	information technology
LMIC	low- and middle-income countries
M&E	monitoring and evaluation
MDR-TB	multidrug-resistant TB
MoH	ministry of health
MTBC	Mycobacterium tuberculosis complex
NGS	next-generation sequencing
NSP	national strategic plan
NTP	national TB programme
NTRL	national TB reference laboratory
QA	quality assurance
QC	quality control
RR-TB	rifampicin-resistant TB
SOP	standard operating procedure
SRL	supranational reference laboratory
TB	tuberculosis
tNGS	targeted next-generation sequencing
TWG	technical working group
UI	uncertainty interval
WHO	World Health Organization
WGS	whole genome sequencing
WRD	WHO-recommended rapid diagnostic test

Glossary of terms related to next generation sequencing

Amplicon – A specific fragment of DNA that is replicated millions of times using the polymerase chain reaction (PCR) amplification process. Amplicons are the starting material for library preparation during targeted next-generation sequencing (tNGS).

Bioinformatics pipeline – A series of analyses, algorithms and programs executed in a predefined sequence to process, analyse, interpret and report NGS results.

Breadth of coverage –The percentage of the intended reference genome for which the genomic positions (bases) were sequenced with minimal selected coverage.

Depth of coverage – The number of sequencing reads at a given position in the sequenced genome. The more reads there are, the more confidence there is that the sequence is accurate.

DNA library – The processed sample material that serves as the input material for NGS. A DNA library is obtained by fragmenting and sorting DNA to obtain fragments of a predefined length and attaching oligonucleotide adaptors to the ends of the fragments to enable tNGS or whole genome sequencing (WGS).

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

Next-generation sequencing – A high-throughput sequencing method used to determine the nucleotide sequence of a genome in a single biochemical reaction. NGS is performed by non-Sanger-based sequencing technologies that can sequence multiple DNA fragments in parallel; the sequences are then assembled and mapped to a reference genome using bioinformatics analyses.

NGS laboratory workflow – A series of laboratory procedures required to generate raw NGS data from a sample. The workflow typically includes sample processing, DNA extraction, fragmentation or amplification, and preparation and sequencing of the library.

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. All NGS manufacturers use Phred scores as the measure of sequence quality reporting.

Reference genome – The validated and published sequence of a known genome, gene, or artificial DNA construct. A sequence produced by an NGS instrument may be aligned to a reference sequence to assess NGS accuracy or to find nucleotide changes (mutations).

Sanger sequencing – Technique for DNA sequencing based on the incorporation of chain-terminating dideoxy-nucleotides by DNA polymerase during in vitro DNA replication.

Sequence alignment – An algorithmic approach to match consecutive nucleotide bases in one sequence with another sequence to identify genetic variation between the sequences.

Sequence variants – Differences at specific positions between two aligned sequences. Variants include single-nucleotide polymorphisms, insertions and deletions, 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.

Targeted NGS – NGS of specific regions in a genome. Generally, tNGS is focused on sequencing a select set of genes or gene regions that have known or suspected associations with a specific pathogen, lineage, or a specific phenotype (e.g., drug resistance).

Whole genome sequencing – 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.

1. Introduction

This document provides practical guidance on planning and implementing next-generation sequencing (NGS) technology for characterization of *Mycobacterium tuberculosis* complex (MTBC) bacteria. The aim is to detect mutations associated with drug resistance in the context of a surveillance system for tuberculosis (TB). It complements two other publications on TB:

- *The use of next-generation sequencing technologies for the detection of mutations associated with drug resistance in Mycobacterium tuberculosis complex: technical guide (1)*, which provides an overview of NGS methods and workflows, and a comprehensive review of the scientific evidence on characterization of the genetic basis of phenotypic drug resistance to major anti-TB drugs; and
- *Guidance for the surveillance of drug resistance in tuberculosis (2)*, which provides guidance for establishing continuous surveillance systems for drug-resistant TB (DR-TB) or, in settings where this is not yet possible, for conducting periodic surveys. This includes the incorporation of NGS into laboratory testing algorithms for DR-TB surveillance.

Whole genome sequencing (WGS) is a valuable tool for the surveillance of DR-TB *(3)*. This guide offers a framework for making decisions about NGS-based drug susceptibility testing (DST), a roadmap for implementation and practical guidance for country planning and implementation of NGS-based DST within a DR-TB surveillance system.

The guidance presented here assumes that a country already has a DR-TB surveillance system that meets its current and projected needs and has decided to incorporate the use of NGS-based DST into either a periodic survey or a continuous surveillance system. The document includes the following key components:

- Understanding recommended uses of NGS for surveillance in different settings, including consideration of TB epidemiology in a given country, laboratory system infrastructure, and objectives of the national surveillance system and the national strategic plan (NSP) for TB.
- Assessing country readiness to implement NGS, including why, where, and how to implement it.

- Managing supplies, forecasting, procurement, and distribution.
- Planning site-level readiness assessment, installation, and set-up of sequencing equipment.
- Validating NGS protocols and embedding strong quality assurance (QA) mechanisms at each step.
- Building capacity among laboratory staff, bioinformatics staff, clinicians, and programme staff to incorporate NGS into the DR-TB surveillance system.
- Ensuring coordination among donors and partners supporting implementation of NGS in countries; and
- Stablishing a framework to measure the impact of NGS on the effectiveness of the DR-TB surveillance system.

This guide does not address implementation considerations for the routine use of NGS-based DST to guide patient care decisions.

1.1 Target audience

This guide is intended to inform staff of national TB programmes (NTPs) and ministries of health (MoHs), implementing partners, laboratory managers and technical staff, clinicians, donors, and other stakeholders engaged in DR-TB surveillance and TB laboratory strengthening.

1.2 Global burden of DR-TB

Globally, TB remains an enormous public health problem, with an estimated 10.6 million (95% uncertainty interval [UI]: 9.9-11 million) people developing TB in 2021 *(4)*. A major threat to achieving the goal of ending the global TB epidemic by 2030 is the emergence and spread of DR-TB, more specifically, rifampicin-resistant TB (RR-TB), which includes multidrug-resistant TB (MDR-TB) (i.e., TB with resistance to both rifampicin [RIF] and isoniazid [INH]). In 2021, there were an estimated 450 000 (95%UI: 399 000-501 000) new cases of RR-TB *(4)*.

The World Health Organization's (WHO's) End TB Strategy emphasizes that, to combat the spread of MDR/RR-TB, all bacteriologically confirmed TB patients should receive DST at least for rifampicin and all patients with RR-TB should receive DST at least for fluoroquinolones. In 2021, 70% of patients with bacteriologically confirmed TB were tested for resistance to rifampicin *(4)*. Furthermore, in 2019, an estimated 1.1 million people (95%UI: 0.6-1.5 million) had TB that was resistant to isoniazid and susceptible to rifampicin, referred to as Hr-TB *(5)*. Most cases of Hr-TB are not detected, given that testing for resistance to isoniazid is often performed only for those with RR-TB. Hence, many people with TB do not receive the most appropriate treatment regimen.

Compared with people with drug-susceptible TB, people with Hr-TB who are treated with the standard regimen for drug-susceptible TB have a much higher risk of treatment failure (11% vs 2%), relapse (10% vs 5%) and acquiring additional drug resistance (8% vs 1%) *(6)*.

Detection of DR-TB requires DST using rapid molecular tests, culture-based phenotypic testing methods or sequencing technologies *(7)*. The introduction of WHO-approved rapid molecular tests for the detection of drug resistance has greatly improved the timeliness, reliability, and availability

of DST in resource-limited settings, particularly with respect to the detection of rifampicin resistance. The Xpert MTB/RIF and Xpert Ultra tests (Cepheid Inc., Sunnyvale, CA, United States of America [USA]) and line-probe assays (e.g. GenoType® MTBDRplus, Hain Lifescience, Nehren, Germany) were important in increasing the detection of laboratory-confirmed RR-TB and MDR-TB from 41 000 patients in 2009 to more than 166 991 patients in 2021 *(4)*. Detection of MDR/RR-TB is critical, but comprehensive DST (i.e., DST for all drugs potentially included in a treatment regimen) is also needed to fully understand the burden of DR-TB and ensure effective treatment.

1.3 Current approaches to DR-TB surveillance

DR-TB surveillance systems may include:

- **continuous surveillance**
- **periodic surveys**
- **sentinel surveillance.**

A thorough understanding of the epidemiology of DR-TB, including the spectrum and emergence of drug resistance over time, is needed to plan diagnostic and treatment services for patient care, monitor the effectiveness of TB control interventions and review the performance of WHO-recommended treatment regimens *(8)*. DR-TB surveillance systems are designed to address these issues by collecting and analysing data on anti-TB drug resistance in a systematic and ongoing manner *(2)*. The two main approaches to DR-TB surveillance that produce countrywide data are continuous surveillance systems based on routine DST for all patients with bacteriologically confirmed TB and periodic surveys of a representative sample of patients with TB. A third approach, sentinel site surveillance, may be a useful interim approach for monitoring DR-TB in selected sites in countries where a continuous surveillance system is not yet feasible. However, data from sentinel surveillance are not nationwide and cannot be used to estimate the burden or trends of DR-TB in a country. WHO has developed updated guidance on the establishment of routine continuous surveillance systems or the planning, implementation and analysis of periodic surveys *(2)*.

In high-income countries, most patients with bacteriologically confirmed TB receive DST routinely. An increasing number of low- and middle-income countries (LMICs) are transitioning towards establishing continuous surveillance systems for DR-TB by taking advantage of the expansion of rapid molecular technologies. However, in high TB burden settings, routine universal DST is not yet feasible because of cost and capacity constraints, and the burden of DR-TB is usually estimated through nationwide, periodic surveys using laboratory algorithms that rely on culture-based phenotypic DST or molecular DST, or a combination of the two. Countries working towards a continuous surveillance system may consider a phased approach that starts by targeting certain high-risk groups (e.g., contacts of patients with DR-TB, patients whose treatment failed and previously treated patients) and expands as resources permit.

Historically, DR-TB surveys have relied on culture-based phenotypic DST in liquid or solid media; such surveys take weeks to generate results and have sophisticated biosafety requirements that are difficult to establish and sustain in LMICs. Large numbers of survey samples can overwhelm culture and DST capacity and have a negative impact on routine clinical testing at national reference laboratories or other participating laboratories. Furthermore, culture-based phenotypic DST requires safe and temperature-controlled transport of specimens containing viable bacteria from the collection sites to testing laboratories. It also has limitations related to the reliability

and reproducibility of the test methods for some drugs. These factors make it difficult to repeat surveys using culture-based phenotypic DST at frequent intervals, particularly in resource-limited countries.

Molecular DST holds promise to overcome some of these obstacles. Methods for molecular DST for TB rely on the detection of mutations associated with phenotypic resistance to medicines currently used for the treatment of TB (e.g., rifampicin, isoniazid, and fluoroquinolones). Significant advances in understanding the genetic basis of drug resistance in MTBC bacteria have enabled reliable prediction of most of the clinically relevant and globally prevalent resistant phenotypes, based on results of molecular DST. Currently available WHO-approved rapid molecular drug susceptibility tests can be used to detect specific mutations known to confer phenotypic resistance. The tests can also be used to detect the presence of a mutation by the lack of probe hybridization to the wild-type sequence, which is used to infer phenotypic resistance. However, these tests are limited in their capacity to interrogate all of the known genomic targets associated with resistance (reducing the sensitivity of the test); also, in some cases, they lack the ability to differentiate between mutations that confer resistance and those that do not (e.g., synonymous substitutions that do not alter the protein sequence), which in turn decreases test specificity.

Molecular DST based on NGS can be used to identify resistance-conferring mutations in all known genomic targets. NGS refers to techniques that rely on the sequencing of multiple DNA fragments in parallel, followed by bioinformatics analyses to assemble the sequences. The technologies can be used to determine the nucleotide sequence of an entire genome (i.e., WGS) or part of a genome (i.e., targeted NGS [tNGS]) in a single sequencing run.

The primary use of NGS is to detect mutations known to confer resistance to rifampicin and other first-line and second-line drugs, and thereby categorize a strain as drug-resistant or drug-susceptible. However, NGS can provide much more information than just the prevalence of DR-TB; it can contribute to the surveillance of TB and DR-TB, and to research and development of global importance. WGS data can be used to assess the prevalence, emergence and spectrum of mutations conferring resistance in a population; it can also provide information on the genotypes (clades) of MTBC strains circulating in the community and transmission dynamics, including outbreak identification. Combining WGS data with phenotypic DST data makes it possible to assess the performance (sensitivity and specificity) of different molecular DST tools in given settings; it can also allow the identification of novel mutations and resistance-conferring loci, which in turn could inform the development of new diagnostic tests or improvement of existing tests.

Sequencing has the potential to both expand global comprehensive DST and transform the diagnosis and treatment of DR-TB. Routine use of NGS-based DST has already been implemented in large public health systems in high-resource settings such as England, the European Union and the USA, where it is being used to reduce their reliance on culture-based phenotypic DST *(9)*.

1.4 Incorporating NGS into DR-TB surveillance

NGS methods are well suited for use in DR-TB surveillance systems because they can provide reliable information for many anti-TB drugs in a single test, and they can detect mutations that may be missed by other molecular assays. Depending on the throughput and number of drugs to be tested, NGS may be more cost-effective than other methods for comprehensive DST (e.g., testing for all drugs that might be used in a country's treatment regimens), particularly phenotypic DST.

Advantages of NGS-based DST for DR-TB surveillance:

- Tests many drugs in a single assay.
- Provides results for drugs for which phenotypic DST is unreliable.
- Detects heteroresistance.
- Distinguishes resistance-conferring mutations from other mutations.
- Can be done directly from a sputum specimen (tNGS).
- If done from a culture, it does not require a "pure" culture of MTBC bacteria.
- Provides additional information about molecular epidemiology; and
- Is potentially less costly than phenotypic DST, although start-up costs are high.

In a landmark study of about 12 000 samples from 13 endemic countries, NGS-based DST overcame many of the challenges of phenotypic-based DR-TB surveillance and the limitations of other molecular DST methods by providing resistance profiles for a greater range of drugs in less time and for more countries *(3)*. However, few LMICs currently have the capacity to perform NGS in-country. Recent periodic surveys in countries such as the Democratic Republic of the Congo *(10)*, Djibouti *(11)*, Eritrea *(12)* and Eswatini have used NGS, but the testing was conducted by the relevant WHO TB supranational reference laboratory (SRL). Expanding this approach to more countries brings challenges related to requirements for rigorous sample safety and shipping regulations, and data transmission and storage needs. The uptake of sequencing by LMICs has so far been limited, mainly owing to the perceived technical and cost barriers for integration of NGS into existing laboratory workflows, the need for expert guidance regarding the management and interpretation of sequencing data, lack of availability of NGS distributors and technical support, and lack of readily available standardized workflows including data analysis solutions *(13)*. Uptake has also been slowed by the lack of end-to-end (turnkey) solutions for NGS workflows; that is, solutions that package the discrete elements of the sequencing workflow into a standardized and complete solution from sample collection to data analysis and interpretation. This is the case despite the availability of a target product profile for sequencing to aid manufacturers in their product design *(1)*.

To be an effective approach for DR-TB surveillance, NGS-based DST must be accurately interpreted and used to predict a drug-resistance profile. Understanding of the association of specific mutations with the expression of phenotypic drug resistance has expanded rapidly over recent years. Research consortia including ReSeqTB, CRyPTIC and TB Portals have collated global genotypic and phenotypic data from many thousands of MTBC strains. In a 2018 systematic review *(14)*, a statistical method for grading mutations was shown to be useful for defining the association between specific resistance mutations and phenotypic drug resistance. The clinical grading system was developed using an expert, consensus-driven approach and it provides guidance to assess

the clinical relevance of any detected mutation. Using a similar approach, WHO has developed a comprehensive catalogue of confidence-graded mutations associated with drug resistance to harmonize the interpretation of NGS data *(15)*; this catalogue will be regularly updated as more data become available.

WHO also hosts a TB sequencing database and platform *(16)* to:

- Support standardized analysis of NGS data and associated metadata and provide a repository for storage and safeguarding of global TB datasets from countries, academic institutions and other stakeholders.
- Contribute to the global surveillance of DR-TB, including prevalence of resistance to different drugs; and
- Facilitate the maintenance of the aforementioned WHO *Catalogue of mutations in Mycobacterium tuberculosis complex and association with drug resistance (15)*.

1.5 Overview of NGS methods

WGS is used to sequence an entire genome using NGS technology (Table 1). In addition to investigating drug resistance, this approach is particularly useful for high-resolution investigation of strain relatedness (molecular epidemiology) and for research into novel mutations or gene regions associated with drug resistance, which in turn may lead to identification of novel drug targets and new targets for molecular diagnostics. Currently, WGS can only be reliably and cost-effectively performed using bacteria recovered from cultures, owing to the need for substantial amounts of starting DNA and the complexities arising from the presence of human DNA in clinical samples.

Conversely, tNGS uses an initial MTBC-specific amplification or target capture step. Thus, it can be performed directly from high-quality sputum samples and can yield high coverage sequence for many gene targets at once (e.g., all regions or genes that may contain mutations associated with drug resistance). A small number of all-in-one tNGS assays are commercially available or in late-stage development that can provide comprehensive DST without the need for culture. These assays include:

- The Deeplex-MycTB assay (Geno Screen, Lille, France), which can detect mutations in gene regions associated with resistance to at least thirteen drugs (isoniazid, rifampicin, ethambutol, pyrazinamide, fluoroquinolones, streptomycin, capreomycin, kanamycin, amikacin, ethionamide, linezolid, bedaquiline and clofazimine).
- The NanoTB assay (Oxford Nanopore Technologies, Oxford, United Kingdom of Great Britain, and Northern Ireland), which can detect resistance to ten drugs and has the advantage of working on small devices such as the MinION (a compact instrument with the potential to be decentralized).
- The DeepChek assay (ABL-Advanced Biological laboratory, Luxembourg), which can predict resistance associated to thirteen drugs. The assay relies on deep sequencing and analysis of 13 drugs (rifampicin, isoniazid, pyrazinamide, ethambutol, fluoroquinolones, levofloxacin, amikacin, kanamycin, capreomycin, streptomycin, ethionamide, bedaquiline, clofazimine).

- The Next Gen-RDST assay (Translational Genomics Research Institute, Phoenix, Arizona, USA), which can detect mutations associated with resistance to at least seven drugs (rifampicin, isoniazid, capreomycin, kanamycin, amikacin, moxifloxacin and ofloxacin); and
- The Ion AmpliSeq TB panel is an RUO assay compatible with Illumina platforms, and can detect mutations in 8 genes associated with antimicrobial resistance (*embB*, *eis*, *gyrA*, *inhA*, *katG*, *pncA*, *rpoB*, and *rpsL*).

This is a rapidly evolving field, and more assays may become available.

Currently available NGS platforms for WGS and tNGS use a similar workflow (Fig. 1.1), which involves extracting DNA from MTBC bacteria recovered from a clinical sample or culture, then processing the DNA to generate a library of DNA fragments (including target amplification in tNGS), sequencing the library, and finally using bioinformatics tools to assemble the sequences

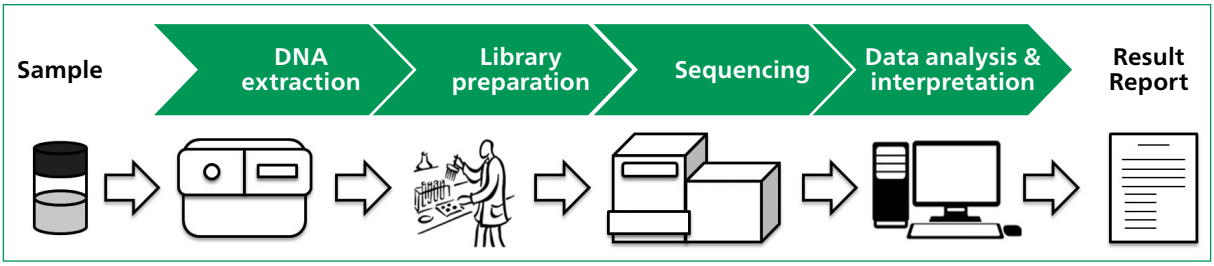
Table 1.1 Comparison between whole genome and targeted sequencing

Whole genome sequencing	Targeted sequencing
The entire genome is sequenced	Only pre-selected targeted loci are sequenced, typically <5% of the genome
WGS provides sequence information on all genes, which may be useful for molecular DST, epidemiology, and identification of novel mechanisms of drug resistance	Provides sequence information on pre-selected gene targets of interest for molecular DST based on currently known resistance-conferring genetic mutations
Adding a newly recognized genetic locus associated with drug resistance would require a relative minor change in the bioinformatics analysis	Adding a newly recognized genetic locus associated with drug resistance may require the development and validation of procedures for amplifying and sequencing a new target
Generation of information on newly identified resistance-conferring loci or for additional drugs would simply require a bioinformatics reanalysis of the existing whole genome sequence	Generation of information on newly identified resistance-conferring loci or for additional drugs would require resequencing of the sample with a new set of targets
WGS typically requires culture to enrich and amplify the MTBC bacteria (adds 5–15 days to the turnaround time and requires a BSL-3 facility); it may also require a specimen referral system that can transport viable MTBC bacteria	In addition to cultured samples, tNGS can be done using MTBC recovered directly from clinical specimens, although the quality of tNGS data can be affected by the quality of the clinical specimen and the number of bacteria in the specimen
Certain WGS platforms require large amounts of high-quality DNA and more stringent extraction as the starting material for library preparation	Requires less DNA and less stringent extraction because the amplification step produces sufficient TB starting material for library preparation; the use of PCR requires precautions to avoid the risk of end-product contamination
Provides a lower depth of coverage than tNGS	Provides a higher depth of coverage than WGS, which may facilitate the detection of heteroresistance
Typically uses a high-throughput instrument that results in higher initial capital and maintenance costs, but potentially has lower costs per sample for larger batches	Typically uses a lower throughput instrument that results in lower initial capital and maintenance costs, but potentially has higher costs per sample for smaller batches
Has more complex bioinformatics and data storage requirements	Has simpler bioinformatics and data storage requirements

BSL: biosafety level; DNA: deoxyribonucleic acid; DST: drug susceptibility testing; MTBC: Mycobacterium tuberculosis complex: NGS: next-generation sequencing; PCR: polymerase chain reaction; tNGS: targeted next-generation sequencing; WGS: whole genome sequencing

and compare them to the sequence of a reference strain, to identify "variants" or mutations relative to the reference strain. Because each position in the genome is sequenced many times (the number of times a position is sequenced is referred to as the depth of coverage), mixtures of mutant and wild-type sequences can be detected; this is similar to the information generated by the proportion method used for phenotypic DST.

Fig. 1.1 Schematic representation of a standard NGS workflow



More detailed information regarding available NGS technologies is available *(1, 9, 17, 18)*.

An overview of the NGS workflow for both tNGS and WGS applications is provided in WHO's *The use of next-generation sequencing technologies for the detection of mutations associated with drug resistance in Mycobacterium tuberculosis complex: technical guide (1)*. That publication describes the available NGS instruments and technologies, potential applications of NGS and considerations for LMICs (current at the time of publication in 2018).

Main messages from WHO's NGS technical guide *(1)*

- NGS can be successfully implemented in TB reference laboratories.
- NGS can provide a comprehensive drug-resistance profile.
- A mutation grading system was developed using an expert, consensus-driven approach to provide guidance for interpreting the clinical relevance of any detected mutation.
- NGS-based DST can reduce the need for phenotypic DST and may be particularly important for drugs for which phenotypic testing is unreliable or in settings that do not have the capacity to reliably perform phenotypic DST.
- In a multicentre study, the costs associated with performing NGS-based DST as part of periodic surveys for eight drugs (US$ 150) was less than for phenotypic testing (US$ 230) *(3)*.
- The cost to procure, install and maintain NGS instruments is substantial and needs to be carefully budgeted.
- Several NGS methods for MTBC may be soon commercially available as sample-to-result solutions.
- NGS-based DST assays have not yet been reviewed or approved by WHO for clinical use.

1.6 How to use this implementation guide

Countries can use this guide to assist in planning for the incorporation of NGS into DR-TB surveillance. The main steps in this process are listed below. A template for a Gantt chart for an implementation roadmap is given in Annex 1 and a high-level checklist for NGS implementation in Annex 2. The main steps are as follows:

1. Define the intended immediate and future use of NGS for DR-TB surveillance in the country, in line with the objectives of the national TB surveillance system, as outlined in a country's NSP for TB. This will have important implications for the choice of technologies and equipment to use, the selection of a site or sites for conducting testing, specimen referral systems and target turnaround times for results.
2. Establish a technical working group to lead planning, including performing a readiness assessment, developing a costed operational plan with timelines and milestones, and overseeing compliance with relevant regulatory processes and procedures.
3. Based on the intended use of NGS in the country, select, procure, and set up equipment in a safe, secure, and functional testing site or sites.
4. Establish forecasting, ordering and distribution procedures to ensure a reliable and timely supply of quality-assured reagents and consumables.
5. Develop and deploy a well-defined, comprehensive set of standard operating procedures (SOPs) to address all aspects of the laboratory testing process, from sample collection to reporting of results. Provide clear decision-making guidance for the selection of patients for NGS-based DST.
6. Secure adequate storage capacity and processes for backup and retrieval of the large amounts of data generated by NGS; select and implement relevant bioinformatic tools to analyse and interpret NGS data; and develop SOPs for data security, sharing and ensuring confidentiality.
7. Implement a comprehensive QA programme that includes quality control (QC), performance indicator monitoring, proficiency testing, rechecking or interlaboratory comparisons, regular onsite supportive supervision with timely feedback, corrective actions, and follow-up for each step of the process.
8. Update surveillance forms and registers to capture the relevant patient and NGS data, ideally through an electronic case-based recording and reporting system. Standardize the recording of NGS results in an easy-to-read format, to facilitate their interpretation.
9. Develop and implement training, mentoring and competency assessment programmes to ensure a well-trained workforce with the knowledge, skills, and abilities to implement NGS.
10. Establish and monitor a set of indicators or milestones to assess the implementation process. Implement a framework for monitoring and evaluation (M&E) to assess the impact of NGS.

2. Country planning

2.1 Define immediate and future use of NGS for DR-TB surveillance

The objectives of DR-TB surveillance in the country must be considered when planning the implementation of NGS. These will guide the choice of methods (WGS vs tNGS) equipment (including placement), batch size requirements (i.e., number of samples per sequencing run), target turnaround times for results and specimen referral systems. Information on setting objectives for a DR-TB surveillance system can be found in WHO's Guidance for the surveillance of drug resistance in tuberculosis *(2)*. Implementation of NGS should take into consideration not only the immediate use of NGS-based DST in the DR-TB surveillance system, but also the long-term uses of these technologies.

Potential NGS uses include:

- detection of drug resistance
- DR-TB surveillance
- patient care
- genotyping
- molecular epidemiology
- outbreak investigation
- pathogen identification
- research.

As described in Table 1.1 (Section 1), tNGS is typically more suited to low-throughput NGS instruments (i.e., benchtop equipment) that require smaller investment and have higher depth of coverage of sequencing, shorter turnaround times for results, less stringent biosafety requirements, and simpler bioinformatics and data storage requirements than WGS applications. However, these beneficial features come at the cost of less information about TB and DR-TB. Countries will need to balance the potential advantages and disadvantages in deciding which technology is best suited to meet the immediate objectives of the DR-TB surveillance system. Fortunately, both WGS and tNGS use similar workflows and techniques, and can be done on the same NGS instrument; thus, switching between a tNGS and WGS application requires straightforward changes in sample processing and library preparation. However, additional bioinformatic expertise and computing power may be needed to interpret WGS data compared to tNGS data.

Assays for tNGS require lower amounts of starting DNA, which in turn makes it possible to use tNGS with pathogens recovered directly from clinical specimens. As such, laboratories may prefer

to select tNGS applications if they plan to sequence MTBC bacteria recovered directly from sputum specimens. Furthermore, tNGS is most suited to settings that plan to investigate only drugs with known molecular mechanisms of resistance, such as rifampicin, isoniazid and fluoroquinolones (see WHO's catalogue of mutations for a more complete list *(15)*).

The performance of tNGS assays may be less effective for smear-negative samples. Thus, in the near term, tNGS on smear-positive samples only may be a more useful and cost-effective approach. The potential future uses of tNGS applications will, in large part, be determined by the availability of well-characterized, informative targets for predicting the drug-resistance profile. However, adding a new target to an existing system may not be straightforward – it could require re-optimization of the amplification or capture process as well as validation of the modifications.

WGS collects information on the entire genome, which opens a wide range of potential applications. For a DR-TB surveillance system, WGS can detect any of the known resistance-conferring mutations in any region of the genome (not just in the targeted regions of tNGS). In addition, if a new region or resistance-conferring mutation is identified for an existing drug or for a new drug, this could be incorporated through a straightforward bioinformatics reanalysis of existing WGS data.

WGS can also provide information on strain relatedness, which could enrich the usefulness of the data generated by the DR-TB surveillance system. The technique has many molecular epidemiological applications including strain evolution, outbreak investigation, cluster identification, lineage detection, transmission dynamics and information on the population structure of circulating strains.

Considerations for selecting an NGS technology for use in a DR-TB surveillance system

- Which drugs are to be included in the surveillance system (immediate and long-term plans)?
- Have the targets for each drug already been defined internationally (e.g., the rifampicin-resistance-determining-region of the rpoB gene)?
- If targets have not yet been defined for a given drug, will phenotypic DST be used? What are the plans if targets are defined in the future for an important drug (e.g., bedaquiline)?
- How many samples are to be tested in a given period?
- Is the turnaround time for results or the batch size important?
- Will culture be performed for all participants? If so, is sufficient culture capacity available?
- Is a specimen referral system available that can safely transport viable MTBC bacteria?
- Is additional molecular epidemiologic information desirable (e.g., information on strain relatedness, lineages, and clusters)?
- Are any research studies planned?

(Table 1.1 in Section 1 lists the features of tNGS and WGS that are relevant when addressing these questions)

WGS is particularly well suited to research studies, including the identification of novel mutations or genes associated with drug resistance, which could inform the development of new drug targets or new diagnostics, or the identification of virulence factors. WGS is also useful for identifying different organisms in a sample through metagenomics approaches (e.g., microbiome analysis). In the future, this may eliminate the need for the many tests often required in a diagnostic laboratory to detect and identify an unknown pathogen in a clinical or environmental sample. In the mycobacteriology laboratory, which might mean one test to identify any *Mycobacterium* species.

Countries should also consider that a proposed investment in NGS-based DST for DR-TB surveillance has potential implications beyond TB. Countries can leverage the installed sequencing machines and capacity for performing NGS for TB as a scalable template for expansion into NGS for other infectious diseases, antimicrobial resistance, outbreak preparedness, noncommunicable diseases (e.g., oncology) and other precision medicine testing. Similarly, experiences and resources developed for the use of NGS for other diseases could be leveraged to facilitate the implementation of NGS for TB.

2.2 Establish an NGS technical working group and define roles and responsibilities

A technical working group (TWG) comprising representatives from different stakeholders should be established to guide the process for implementing NGS-based DST in the country. This may be accomplished through expansion of an existing laboratory TWG. The establishment of the TWG should be led by the MoH, NTP and national TB reference laboratory (NTRL). The TWG should be mandated to:

- Adapt the recommended NGS implementation roadmap to develop a country-specific workplan for NGS adoption in the NTP.
- Provide oversight for monitoring the implementation of the NGS workplan; and
- Assess the impact and success of NGS introduction.

In addition to representatives from the MoH, NTP and NTRL, it will be important to include members with expertise in NGS technologies, data management and information technology (IT). In addition, representatives from the following key stakeholders may also be invited to participate, as appropriate:

- Regulatory and procurement bodies.
- Technical partners such as WHO SRLs, WHO, the Foundation for Innovative New Diagnostics (FIND) or other disease programmes, academic or research institutes with experience using NGS and laboratories with existing sequencing capacity; and
- Implementing partners such as intermediate and peripheral laboratories and clinical facilities that will participate in the NGS programme (e.g., peripheral sites that will submit samples for sequencing) and providers of specimen transport systems.

The TWG should be led by a suitably qualified individual; for example, a laboratory manager or a laboratory focal person from the NTP or NTRL. Defining the roles and responsibilities of members of the implementation team as well as external partners and donors should be an integral component of the planning process. Suggested roles and responsibilities of entities involved in the implementation of NGS are provided in Table 2.1, and a high-level checklist for NGS implementation is provided in Annex 2.

The TWG members should familiarize themselves with contents of *The use of next-generation sequencing technologies for the detection of mutations associated with drug resistance in Mycobacterium tuberculosis complex: technical guide (1)* and *Guidance for the surveillance of drug resistance in tuberculosis (2)*.

The TWG should also coordinate and harmonize planning of NGS implementation with the existing surveillance system, based on routine methods of DST for patients with bacteriologically confirmed TB or periodic surveys of representative samples of patients. Considerations should include, for example, whether it is feasible to incorporate NGS into the current surveillance system or the next planned survey based on the applicable timeline and budget; and which NGS system is best suited to provide the information needed.

Table 2.1 Roles and responsibilities of a TWG for the implementation of NGS for DR-TB surveillance

TWG member	Roles and responsibilities
National / regional level	
MoH and NTP	• Define the objectives and scope of the surveillance system, including the role of NGS. • Engage with technical and funding partners to enable planning for NGS implementation. • Appoint staff to conduct readiness assessment of the clinical, laboratory, programmatic, bioinformatic and procurement activities required. • Establish indicators to monitor the implementation and impact of NGS. • With the NTRL, modify diagnostic algorithms to incorporate NGS. • With the NTRL, select sites to implement NGS. • Develop or update essential documentation (e.g., implementation plans, monitoring plans, and decision-making protocols) to reflect the use of NGS. • Implement a system to ensure that a unique identification number is used on all forms and across all linked databases (with the NTRL); each identification number should be linked to only one person and each participant should be identified by only one number. • Train and supervise staff at clinical sites on the selection of patients to include for NGS and on the procedures for submitting samples for NGS. • Establish a clinical management expert group to be responsible for reviewing all DST results (including results of NGS, phenotypic and other testing methods) and providing advice for patient care if needed
NTRL	• Assess laboratory and network capacities and capabilities for NGS and specimen referral. • Develop essential documentation such as implementation plans, and SOPs (including those for specimen collection, processing and referral, testing, reporting and QA) related to the use of NGS. • Update data collection forms and results reporting forms to accommodate the use of NGS.

TWG member	Roles and responsibilities
	• Ensure that a unique identification number is used on all patient forms and all linked databases (with the NTP); each identification number should be linked to only one person, and each participant should be identified by only one number, • Assist with updates to laboratory information systems and electronic recording and reporting systems, if necessary • Train and supervise laboratory staff at NGS testing sites, with the NTP and technical partners (e.g., SRL) • Train and supervise staff at specimen collection and referral sites on collecting and transporting specimens for NGS. • Provide technical assistance to sites. • Establish a communication channel for participating laboratories to contact if experiencing any problems and provide troubleshooting. • Implement an EQA system.
Regulatory bodies	• Define and communicate regulatory or importation requirements to the implementation team and manufacturers. • Develop a framework for evaluation and approval of this class of technologies, and provide this information to manufacturers
Government procurement bodies	• Forecast the consumables needed (with the NTP) • Integrate new consumables into the national supply chain. • Monitor stock usage and provide timely feedback
Technical partners	• Provide guidance and technical assistance. • Support implementation of country policies and plans, with the NTP leadership.
TWG member	**Roles and responsibilities**
Implementing partners including NGS testing site (possibly NTRL) and specimen collection sites	
NGS testing site manager	• Provide information during the readiness assessment. • Perform site-level implementation activities. • Inform referral and clinical managers when NGS for DR-TB surveillance has started. • Monitor and evaluate implementation of activities. • Implement systems for internal QC and EQA
Laboratory staff	• Provide information during the readiness assessment. • Perform onsite verification with NGS and report any troubleshooting issues to the NTP and NTRL
Specimen collection and referral sites	• Implement SOPs for specimen collection, processing, and referral. • Coordinate specimen referral procedures with the NGS testing site or sites

DR-TB: drug-resistant TB; EQA: external quality assessment; MoH: ministry of health; NGS: next-generation sequencing; NTP: national TB programme; NTRL: national TB reference laboratory; QA: quality assurance; QC: quality control; SOP: standard operating procedure; SRL: supranational reference laboratory; TB: tuberculosis; TWG: technical working group.

The TWG should engage with NGS platform providers, who can provide relevant up-to-date information on NGS instruments, reagents, and consumables as well as advice on technical issues and training.

It may also be necessary to establish a clinical management expert group to be responsible for reviewing NGS results and providing advice for patient care, in view of other results from parallel testing with phenotypic DST or other tests. The advice of a clinical management group may be particularly important if the NGS-based DST detects resistance to a drug that has previously not been detected or is not tested by routine DST methods used in the country. This may result in modifications to routine DST algorithms or to recommended treatment regimens.

2.3 Integrate NGS into national plans and algorithms for DR-TB surveillance

The TWG should make recommendations to the MoH or NTP on the intended use of NGS, considering TB epidemiology in the country, global knowledge on mutations conferring drug resistance, current testing algorithms and existing sample referral systems. An important consideration is which drugs will be investigated in the context of surveillance or survey activities. Although molecular tests are available for each of the first-line drugs, they are not currently available for all the second-line drugs (e.g., bedaquiline) and the testing algorithm will need to include both phenotypic and molecular DST for these (Fig. 2.1). Priority should be given to first-line anti-TB drugs and Group A drugs (bedaquiline, linezolid, fluoroquinolones).

In the context of DR-TB surveillance, the starting point for any algorithm is usually the collection of a specimen from a patient with bacteriologically confirmed TB. The algorithms are then defined by whether tNGS or WGS will be used (Fig. 2.2). Although both WGS and tNGS can be performed on bacterial culture isolates, tNGS can also be performed directly using the sputum specimen.

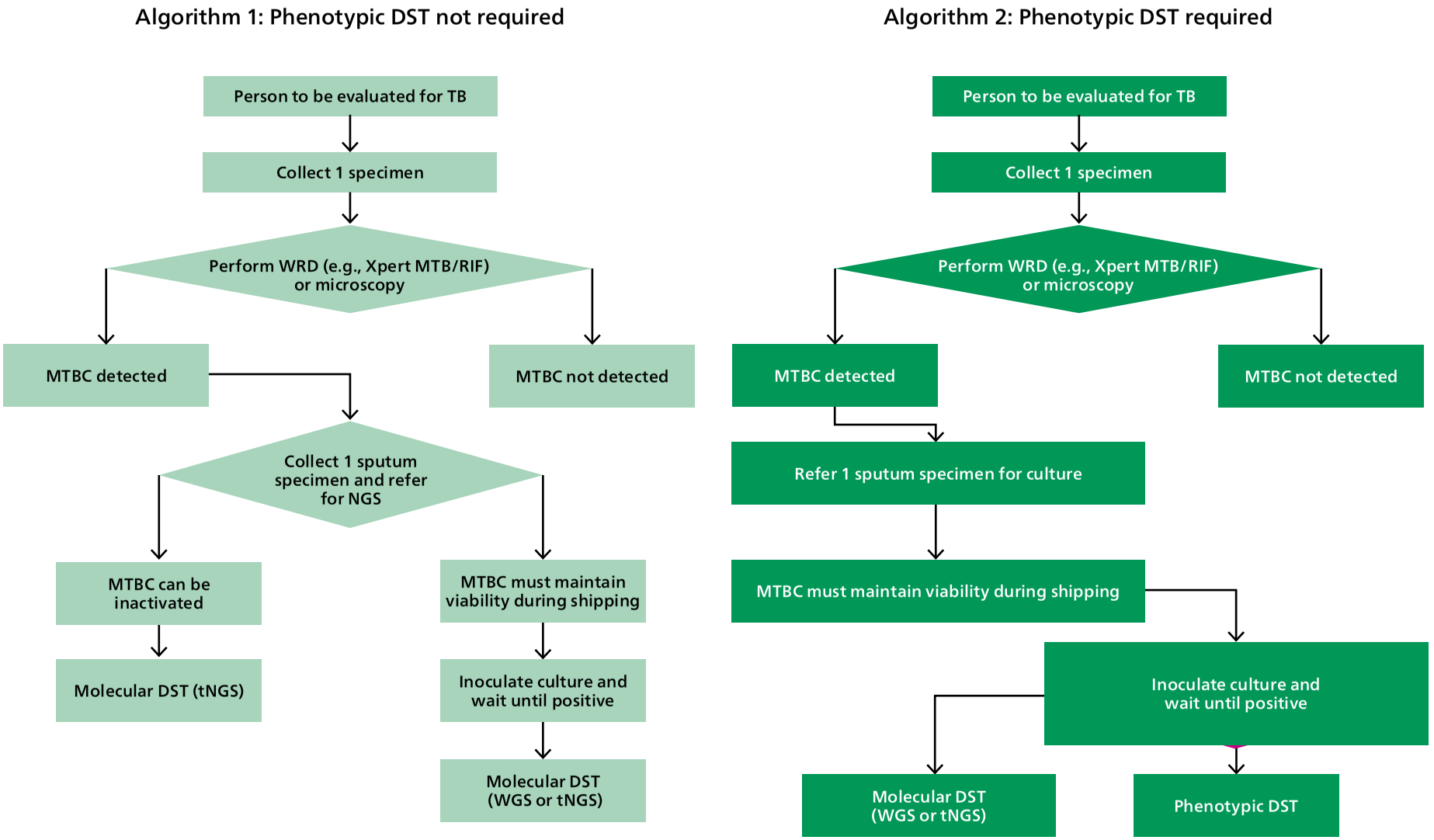
National guidelines for the surveillance of DR-TB may need to be updated to address ethical issues *(19)*. Such issues include confidentiality (sensitive patient information should be kept confidential and shared and revealed to others only where it is strictly necessary for the functioning of the surveillance system) and informed consent (informed consent should be sufficiently detailed to describe the use and storage of an individual's data and specimens). For laboratories, informed consent should include the timeline of specimen storage, specimen transfer agreements and international shipment of specimens for further testing, and inclusion of specimens in any future studies. Informed consent may also be needed for the sharing of raw NGS data, the storing of NGS data on publicly accessible databases or follow-up use of clinical samples. In most bioinformatic pipelines, the first step of data processing is the removal of all non-TB sequences, eliminating any risk of human DNA sequences being shared.

Local ethical review and approval by national ethics committees or institutional review boards should be sought, in accordance with local and national policies.

2.4 Conduct a readiness assessment

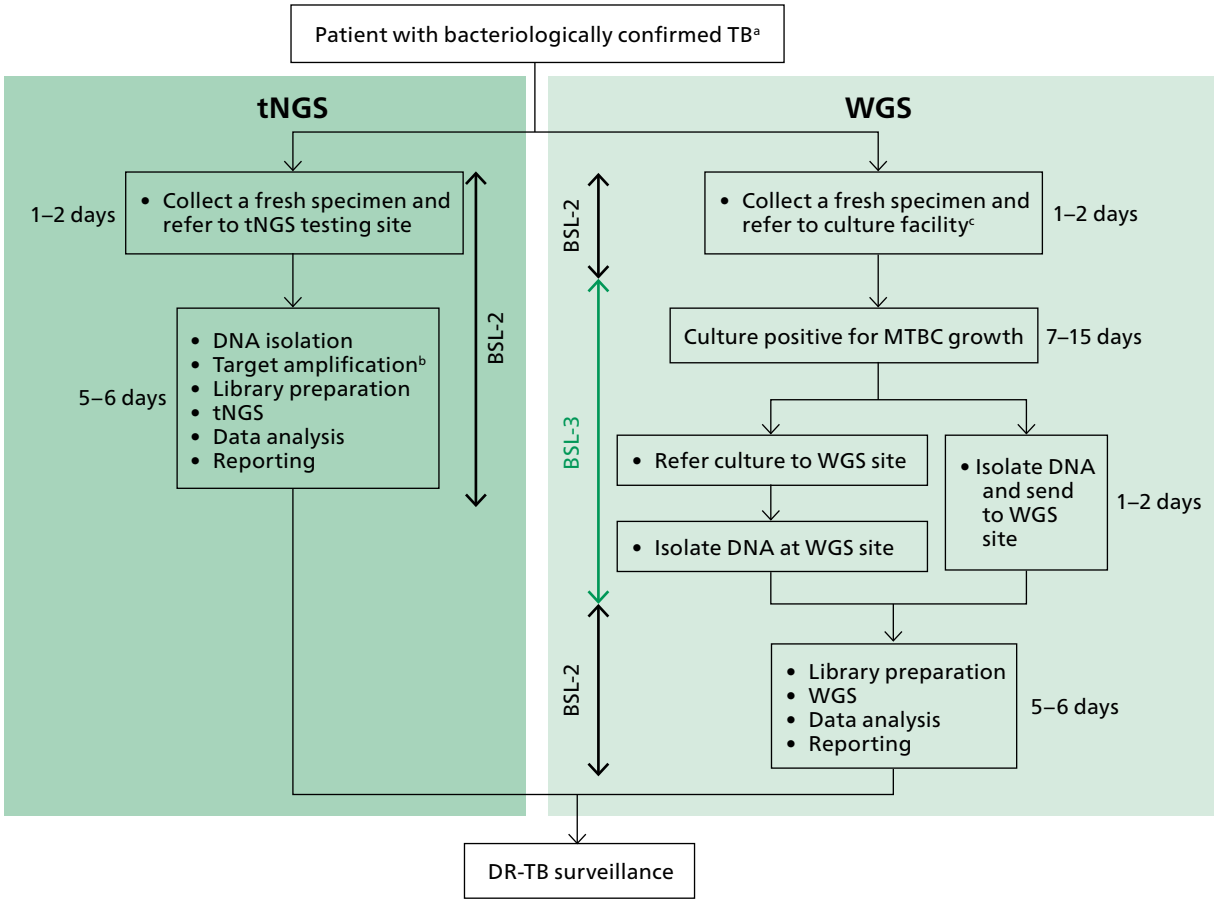
A readiness assessment of the existing laboratory network, availability of NGS capacity and on-going utilization, should be conducted to inform the plans for implementation. The NGS assessment should include the elements listed in the NGS implementation high-level checklist (Annex 2) and the readiness assessment checklists (Annex 3). The main elements to be assessed are regulatory requirements; laboratory and network infrastructure, expertise and experience, IT and bioinformatics capabilities, availability and adequacy of SOPs, availability of ongoing support for NGS instrument operation and bioinformatics analysis, supply chain, financial resources, and QA systems. The assessment should also determine the need for any revisions to training, recording, and reporting forms, and M&E tools to inform the operational plan development and implementation roadmap (Annex 1). For the prospective NGS testing site, detailed assessment of the laboratory's readiness with respect to physical facilities, staffing and bioinformatics will be needed (see the checklist in Annex 3, Part B). Technical assistance from an external technical expert

Fig. 2.1 Examples of testing algorithms employing NGS-based DST for DR-TB surveillance



DR-TB: drug-resistant TB; DST: drug susceptibility testing; MTBC: *Mycobacterium tuberculosis* complex; NGS: next-generation sequencing; TB: tuberculosis; tNGS: targeted NGS; WGS: whole genome sequencing; WHO: World Health Organization; WRD: WHO-recommended diagnostic test.

Fig 2.2 Laboratory algorithms for tNGS and WGS



AFB: acid-fast bacillus; BSL: biological safety level; DNA: deoxyribonucleic acid; DR-TB: drug-resistant TB; MTBC: *Mycobacterium tuberculosis* complex; TB: tuberculosis; tNGS: targeted next-generation sequencing; WGS: whole genome sequencing.

[a] The starting point for both tNGS and WGS for DR-TB surveillance is a sputum sample from a patient with bacteriologically confirmed TB identified using a WHO-recommended rapid diagnostic test, smear microscopy or culture. Available evidence indicates that tNGS can be efficiently performed from AFB smear-positive samples. The performance of tNGS on paucibacillary samples (e.g., Xpert MTB/RIF and Xpert Ultra positive samples with trace, low or very low results) has not yet been established. In this case, an initial culture step may be required before sequencing.

[b] The probes to be used for the capture or target amplification step will depend on the which drugs are to be tested.

[c] In some settings, culture and WGS may be done at the same facility. The initial processing of a sample to inoculate a culture is considered a moderate-level bio-risk activity (requiring a BSL-2 laboratory), whereas manipulation of a positive culture is a considered a high-level bio-risk activity (BSL-3 laboratory). Culture facilities may inactivate the bacteria in the culture before shipment to the WGS site, which would reduce the biosafety measures required for transport to, or manipulation at, the WGS site.

(e.g., from a WHO SRL or an experienced NGS user) may be needed to assist with the readiness assessment. An example of an NGS readiness assessment is given in Annex 4.

The TWG should coordinate the assessment, and delegate responsibility for conducting the assessment usually to the NTP and/or NTRL. The purpose of the exercise should be made clear to all stakeholders involved in the assessment, including testing, referral, and clinical site staff, implementing partners and those responsible for data collection and reporting.

The assessment will also need to evaluate the relevant portions of the TB diagnostic network. Of relevance are the steps for the initial detection of MTBC bacteria at the peripheral diagnostic

health centre (i.e., identifying patients with presumptive TB, collecting quality specimens, and conducting tests for bacteriologic confirmation of TB) and the specimen referral system. A checklist for evaluation of a specimen referral system can be found in the *GLI guide to TB specimen referral systems and integrated networks (20)*.

All NGS workflows require a molecular biology infrastructure that involves the use of at least three separate wet laboratory areas. One clean space is needed for all pre-amplification procedures, another for sample preparation and DNA extraction, and another for all amplification and post-amplification procedures. Key requirements of these facilities include:

- The space being designed to ensure a unidirectional workflow and separation of activities to minimize the potential for cross-contamination.
- Adequate bench space to support the size and weight of the NGS instrument for general molecular biology techniques and for storage of consumables, reagents, and kits.
- An adequate electrical service that meets power specifications, and safety measures and an uninterruptible power supply appropriate for the selected technology; and
- An appropriate working and storage environments that address temperature (air conditioning or heating), humidity, elevation, air quality, ventilation, and vibration-dampening requirements.

Sample preparation involves processing clinical samples or cultures containing viable MTBC bacteria. It must be performed in laboratories that comply with applicable biosafety level (BSL) standards and all steps must be conducted in accordance with appropriate biosafety standards. For example, the processing of sputum specimens for DNA extraction should be performed in a biological safety cabinet under at least BSL-2 conditions, and the processing of cultures should be performed in a TB containment laboratory (BSL-3) *(21)*. A thorough assessment of biosafety practices and procedures will be particularly important for NGS laboratories that do not routinely handle TB specimens or cultures.

The capacity to analyse and store large amounts of data is a critical component of NGS applications. IT requirements include:

- Adequate computing resources including the instrument computer and computers for data analysis.
- Sufficient data storage capacity (local or cloud-based services) including external storage for data backups.
- Appropriate data security measures.
- Access to data analysis tools.
- Staff trained in the use of the analytic tools for NGS data.
- IT resources and expertise for the installation and ongoing maintenance of hardware, software, and networks.
- A network and high-speed internet connection; and
- Data connectivity solutions to link NGS data to other relevant patient data.

Once the site successfully completes the readiness assessment, the TWG and the NTP and/or NTRL should inform all relevant stakeholders involved.

2.5 Develop a costed operational plan for implementation

A detailed costed operational plan should be developed based on the strategic objectives. The implementation of NGS must overcome potential obstacles such as the cost of instruments, ancillary equipment and consumables; requirements for improving or establishing the necessary laboratory and network infrastructure (e.g. a specimen transport system); specialized staff; readily available data analysis (bioinformatics) and data storage solutions; expert technical assistance for data management and clinical interpretation of data; maintenance of confidentiality of patient information; and establishment of a QA system.

Successful implementation of the plan will require financial and human resource commitments from the MoH and NTP, with support from implementing partners. A budget should be developed to address activities in collaboration with key partners (Annex 5).

Supporting resources can be found in Annexes 1–5.

3. Regulatory

3.1 Determine importation requirements

National authorities should be consulted to determine the relevant processes to be followed for importation. Countries should work closely with the manufacturers or authorized service providers of equipment and consumables to determine importation and registration requirements and to enable initiation of country verifications, if required.

3.2 Conduct country verification study as required

Validation[1] studies are large-scale studies undertaken to establish the performance characteristics of a test (e.g., sensitivity, specificity, accuracy, positive and negative predictive values, and robustness). For NGS-based DST methods, multi-country studies have recently been undertaken to validate the performance of NGS-based DST. The results of these studies will establish the target performance values of the method. Depending on national accreditation requirements, some countries may need to conduct additional country-specific verification studies.

Laboratories that are implementing the method usually do not need to repeat large-scale studies, but can instead conduct small-scale verification *(22)* studies. Such studies can demonstrate that the laboratory can achieve the same performance characteristics that were obtained during the validation studies when using the test as described in the validation studies and that the method is suitable for its intended use in the population of patients being assessed.

When planning an NGS verification study *(23)*, the following should be considered:

- A detailed protocol is required, outlining the number and types of samples to be tested and defining the criteria that must be met to demonstrate that the laboratory can achieve the targeted performance characteristics. Samples should be chosen to assess the performance of the entire NGS process, from DNA isolation to interpretation of results.

[1] Note that the terms validation and verification are used almost interchangeably in the literature which may generate confusion. During implementation, the focus should be on small-scale studies to verify the performance of NGS-based DST in the NGS laboratory.

- If NGS is to be implemented in several laboratories, a more extensive verification study may be done at the NTRL (e.g., 30–50 samples), with limited verification studies done at the other laboratories, for cost saving and efficiency.
- For verification, a mix of samples should be selected that will give results at test thresholds (e.g., a mix of positive and negative results) and will give a variety of semiquantitative results. Samples for verification could be leftover sputum or frozen sputum samples with known results, stored clinical isolates or proficiency testing panels. Countries should select a variety of strains for verification, based on their local epidemiology.
- Reproducibility and repeatability could be assessed by testing three reference samples three to five times each.
- A verification report should be compiled, the observed performance parameters compared with the published performance and a determination of acceptance made.

3.3 Complete national regulatory processes

Countries should work closely with the relevant government authorities, manufacturers, or authorized service providers as necessary to meet any requirements of the national regulatory authority. There must be an appropriate length of time for submitting the application and providing any required supplementary evidence.

4. Equipment

4.1 Select, procure, install, and set up equipment

An essential step in the implementation process is the selection of an appropriate sequencing instrument to fit the required NGS applications. NGS instruments differ widely in chemistry, throughput, price, time to completion of a sequencing run and NGS read length. The most suitable instrument for a country will depend on the intended use of NGS. In general, it is best to choose a sequencing instrument that is widely available and has good local supply distribution and support. The chosen instrument should also have reliably low error rates and be capable of high or low-throughput processing, depending on individual laboratory needs. Annex 6 provides a list of the current commercially available NGS instruments. Summaries and reviews of NGS instruments can be found in recent publications *(1, 17, 24)*.

In addition to the sequencing instrument, specialized equipment will also be needed for DNA extraction, library preparation and quality checks. Important ancillary instruments include:

- An automated DNA extraction instrument (extraction can also be done manually).
- An instrument for DNA quantity and quality assessment.
- A DNA fragment analyser; and
- A thermal cycler.

Whichever NGS technology is selected, most require installation by the manufacturer. Issues to consider at the time of set-up include power supply and backup options, electrical connections, computing hardware and software, a maintenance plan (e.g., weekly, monthly or pre-run checks), equipment warranty and necessary training. Annex 6 provides links to current installation resources for the various NGS instrument manufacturers.

4.2 Assess site readiness and ensure a safe and functional testing site

The selection of one or more sites to conduct NGS testing is usually determined by the NTP or NTRL in consultation with the TWG, based on factors such as intended use of NGS (it is important to highlight that such technology can be implemented for other diseases program as well like HIV, AMR etc.), TB epidemiology and caseload, geographical considerations, anticipated NGS

workload, laboratory and IT capacity, and efficiency of referral networks. Each testing site should be evaluated for readiness (see example in Annex 4) before the testing of clinical specimens. In addition, existing testing sites should be regularly assessed for safety and operational functionality.

Countries may decide to implement NGS-based DST for DR-TB surveillance at a central facility initially (e.g., the NTRL) because of the capital costs and required computational expertise and resources. Starting with implementing NGS-based DST for DR-TB surveillance at the NTRL will give the country the opportunity to build knowledge, skills, abilities, and experience at the NTRL, and establish a technical and bioinformatic infrastructure that can serve as a solid foundation for expanding NGS services to additional sites or for additional uses in the future.

In a functional testing site, testing instruments will be properly positioned in a clean, secure, and suitable location (e.g., placed on a vibration-free bench that is not directly under an air conditioning unit). Most instruments will require an uninterrupted supply of power, and appropriate working and storage conditions (humidity and temperature controlled). In a safe environment, WHO biosafety recommendations *(21)* for conducting the diagnostic test will be followed with adequate ventilation, appropriate personal protective equipment will be used, and biological waste will be disposed of safely and in accordance with regulations. Failure to provide a safe and functional work environment can affect the quality and reliability of testing. An installation checklist is provided in Annex 7.

5. Supplies

5.1 Review forecasting, ordering and distribution procedures

Uninterrupted availability of reagents at the testing site is essential to ensure the development of technical capacity in the early stages of implementation (i.e., avoiding long delays between training and availability of reagents) and to ensure consistent service during routine use for surveillance or clinical care.

Measures required to ensure uninterrupted supply of reagents are as follows:

- Ensuring that qualified laboratory staff define requirements for reagents, consumables, and equipment.
- Carefully timing importation and in-country distribution procedures to ensure there is sufficient remaining shelf life of reagents once consumables reach testing sites.
- Monitoring consumption rates, tracking reagent-specific shelf-lives and forecasting needs to avoid expirations or stock-outs.
- Ensuring that sites have received training and equipment has been installed before shipment of reagents; and
- Ongoing monitoring of all steps in the procurement and supply chain to ensure adherence to the planned schedule.

Purchasing and distribution strategies should be reassessed at regular intervals to ensure they are responsive to the needs and current situation. NGS manufacturers typically maintain regional distribution and support centres that help to facilitate the procurement process and delivery of commodities for countries within that region.

5.2 Develop procedures to monitor reagent quality and shelf life

Depending on the selected sequencing instrument and workflow, an implementing laboratory will need to procure and maintain a variety of supplies from different commercial partners (Annex 8 lists essential equipment and reagents). NGS laboratory managers should develop procedures to monitor reagent quality and shelf life based on manufacturer recommendations, to ensure that high-quality sequencing data are generated. Additionally, the laboratory must establish SOPs for handling the reagents and chemicals to ensure both quality and safety.

6. Essential documents and procedures

6.1 Develop essential documents

High-level essential documentation on governance arrangements, communication, monitoring, responsibilities, accountability, and delegation logs should be developed or updated to reflect the use of NGS-based DST in the DR-TB surveillance system. A detailed list of essential documents for a DR-TB surveillance system can be found in WHO's *Guidance for the surveillance of drug resistance in tuberculosis (2)*.

6.2 Develop SOPs

During the initial planning process, the intended use, or uses of NGS in DR-TB surveillance will be defined (Section 2) and the NGS instrument and system selected (Section 4). Based on these selections, procedures must be defined, selected, developed, or customized for:

- Identifying patients to be included in NGS-based DR-TB surveillance.
- Collecting, processing, storing and transporting specimens to the NGS laboratory.
- Laboratory testing (e.g., sample processing, DNA extraction, library preparation and sequencing).
- Data analysis and sharing (Section 7).
- Process controls (internal QC) and EQA (Section 8); and
- Recording and reporting results (Section 9).

A well-defined, comprehensive set of SOPs is essential (Table 6.1) The SOPs should address all aspects of the laboratory testing processes, from sample collection to results reporting. Given the complexity of the workflow and potentially high cost of errors, strict adherence to the SOPs is essential to ensure high-quality results. In addition to laboratory-related SOPs, guidance and related SOPs will be needed for the selection of patients for NGS and collection, recording and reporting of other metadata.

Table 6.1 SOPs related to laboratory testing.

Process	Description of SOP
Specimen collection	Defines the types of specimens, collection procedures, volume and quality requirements, and labelling requirements
Processing and storage	Describes procedures for processing and storing specimens before transport to the NGS testing site
Specimen referral	Defines packaging and transport requirements and procedures – see the *GLI guide to TB specimen referral systems and integrated networks (20)*
Specimen receipt and accessioning	Describes procedures for performing quality checks, assigning a laboratory sample number, entering the relevant sample and patient information into the laboratory information system and, if necessary, processing and storing of the sample before beginning the NGS process
Sample processing	Defines procedures for processing samples (e.g., sputum or culture) in preparation for the DNA extraction step, and quality and quantity requirements
DNA extraction	Defines procedures for extracting DNA for use in the library preparation, and quality and quantity requirements
Library preparation	Defines procedures for preparing the library of fragments, processing samples for sequencing, and quality and quantity requirements
NGS	Describes the protocol for NGS and quality checks and data requirements (e.g., format or transmission) for bioinformatic procedures
Data analysis	Defines programmes and procedures used for data analysis, interpretation of sequence differences, quality checks and ensuring data security and confidentiality
Recording and reporting	Defines procedures and forms for recording and reporting results
EQA	Describes procedures for conducting and participating in an EQA programme

DNA: deoxyribonucleic acid; EQA: external quality assessment; NGS: next-generation sequencing; SOP: standard operating procedure; TB: tuberculosis.

Some NGS procedures will rely on the manufacturer's protocols included with the commercial kits (e.g., a library preparation kit). Other SOPs will need to be developed. Countries may require technical assistance for SOP development from a laboratory that is currently conducting NGS.

7. Data

This section briefly summarizes data storage requirements, analytic tools and data-sharing protocols. An in-depth discussion of these critical elements of the NGS processes may be found in *The use of next-generation sequencing technologies for the detection of mutations associated with drug resistance in Mycobacterium tuberculosis complex: technical guide (1)*. Technical assistance from an IT expert or department may be needed. A checklist to assess IT and data readiness is included in the site readiness checklist in Annex 3.

7.1 Plan for data storage and computing

Adequate secure data storage is essential for successful implementation of NGS. Each NGS run can generate thousands of megabytes of data (Annex 9). For example, when "benchtop" NGS platforms are used, 7.5–15 gigabytes (GB) of data are generated during a 24–48 hour run. A sequencing run may include as many as 24–48 samples for WGS or 96 samples for targeted NGS. For WGS, the raw sequence files (FASTQ files) for each sample usually have a total unzipped size of more than 350 megabytes (MB), to ensure a minimum average coverage depth of 30-fold, if more than 95% of reads map to the TB reference genome. FASTQ files for each sample subjected to targeted NGS are usually smaller. The large amount of data generated for each sample must be securely stored (usually as compressed or zipped FASTQ files), routinely backed up (ideally in two locations) and processed on secure systems to ensure data availability, security, and traceability. The hard drives on the NGS platforms (typically 500 GB) are not sufficient for storage of raw sequencing data generated in multiple runs.

Options for data storage include computer hard drives or external hard drives (a 1 terabyte hard drive may be able to store WGS files for up to 1500 samples); high-capacity flash drives (also known as memory sticks or thumb drives); a scalable, local computing cluster; and cloud-based systems (e.g., Illumina BaseSpace, Google Cloud and Amazon Drive). Cloud-based systems are the preferred option. Online public databases (e.g., the Sequence Read Archive or the European Nucleotide Archive) can be used for archiving sequences. Data storage solutions vary in cost, ease-of-use, hardware and software requirements, remote accessibility, data transmission needs (e.g., internet speed), data encryption protocols, access control, suitable file formats and need for local IT support. Certain data storage solutions may require data-sharing agreements and a legal framework, particularly if the data are to be stored outside the country.

Current experiences in implementing NGS in LMIC suggest that maintaining local in-country servers for secure and reliable data storage has both advantages and disadvantages. For data storage, local services are likely to be more expensive and complex to maintain than cloud-based services. However, performing analysis of NGS data on local servers is likely to be less costly than using could-based services.

Local servers ensure high levels of data security and high speed to upload or download data without the need for an Internet connection. The system set-up and backups are completely customizable, as is the computing power, where a central processing unit can work with a graphics processing unit to increase the throughput of data and the number of concurrent calculations within an application (i.e., simultaneous analysis of multiple sequencing files). Local servers require a certain capital investment in hardware and infrastructure; also, they need appropriate physical facilities, ongoing support, and maintenance.

A cloud is a "virtual" environment that is accessible via an internet connection. A user rents the server space from a provider. This enables an easy adjustment of storage space and computing capacity with a pay-as-you-go approach. Resources can easily be scaled up or down, without the need for investment in infrastructure, physical space, maintenance, and upgrades. However, users cannot access data in the cloud without an internet connection; also, storing or analysing a large amount of sequencing data may have important costs. Table 7.1 summarizes the features of NGS data storage options.

Regardless of the data storage solution selected, all laboratories must ensure that adequate data protection processes are in place to ensure the confidentiality of patient data. IT experts should be consulted for the design and selection of a solution that best fits the needs of the laboratory and programme and adheres to applicable national regulations and guidelines.

7.2 Select analytic tools

In recent years, many commercially and publicly accessible analytic pipelines have become available that make the analysis of MTBC NGS data accessible to nonexperts and bioinformatic experts alike *(1)*. Some of the commonly used bioinformatics pipelines for TB NGS data include webservers such as PhyResSE, TBProfiler, ResFinder, Mykrobe, genTB and software solutions such as KvarQ, Clockwork (CRyPTIC pipeline) and the ReSeqTB platform *(23)*. For advanced users, many commercial or free individual programs are available to analyse sequence data. Most of these tools can analyse local or cloud-based raw sequencing data, to identify mutations and relate those mutations to drug-resistance phenotypes for users with minimal expertise. The methods chapter of WHO's *Catalogue of mutations in Mycobacterium tuberculosis complex and association with drug resistance (15)* provides a consensus for selected parameters for bioinformatic analyses using the Clockwork pipeline.

The interpretation and reporting of sequence variants should follow international recommendations and standards such as those described in the WHO catalogue *(15)*, an updated version of which will be published in 2023.

Factors to be considered in selecting the analytic tool or tools that are best suited for the intended use of the NGS data include compatibility of the tool with the data output by the NGS instrument,

Table 7.1 Features of NGS data storage options

Features of data storage options	Local drives	Scalable local computer cluster	Cloud
Hardware	External drives or USB drives	Multiple computers and ethernet connections	Hardware for internet connection
Initial capital cost	Low	Depends on the number of computers and connections	Low (internet connection)
Local IT infrastructure and expertise needed	Minor	Sophisticated and intensive for set-up and maintenance of network and physical facilities for computer cluster	Support for internet access; continuous support from software and hardware owners
Operational costs	Low	Maintenance of network by local IT staff	Subscription fee for storage; internet fees
Capacity	Relatively low	Medium	High
Ease-of-use	Requires computer expertise and hands-on time to perform daily or weekly backups and retrieval of data	Easy for the end-user but intensive for IT staff	Easy
Risk of loss of data	Loss can be a risk if the drive is damaged or stolen	Depends on local IT infrastructure	Loss is not a significant risk; storage is highly reliable
Backup	Can be automated; responsibility of laboratory staff	Responsibility of cluster manager	Automatically performed by cloud service provider
Security and access control	User dependent. Drives must be password protected	Dependent on local network and must be password protected	Strong security and access controls
Remote access	No	Only if local cluster can be accessed remotely	Yes
Requires stable high-speed internet for data transmission	No	No	Yes
Susceptible to viruses	Yes	Depends on local IT	No
Data sharing	No concerns	Controlled by local IT	May require a special agreement to comply with national standards for confidentiality and use of patient data
Out-of-country data storage	Not applicable	Not applicable	May not be allowed; but cloud service providers may be able to provide a storage centre located in the country
Computing power	Not applicable	Flexible – modules can be added according to computing requirements; possibility of high-performance computing	High-capacity, increased flexibility, scalable and on-demand

the type of information that is returned by the analytic tool, computational resources and bioinformatics expertise needed at the local site, and time required to complete the analysis.

For laboratories with strong bioinformatics support, NGS data analysis can be performed using a laboratory-tailored collection of open-source software programmed to function together in an analytic pipeline (e.g. MTBSeq) *(25)*. This provides the opportunity for the laboratory to customize the analytic pipeline and conduct analyses that may not be offered by the available pipelines.

A verification study of the bioinformatics pipeline, especially customized pipelines, should be performed before it is used with patient samples. Guidance on conducting a verification study for a bioinformatics pipeline is available *(26)*. Re-verification may be needed when significant modifications (e.g., adding a new gene target or mutations associated with resistance) are made to the bioinformatic pipeline.

7.3 Develop procedures for data sharing and ensuring confidentiality

Because NGS generates sequences of whatever DNA is present in a sample, laboratories must be particularly careful with sequence data generated from human clinical samples (e.g., sputum specimens, which may contain considerable amounts of human DNA). Currently, two features of the NGS procedures for TB mitigate this concern: the process of isolating bacteria by culture should eliminate the risk of sequencing any human DNA; and the amplification or capture step used in the processing of clinical specimens for tNGS also serves to minimize the amount of human DNA that would get into the sequencing reaction. Furthermore, most bioinformatics pipelines include steps to discard any sequence reads that map to the human genome before sharing TB sequencing data.

NGS-base surveillance allows monitoring the prevalence and spread of mutations associated with resistance to existing drugs, identification of rare variants, and provides data to help uncover novel mutations associated with phenotypic resistance to new drugs. To obtain such information: data derived from surveillance efforts are typically analysed and published as aggregated data, rather than individual case-based data. Nevertheless, procedures must be in place to inform patients of all plans for the use of the NGS data and any associated metadata, and their rights with respect to the protection of the data. The rights of the patient with respect to the use of their personal genetic information must be safeguarded. Informed consent may be needed for the sharing of their NGS data, storing of their data on publicly accessible databases or any future use of their clinical samples. Local ethical review and approval by local or national ethics committees or institutional review boards should be sought, and local and national policies and rules should be followed.

It is critical that sequencing data can be linked to other relevant metadata to enable the required epidemiological analyses. Data connectivity solutions should be in place before implementation of NGS, and a unique survey identification number should be used on all forms and across all databases. Each survey identification number should be linked to only one person and each participant should be identified by only one number.

8. Quality assurance, control, and assessment

8.1 Implement a comprehensive QA programme

A comprehensive QA or quality management programme is needed to ensure the accuracy, reliability, and reproducibility of laboratory results. The essential elements of a QA system include:

- SOPs, training, and competence assessment.
- Instrument verification.
- Equipment maintenance.
- Method validation or verification.
- QC.
- Lot testing (also known as incoming QC or new batch testing).
- EQA.
- Quality indicator monitoring; and
- Continuous quality improvement.

A comprehensive discussion of these elements may be found in the *GLI practical guide to TB laboratory strengthening (27)*. For examples of quality and progress indicators relevant to DR-TB surveillance and surveys specifically, see WHO's *Guidance for the surveillance of drug resistance in tuberculosis (2)*. This section describes NGS-specific features of QC, EQA and quality indicator monitoring.

8.2 Establish and monitor QC

Because of the complexity of the NGS workflow and the current lack of commercially available end-to-end products, it is particularly important to conduct quality checks after each of the main steps in the process (Annex 10 provides details). Checks include the following:

- *Specimens* – assess the source, quantity, and quality of the source sample (e.g., sputum specimen).

- *DNA extraction* – assess the quality and quantity of the extracted DNA.
- *Library preparation* – assess the quality and quantity of the generated library.
- *Sequencing* – assess the quality of the run and base calling.
- *Sequence assembly and analysis* – assess the proportion of coverage, depth of coverage and quality scores of the mapping; and
- *Variant calling* – assess the variant call quality score, strand bias and allele frequencies.

The target value or threshold (e.g., minimum amount of DNA required) for the quality checks will vary according to the method (tNGS or WGS), library preparation kit, NGS instrument and analytic pipeline. A detailed discussion of NGS quality checks can be found in *The use of next-generation sequencing technologies for the detection of mutations associated with drug resistance in Mycobacterium tuberculosis complex: technical guide (1)*. Technical assistance may be needed from an experienced sequencing laboratory to assist the newly implementing laboratory to define target values.

Internal QC should include the use of a DNA-free (e.g., water or buffer only) sample as a negative control in every batch of DNA extractions and the use of genomic DNA of H37Rv or other reference genome (e.g., *M. bovis* bacillus Calmette-Guérin [BCG] or EQA reference strain) as a control for library preparation and sequencing.

8.3 Implement an EQA programme

An EQA programme includes proficiency testing, rechecking or interlaboratory comparisons (one of these approaches is usually sufficient); regular onsite supportive supervision; and timely feedback, corrective actions, and follow-up. An integral part of an EQA programme is (onsite) support provided to sites performing poorly in the EQA.

Proficiency testing

Proficiency testing compares test results from a testing site with a reference result (and with other laboratory results for the same panel of samples) to determine comparability among testing sites. The purpose is to:

- Identify testing sites with serious testing deficiencies.
- Target support to the most poorly performing testing sites; and
- Evaluate the proficiency of users after training.

For many laboratory tests, the EQA programme includes proficiency testing to determine the quality of the results generated at the testing site. Although several well-characterized MTBC strains are available (e.g., strains used by the SRL network for testing of phenotypic DST), a formal proficiency testing programme for NGS of MTBC has not yet been established. Countries should contact their SRL to discuss opportunities for proficiency testing.

Recently, the European National Reference Laboratory Network for Tuberculosis, with funding from the European Centre for Disease Prevention and Control, developed a proficiency testing

programme for WGS for TB. This programme assesses proficiency for detecting drug resistance and relatedness of strains (Annex 11); it involves 10 WGS-grade DNA specimens and five electronic data sets (FASTQ files) being distributed to participating European reference laboratories. DNA specimens are from mycobacterial cultures and include a sample that contains a 50:50 mixture of a rifampicin-susceptible isolate and a rifampicin-resistant isolate. Laboratories are expected to report mutations associated with resistance to TB drugs and whether these mutations are likely to be associated with high-level or low-level resistance; any highly similar isolates (indicating epidemiological linkage); and the genotype of the strains. All participating laboratories receive an individualized report detailing their performance. Laboratories must score 80% to receive a certificate.

Rechecking of samples

Comparisons among laboratories may also be used as an external assessment of quality. This usually involves the retesting of samples in another quality-assured laboratory. Many TB laboratories are familiar with this approach (referred to as blinded rechecking) because it is performed routinely for EQA for acid-fast bacillus (AFB) smear microscopy. Countries should contact their SRL to discuss opportunities for interlaboratory comparisons.

Retesting with a reference method

A variation of the rechecking approach is to test a subset of specimens with NGS and with a reference method (e.g., phenotypic DST) in the same or a different laboratory. Testing of specimens with both tests commonly uses 10% of samples. This approach does not directly assess the quality of the sequencing; rather, it assesses the agreement between the two tests with respect to detecting drug resistance. Discordant results must be carefully interpreted because discordance could be due to limitations of either the NGS method or the reference method.

Onsite supervisory visits

Onsite supervisory visits for assessment and training are especially critical during the early stages of implementing a new test or procedure because they provide motivation and support to staff. Such visits are also good opportunities to provide refresher training, mentoring, troubleshooting advice and technical updates. Strong relationships between NGS users encourage rapid reporting of any problems and enable rapid troubleshooting, retraining and corrective actions. Onsite supervisory assessments should be documented using standardized checklists to ensure consistency and completeness of information, and to enable monitoring of trends and follow-up on recommendations and corrective actions. An onsite supervisory programme requires substantial planning and resources (both financial and human).

For countries that are establishing NGS at the national laboratory, onsite supervisory evaluations may be conducted by experts from an SRL, or other local or international NGS experts (e.g., from technical partners, academic centres, or other disease programmes).

8.4 Monitor and analyse quality indicators

Routine monitoring of quality indicators, also known as performance indicators, is a critical element of assuring the quality of any diagnostic test. General laboratory quality indicators (Table 8.1) are recommended in the *GLI practical guide to TB laboratory strengthening (27)*, and quality indicators applicable to laboratory testing for DR-TB surveillance are recommended in WHO's *Guidance for the surveillance of drug resistance in tuberculosis (2)*. In addition, laboratories should monitor quality indicators specific to NGS (Table 8.2). Quality indicators should be collected and analysed on a monthly or quarterly basis and disaggregated according to tests. These indicators are provided as a guide; laboratories should review and set locally appropriate targets.

Table 8.1 General laboratory quality indicators

Indicator	Target
Number of tests performed, by type of test	Setting specific
Service interruptions	No interruptions
– Stock-outs	No stock-outs leading to service interruption
– Equipment down time	No equipment downtime leading to service interruption
Turnaround time	90% of results meet the test-specific turnaround time
Test statistics (quality indicator) report	100% of reports are completed by the defined due date
EQA results	>90% EQA panels are passed
QC results	>90% QC results meet the expected criteria
Customer satisfaction	>80% of surveyed customers are satisfied
Technician productivity	Report average number of tests performed per month per technician; the target is setting specific

Adapted from Global Laboratory Initiative (2017) *(20)*

Table 8.2 NGS quality indicators

Indicator (analyse monthly)	Description	Target	Comment
Number and proportion of samples that generate interpretable data for the targeted drugs	Number of specimens that generated interpretable data divided by the total number of specimens tested	>90%	Stratifying this indicator by drug may identify issues with individual gene targets or drugs
Number and proportion of samples tested by NGS that failed QC at any stage of the NGS process	Number of samples that failed the QC criteria divided by total number of samples processed	<10%	Disaggregated by stage of protocol at which failure occurred
Number and proportion of specimens with discordant results when NGS-based DST is compared with phenotypic DST[a]	Number of specimens in which the NGS DST result is different from the phenotypic DST result divided by the number of specimens tested by NGS and phenotypic DST	Varies depending on the drug and population; targets to be set for each drug tested (e.g., for rifampicin, expect <5%) *(15)*	Disaggregate by drug and phenotypic DST method

DST: drug susceptibility testing; NGS: next-generation sequencing; QC: quality control.

[a] In Algorithm 2 of Fig. 2.1 (in Section 2), samples are routinely tested with NGS and phenotypic DST. In algorithms that do not include routine testing with a second method, retesting of 10% of samples tested by NGS using a second method (e.g., phenotypic DST) is recommended as part of QC.

A quality indicator framework includes data collection tools. Countries should review their existing tools to determine which data are already being collected and which existing tools could be revised to enable collection of the required data. In some cases, additional data collection tools may be required. A list of data and quality indicators for NGS-based DST is given in Annex 12.

The frequency of data collection for these indicators should be informed by the resources required and the schedule of meetings during which these data may be reviewed. Frequent review of indicators is essential to ensure that any nonconformities or lack of progress towards targets can be acted upon in a timely manner, and that operational changes can be applied.

Programmes should establish a baseline for all indicators, bearing in mind that this may only be possible for some indicators after new data collection tools have been developed and implemented. Targets should be set for each indicator; a target may be an absolute number or a proportion of sites meeting a defined criterion. At the national level, there should be periodic review of progress towards meeting targets. Also, the programme should critically evaluate reasons for not meeting targets, put in place corrective actions and revise the targets or timelines as needed.

Supporting resources for this section are available in Annexes 10–12.

9. Recording and reporting

9.1 Review and revise data collection and test requisition forms

Depending on the current format of the country's data collection tools and test requisition forms used in DR-TB surveillance, revisions to accommodate NGS testing may be necessary.

Because patient data (also known as metadata) are critical for DR-TB surveillance and the correct interpretation of the NGS results, programmes should ensure that they capture such information on the data collection and test request forms. In many countries, fields for such data are already on the forms but those fields are incompletely or inconsistently completed. Refresher training for clinical and laboratory staff should be conducted to ensure that forms are filled out correctly and completely. Countries should determine the most efficient way to update forms based on their own situation.

A unique survey identification number should be used on all forms and across all databases. Each survey identification number should be linked to only one person and each participant should be identified by only one number.

9.2 Create or review and revise NGS-based DST reporting forms

A crucial first step for the TWG will be to define the information to be provided to the DR-TB surveillance system. The type of information could range from phenotypic resistance prediction (analogous to what is in a phenotypic DST report in the form of susceptible or resistant) to a list of the specific mutations detected (e.g., to monitor trends in resistance-conferring mutations, identify low-frequency variants or assess distribution of minimum inhibitory concentrations [MICs]), to a comprehensive list of molecular epidemiological information (to monitor clustering and transmission dynamics). The forms used for reporting NGS-based DST results must balance the need to convey the large amount of information generated by NGS and the information that is essential for interpreting and using the results in the DR-TB surveillance system.

The decision on what information to include in the NGS report form should be guided by the analyses defined in the DR-TB surveillance protocol. To address the information needs, the reporting template may include sections that provide details of the following (Annex 13):

- Sequencing approach:
  - sequencing type (WGS or tNGS).
  - sequencing platform used.
  - Bioinformatics software, pipeline and version used for analysis.
  - Information on the quality of the NGS results (e.g., average depth of coverage or quality score).
- Drugs and genes tested, and mutations identified:
  - drugs of interest included in the resistance profile.
  - known drug resistance-conferring target gene or genes and respective loci identifications.
  - mutations detected, including:
    - proportion (%) of resistance alleles at loci of interest (to identify mixed infections when present).
    - type of mutation (e.g., amino acid change, promoter mutation, or insertion or deletion).
    - coding effect (synonymous, nonsynonymous or frameshift).
    - amino acid changes (when nonsynonymous).
- Phenotypic resistance prediction:
  - confidence grading of detected mutations (e.g., associated with resistance, not associated with resistance or uncertain significance).
  - resistance profile (final classification as susceptible or resistant).
  - prediction of low-level or high-level resistance where applicable.
- The genotype of the strain:
  - phylogenetic lineage, sub-lineage, or local strain information.
  - detected genomic clustering (if any); and
- Specific comments (e.g., for disputed or uncertain mutations).

For a DR-TB survey, many of these details (e.g., the sequencing process or genes sequenced) will be defined in the survey protocol and may not need to appear in every report.

It may be helpful for the TWG to convene a workshop with programme, laboratory, and clinical subject matter experts, to standardize the reporting form to meet the needs of the DR-TB surveillance system. At a minimum, the reporting form and DR-TB surveillance database should capture the unique patient identifier, quality statistics (e.g., breadth and depth of coverage) and lineage classification for each sample. For each drug analysed, the report form and database should include information on the genes analysed, any mutations detected and the corresponding confidence grading and resistance profile for each drug.

A global consortium *(15, 28)* previously led a consensus process to standardize language for reporting of NGS-based DST results and generate a generic reporting form for MTBC NGS-based

DST results (Annex 13). Although the consensus forms were intended to be used to report NGS-based DST results to clinicians, for use in patient care decisions, they may be useful guides for developing forms for reporting information for a DR-TB surveillance system.

9.3 Review and revise laboratory and surveillance registers

Current laboratory and surveillance registers that are based on the WHO reporting framework *(29)* (a revised version will be published in 2023) will need to be modified to record the results of NGS-based DST. Countries should implement a standardized approach to recording NGS results in laboratory and surveillance registers and use it consistently across all testing and clinical sites.

Forms for laboratory records may also need to be modified. Because of the nature of the NGS workflow, it is important that the methods, instruments, reagents, and bioinformatics pipeline used for the testing of each patient sample can be identified and traced.

9.4 Contribute MTBC sequences to the WHO TB Sequencing database

Countries are encouraged to submit sequences and relevant metadata to the TB sequencing database hosted by WHO *(15)*. This may require data-sharing agreements and a legal framework. The database is a repository of MTBC sequences that informs ongoing updates of the WHO catalogue of resistance-conferring mutations *(15)*, guides the development of rules for interpreting NGS results, generates a global picture of the patterns of resistance to TB drugs, and informs improvements in existing molecular tests and development of future diagnostic tests.

10. Human resources and training

10.1 Develop terms of reference and position descriptions

The successful implementation of NGS will depend on the expertise, training and experience of the laboratory personnel involved. Available staff should be assessed regarding molecular biology (for performing and troubleshooting NGS) and bioinformatics (for sequence analysis).

Position descriptions with clearly defined roles and responsibilities and required competencies and skills will be needed for the staff involved in undertaking NGS. Descriptions are needed for laboratory technicians conducting the sequencing (including DNA extraction, library preparation and sequencing steps); staff conducting the data analysis (including bioinformatics staff); and a senior scientist or laboratory supervisor to oversee the full NGS process, review the results and interpretations, and authorize release of results to the DR-TB surveillance system (Examples of terms of references can be found in the Annex 14).

10.2 Develop training curricula for key staff cadres

The focus of training efforts will be on laboratory and bioinformatics staff conducting the NGS procedures and data analyses. However, successful implementation of NGS-based DST will also require the training of other laboratory, programme, and clinical staff on all aspects of the pathway, from selecting patients to include for NGS-based DST to using the NGS data to meet the objectives of the surveillance system.

NGS applications are cutting-edge technologies with many facets that will be unfamiliar to mycobacteriology technicians who have received training on classical microbiology techniques. Training or experience in molecular biology will be needed to enable technicians to perform and troubleshoot NGS laboratory techniques. For laboratory staff, an NGS training curriculum may include the following (Annex 15):

- Basic molecular biology and computer skills.
- Background on the molecular basis of drug resistance in MTBC.

- The theory and scientific basis of NGS.
- The diagnostic cascade and testing algorithms.
- SOPs.
- Hands-on experience with wet laboratory processes (e.g., sample preparation, DNA extraction, library preparation and sequencing).
- Use and evaluation of QCs and quality checks.
- EQA programmes.
- Basic data analysis skills including assessing the quality of the sequencing run, determining the drug-resistance profile for all tested drugs, and identifying strain lineages.
- Use of laboratory forms and registers, accessioning samples, and recording and reporting results.
- Good laboratory practices including equipment maintenance and cleaning, reagent storage, waste disposal, and chemical and biological safety; and
- Troubleshooting.

For bioinformatics and IT staff, training curricula may include:

- Background on the molecular basis of drug resistance in MTBC.
- The theory and scientific basis of NGS.
- Requirements for computing resources (hardware and software), internet and network connections, and data storage.
- Procedures and options for acquiring, processing, transmitting, and storing data.
- The theory of approaches to analysing NGS data and available analytic tools.
- Hands-on training with analytic tools; and
- Data security and confidentiality of patient data.

For programme staff involved in using and analysing NGS results for surveillance, training curricula may include:

- Background on the molecular basis of drug resistance in MTBC.
- The theory and scientific basis of NGS.
- The diagnostic cascade and testing algorithms.
- Guidelines for the selection of patients for NGS-based DR-TB surveillance.
- Use of data collection forms, test requisition forms and registers.
- Recording and reporting of results including the NGS-based DST result form; and
- Guidelines for interpreting NGS-based DST results with respect to DR-TB surveillance.

10.3 Conduct training

Training should be scheduled well in advance of the introduction of NGS. This should include training of staff from all facilities that will be selecting patients to include in NGS-based DST.

Often, manufacturers of NGS instruments or authorized service providers will provide training on the technical aspects of the NGS instrument and technology. However, such training will not include other locally relevant aspects (e.g., the national testing algorithm, specimen collection, specimen referral, recording and reporting of results, forms, and registers), which remain the responsibility of the NTP and NTRL.

10.4 Assess competency

Competency assessments should be performed after training and periodically thereafter (e.g., annually). Competency assessments should include evaluation of the knowledge and skills for performing each of the tasks involved in an NGS assay including:

- Preparation of equipment and reagents.
- Sample access and data entry.
- Sample processing.
- DNA extraction.
- Library preparation.
- Sequencing.
- Data processing, analysis, and interpretation; and
- Good laboratory practices including equipment maintenance and cleaning, reagent storage, waste disposal and safety.

Assessments should be conducted by an experienced NGS user or trainer and should include observation of the person being trained as they independently conduct each of the required tasks. A template for a competency assessment for NGS is given in Annex 16. As with proficiency testing, processing of three samples from DNA extraction to results interpretation should be sufficient to assess competency.

10.5 Provide for post-training mentoring and support

Post-training mentoring and support that builds on the initial training will help to ensure success in the implementation of NGS and enable the programme to keep abreast of the latest advances in this rapidly evolving field. A support programme will also facilitate troubleshooting during the implementation of any new technology. Potential sources of mentorship and support are as follows:

- Several NGS companies and their distributors offer service contracts that can provide technical support on instrument installation, operation, and maintenance; on DNA extraction procedures and library preparation kits; and for troubleshooting of all aspects of the NGS workflow.

- The WHO SRL network provides support to NTRLs in implementing novel methods and tools for TB diagnosis and surveillance (now including NGS), and in assessing the quality of the (molecular) methods introduced.
- Public online NGS forums[1] and communities can help users in designing and performing targeted NGS experiments, and in interpreting results and troubleshooting; and
- In some countries, staff in other programmes or research facilities that use NGS technologies may be able to provide support for the NGS application and troubleshooting.

[1] Online forums include SEQ answers (https://seqanswers.com/), Biostars (https://www.biostars.org/) and LinkedIn's (LinkedIn Corporation, Sunnyvale, California, USA) genomics forum (https://www.linkedin.com/groups/1907871/profile).

11. Monitoring and evaluation

11.1 Monitor the implementation of NGS

During the initial planning phase, countries should establish a set of milestones that can be used to monitor the implementation process. The readiness assessment checklist in Annex 3 may be useful in selecting milestones and indicators. Using information given in the previous sections, indicators should be developed to monitor the following:

- Development of a costed implementation plan.
- Acquisition of required financial resources for implementation and ongoing costs.
- Addressing of any regulatory, importation and ethical issues.
- Preparation of the testing site for performing NGS:
  - facility upgrades (e.g., number of rooms, electrical supply, and environmental controls).
  - equipment procurement and installation.
  - bioinformatics support.
  - procurement of supplies and reagents.
- development of SOPs to address all aspects of the diagnostic process (e.g., patient selection, specimen collection, specimen transport, NGS, and recording and reporting).
- Development or acquisition of adequate data storage capacities and required bioinformatics and analytic tools and expertise.
- Development and implementation of QC and EQA systems.
- Development and dissemination of required forms (e.g., test requisition forms, laboratory and clinical registers, and reporting forms).
- Development of human resources including the training and competency assessment of sufficient laboratory and clinical staff with the required skills.
- Development of a plan to monitor implementation; and
- Development of a plan to monitor and evaluate the impact of NGS.

Once launched, use of the NGS services in the DR-TB surveillance system could be tracked by assessing:

- the number of NGS tests conducted as part of the DR-TB surveillance system; and
- the number of patients for whom NGS-based DST results were reported to the DR-TB surveillance system.

The quality of the NGS services can be evaluated by monitoring the quality checks and performance indicators described in Section 8.

11.2 Monitor and evaluate the impact of NGS

A framework for M&E of the impact of NGS is essential to inform decision-making. NGS-based DST can improve DST, especially for drugs for which there is no reliable phenotypic DST method and in laboratories that do not have the facilities and technical expertise to conduct the technically demanding phenotypic DST methods. With respect to a DR-TB surveillance system, this should affect the number and proportion of people with TB for whom DST results are recorded in the system, and the number and proportion of WHO-recommended drugs for which DST results are recorded in the system.

The TWG should define the objectives of incorporating NGS-based DST into the DR-TB surveillance system, whether for continuous routine surveillance or for a periodic survey. For each objective, the TWG should develop outcome indicators to assess impact. For each outcome indicator, the TWG should define its purpose, target, data elements, data sources, how it should be calculated, process indicators and corresponding data elements that contribute to it. Indicators are typically analysed quarterly or annually, and trends are identified and investigated. The baseline for each indicator may be what was observed when the previous laboratory testing algorithm (e.g., phenotypic DST) was used. It is anticipated that the incorporation of NGS-based DST into the DR-TB surveillance system will lead to increases in each of the indicators.

Typically, a major objective of implementing NGS-based DST is to improve the completeness of the DST results recorded in the DR-TB surveillance system. Possible outcome indicators to assess this objective are given in Table 11.1. Annex 17 provides an example of an outcome indicator that describes its purpose, target, data elements, data sources, calculation, process indicators and corresponding data elements that contribute to the main indicator. In-depth analyses of the process indicators may be useful as follow-up investigations to further elucidate the contribution of NGS-based DST to the outcome and identify opportunities for interventions to increase impact.

Additional objectives and indicators may be developed to assess the impact on the identification of people with DR-TB, the timeliness of reporting of DST results to the DR-TB surveillance system or the detection of strain clusters or outbreaks. For example, NGS-based DST might improve the timeliness of the reporting of the DST results (particularly if tNGS testing of sputum specimens is used), which in turn could allow more rapid detection of changes in DR-TB trends or detection of clusters suggestive of DR-TB outbreaks.

Table 11.1 Examples of impact measures and outcome indicators

Objective: to improve the completeness of surveillance for drug-resistant TB	
Outcome Indicator 1	Number and proportion of cases recorded in a DR-TB surveillance system with DST results reported (stratified by drug)
Outcome Indicator 2	Number and proportion of cases identified as having DR-TB (stratified by drug)
Outcome Indicator 3	Number and proportion of cases with complete DST results, i.e., with results entered for each of the drugs included in the DR-TB surveillance system (and compared to the list of WHO-recommended drugs to assess comprehensiveness of the DST)
Process indicators to assess the contribution of NGS-based DST to the outcome indicators	
Process Indicator 1	Number and proportion of cases with DST results (any method) for which NGS-based DST results were recorded in the DR-TB surveillance system
Process Indicator 2	Number and proportion of cases identified as having DR-TB based on NGS-based DST results (stratified by drug)
Process Indicator 3	Number and proportion of cases with NGS-based DST results recorded in the DR-TB surveillance system stratified by 1) individual drug, 2) completeness of DST results

As part of demonstrating the impact of NGS and to assist with future planning and policymaking, countries should consider evaluating the cost, cost–effectiveness and end-user perspective of implementing NGS-based DST in the DR-TB surveillance system 1 year after implementation, and regularly thereafter.

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Annex 1

Template Gantt chart for an implementation roadmap

Steps	Timeline											
Policy and planning												
Establish and define roles and responsibilities of a TWG												
Review WHO/FIND technical guide and implementation considerations												
Define immediate and future purposes of NGS												
Update relevant national TB strategic plans, diagnostic algorithms and guidelines												
Identify laboratories that will perform NGS												
Perform a programmatic readiness assessment												
Assess NGS site readiness – laboratory facilities												
Assess NGS site readiness – IT/data												
Develop a costed operational plan												
Regulatory												
Determine importation requirements												
Conduct country verification, if required												
Complete national regulatory processes												
Equipment												
Perform necessary upgrades to testing site												
Select equipment												
Install and set up equipment												
Supplies												
Review forecasting, ordering and distribution procedures												
Develop procedures to monitor reagent quality and shelf-life												
Procedures												
Develop standard operating procedures												

Data												
Design an appropriate data storage solution, including linkage with other relevant databases												
Acquire and install data storage solution												
Select analytic tools												
Acquire analytic tools and training and implement												
Verify the performance of the bioinformatics pipeline												
Develop data sharing policies and procedures												
Quality assurance												
Implement or strengthen quality assurance programmes in NGS laboratories												
Develop policies and procedures for quality control of NGS												
Develop and implement an EQA programme												
Develop tools and system for routine monitoring of quality indicators												
Recording and reporting												
Review, revise and disseminate request for examination forms												
Review, revise and disseminate test requisition, recording and reporting forms, as needed												
Modify and disseminate laboratory and clinical registers												
Human resources and training												
Develop terms of reference and job descriptions for key staff												
Develop training curricula for different staff cadres												
Conduct training												
Conduct competency assessments after training and periodically thereafter												
Establish a post-training mentoring and support system												
Monitoring and evaluation												
Develop key indicators or milestones for implementation												
Implement monitoring												
Develop a framework to monitor and evaluate impact												
Monitor and evaluate impact												

EQA: external quality assessment; FIND: Foundation for Innovative New Diagnostics; NGS: next-generation sequencing; TB: tuberculosis; TWG: technical working group; WHO: World Health Organization.

Annex 2

Next-generation sequencing implementation high level checklist

1. Policy and planning

- Have roles and responsibilities for coordinating the implementation of next-generation sequencing (NGS) been clearly defined?

- Do the objectives of the national surveillance system or the protocol of a planned drug-resistant tuberculosis (DR-TB) survey include the use of NGS-based drug susceptibility testing (DST)?

- Has funding been mobilized, and do the timing and budget for NGS implementation align with the timing and budgeting of DR-TB surveillance?

- Has a stakeholder mapping process been conducted, including all key internal stakeholders (within government) and external stakeholders (local and international)?

- What support can partners provide for the implementation process?

- Has the intended use of NGS been decided? Have projections been made for the number of samples to be tested per year or per site? For surveys, has World Health Organization (WHO) guidance been followed for survey design and sample size estimation?

- Has a costed implementation plan been developed?

- Have adequate financial resources for capital investments, implementation and projected on-going costs been secured?

2. Regulatory

- What are the importation requirements for instruments, reagents and supplies for NGS?
- What is the regulatory process required for NGS?
- Is verification of NGS needed for regulatory approval for use in DR-TB surveillance? If so, what type of protocol and number of samples are required, what is the timeline and where will verification studies be conducted?

- Is the designated authority (national TB programme [NTP], procurement agency or partners) engaged with manufacturers to support regulatory processes?

3. Site readiness

- Are adequate laboratory facilities, space, and infrastructure available?
- Do facilities, equipment, policies, and practices meet the TB biosafety standards?
- Are safe facilities for culturing Mycobacterium tuberculosis complex (MTBC) available, if needed?

- Are appropriately trained and competent staff available to conduct NGS and bioinformatic analyses?

- What IT resources are available and what else will be needed?

- Which NGS instrument has been selected and what are the requirements for its installation and maintenance?

- Are adequate systems available for specimen referral and reporting of results?

4. Procurement and supply chain

- Which partners support NGS in the country, and what is their scope of activities (i.e., how can they contribute to the transition)?

- Which partners procure instruments and consumables?

- Have manufacturers or distributors been identified who can support implementation, equipment maintenance (warranties or service contracts) and commodities?

- Is a procurement system available to ensure the availability of reagents and supplies those accounts for procurement times, consumption rates and shelf-life of reagents?

- What is the planned procurement by the ministry of health (MOH) and partners for this year?

5. Procedures

- Which standard operating procedures (SOPs) and forms will need to be updated or developed?

6. Data

- What data storage solutions are available at the testing site?

- What upgrades to data systems will be needed?

- Which data analysis tools have been selected?

- Who will conduct the bioinformatics analyses?

- Is sufficient bioinformatics expertise available at the testing laboratory?

- What support are partners able to provide for bioinformatics and data analysis?

- Are procedures in place to define data-sharing protocols and ensure the confidentiality?

- Are procedures in place to link NGS data to laboratory information systems, electronic registers, and so on?

7. Quality assurance

- Are the essential elements of a quality assurance system in place at the testing site?

- Are protocols in place to conduct and document the quality checks of each step of the NGS process, and to ensure the use of positive and negative controls?

- Is an external quality assessment programme in place?

- Which partners can assist with proficiency testing, supervisory visits and rechecking of samples?

- Have quality (performance) indicators been defined and appropriate data collection tools developed?

8. Recording and reporting

- Are revisions of the current data collection form and request for laboratory testing form required for the introduction of NGS into the DR-TB surveillance process?

- Is there a need for revision of:
  - forms for reporting the results of laboratory testing to the surveillance programme.
  - Laboratory, clinical or surveillance registers; or
  - the forms used for laboratory records, to ensure the traceability of methods, reagents, instruments, bioinformatics pipeline etc.

- If an electronic laboratory information system is in use, what updates will be required?

- If an electronic recording and reporting system is in place, what updates will be required?

9. Training

- Have terms of reference and competency-based job descriptions been developed for key staff (e.g., laboratory technicians, bioinformatics officer and clinicians)?

- Is a national approved training curriculum available?

- Who is responsible for updating training materials for laboratory, clinical and programme staff, and what is the process for updating the materials?

- Is the approved curriculum used for all training, including training delivered by partners?

- Are standard procedures used to assess and document the competence of all staff involved in NGS?

10. Monitoring the transition

- What changes to monitoring and evaluation (M&E) tools and processes would be required to enable monitoring of additional indicators (i.e., progress indicators and laboratory indicators)?

- What support can partners provide in monitoring of new algorithms and adherence to guidelines at sites?

Annex 3

Situational Analysis Checklists

A situational analysis should be conducted to determine which NGS activities are being implemented at the national and testing site levels and to identify gaps and obstacles to producing quality assured NGS data. The situational analysis should be used to inform recommendations on how to implement NGS activities and to develop a roadmap and timeline for NGS implementation.

Part A assesses the national-level NGS-specific aspects the laboratory system that are required to perform NGS-based DST to aid in the diagnosis and treatment of TB. Part B assesses NGS-specific activities at the testing site. Part B can also be used as a standalone checklist to assess the readiness of a laboratory to conduct NGS-based DST for TB.

Note that this checklist does not address many generic aspects of the TB diagnostic network (e.g., specimen referral, workforce development, network coverage, etc.). The TB-NET tool can be used to conduct a comprehensive evaluation of the TB diagnostic network.

Part A. National-Level NGS Situational Analysis Checklist

At the national level, key NGS activities include planning, budgeting, allocating resources, developing policies, standardizing procedures, and forms, facilitating regulatory compliance and procuring equipment and consumables. The national programme is also responsible for ensuring that all laboratories that conduct NGS testing for TB follow the same policies and procedures. However, at least initially in many countries, there may be only one laboratory conducting NGS testing for TB. In that case, some national-level activities (e.g., development of SOPs, training, procurement, etc.) may be delegated to the NGS laboratory and many questions in this checklist can be answered by the NGS laboratory. Questions directly related to national-level responsibilities (e.g., national policies and guidelines) are marked with an '□' in the 'symbol' column.

Most questions are to be answered with a 'Yes' (achieved), No' (not achieved) or 'Partial' (partially achieved). Some questions (e.g., how many instruments?) will be answered by providing numbers) and some (e.g., who is responsible for …?) will have text answers. Space is provided to provide comments for the responses for each question.

To aid in the assessment, a suggested approach to assessment is provided for each question.

Symbol	Approach
🕮	Review applicable documents, e.g., policies, SOPs, guidelines, and data
?	Ask staff members or clients for their views or level of understanding
😐	Objective observations or conclusion
☝	Test the functionality of the equipment or system
□	Question to be addressed by national programme

Country name:	
Assessor name:	
Assessor contact details:	
Date of assessment:	

General	
How many testing sites are or will be providing NGS testing for TB?	
How many NGS instruments have been placed in-country:	
• For diagnostic testing for TB?	
• For diagnostic testing for other diseases or organisms? Specify which diseases.	
• For research on TB?	
• For research on other diseases or organisms? Specific which diseases or organisms	
Are additional NGS instruments planned for use for TB, and if so, how many?	
Where are the current NGS instruments that are being used for TB located and are they intended for TB use only or across diseases?	

In addition to noting the presence of documentation, assessors must collect, where possible, a copy of the policy, document, SOP, or form.

		Yes	No	Partial	Comments
1. Policies, Governance, Strategic Planning and Resources					
a. Are the following national guidelines and policies in place (i.e., approved, accessible and implemented):	😐, ? □	Y	N	P	
• National Health Strategic Plan	🕮	Y	N	P	
• National TB Strategic Plan	🕮	Y	N	P	
• National TB Laboratory Strategic Plan	🕮	Y	N	P	
• NGS Implementation Plan	🕮	Y	N	P	
b. Are national policies in place that address the following aspects of NGS:	🕮, ?, □	Y	N	P	
• Intended use of NGS (e.g., NGS-based DST for TB patient care)?		Y	N	P	
• Selection of laboratories that will conduct all or part (e.g., bioinformatics analyses) of the NGS process?		Y	N	P	
• Validation of all NGS methods including the wet bench processes and data analysis pipeline?		Y	N	P	
• Implementation and documentation of upgrades to the bioinformatics pipeline and wet bench processes (e.g., instruments or data analysis tools)?		Y	N	P	

		Yes	No	Partial	Comments
• Use of nationally approved, standardized procedures, documents, records and forms at NGS testing sites?		Y	N	P	
• Interpretation of sequence variants according to international standards as indicated in the" Catalogue of mutations in Mycobacterium tuberculosis and their association with drug resistance"[a]?		Y	N	P	
• Reporting of sequence findings unrelated to purpose of the NGS (e.g., finding virulence factors when conducting NGS-based DST)?		Y	N	P	
• Indications and procedures for confirmatory testing?		Y	N	P	
• Tracing methods, instruments and reagents used for testing each patient sample?		Y	N	P	
• Documentation of non-conformities and exceptions in the testing process for a patient sample?		Y	N	P	
• Confidentiality and security of NGS data and patient information?		Y	N	P	
• Maintenance, servicing and verification of NGS instruments?		Y	N	P	
• Training and competency assessments?		Y	N	P	
• Participation in an NGS proficiency testing program?		Y	N	P	
• Procurement and supply of diagnostic reagents for NGS?		Y	N	P	
• Monitoring and evaluation?		Y	N	P	
c. Has a national governance structure been established for NGS-based DST? If yes,	😐, □, ?	Y	N	P	
• Is there a national regulatory body to approve the use of NGS-based diagnostic testing?	😐, ?	Y	N	P	
• Is there a Technical Working Group specifically responsible for guiding the implementation of NGS-based DST?	😐, ?	Y	N	P	
• Is there an NGS Focal Person or equivalent to oversee the implementation of NGS-based DST?	😐, ?	Y	N	P	

[a] Catalogue of mutations in *Mycobacterium tuberculosis* complex and their association with drug resistance. Geneva: World Health Organization; 2021. License: CC BY-NC-SA 3.0 IGO. https://www.who.int/publications/i/item/9789240028173

		Yes	No	Partial	Comments
d. Has a strategic planning process been conducted for NGS-based DST for TB? If yes, did it include:	😐, ? 📖	Y	N	P	
• Engagement of key partners (e.g., WHO, implementing partners, etc.) in policy development for TB testing?	😐, ?	Y	N	P	
• Engagement of other disease programs who are using NGS in policy development for TB testing?	😐, ?	Y	N	P	
• A stakeholder mapping process that included all key internal (within government) and external stakeholders? (Local and international)?	😐, ?	Y	N	P	
• Definition of roles and responsibilities for coordinating the transition process?	😐, ?	Y	N	P	
• Definition of the intended use of NGS? If yes, what is the intended use?	😐, ?	Y	N	P	
e. Which partners support NGS in the country and what is their scope of activities (how can they contribute to the transition)?	😐, ?	Y	N	P	
f. Does a current costed national plan exist for NGS implementation?	📖	Y	N	P	
g. Have projections been made for the number of samples to be tested per year or per site?	📖	Y	N	P	
h. Are resources (e.g., funding, staff, laboratory infrastructures, etc.) available at the national level to support NGS:	😐, ? 📖	Y	N	P	
• NGS-related implementation activities?	😐, ?	Y	N	P	
• Infrastructure improvements?	😐, ?	Y	N	P	
• Capital investments?	😐, ?	Y	N	P	
• Equipment, procurement, installation and maintenance?	😐, ?	Y	N	P	
• Training according to the national plan for the current year?	😐, ?	Y	N	P	
• On-site supervisory visits?	😐, ?	Y	N	P	
• Proficiency testing activities?	😐, ?	Y	N	P	
• Monitoring and evaluation activities?	😐, ?	Y	N	P	
• Analysis of quality and performance indicators?	😐, ?	Y	N	P	
• Procurement activities? • Projected on-going costs?	😐, ?	Y	N	P	

		Yes	No	Partial	Comments
2. Regulatory					
a. What are the importation requirements for instruments, reagents, and supplies for NGS-based DST?	😐, ?	Y	N	P	
b. What is the regulatory process required for NGS-based DST?	😐, ?	Y	N	P	
c. Is country verification of NGS-based DST needed for regulatory approval? If yes,	😐, ?	Y	N	P	
• What type of protocol and number of samples are required?					
• Where will verification studies be conducted?					
• What is the timeline for the studies?					
d. Is the designated authority (NTP, NTRL, procurement agency or partners) engaged with manufacturers to support regulatory processes?	😐, ?	Y	N	P	
3. A safe and functional testing site					
a. Are there national requirements for the laboratory infrastructure needed for NGS-based DST for TB?	🕮, ?	Y	N	P	
b. Is there a mechanism for assessing each NGS testing site for readiness?	🕮, ?	Y	N	P	
• If yes, is a standardized checklist used?	🕮, ?	Y	N	P	
d. Is there a mechanism for upgrading facilities as needed to create a safe functional work environment?	🕮, ?	Y	N	P	
e. Are there partners and identified funding sources to support upgrades? If so, who?	🕮, ?	Y	N	P	
4. Equipment and supplies					
4.1 Equipment, service and maintenance					
a. Has the NGS instrument been selected? If yes,	😐, ?	Y	N	P	
• Which instrument?					
• If there is more than one NGS DST site for TB, will the same instrument be used? If not, why not?					
b. Is a list of approved ancillary NGS equipment available? e.g., DNA extraction instrument, fluorometer or spectrophotometer, DNA fragment analyzer, thermocycler	🕮, ?	Y	N	P	
c. Are there partners in the country that can support NGS implementation, installation and on-going use?	😐, ?	Y	N	P	
d. Are policies in place requiring testing sites to maintain and service all NGS instruments?	🕮	Y	N	P	

		Yes	No	Partial	Comments
e. Have manufacturers or distributors been identified who can support installation and equipment maintenance (warranties or service contracts)?	😐, ?	Y	N	P	
f. Is there a mechanism in place to monitor if testing sites are performing maintenance and servicing of all NGS instruments?	😐, ?	Y	N	P	
4.2 Procurement & supply chain					
a. Is there a national policy in place for procurement of NGS instruments and reagents?	🕮	Y	N	P	
b. Are TB laboratory and sequencing experts involved in setting specifications for procurement?	😐, ?	Y	N	P	
c. Is procurement of NGS instruments and reagents managed centrally (e.g., NTRL, Central Medical Store etc.)?	😐, ?	Y	N	P	
d. Is a procurement system available to ensure the availability of NGS reagents and supplies that consider procurement times, consumption rates and shelf-life of reagents?	😐, ?	Y	N	P	
e. Is there a mechanism to provide NGS test consumption data to inform procurement practices?	😐, ?	Y	N	P	
f. Are there partners in the country that can support procurement of NGS commodities?	😐, ?	Y	N	P	
g. Is there a practice in place to quality check new lots of NGS reagents coming into the country?	😐, ?	Y	N	P	
h. What is the planned procurement by MOH and partners for this year?	?				
• NGS instruments and ancillary equipment					
• Consumables					
5. Procedures and documentation					
a. Are the following standardized documents, records and forms available, approved and up to date (i.e., incorporates NGS testing)?	🕮	Y	N	P	
• TB diagnostic algorithm		Y	N	P	
• NGS-DST requisition form		Y	N	P	
• Sample collection and transport forms		Y	N	P	
• Laboratory register		Y	N	P	
• NGS instrument maintenance log		Y	N	P	
• NGS reporting codes		Y	N	P	
• NGS performance indicator reporting form		Y	N	P	

		Yes	No	Partial	Comments
• NGS test reporting form		Y	N	P	
• Non-conformity, exception and corrective action logs		Y	N	P	
• NGS PT results form & failure follow-up form		Y	N	P	
b. Are standard operating procedures (SOPs) available, approved and up to date that document all steps in the:	🕮	Y	N	P	
• Wet bench processes used to generate NGS data?		Y	N	P	
• Bioinformatics pipeline used to analyze, interpret, and report NGS results		Y	N	P	
c. Is there a mechanism to review and update standardized documentation?	🕮	Y	N	P	
6. Data storage and analysis tools					
a. Are policies and procedures in place for NGS activities that:	🕮, ? □	Y	N	P	
• Define data sharing protocols and transfer strategies?		Y	N	P	
• Maintain relevant intellectual property rights?		Y	N	P	
• Ensure that internal and external storage and transfer of sequencing data maintains patient confidentiality and security?		Y	N	P	
• Link NGS data to laboratory information systems, electronic registers, MMIS2, etc.?		Y	N	P	
• Identify which data system(s) will be used?		Y	N	P	
• Specify if the data system will be specific for TB or if it will be integrated across diseases?		Y	N	P	
• Define who will have access to the data?		Y	N	P	
• Define storage requirements for the input, intermediate, and final data files generated during data analysis?		Y	N	P	
• Define processes for validating the bioinformatics pipeline and revalidating the pipeline or components of it when modifications are made?		Y	N	P	
• Monitor, document and implement patch-releases, upgrades and other updates to the bioinformatics pipeline		Y	N	P	
• Describe how data will be secured?		Y	N	P	
• Specify how, and at what frequency, data will be backed-up?		Y	N	P	

		Yes	No	Partial	Comments
• Describe who is responsible for technical support, IT-support and updates		Y	N	P	
b. Has an assessment of existing data storage solutions, infrastructure (e.g., internet access) and analytic tools for NGS been conducted? If yes, did it assess:	😐, ?	Y	N	P	
• adequacy of available data storage solutions and if upgrades are needed?		Y	N	P	
• adequacy of data transfer tools?		Y	N	P	
• If upgrades to infrastructure (e.g., internet) will be needed?		Y	N	P	
• Availability of data analysis tools?		Y	N	P	
• Availability of bioinformatics expertise?		Y	N	P	
c. Does a current costed plan exist for implementation of an NGS data system and performing necessary upgrades, replacement, and maintenance?	🕮	Y	N	P	
d. If yes, does the implementation plan address:	🕮	Y	N	P	
• Upgrades to infrastructure?		Y	N	P	
• Installing and maintaining hardware and software at the testing site?		Y	N	P	
• Setting up the data processing, transfer and storage systems at the testing site?		Y	N	P	
• Training for new users and refresher training for existing users?		Y	N	P	
• Providing operational costs of the system?		Y	N	P	
• Developing and disseminating SOPs for access, reporting, data entry, data security including patient meta-data and data back-up?		Y	N	P	
e. Have SOPs for access, reporting, data entry, data security and data back-up been developed and approved?	🕮, ?	Y	N	P	
f. Are any partners able to provide support for bioinformatics and data analysis?	😐, ?	Y	N	P	
7.1 Quality Assurance					
a. Are national policies in place requiring the essential elements of a quality assurance system to be in place at the testing site?	😐, ? □	Y	N	P	
• SOPs, training, and competence assessment		Y	N	P	
• Instrument verification		Y	N	P	
• Equipment maintenance		Y	N	P	
• Method validation or verification		Y	N	P	

		Yes	No	Partial	Comments
• Quality control (QC)		Y	N	P	
• Lot testing (also known as incoming quality control or new batch testing)		Y	N	P	
• External quality assessment (EQA)		Y	N	P	
• Quality indicator monitoring		Y	N	P	
• Continuous quality improvement (CQI) including the identification and documentation of non-conformities, exceptions, and corrective actions		Y	N	P	
b. Does the quality assurance policies address all steps in the NGS wet bench processes and the bioinformatics pipeline?	☹, ?	Y	N	P	
7.2 Establish and monitor quality controls					
a. Are protocols in place to conduct and document quality checks of each step of the NGS-based DST process and ensure the use of positive and negative controls?	☹, ?	Y	N	P	
7.3 External quality assessment					
a. Is an external quality assessment programme in place?	☹, ?	Y	N	P	
• Proficiency testing (PT)?		Y	N	P	
• Re-checking samples?		Y	N	P	
• On-site supervisory visits?		Y	N	P	
b. Have partners been identified who can provide technical assistance or assist with proficiency testing, supervisory visits and re-checking of samples? If so, who?	☹, ?	Y	N	P	
c. Do all NGS testing sites currently participate in a PT programme for NGS?	☹, ?	Y	N	P	
d. Who provides the PT panels?	☹, ?				
e. Does NTRL, or designee provide oversight of the PT programme and performance of testing sites?	☹, ?	Y	N	P	
f. Are mechanisms in place to follow-up sites that produce incorrect PT results?	☹, ?	Y	N	P	
g. Do the NGS laboratories participate in inter-laboratory comparisons (re-checking of samples)? If yes, with which laboratories?	☹, ?	Y	N	P	
h. Are there national policies requiring on-site supervisory visits to NGS testing sites?	🕮	Y	N	P	
i. Are supervisory visits conducted by trained supervisors?		Y	N	P	
j. Are approved standardized checklists available for conducting supervisory visits of NGS laboratories?	🕮, ?	Y	N	P	

		Yes	No	Partial	Comments
k. Is there a mechanism to provide feedback to testing sites following a supervisory visit?	?	Y	N	P	
l. Are the outcomes of supervisory visits reviewed at the central level (e.g., NTRL)?	? □	Y	N	P	
7.4 Monitor and analyze quality indicators					
a. Is there a national policy in place requiring the collection of performance indicators?	& □	Y	N	P	
b. Does the NTP/NTRL collect, analyze and use NGS testing performance indicators for decision making?	😐, ? □	Y	N	P	
c. Have appropriate data collection tools developed and disseminated?	😐, ? □	Y	N	P	
8. Recording and reporting					
a. Is revision of the current request for examination form required for introduction of NGS-based DST?	🕮, ?	Y	N	P	
b. Is revision of reporting forms required for introduction of NGS-based DST?	🕮, ?	Y	N	P	
c. Does the reporting form indicate that. "the, Catalogue of mutations in Mycobacterium tuberculosis and their association with drug resistance"[a] was used to interpret and report sequence variants?	🕮, ?	Y	N	P	
d. Is a revision of laboratory or clinical registers needed required for introduction of NGS-based DST?	🕮, ?	Y	N	P	
e. Is a revision of forms for laboratory records needed to identify the following for each sample tested:	🕮, ?	Y	N	P	
• the methods, instrument(s) and reagents used for processing and sequencing?		Y	N	P	
• the version of the bioinformatics pipeline used to generate, analyse and interpret the NGS data?		Y	N	P	
• any non-conformities and exceptions in the testing process?		Y	N	P	
f. If an electronic laboratory information system is in use, what updates will be required?	?				
g. If an electronic recording and reporting system is in place, what updates will be required? If not, what system will be used to inform clinicians promptly about NGS-based DST results?	?				

[a] Catalogue of mutations in *Mycobacterium tuberculosis* complex and their association with drug resistance. Geneva: World Health Organization; 2021. License: CC BY-NC-SA 3.0 IGO. https://www.who.int/publications/i/item/9789240028173

		Yes	No	Partial	Comments
9. Training and competency assessment					
a. Have terms of reference been developed for key staff involved in NGS?	🕮, ?	Y	N	P	
• laboratory technicians		Y	N	P	
• bioinformatics officer		Y	N	P	
• clinicians and health care workers		Y	N	P	
b. Is approved standardized training for NGS-based DST available for:	🕮, ?	Y	N	P	
• laboratory technicians?		Y	N	P	
• bioinformatics officer?		Y	N	P	
• clinicians and healthcare workers?		Y	N	P	
• TB programme staff?		Y	N	P	
c. Is the approved training curriculum used for all trainings, including those delivered by partners?	😐, ?	Y	N	P	
d. Are standard procedures used to assess and document the competence of all staff involved in NGS-based DST?	🕮 😐, ?	Y	N	P	
• laboratory technicians?		Y	N	P	
• bioinformatics officer?		Y	N	P	
• clinicians and healthcare workers?		Y	N	P	
• TB programme staff?		Y	N	P	
e. Are competency assessments for NGS users conducted at least annually?	🕮	Y	N	P	
f. Are records of training kept centrally?	🕮	Y	N	P	
g. Is there a national annual training plan?	🕮 □	Y	N	P	
10.1 Monitoring and evaluation of the implementation of NGS testing					
a. Are there national policies and plans in place to monitor and evaluate the implementation of NGS testing?	🕮 □	Y	N	P	
b. Have key indicators and milestones been established to monitor the implementation process?	😐, ? □	Y	N	P	
c. Are the following implementation indicators monitored and analyzed by the national programme at least annually	😐, ? □	Y	N	P	
• Total number of NGS tests performed (disaggregated by population, e.g., HIV+, children, vulnerable, EPTB)		Y	N		
• Number and proportion of NGS testing sites relative to projected need		Y	N		
• Number and proportion of clinical sites with access to NGS testing on site or via a functional referral network		Y	N		
d. Are partners available that can assist with monitoring of the implementation of NGS?	😐, ?	Y	N	P	

		Yes	No	Partial	Comments
10.2 Monitoring and evaluation of impact of NGS-based DST					
a. Are there national policies and plans in place to monitor and evaluate the impact of NGS-based DST?	🕮	Y	N	P	
b. Are the following indicators monitored and analyzed by the national programme at least annually	😐, ?	Y	N	P	
• Number and proportion of cases recorded in a DR-TB surveillance system with DST results reported (stratified by drug)		Y	N		
• Number and proportion of cases identified as having DR-TB (stratified by drug)		Y	N		
• Number and proportion of cases with complete DST results, i.e., with results entered for each of the drugs included in the DR-TB surveillance system (and compared to the list of WHO-recommended drugs to assess comprehensiveness of the DST) -recommended first- and second-line drugs		Y	N		
• Number and proportion of cases with DST results (any method) for which NGS-based DST results were recorded in the DR-TB surveillance system		Y	N		
• Number and proportion of cases identified as having DR-TB based on NGS-based DST results (stratified by drug)		Y	N		
• Number and proportion of cases with NGS-based DST results recorded in the DR-TB surveillance system stratified by 1) individual drug, 2) completeness of DST results					
c. Are partners available that can assist with monitoring the impact of NGS-based DST?	😐, ?	Y	N	P	

Part B. Site-Level NGS Situational Analysis Checklist

This checklist is used to assess the readiness of a laboratory to conduct NGS-based DST for TB. The numbering of the sections in this checklist corresponds with the numbering of the sections in the implementation guide. Most questions are to be answered with a 'Yes' (achieved), No' (not achieved) or 'Partial' (partially achieved). Some questions (e.g., how many instruments?) will be answered by providing numbers) and some (e.g., who is responsible for …?) will have text answers. Space is provided to provide comments for the responses for each question. To aid in the assessment, a suggested approach to assessment is provided for each question.

Symbol	Approach
🕮	Review applicable documents, e.g., policies, SOPs, guidelines, and data
?	Ask staff members or clients for their views or level of understanding
😐	Objective observations or conclusion
☝	Test the functionality of the equipment or system

Country name:

District:

Testing site:

Assessor name:

Assessor contact details:

Date of assessment:

In addition to noting the presence of documentation, assessors must collect, where possible, a copy of the policy, document, SOP, or form.

		Yes	No	Partial	Comments
1. Policies, Governance, Strategic planning, and resources					
a. Are the following national guidelines, policies and plans accessible at the testing site:	🕮	Y	N	P	
• Use of NGS-related standardized documents, records, and forms?		Y	N	P	
• Maintenance, servicing, and verification of NGS instruments?		Y	N	P	
• Training and competency assessments?		Y	N	P	
• Quality Assurance and external quality assessment?		Y	N	P	
• Procurement & supply of NGS-DST reagents?		Y	N	P	
• Monitoring and evaluation?		Y	N	P	
• National strategic plan for NGS implementation?		Y	N	P	

		Yes	No	Partial	Comments
b. Has a staff member (part-time or full-time) responsible for overseeing the implementation of NGS been appointed?	😐, ?	Y	N	P	
c. Has a specific laboratory plan been developed for the implementation of NGS testing? If yes, does the plan:	🕮	Y	N	P	
• Define the intended use of NGS?		Y	N	P	
• Describe which processes will be conducted in the laboratory and which, if any, will be referred to other service providers (e.g., bioinformatic analyses)?		Y	N	P	
• Describe an implementation roadmap, timeline, and milestones?		Y	N	P	
d. Which partners support NGS in the country and how can they contribute to NGS implementation?	?				
e. Does a current costed plan exist for NGS implementation in the laboratory?	🕮	Y	N	P	
f. Are adequate resources (e.g., funding, staff, laboratory infrastructures, etc.) available to support NGS implementation activities including:	😐, ?	Y	N	P	
• Infrastructure upgrades?		Y	N	P	
• Purchase (or lease) and installation of equipment?		Y	N	P	
• Purchase and installation of data storage solutions?		Y	N	P	
• Acquisition of data analysis tools?		Y	N	P	
• Training and competency assessments of laboratory workers?		Y	N	P	
• Training of clinicians and healthcare workers?		Y	N	P	
• NGS-related documentation activities?		Y	N	P	
g. Are adequate resources (e.g., funding, staff, laboratory infrastructures, etc.) available to support on-going costs of NGS diagnostic activities including:	😐, ?	Y	N	P	
• Equipment maintenance and service contracts?		Y	N	P	
• Data storage costs?		Y	N	P	
• Analytic tools (e.g., subscription fee or internet fee)?		Y	N	P	
• Referred services (e.g., bioinformatic analyses), if any?		Y	N	P	
• On-going training and competency assessments?		Y	N	P	
• Quality assurance (proficiency testing, monitoring indicators etc.)?		Y	N	P	

		Yes	No	Partial	Comments
• Projected on-going costs related to NGS diagnostic testing (staff, reagents, consumables, etc.)?		Y	N	P	
h. Are a sufficient number of qualified staff available for NGS diagnostic testing?	😐, ?	Y	N	P	
• Laboratory technicians		Y	N	P	
• Bioinformaticians		Y	N	P	
2. Regulatory					
a. Will the laboratory conduct a verification study of NGS-based DST? If yes,	😐, ?	Y	N	P	
• What type of protocol and number of samples are required?					
• What is the timeline for the studies?					
3.1 A safe and functional NGS testing site					
a. Is the physical facility of sufficient space and design to enable safe working practices for NGS?	😐, ?	Y	N	P	
• Appropriate space for processing specimens or cultures that meets biosafety requirements?		Y	N	P	
• Separate room for pre-amplification procedures?		Y	N	P	
• Separate room for sample preparation and DNA extraction?		Y	N	P	
• Separate room for amplification and post-amplification procedures		Y	N	P	
• Adequate bench space to support the size and weight the NGS instrument; ancillary equipment; and for general molecular biology techniques?		Y	N	P	
• Is suitable secure space available for conducting the bioinformatic analyses?		Y	N	P	
b. Is the NGS instrument placed on a vibration free, dedicated surface?	😐, ?	Y	N	P	
c. Is the NGS instrument and computer safe from theft?	😐, ?	Y	N	P	
d. Are the NGS workstations clean, free of clutter, and organized for efficient operation?	😐, ?	Y	N	P	
e. Is an adequate electric service available that meets power specifications and safety measures required for the NGS instrument?	😐, ?	Y	N	P	
f. Is an uninterrupted power supply (UPS)in use?	😐, ?	Y	N	P	
g. Is there sufficient, secured, and organized storage space for reagent kits and supplies?	😐, ?	Y	N	P	

		Yes	No	Partial	Comments
h. Is there documented monitoring and review of environmental temperatures at the testing and storage areas?	😐, ?	Y	N	P	
i. Does the testing site use appropriate disinfectants and are they prepared correctly?	😐, ?	Y	N	P	
j. Does the testing site ensure an optimal working temperature (19–25 °)	😐, ?	Y	N	P	
k. Is the humidity level monitored (range 30–75%)					
l. Does the testing site perform regular risk assessments?	😐, ?	Y	N	P	
m. Does the testing site provide sufficient ventilation for NGS testing procedures?	😐, ?	Y	N	P	
n. Is suitable personal protective equipment (PPE) provided at the testing site and are staff trained in its correct use?	😐, ?	Y	N	P	
o. Does the testing site segregate waste and dispose of it by incineration or as per national regulations or guidelines?	😐, ?	Y	N	P	
4. Equipment and supplies					
4.1 Equipment service and maintenance					
a. Have the NGS instrument and other equipment been installed in the laboratory?	🕮	Y	N	P	
b. Was the NGS instrument verified on site prior to routine use for patient testing?	😐,?				
c. Are all NGS instruments maintained and in good working condition?	😐, ?	Y	N	P	
• Sequencer (e.g., Illumina MiSeq)		Y	N	P	
• automated nucleic acid extraction instrument		Y	N	P	
• Fluorometer/spectrophotometer		Y	N	P	
• DNA fragment analyzer (e.g., Bioanalyzer)		Y	N	P	
• Thermocycler		Y	N	P	
• Automated liquid handling system					
d. Does the testing site perform and document preventive maintenance on all NGS instruments as required?	😐, ?	Y	N	P	
e. Are routine maintenance (daily, weekly, and monthly) procedures performed and recorded for all NGS instruments?	🕮, ?	Y	N	P	
f. Are NGS instrument maintenance records reviewed regularly (at least monthly) by the supervisor or designee with root cause analysis conducted following equipment malfunction, and corrective action taken?	😐, ?	Y	N	P	
g. Is there an SOP in place to obtain repairs or service for all NGS instruments?	🕮, ?	Y	N	P	

		Yes	No	Partial	Comments
h. Are all NGS instrument warranties and service contracts in place and adhered to?	😐, ?	Y	N	P	
4.2 Procurement & supply chain					
a. Is there a national procurement and distribution system for NGS reagents and consumables?	😐, ?	Y	N	P	
b. Does the testing site monitor consumption of NGS consumables?	😐, ?	Y	N	P	
c. Are NGS testing supplies available at the testing site, are in-date, labeled with receive date, organized, and stored at recommended storage conditions?	😐, ?	Y	N	P	
d. Is quality control testing (QC) performed on new lots of NGS reagents prior to their use for testing patient samples to ensure that they perform as expected?	🕮, ?	Y	N	P	
e. Are NGS supplies inventoried (physical count) at least monthly?	🕮, ?	Y	N	P	
f. Is cold chain required for reagents shipment?					
g. Does the testing site adequately store NGS reagents?	😐, ?	Y	N	P	
h. Is there a designated person at the testing site that is responsible for forecasting and procurement?	?	Y	N	P	
• If yes, who at the testing site is responsible?					
• If not, who or what organization is responsible?					
• Who controls the budget?					
5. Procedures and documentation					
a. Are all the needed standardized documents, records and forms related to NGS testing readily accessible to all staff?	🕮	Y	N	P	
• TB diagnostic algorithm		Y	N	P	
• NGS-DST requisition form		Y	N	P	
• Sample collection and transport forms		Y	N	P	
• Laboratory register		Y	N	P	
• NGS instrument maintenance log		Y	N	P	
• Stock cards		Y	N	P	
• Temperature monitoring records		Y	N	P	
• Nonconformity and corrective action log		Y	N	P	
• Training records forms		Y	N	P	
• NGS reporting codes		Y	N	P	
• NGS performance indicator reporting form		Y	N	P	
• NGS test reporting form		Y	N	P	

		Yes	No	Partial	Comments
• NGS PT results form & failure follow-up form		Y	N	P	
b. Are the following NGS-related standard operating procedures (SOPs) approved and accessible at the testing site?	🕮	Y	N	P	
• NGS-DST requisition		Y	N	P	
• Specimen collection		Y	N	P	
• Specimen processing and storage		Y	N	P	
• Specimen referral		Y	N	P	
• Specimen receipt and accessioning		Y	N	P	
• Sample processing for DNA extraction		Y	N	P	
• DNA extraction		Y	N	P	
• Library preparation		Y	N	P	
• Next generation sequencing		Y	N	P	
• Data analysis		Y	N	P	
• Confirmatory testing – indications and procedures		Y	N	P	
• Recording and reporting		Y	N	P	
• External quality assessment (PT)		Y	N	P	
• Quality indicator monitoring and data analysis		Y	N	P	
• Waste management		Y	N	P	
• Spill management		Y	N	P	
c. Does the testing site perform an annual review of all SOPs, documents, and forms?	🕮	Y	N	P	
d. Is there evidence that the personnel have read all SOPs, documents, and forms?	🕮	Y	N	P	
6. Data storage and analytic tools					
a. Are approved SOPs in place for each step in the bioinformatics pipeline used to analyze, interpret and report NGS results?	🕮	Y	N	P	
b. Do the approved SOPs:	🕮	Y	N	P	
• define data sharing protocols?		Y	N	P	
• Ensure the confidentiality of data and patient information (e.g., measures of pseudonymization)?		Y	N	P	
• Ensure that internal and external storage and transfer of sequencing data maintain confidentiality and security?		Y	N	P	
• Link NGS data to laboratory information systems, electronic registers, MMIS2, etc.?		Y	N	P	
• Define who will have access to the data?		Y	N	P	

		Yes	No	Partial	Comments
• Define processes and storage requirements for the input, intermediate, and final data files generated during data analysis?		Y	N	P	
• Define the process for validating or revalidating the bioinformatics pipeline and describing changes when modifications are made to the entire pipeline or components of the pipeline?		Y	N	P	
• Define processes to monitor, document and implement patch-releases, upgrades, and other updates to the bioinformatics pipeline?		Y	N	P	
• Describe how data will be secured?		Y	N	P	
• Specify how, and at what frequency, data will be backed-up?		Y	N	P	
• Describe who is responsible for technical support, IT-support, and updates?		Y	N	P	
c. Are procedures in place that ensure the confidentiality of patient information and for obtaining informed consent as needed?	😐, ?	Y	N	P	
d. Is suitable secure storage available for the compressed FASTQ sequence files? If yes, which option is used.	😐, ?	Y	N	P	
• External hard drives? Describe capacity.	😐, ?	Y	N	P	
• Local servers? Describe capacity	😐, ?	Y	N	P	
• Cloud-based storage? Describe capacity	😐, ?	Y	N	P	
e. Will analysis the sequence data be done on site? if yes:	😐, ?	Y	N	P	
• Are the needed analytic tools available on site?	😐, ?	Y	N	P	
— If yes, describe the tools					
— If not, describe plan for obtaining tools					
• Is there a suitable dedicated desktop or laptop PC for analyzing sequence data? Recommended specifications: — 64-bit processor, at least quad core — ≥ 16 GB RAM (≥ 32 GB suggested) — Hard drive: ≥ 2 TB (2 Solid-State/ RAID hard drives, 1TB each, suggested) — Operating System: Unix-Like or Ubuntu	😐, ?	Y	N	P	

		Yes	No	Partial	Comments
f. Will analysis of the sequence data be outsourced? If yes, describe the outsourcing plans	😐, ?	Y	N	P	
g. Will the interpretation of sequence variants follow international recommendations as indicated in the: "Catalogue of mutations in Mycobacterium tuberculosis and their association with drug resistance"[a])?	😐, ?	Y	N	P	
h. Is adequate bioinformatics expertise available on site?	😐, ?	Y	N	P	
i. Are any partners able to provide support for bioinformatics and data analysis?	😐, ?	Y	N	P	
j. Does a current costed plan exist for implementation of an NGS data system and performing necessary upgrades?	🕮	Y	N	P	
k. If yes, does the implementation plan address:	🕮	Y	N	P	
• Upgrades to infrastructure?		Y	N	P	
• Installing and maintaining hardware and software at the testing site?		Y	N	P	
• Setting up the data system at the testing site?		Y	N	P	
• Training for new users and refresher training for existing users?		Y	N	P	
• Providing operational costs of the system?		Y	N	P	
• Developing and disseminating SOPs for access, reporting, data entry, data security and data back-up?		Y	N	P	
l. Are adequate resources available for projected on-going costs of data storage and analysis?	😐, ?	Y	N	P	
7.1 Quality Assurance					
a. Are the essential elements of a quality assurance system in place?	😐, ?	Y	N	P	
• SOPs, training, and competence assessment		Y	N	P	
• Instrument verification		Y	N	P	
• Equipment maintenance		Y	N	P	
• Method validation or verification		Y	N	P	
• Quality control (QC)		Y	N	P	
• Lot testing (also known as incoming quality control or new batch testing)		Y	N	P	
• External quality assessment (EQA)		Y	N	P	
• Quality indicator monitoring		Y	N	P	

[a] Catalogue of mutations in *Mycobacterium tuberculosis* complex and their association with drug resistance. Geneva: World Health Organization; 2021. License: CC BY-NC-SA 3.0 IGO. https://www.who.int/publications/i/item/9789240028173

		Yes	No	Partial	Comments
• Continuous quality improvement (CQI) including the identification and documentation of non-conformities and corrective actions		Y	N	P	
7.2 Establish and monitor quality controls					
a. Are protocols in place to conduct and document quality checks of each step of the NGS-based DST process?	😐, ?	Y	N	P	
b. Are protocols in place that ensure the use of positive and negative controls?	😐, ?	Y	N	P	
7.3 External quality assessment					
a. Is an external quality assessment programme in place?	😐, ?	Y	N	P	
• Proficiency testing (PT)?		Y	N	P	
• Re-checking samples?		Y	N	P	
• On-site supervisory visits?		Y	N	P	
b. Have partners been identified who can assist with proficiency testing, supervisory visits, and re-checking of samples? If so, who?	😐, ?	Y	N	P	
c. Does the NGS site currently participate in a PT programme for NGS? if yes,	😐, ?	Y	N	P	
• Who provides the PT panels?					
• Who oversees the PT programme and provides feedback on the performance of testing site?					
d. Does the NGS laboratory participate in inter-laboratory comparisons (re-checking of samples)? If yes, with which laboratories?	😐, ?	Y	N	P	
e. Does the laboratory receive on-site supervisory visits? If yes,	🕮	Y	N	P	
• Who conducts the supervisory visits?					
• Is feedback provided to the testing site following a supervisory visit?	🕮, ?	Y	N	P	
• When was the last supervisory visit and what was the feedback?					
7.4 Monitor and analyze quality indicators					
a. Are appropriate data collection tools for NGS quality indicators available?	😐, ?	Y	N	P	
b. Are the following NGS Testing Quality indicators monitored and analyzed by the NGS testing site and reported:	😐, ?	Y	N	P	
• Number of specimens tested with NGS (Disaggregated by HIV status, MDR risk, extra-pulmonary TB, pediatric)		Y	N	P	
• Number and proportion of NGS assays that generated no results		Y	N	P	
• Number and proportion of NGS assays that failed QC		Y	N	P	

		Yes	No	Partial	Comments
• Number and proportion of specimens tested with NGS for which a result was reported within the target turnaround time (i.e., time from receipt of specimen to reporting of results)		Y	N	P	
• Number and proportion of specimens with discordant results when NGS-based DST is compared with phenotypic DST		Y	N	P	
• Number and proportion of specimens with discordant results when NGS-based DST is compared with other molecular tests (e.g., Xpert MTB/RIF or LPA)		Y	N	P	
8. Recording and reporting					
a. Is an approved request for examination form available to request NGS-based DST?	🕮, ?	Y	N	P	
b. Is an approved reporting form available to report the results of NGS-based DST?	🕮, ?	Y	N	P	
c. Does the reporting form indicate that the: "Catalogue of mutations in Mycobacterium tuberculosis and their association with drug resistance"[a]) was used to interpret and report sequence variants?	🕮, ?	Y	N	P	
d. Is there an SOP for reporting sequence findings unrelated to the purpose of the NGS (e.g., finding virulence factors when conducting NGS-based DST)?	🕮, ?	Y	N	P	
e. Are the laboratory and clinical registers suitable for recording the results of NGS-based DST?	🕮, ?	Y	N	P	
f. For each sample, do laboratory records identify.	🕮, ?	Y	N	P	
• The methods, instrument(s) and reagents used for processing and sequencing?		Y	N	P	
• The version of the bioinformatics pipeline used to generate, analyze, and interpret the NGS data?		Y	N	P	
• Any non-conformities and exceptions in the testing process?		Y	N	P	
g. If an electronic laboratory information system is in use, can NGS-based DST results be entered into it?	😐, ?	Y	N	P	
h. If an electronic recording and reporting system is in place, can NGS-based DST results be recorded and reported using it?	😐, ?	Y	N	P	

[a] Catalogue of mutations in *Mycobacterium tuberculosis* complex and their association with drug resistance. Geneva: World Health Organization; 2021. License: CC BY-NC-SA 3.0 IGO. https://www.who.int/publications/i/item/9789240028173

		Yes	No	Partial	Comments
9. Training and competency assessment					
a. Are terms of reference and competency -based job descriptions available for key staff involved in NGS?	🕮,?	Y	N	P	
• laboratory technicians		Y	N	P	
• bioinformatics officer		Y	N	P	
b. Are records in place documenting that all staff have been trained on assigned work processes, procedures, and tasks?	🕮,?	Y	N	P	
c. Are standard procedures used to assess and document the competence of all staff involved in NGS-based DST?	🕮	Y	N	P	
• laboratory technicians?		Y	N	P	
• bioinformatics officer?		Y	N	P	
d. Are competency assessments for NGS users conducted annually?	🕮	Y	N	P	
e. Are records in place documenting that all NGS users have been assessed for competency?	🕮	Y	N	P	
f. Does the site provide NGS training for clinicians and healthcare workers on:	🕮	Y	N	P	
• Diagnostic algorithm incorporating NGS?		Y	N	P	
• Ordering NGS tests?		Y	N	P	
• Sample requirements for NGS testing?		Y	N	P	
• Sample transport?		Y	N	P	
• Interpretation of NGS test results?		Y	N	P	
10.1 Monitoring and evaluation of the implementation of NGS testing					
a. Is the accomplishment of key indicators and milestones for NGS implementation reported to the national programme?	😐, ?	Y	N	P	
b. Are the following implementation indicators reported to the national program at least annually	😐, ?	Y	N	P	
• Total number of NGS tests performed (disaggregated by population, e.g., HIV+, children, vulnerable, EPTB)		Y	N		
• Number and proportion of NGS testing sites relative to projected need		Y	N		
• Number and proportion of clinical sites with access to NGS testing on site or via a functional referral network		Y	N		
10.2 Monitoring and evaluation of impact of NGS-based DST					
a. Are the following indicators monitored and reported to the national programme at least annually	😐, ?	Y	N	P	
• Number and proportion of notified bacteriologically confirmed TB cases with reported NGS-based DST results for rifampicin susceptibility		Y	N	P	

		Yes	No	Partial	Comments
• Number and proportion of notified rifampicin-resistant TB cases with reported NGS-based DST results for fluoroquinolone resistance		Y	N	P	
• Number and proportion of notified bacteriologically confirmed TB cases with reported NGS-based DST results for WHO-recommended first- and second-line drugs		Y	N	P	
• Number and proportion of bacteriologically confirmed TB patients who were placed or continued a treatment regimen based on NGS-based DST results		Y	N	P	
• Number and proportion of bacteriologically confirmed TB patients included in a surveillance system (survey) for all WHO-recommended first- and second-line drugs		Y	N	P	
11. Specimen referral system					
a. Are all people involved in specimen referral trained in TB specimen (defined as: genomic DNA; viable or inactivated sputum sediments/culture isolates) collection, referral, transportation, and reception?		Y	N	P	
b. Is triple packaging used for all national and international TB specimen transportation?		Y	N	P	
c. Are there standardized procedures for national and international TB specimen transportation (including defined roles and responsibilities)?		Y	N	P	
d. Are TB specimen referral and transportation systems in place at the local, regional, and national levels?		Y	N	P	
e. Are there Material Transfer Agreements (MTAs), Memoranda of Understanding (MoUs) and an international specimen referral system in place for TB specimens (genomic DNA; viable or inactivated sputum sediments/culture isolates) that require testing outside of the country or for importation of quality assessment and control materials?		Y	N	P	

		Yes	No	Partial	Comments
12. Biosafety					
a. Are there national laboratory building requirements that include detailed standards for TB laboratories (applicable to biosafety containment and molecular biology laboratories)?					
b. Are TB laboratory-specific building requirements consistently applied to all laboratory facilities?					
c. Are TB laboratory facilities regularly maintained and is there uninterrupted availability of general utilities (water, energy, communication lines)?					
d. Does the biosafety and biosecurity manual cover key requirements for the safe handling of TB (specimens for testing, isolates/ strains) based on bio-risk assessment? The requirements may be addressed as part of the national laboratory biosafety and biosecurity manual or in a separate TB laboratory biosafety and biosecurity manual					
e. Is the national laboratory biosafety and biosecurity manual implemented and incorporated into standard operating (SOP) procedures that contain adequate information on TB laboratory biosafety?					
f. Are designated safety officers available in all facilities? (part-time or full time)					
g. Is safety equipment needed for safely working with TB specimens and isolates available (e.g., PPE)?					
h. Are certified biosafety cabinets (BSC) available according to the facility biosafety level (BSL) wherever needed?					
i. Are basic occupational health services available to all laboratory workers?					
j. Are standardized procedures for collecting, storing and disposal of identified categories of waste implemented according to national standards?					
k. What are the methods used to safely dispose of infectious waste?					

		Yes	No	Partial	Comments
13. NGS TESTING: Pre-testing, Testing, Post-testing Phases					
a. Are standardized forms, registers, logbooks or electronic files for recording patient and specimen information available at the site?	🕮	Y	N	P	
b. Are standardized forms, registers, logbooks, or electronic files for recording NGS results available at the site?	🕮	Y	N	P	
c. Are all forms, registers, logbooks, or electronic files complete and legible?	🕮	Y	N	P	
d. Are all forms/registers/logbooks or electronic files properly labelled, organized, and kept in a secure location?	🕮	Y	N	P	
e. Are the NGS operator manual and NGS package insert available at the site and accessible to all testing staff (hard or soft copy)?	🕮	Y	N	P	
f. Are NGS pre-testing (pre-analytical) procedures adequately followed?	🖗	Y	N	P	
g. Are NGS testing (analytical) procedures being adequately followed?	🖗	Y	N	P	
h. Are the correct safety practices and procedures adequately followed before, during, and after NGS testing?	🖗	Y	N	P	
i. Does the site monitor performance of internal quality controls?	😐, ?	Y	N	P	
j. Are the NGS reagents being used for patient testing in-date and labelled with new expiry date when kit was opened?	😐, ?	Y	N	P	
k. Are NGS post-testing (post-analytical) procedures being adequately followed?	🖗	Y	N	P	
l. Are NGS results recorded in an appropriate register in a timely manner?	🕮	Y	N	P	
m. Has the site put measures in place to prevent unauthorized access to NGS test data?		Y	N	P	
n. Does the site monitor and meet its target test results turnaround time (TAT) in >80% of the cases in the last 3 months? What is the site's target test results TAT?	😐, ?	Y	N	P	

Annex 4

Example of a next-generation sequencing readiness assessment – the South African experience

The answers given here about a tuberculosis (TB) next-generation sequencing (NGS) readiness assessment were contributed by Shaheed Vally Omar, Farzana Ismail, Centre for Tuberculosis, National Institute for Communicable Diseases, National Health Laboratory Service, Johannesburg, South Africa.

1. Policy and planning: How many testing sites are or will be providing NGS testing for diagnostic testing for TB (and other diseases or organisms)?
   This service is currently centralized at the NICD. However, there are plans to capacitate 4 tertiary laboratories for NGS in this financial year.
2. Are additional NGS instruments planned for use for TB?
   No
3. Has a strategic planning process been conducted for NGS-based drug susceptibility testing (DST) for TB?
   Yes, discussion are ongoing together with the National TB Control and Management cluster.
4. Does a current costed national plan exist for NGS implementation?
   No, there is no national plan.
5. Have projections been made for the number of samples to be tested per year or per site?
   With the introduction of newer shorter DR-TB regimens we estimate that all Rif-R cases would required NGS for select drugs
6. Are resources (e.g. funding, staff and laboratory infrastructure) available at the national level to support NGS?
   No
7. Regulatory: Has a national governance structure been established for NGS-based drug susceptibility testing (DST)?
   No
8. What is the regulatory process required for NGS-based DST?
   The National TB Programme is closely aligned with WHO recommendation. Once endorsement has been received from WHO this would trigger local regulatory approval through The South African Health Products Regulatory Authority (SAHPRA)

9. Is country verification of NGS-based DST needed for regulatory approval?
   Yes
10. Are there national requirements for the laboratory infrastructure needed for NGS-based DST for TB?
    No
11. Is there a mechanism for assessing each NGS testing site for readiness?
    Yes, the NTBRL current runs external quality assessment (EQA) programmes for validation and verification for both TB genotypic and phenotypic test methods, both in-country. WGS would be added to the schedule of test methods using the panel supplied by the WHO. However, an assessment tool needs to be developed to ensure that all the required skills, procedures and equipment are in place and functional for implementation.
12. Equipment: Which NGS instrument been selected?
    Illumina
13. Are there partners in the country that can support NGS implementation, installation and ongoing use?
    Yes, the NICD has sufficient expertise to support
14. Have manufacturers or distributors been identified who can support installation and equipment maintenance (warranties or service contracts)?
    Yes
15. Is there a mechanism in place to monitor if testing sites are performing maintenance and servicing of the NGS instrument?
    Yes, equipment maintenance is a key component of medical laboratory ISO certification.
16. Supplies: Is there a national policy in place for procurement of NGS instruments and reagents?
    No
17. Is a procurement system available to ensure the availability of NGS reagents and supplies that consider procurement times, consumption rates and shelf-life of reagents?
    Yes
18. Laboratory procedures: Are the standardized documents, records and forms available, approved and up to date (i.e. incorporates NGS testing)? Algorithms, requisition form, laboratory register and reporting form.
    No
19. Data handling: Are national policies and procedures in place for NGS activities that define data sharing protocols; ensure the confidentiality of data; link NGS data to laboratory information systems, electronic registers, MMIS2, etc.; and describe how will data be secured? Has an assessment of existing data storage solutions, infrastructure (e.g., internet access) and analytic tools for NGS been conducted?
    No

20. Quality assurance, control, and assessment: Are national policies in place requiring the essential elements of a quality assurance system to be in place at the testing site?
    Yes, all sites are expected to have ISO 15189 accreditation, and this accreditation ensures the necessary quality assurance system for a medical diagnostic laboratory.
21. Are protocols in place to conduct and document quality checks of each step of the NGS-based DST process and ensure the use of positive and negative controls?
    No
22. Is an EQA programme in place?
    Yes, only for the WHO TB Supranational Reference Laboratory (WHO SRL EQA).
23. Is there a national policy in place requiring the collection of performance indicators?
    No
24. Recording and reporting: Is revision of the current request for examination form required for introduction of NGS-based DST? If an electronic laboratory information system is in use, what updates will be required?
    Yes revision of the current request form will be required. The LIS would require development, which would include creation of a test code – if the instrument/analysis tool allows for transmission of results to the LIS through an interphase this could be developed as well.
25. Human resources and training: Have terms of reference been developed for key staff involved in NGS?
    No
26. Is approved standardized training for NGS-based DST available?
    No
27. Are competency assessments for NGS users conducted annually?
    No
28. Monitoring and evaluation (M&E): Are there national policies and plans in place to monitor and evaluate the impact of NGS-based DST? Are indicators monitored?
    No

Annex 5

Budgetary considerations for implementation of next-generation sequencing

	Budgetary considerations
Policy and planning	• Workshop for stakeholder engagement and planning. • Technical workshop for guideline and algorithm update. • Readiness assessment cost – HR, travel and report writing. • Printing and distribution costs for revised guidelines and algorithms
Regulatory	• Regulatory submission costs, if applicable • Local travel costs to regulatory authority • Verification study – samples, reagents, and HR.
Procedures	• Workshop and HR for the development of SOPs • Printing and dissemination of revised procedures
NGS Laboratory	• Costs of upgrading laboratory facilities and infrastructure (e.g., electricity and air conditioning)
Purchase and installation of NGS instrument	• Purchase (or lease) of NGS instrument, workstation, uninterruptable power supply • Delivery and importation costs • Installation by manufacturer or authorized service provider • Training • Extended warranty or service contract
Purchase and installation of ancillary equipment	• Purchase of ancillary equipment • Installation of ancillary equipment, if needed
Verification studies	• Verification of wet bench process • Verification of bioinformatics pipeline
Data storage solution	• Purchase or acquisition (e.g., subscription fee) of data storage solution • Costs of data transmission (e.g., high-speed internet service)
Bioinformatics	• Purchase or acquisition of analytic tools • Installation and training • Travel and per diems for site visits for installation and troubleshooting • Cost of technical assistance from service provider or bioinformatics experts • HR
Procurement and supply chain	• Workshop for stakeholders involved in procurement
Specimen collection and transport	• Workshop and HR to update data collection and test requisition forms and procedures. • Printing and distribution costs of updated materials • Establish procedures and mechanisms for transporting specimens from collection sites to testing laboratories
Recording and reporting	• Workshop and HR to update reporting and recording forms, registers. • Printing and distribution of updated materials

	Budgetary considerations
Training	Workshop and HR to update training packages for laboratory and clinical staff. Train-the-trainers workshop, participant and instructor travel, onsite trainings, and sensitization meetings Printing and distribution costs of updated training manuals
Partner coordination	Meetings for stakeholder engagement and planning (see Policy and planning)
Quality assurance	Establish or subscribe to a proficiency testing programme. Arrange interlaboratory comparisons. Costs of supervisory visits.
Monitoring	Meetings to update M&E system and regular meetings to review impact of transition and re-plan. M&E refresher training.
Annual ongoing costs of an NGS servicea	Consumables. HR. Equipment calibration and servicing. Data storage and bioinformatics. Specimen referral and results reporting system. External quality assessment.

HR: human resources; M&E: monitoring and evaluation; NGS: next-generation sequencing; SOP: standard operating procedure.

[a] For a detailed cost analysis of NGS-based DST for *Mycobacterium tuberculosis* complex, see Pankhurst et al. (2016) *(1)*.

Reference for Annex 5

1 Pankhurst LJ, del Ojo Elias C, Votintseva AA, Walker TM, Cole K, Davies J et al. Rapid, comprehensive, and affordable mycobacterial diagnosis with whole-genome sequencing: a prospective study. Lancet Respir Med. 2016;4:49–58. doi: https://doi.org/10.1016/s2213-2600(15)00466-x.

Annex 6

List of commercially available instruments for next-generation sequencing

This annex provides a list of the main instruments for next-generation sequencing (NGS) instruments that are currently commercially available and their characteristics; it was adapted from a technical guide produced by the World Health Organization (WHO) and Foundation for Innovative New Diagnostics (FIND) *(1)*. More comprehensive summaries and reviews of NGS instruments can be found in recent publications *(2, 3)* and in the WHO/FIND technical guide *(1)*.

Manufacturer	Instrument(s)	Run time (hours)	Data output (gigabytes, GB)
Illumina	NovaSeq System HiSeq 2500 NextSeq System MiniSeq MiSeq iSeq 100	10–55	1.2–6000
Thermo Fisher Scientific	Ion GeneStudio S5 System Ion Torrent Genexus System	2–24	0.03–50
MGI Tech	DNBSEQ-G50 DNBSEQ-G400 DNBSEQ-T7	10–109	150–6000
Pacific Biosciences	Sequel IIe System Sequel II System Sequel System	Up to 20–30	20–160
Oxford Nanopore Technologies	Flongle MinION GridION PromethION	Up to 48	2–245

References for Annex 6

1 The use of next-generation sequencing technologies for the detection of mutations associated with drug resistance in *Mycobacterium tuberculosis* complex: technical guide. Geneva: World Health Organization; 2018 (https://apps.who.int/iris/handle/10665/274443).

2 Besser J, Carleton HA, Gerner-Smidt P, Lindsey RL, Trees E. Next-generation sequencing technologies and their application to the study and control of bacterial infections. Clin Microbiol Infect. 2018;24:335–41. doi: https://doi.org/10.1016/j.cmi.2017.10.013.

3 Next generation sequencing instrument guide [website]. Austin, USA: Genohub; 2018 (https://genohub.com/ngs-instrument-guide/).

Annex 7

Installation checklist and resources

Before installing the next-generation sequencing (NGS) instrument in the dedicated tuberculosis (TB) laboratory area, it is important to ensure that all requirements for an appropriate installation and functioning of the selected NGS systems are satisfied. These requirements include delivery of the instrument for installation and any other devices and reagents required for this process, laboratory requirements (e.g., dimensions, placement, laboratory bench, vibration, and set-up for molecular procedures), electrical requirements, environmental requirements, and network considerations. All requirements are reported in the site preparation guides provided by each NGS manufacturer.

Opposite is an example of a site preparation checklist; this checklist is from the Ion Torrent Genexus Integrated Sequencer site preparation guide *(1)*.

Acceptance testing (including technical, administrative, and performance-related procedures to ensure a safe introduction of the new instrument) should be conducted by the (clinical) engineering service before the instrument is installed in the laboratory.

The links listed below connect to the main supporting documentation provided by the manufacturers of the NGS platforms listed in Annex 6. These documents provide an overview and instructions for operating and maintaining the sequencers (i.e., sequencer guide, manuals and protocols, and product literature).

- **Illumina** (http://support.illumina.com/downloads.html):
  - iSeq 100: http://support.illumina.com/downloads/iseq-100-system-guide-1000000036024.html?langsel=/it/
  - MiniSeq: http://support.illumina.com/downloads/miniseq-system-guide-1000000002695.html?langsel=/it/
  - MiSeq: https://emea.support.illumina.com/sequencing/sequencing_instruments/miseq/documentation.html
  - NextSeq 500: http://support.illumina.com/downloads/nextseq-500-user-guide-15046563.html?langsel=/it/
  - HiSeq 2500: http://support.illumina.com/downloads/hiseq_2500_user_guide_15035786.html?langsel=/it/
  - Nova Seq 6000: http://support.illumina.com/downloads/novaseq-6000-system-guide-1000000019358.html?langsel=/it/

✓	Date	Initials	Site preparation requirement
☐			Customer responsibilities have been reviewed.
☐			Personnel have been assigned tasks and responsibilities.
☐			The installation site is identified and meets the following requirements:
			☐ Space and clearance
			☐ Environmental
			☐ Electrical
			☐ Network
			☐ Instrument-to-computer connection
			☐ Safety
☐			The shipment was received and inspected as follows:
			☐ The items shown on the shipping list are the items that were ordered at the time of purchase.
			☐ Damage to shipping containers was reported to the shipping company that delivered the shipment and to your service representative.
			☐ Damage or mishandling was recorded on the shipping documents.
			☐ If provided with the shipment, all reagents and plates are unpacked and stored as specified on package labels.
☐			The installation site is cleared and ready for the installation.
☐			The packaged shipping containers are moved to the installation site.
☐			All materials for installation, qualification, and operation are available.
☐			The *Genexus™ Integrated Sequencer IT Checklist* (Pub. No. MAN0018466) has been completed and returned according to the checklist instructions.
Customer signature:			

- **Thermo Fisher Scientific** (https://www.thermofisher.com/search/results?docTypes=Manuals&persona=DocSupport&linkIn=true):
  - Ion GeneStudio S5 System: https://www.thermofisher.com/order/catalog/product/A38194?SID=srch-srp-A38194#/A38194?SID=srch-srp-A38194
  - Genexus Integrated Sequencer: https://www.thermofisher.com/order/catalog/product/A45727?SID=srch-srp-A45727#/A45727?SID=srch-srp-A45727

- **MGI Tech** (https://en.mgi-tech.com/download/files/):
  - DBNSEQ-G400: https://en.mgi-tech.com/download/files/yi_id/2/type_id/1
  - DBNSEQ-T7: https://en.mgi-tech.com/download/files/yi_id/1/type_id/1
- **Pacific Biosciences** (https://www.pacb.com/support/documentation/):
  - Sequel II and Sequel IIe Systems: https://www.pacb.com/wp-content/uploads/Operations-Guide-Sequel-II-and-Sequel-IIe-Systems.pdf
  - Sequel System: https://www.pacb.com/wp-content/uploads/Operations-Guide-The-SMRT-Sequencer-Sequel-System.pdf
- **Oxford Nanopore Technologies** (https://community.nanoporetech.com/technical_documents) – registration to the website required
  - Laboratory and IT requirements: https://nanoporetech.com/community/lab-it-requirements
  - Flongle: https://nanoporetech.com/products/flongle
  - MinION: https://nanoporetech.com/products/minion
  - GridION: https://nanoporetech.com/products/gridion
  - PromethION: https://nanoporetech.com/products/promethion

Reference for Annex 7

1 Ion Torrent site preparation guide: Genexus™ Integrated Sequencer, Publication Number MAN0017918 Revision F.0. Waltham, MA: Thermo Fisher Scientific;(https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0017918_GenexusIntegratedSequencer_SPG.pdf).

Annex 8

List of essential equipment and reagents required for next-generation sequencing

This annex provides a list of equipment, infrastructure, reagents, and consumables required for next-generation sequencing (NGS). It is based on a generic NGS workflow for use with the Illumina Nextera XT DNA Library Prep Kit and MiSeq system. The mention of specific manufacturers in this list is for information; it does not constitute endorsement.

Activity	Equipment or infrastructure
General[a]	• Areas: BSL3/2+, molecular biology laboratories (pre-PCR and post-PCR) • Freezer (–20 °C) – all areas • Refrigerator (+4 °C) – all areas • Heating block – all areas • Vortex mixer – all areas • Exclusive sets of single-channel pipettes (0.5–2 µL, 1–10 µL, 2–20 µL, 20–200 µL and 100–1000 µL) – all areas • Exclusive sets of multichannel pipettes – all areas • Microcentrifuge (13 000 rpm) – all areas • Microcentrifuge racks – all areas • Discard jar – all areas
DNA isolation and purification	• Centrifuge for conical tubes including aerosol-containment cups (BSL3/2+)
	• Centrifuge racks (BSL3/2+)
	• Electrophoresis apparatus
	• Spectrophotometer (e.g., Thermo Fisher Scientific NanoDrop™ 2000/2000c)
	• Fluorometer (e.g., Invitrogen Qubit 4 Fluorometer)
	• Automated electrophoresis solution (e.g., Agilent 2100 Bioanalyzer System) (optional)
	• Automated DNA isolation instrument (optional)
	• Equipment for DNA purification; for example: — CTAB (N-cetyl-N,N,N-trimethyl ammonium bromide)/NaCl protocol — Promega Maxwell 16 System — Qiagen QIAcube Connect
Library preparation	• Minicooler (4 °C)
	• Magnetic stand for 96-well plates
	• Magnetic stand
	• 96-well thermal cycler
	• Small microcentrifuge for PCR workstation (e.g., Eppendorf MiniSpin) (optional)
	• High-speed microplate shaker
MiSeq sequencing	• Illumina MiSeq sequencer
	• Hard drives for data storage
	• High-speed internet connection (at least 10 Mbps upload speed for internal network uploads)

Activity	Reagents or consumables
General	• Laboratory coats • Laboratory tissues • Disposable gloves • Pasteur pipettes • Sterile disposal loops • Conical tubes • (Micro)centrifuge tubes (screw cap) • Single-channel and multichannel pipettes tips (0.5–2 µL, 1–10 µL, 2–20 µL, 20–200 µL and 100–1000 µL) • 96-well storage plates • 96-well PCR plates • Multichannel reagent reservoirs • Microseal adhesive seals • Plastic troughs for use with multichannel pipettors • Molecular biology grade reagents: water, ethanol, PBS, saline solution, sodium hydroxide and Tween-20
DNA isolation and purification	• Tubes for fluorometer and spectrophotometer
	• Reagent kits for DNA purification system
Library preparation	• Illumina Nextera XT DNA library prep kit
	• Illumina Nextera XT index kit
	• Beckman Coulter Genomics Agencourt AMPure XP
	• Tubes for fluorometer and spectrophotometer
MiSeq sequencing	• Illumina MiSeq Reagent Kit
Special notes for good laboratory practice	• Color-coded gloves for pre-PCR and post-PCR areas
	• Different colour laboratory coats for post-PCR area (library normalization and pooling)
	• Cleaning of DNA workbench with 1% bleach and 70% EtOH

BSL: biosafety laboratory; DNA: deoxyribonucleic acid; EtOH: ethyl alcohol; PCR: polymerase chain reaction; Mbps: megabytes per second; PBS: phosphate buffered saline.

[a] Lists of infrastructure and equipment for general laboratory activities are available *(1, 2)*.

References for Annex 8

1 Barnard M, Parsons L, Miotto P, Cirillo D, Feldmann K, Gutierrez C et al. Molecular detection of drug resistant tuberculosis by line probe assay – laboratory manual for resource-limited settings. Geneva: Foundation for Innovative New Diagnostics; 2012 (https://assets.publishing.service.gov.uk/media/57a08a68ed915d3cfd00074c/LPA_LaboratoryManual_22Mar2012.pdf).

2 GLI practical guide to TB laboratory strengthening. Geneva: Global Laboratory Initiative; 2015 (https://stoptb.org/wg/gli/assets/documents/GLI_practical_guide.pdf).

Annex 9

Estimated data storage needs based on anticipated next-generation sequencing workload

Storage solutions for the sequence data will be needed because the hard drive on the computer associated with the next-generation sequencing (NGS) workstation will quickly fill up. For example, the hard drive on the Illumina MiSeq platform is only 500 gigabytes (GB) and will fill up within about 10 sequencing runs. This is important because the files on the hard drive include files that are needed for troubleshooting a run and for the FASTQ sequence files.

For whole genome sequencing (WGS), the raw sequence files (FASTQ files) for each sample usually have a total size (when unzipped) greater than 350 megabytes (MB) to ensure a minimum average coverage depth of 30-fold, if more than 95% of reads map to the tuberculosis (TB) reference genome. FASTQ files for each sample subjected to targeted NGS are usually smaller, depending on the number of samples per run, number of sequencing cycles and depth of coverage. For storage, FASTQ files are usually compressed or zipped. For WGS, a storage space of about 300 MB per strain is needed for two FASTQ files per strain (assuming paired-end sequencing, e.g. Illumina).

WGS sequencing runs may include 24–48 samples, and each sequencing run takes 24–48 hours to complete. Assuming two sequencing runs per week, 15–30 GB of storage would be needed to accommodate the zipped FASTQ files for the 48–96 samples sequenced each week. One run a week of 24 samples would require about 7.5 GB of storage (only raw FASTQ files).

External hard drives with a 1 terabyte (TB) capacity may be able to hold up to 1500 sequenced samples (considering only raw sequencing files; that is. two FASTQ files per sample, with a zipped size of 150 MB each). Thus, a 1-TB hard drive may fill up within 16 weeks if the laboratory is sequencing 96 samples a week, or within 64 weeks if it is sequencing 24 samples a week. Laboratories using external hard drives should consider storing the sequences from each sample on two separate hard drives (i.e., double back-up). In this case, two 1-TB hard drives would be needed to store the sequence data generated in about 16 weeks of sequencing ninety-six samples per week. One of the risks of adopting this solution is that the hard drive device may become physical damaged or may be lost.

Cloud-based options provide an alternative, and several such systems are available (e.g., Illumina BaseSpace Sequence Hub, Amazon Web Services, Microsoft Azure, and Google Cloud Platform), with different storage prices and sharing or protection policies that need to be carefully considered before implementation. The computing environment offered by these services makes it possible to manage, analyse and share sequencing data. Certain data storage solutions may require data-

sharing agreements and a legal framework, particularly if the data are to be stored outside the country. Local or remote computing servers can be implemented for the NGS laboratory with the support of bioinformatics staff.

In general, a stable and fast internet connection (at least 10 Mbps upload speed for internal network uploads) is required, when the adopted NGS instruments and storage or sharing solutions cannot work offline.

Annex 10

Quality indicators and quality control considerations for next-generation sequencing workflows

This annex is based on Table 4 from the technical guide produced by the World Health Organization (WHO) and Foundation for Innovative New Diagnostics (FIND) *(1)*.

NGS step	Standardization process	Quality metric	Comment
DNA extraction	Specimen or sample quality	Adequate sample purity (with consideration for other organism DNA, human DNA or inhibitors in the sample)	
	Specimen or sample quantity	Sufficient starting material (with consideration for targeted NGS or WGS application)	
	DNA quality	Adequate DNA purity	Quantify DNA using a spectrophotometer, a fluorescence detection system or qPCR
	DNA quantity	Sufficient starting material for library preparation	Examine DNA size and Integrity by agarose gel electrophoresis or by microfluidic instruments
		Adequate volume	
Library preparation	DNA quality	Correct fragment size	Examine DNA size and Integrity by agarose gel electrophoresis or by microfluidic instruments
		Adequate DNA purity	
	DNA quantity	Sufficient starting material for sequencing	Quantify DNA using a spectrophotometer, a fluorescence detection system or qPCR
		Adequate volume	
Sequencing	Quality of the run	Base quality score	Calculate Phred Q score
		Median base quality by cycle and percentage of bases above the quality threshold (with considerations for adaptor trimming and GC content)	
		Indexed sequence capture percentages	
	Sequence base calling	Number of reads	
		Read length	
		Percentage of unique reads	
		Percentage of duplicate reads	

NGS step	Standardization process	Quality metric	Comment
Assembly	Reference-guided assembly (alignment of sequence reads to reference genome)	Appropriate reference	
		Percentage of reads correctly mapped to the reference genome or target	
		Average coverage of genome or target region	
		Average depth of coverage	
		Read duplication	
		Mapping quality scores	Calculate percentage MTBC by Kraken taxonomic sequence classification system or percentage mapping by Burrows-Wheeler Aligner
	De novo assembly (assembly of reads based on sequence overlap in the absence of a reference genome)	Number of contigs	
		Contig length, N50	
		Number of scaffolds	
		Complete or partial assembly (percentage assembly size)	
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 (including different variant types and portions and ratios of base substitutions)	
		Variant allele frequency (heterozygous and homozygous calls)	

DNA: deoxyribonucleic acid; MTBC: Mycobacterium tuberculosis complex; NGS: next-generation sequencing; qPCR: quantitative polymerase chain reaction; WGS: whole genome sequencing.

Reference for Annex 10

1 The use of next-generation sequencing technologies for the detection of mutations associated with drug resistance in *Mycobacterium tuberculosis* complex: technical guide. Geneva: World Health Organization; 2018 (https://apps.who.int/iris/handle/10665/274443).

Annex 11

Development of a proficiency testing programme for next-generation sequencing for tuberculosis

This annex describes the development of an external quality assessment (EQA) scheme for tuberculosis (TB) whole genome sequencing (WGS); that is, the European Reference Laboratory Network for TB (ERLTB-Net) *(1)*. It is based on text contributed by Richard Anthony, Vlad Nikolayevskyy and Dick van Soolingen.

Established genotyping typing systems (e.g. mycobacterial interspersed repetitive units [MIRU]-variable number of tandem repeats [VNTR], Spoligotyping) lack resolution compared with WGS *(2)*. The utility of TB WGS for resistance prediction, combined with rapid developments in genome sequencing technology *(3)*, has led to the rapid uptake of WGS by European national TB reference laboratories (NRLs) for Mycobacterium tuberculosis (MTB) genotyping and resistance detection. The ERLTB-Net (currently ERLTB-Net-2) is sited within a series of projects funded by the European Centre for Disease Prevention and Control (ECDC). The ECDC previously developed and coordinated an EQA scheme for mycobacterial genotyping based on multilocus sequence typing (MLST) of variable regions (MIRU-VNTR). Recognizing a value of WGS in TB laboratory diagnosis and surveillance, a European-wide TB WGS EQA scheme was developed within ERLTB-Net, starting with a pilot round starting in 2016. In 2017 and 2018, a total of 13 European laboratories joined the EQA scheme.

The first pilot of the scheme was undertaken in 2016 and in 2017; in 2018, the scheme was developed further. Most laboratories arrived at similar results for the prediction of resistance to first-line drugs and recognizing epidemiologically linked cases, even though they used independently developed WGS analysis pipelines. However, considerable variations in the number of single nucleotide polymorphisms (SNPs) measured genetic distances, and the general interpretation and reporting of the complex WGS results were observed between laboratories, highlighting an urgent need to establish an EQA scheme for TB WGS.

The scheme includes 10 WGS-grade DNA specimens from mycobacterial cultures. These DNA specimens are prepared and distributed to European reference laboratories. Specimens include sets of DNA extracts derived from duplicate and closely related samples (i.e., isolates from proven epidemiologically linked cases), and isolates bearing common and rare mutations associated with drug resistance. In addition to the 10 DNA extracts, electronic data sets from five isolates (FASTQ files) are provided to the participating laboratories, which are expected to report the following:

- mutations associated with resistance to anti-TB drugs and whether these mutations are likely to be associated with high-level or low-level resistance.

- any highly similar isolates (indicating epidemiological transmission or laboratory cross contamination); and
- genotype (phylogenetic lineage) of the strains.

Within a scoring system developed alongside the EQA scheme, a 10% penalty is given for missed epidemiological links, incorrect or missed mutations associated with first-line drug resistance, or an incorrect genotype. A 5% penalty is given for minor errors such as an incorrect level of resistance (high or low level) or failure to mention low-level resistance where this is of clinical relevance (because these are counted as minor errors), and more complex characteristics of the panel (described below) that are incorrectly reported or missed. A penalty of 25% is given for any missed multidrug-resistant TB (MDR-TB) isolates. All participating laboratories receive an individualized report detailing their performance. Laboratories must score 80% to receive a certificate.

The EQA panels also include samples specifically selected to assess aspects of the laboratory's performance. For example, panels in 2016–2018 contained a Mycobacterium canetti isolate, a closely related pair of isolates separated by more than twenty but fewer than 100 SNPs, and a sample comprising a 50:50 mixture of two other isolates (with one harbouring a mutation associated with rifampicin resistance). The inclusion of such isolates enables the network to identify areas where laboratories are performing adequately and areas where improvements are needed. Correct identification and interpretation of mixed isolates and minority variants (especially in loci strongly associated with drug resistance) is of particular importance. Rapid development of analytical tools is expected to provide information that can be used to improve future EQA schemes and panel composition. Based on the experience with MIRU-VNTR typing EQA rounds, highlighting these problems is expected to result in efforts to address these weaknesses.

A significant issue with the use of WGS is the comparison of results between laboratories. The inclusion of FASTQ files in the latest EQA round made it possible to assess the ability of laboratories to compare data generated in their laboratory with data from a different laboratory. Identification of clusters between laboratories is currently complex; for example, it requires FASTQ files or isolates to be shared. Strategies to provide an identification that could be used would include the use of type strains or sequences, the identification of curated strain-specific SNP lists, or a standardized nomenclature using MLST *(4, 5)*. This issue is being addressed – for example, by the EUSeqMyTB *(6)* project for MDR-TB – and developments will influence future EQA panels and activities.

In conclusion, there is rapid uptake and developments in the use and standard interpretation of WGS for MTB genotyping and resistance detection. Introducing these developments and ensuring that they translate into robust routine protocols is challenging for laboratories; however, it can be monitored and supported by the use of internationally recognized EQA schemes using carefully selected strain panels.

References for Annex 11

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Annex 12

Data and quality indicators for drug susceptibility testing based on next-generation sequencing

Data element	Variable	Result	Comment
Number of samples that were tested by NGS-based DST	A		
Number of samples that were tested by NGS-based DST that failed QC criteria at any stage of the NGS process:	B		Disaggregate by stage of protocol where failure occurred
• Number that failed QC criteria during specimen collection and accessioning			
• Number that failed QC criteria during the DNA extraction process			
• Number that failed QC criteria during the library preparation process			
• Number that failed QC criteria during the sequencing process			
• Number that failed QC criteria during the sequence assembly and analysis process			
• Number that failed QC criteria during the variant calling and mutation identification process			
Number of NGS assays that generated interpretable data for the targeted drugs. Data should be collected for each drug for which NGS-based DST data are used in the DR-TB surveillance system:	C		
• Number with interpretable data for rifampicin			
• Number with interpretable data for isoniazid			
• Number with interpretable data for ethambutol			
• Number with interpretable data for pyrazinamide			
• Number with interpretable data for fluoroquinolones			
• Number with interpretable data for linezolid			
• Number with interpretable data for bedaquiline			
• Number with interpretable data for clofazimine			
• Number with interpretable data for cycloserine			
• Number with interpretable data for amikacin			
• Number with interpretable data for delamanid			

Data element	Variable	Result	Comment
• Number with interpretable data for ethionamide			
Number of NGS assays that generated no results or invalid results	D		
Number of specimens tested with NGS for which a result was reported within the target turnaround time (i.e., time from receipt of specimen to reporting of results)	E		
Data for quarterly analysis			
Number of patients with TB whose samples were tested by NGS-based DST and phenotypic DST	F		
Number of patients with discordant results between NGS-based DST and phenotypic DST	G		
Number of patients who were tested by NGS-based DST and another molecular test (e.g., Xpert MTB/RIF)	H		
Number of patients with discordant results between NGS-based DST and another molecular DST	I		Stratify by susceptible vs resistant and wild-type vs mutant

DR-TB: drug-resistant TB; DST: drug susceptibility testing; NGS: next-generation sequencing; QC: quality control; TB: tuberculosis.

Analysis and calculations for NGS quality indicators

Indicator	Formula[a]	Result	Target
Proportion of samples that were tested by NGS-based DST that failed QC criteria at any stage of the NGS process	[B/A] ×100		<10%
Proportion of samples that generate interpretable data for all of the targeted drugs	[C/A] ×100		Disaggregate by drug
Proportion of samples that generated no results	[D/A] ×100		
Proportion of samples tested with NGS for which a result was reported within the target turnaround time	[E/A] ×100		
Proportion of samples with discordant results when NGS-based DST is compared with phenotypic DST	[G/F] ×100		Disaggregate by drug
Proportion of samples with discordant results when NGS-based DST is compared with other molecular tests	[I/H] ×100		Disaggregate by drug

DST: drug susceptibility testing; NGS: next-generation sequencing; QC: quality control.

[a] Letters A–I are from the table above

Annex 13

Examples of reporting forms for surveillance using drug susceptibility testing based on next-generation sequencing

A working group that includes representatives of the national tuberculosis (TB) programme (NTP), national TB reference laboratory (NTRL), drug-resistant TB (DR-TB) surveillance database and the next-generation sequencing (NGS) laboratory should be convened to design a template for the NGS-based drug susceptibility testing (DST) report that will be used in the DR-TB surveillance system.

The information to be included in an NGS-based DST report will depend on what information is to be included in the DR-TB surveillance database. At a minimum, the report should include the unique survey participant identifier, complete drug names, category (i.e., resistant or susceptible) of each drug evaluated, the specific mutation or mutations detected (i.e., nucleotide change and position, or three-letters amino acid change) and its frequency among the reads examined as well as information on the laboratory and patient demographics. Additional information may be included such as technical details of the NGS method, NGS test statistics (e.g., depth of reads and breadth of coverage), genes or gene regions sequenced, confidence of resistance interpretation (i.e., high-confidence mutations vs low-confidence mutations) and strain lineage. An example of such a reporting form is shown below.

The working group may also consider basing the form on existing templates for reporting NGS-based DST results to clinicians for clinical management of patients, where such templates were built around recommendations of an expert group including laboratory staff, epidemiologists, clinicians, bioinformaticians, government representatives and public health partners from different world regions *(1, 2)*. For example, Tornheim et al. *(3)* designed a template for a one-page report that provides laboratory and patient demographics, assay details, results for first-line and second-line anti-TB drugs, and a summary of results specifying the high-confidence resistance-associated mutations identified – according to standardized methods for interpreting the association between mutations and phenotypic drug resistance in *Mycobacterium tuberculosis (4, 5)* – and a longer template that includes technical details, mutations with less definitive interpretation and a section for resistance prediction of new or repurposed anti-TB drugs.

Mycobacterium tuberculosis report for drug-resistance surveillance using next-generation sequencing based on drug susceptibility testing.

Patient name:	Patients ID:
Birth date:	Location:
Sample type:	Sample collection date:
Sample ID:	Sample received date:
Requested by:	Sequenced from: ☐ culture ☐ specimen
Requester contact:	Report date:

Assay details

Sequencer:	Method: ☐ WGS ☐ tNGS
Pipeline:	Reference sequence: ☐ H37Rv ☐ .....

Lineage

Mycobacterium tuberculosis lineage:

Resistant to:

Resistant profile:

☐ No mutation detected[a]

☐ Multi drug resistance predicted

☐ Extensive drug resistance predicted

[a] No mutation detected does not exclude the possibility of resistance.

Drug	Locus	# Reads mapped	Breadth of coverage	Mutation(s): codon, amino acid change, allele (%)	Predicted phenotype (resistant or susceptible)	Confidence in resistance association	Comment
Rifampicin	*rpoB*						
Isoniazid	*katG*						
	inhA						
Ethambutol	*embA*						
	embB						
Pyrazinamide	*pncA*						
Fluoroquinolone (LFX or MFX)	*gyrA*						
	gyrB						
Bedaquiline, Clofazimine	*atpE*						
	mmpL5						
Linezolid	*rplC*						
Delamanid	*ddn*						
Amikacin	*rrs*						
	eis						

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Drug	Locus	# Reads mapped	Breadth of coverage	Mutation(s): codon, amino acid change, allele (%)	Predicted phenotype (resistant or susceptible)	Confidence in resistance association	Comment
Streptomycin	*rpsL*						
	rrs						
	gid						
Ethionamide	*inhA*						
	ethA						

Authorized by

Name:	Position:
Signature:	Date:
Reporting laboratory:	Contact information:

ID: identify; NGS: next-generation sequencing; tNGS: targeted NGS; WGS: whole genome sequencing.

References for Annex 13

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Annex 14

Example terms of reference for a senior next-generation sequencing scientist, molecular biologist, and bioinformatics officer

This annex provides suggested terms of reference (TOR) for the senior staff involved with next-generation sequencing (NGS).

Senior NGS scientist TOR

- Supervise and manage the NGS workflow and bioinformatics pipeline.
- Oversee quality assurance and quality control for the NGS process.
- Review and interpret NGS results.
- Review and authorize reporting of NGS results.
- Serve as liaison between staff of the NGS laboratory and staff of the drug-resistant tuberculosis (DR-TB) surveillance system.

Molecular biologist (laboratory technician) TOR

- Become acquainted with the principles, basics, and laboratory workflow of the NGS technology.
- Perform all the protocols of the NGS laboratory workflow (e.g., sample preparation, DNA library preparation and sequencing reaction) according to the manufacturer's instructions and standard operating procedures (SOPs) in use, including quality control steps during the different steps of the workflow.
- Understand the challenges of the NGS laboratory procedures and facilitate troubleshooting.
- Have an overview of the post-sequencing processes, with regard to how to demultiplex and convert the raw sequencer data files to the FASTQ format for downstream analysis and the use of user-friendly NGS data analysis tools.

Bioinformatics officer TOR

- Become acquainted with the basics of the NGS data analysis and interpretation (particularly for bacteria).
- Become proficient in the use of the text-based command line interface of UNIX or Linux operating systems and of software programs commonly used for NGS.
- Apply the existing bioinformatics pipelines for NGS sequence data analysis of *Mycobacterium tuberculosis* complex (MTBC) isolates and user-friendly NGS data analysis tools and adapt to local needs.
- Identify and apply the best computation tools and analytical methods available to manage the sequencing data (handling, storage).
- Understand the challenges of the NGS data analysis and facilitate troubleshooting.

Annex 15

Suggested agenda for next-generation sequencing training programmes

A next-generation sequencing (NGS) training programme would be expected to include the following:

- overview and principles of the NGS technologies for targeted NGS and whole genome sequencing (WGS).
- Review of standard operating procedures (SOPs) and protocols.
- Hands-on experience with wet-laboratory processes (e.g., sample preparation, DNA extraction, library preparation and sequencing).
- Familiarization with using and evaluating quality controls and quality checks.
- Basic data analysis skills for assessing the quality of the sequencing run (FASTQC); and
- (optional) basic understanding of the bioinformatics tools for analysing NGS data for determining the drug-resistance profile of all tested drugs and identifying strain lineages.

Day	Content
1	• (Theory and lectures) • Introduction to NGS and its applications • SOPs for NGS workflow • QC essentials – data labelling and data entry • Trouble shooting of potential NGS problems
2	• Genomic DNA isolation procedures from different sample sources (theory) • Genomic DNA isolation (instructor demonstrations and participant hands-on practice)
3	• Library preparation procedures: — theory — instructor demonstrations and participant hands-on practice • NGS run set-up on the instrument (wet laboratory)
4	• Monitoring the sequencing run (dry laboratory) • Assessing the quality of the run and sequencing QC (sample data, theory)
5	• Data analysis (sample data, dry laboratory) • Post-training mentoring and support programme • Wrap-up
6 (optional)	• Bioinformatics and user-friendly tools for NGS-based relatedness and drug-resistance analysis
7 (optional)	• NGS workflow performed independently by trainees with support from trainers • Post-training mentoring and support programme

DNA: deoxyribonucleic acid; NGS: next-generation sequencing; QC: quality control; SOP: standard operating procedure.

Estimates of training time are shown in the table below.

Content	Estimated time
Lectures and theory	8 hours
Wet laboratory hands-on	16 hours (plus 8 hours for optional day 7)
Dry laboratory hands-on	8 hours (plus 8 hours for optional day 6)
Demonstrations	4 hours
Q&A (discussion)	4 hours
Total	**40 hours** (plus 16 hours for the optional days)

The NGS manufacturers deliver a wide range of personalized training courses (locally, at the company location or online) and workshops that cover, for example, sample preparation, library preparation, sequencing and analysis components. The main manufacturers are:

- Illumina (https://support.illumina.com/training.html);
- Thermo Fisher Scientific (https://www.thermofisher.com/it/en/home/products-and-services/services/training-services/application-instrument-training-courses.html);
- MGI & BGI Tech (https://en.mgi-tech.com/Resource/video/category_id/7);
- Pacific Biosciences (https://www.pacb.com/support/training/); and
- Oxford Nanopore Technologies (https://store.nanoporetech.com/training/).

Annex 16

Competency assessment

This annex lists the various performance criteria used to assess staff for competency in the use of next-generation sequencing (NGS).

1. Preparation of equipment and reagents
    1. Set up of equipment and 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 labelling 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 (SOPs)
    3. Performance of quality checks
    4. Storage of samples or DNA in accordance with requirements
    5. 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 SOPs for library preparation
    5. Prevention of cross-contamination
4. Sequencing
    1. Set up of the sequencing instrument
    2. Following of the SOP for entering sample information, loading samples and conducting the sequencing run
    3. Performance of quality checks
    4. Exporting of data to bioinformatics programs and storage of FASTQ files
    5. Post-run equipment cleaning and maintenance

5. Data processing
    1. Ensuring results are consistent with expectations and reference standards (quality checks)
    2. Reporting of atypical observations
    3. Recording and reporting of results in accordance with test output
    4. Interpretation of data trends or 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
6. Safe work environment
    1. Use of safe work practices and appropriate personal protective equipment (PPE)
    2. Minimization of waste generation
    3. Storage of equipment and reagents
    4. Appropriate cleaning of equipment and safe use of reagents
    5. Preventative maintenance of equipment
    6. Safe disposal of waste including hazardous waste and tested samples

Annex 17

Impact measures

Successful implementation of drug susceptibility testing (DST) based on next-generation sequencing (NGS) for drug-resistant TB (DR-TB) surveys should improve the completeness of surveillance for DR-TB. Targets for the impact indicators and outcome measures will vary depending on the setting, testing algorithm, and use of NGS and other diagnostic tests. In general, trends in the indicators should be monitored and reviewed.

High-level outcome indicators include the:

- number and proportion of participants in a DR-TB surveillance system with DST results reported (stratified by drug);
- number and proportion of participants identified as having DR-TB (stratified by drug);
- number and proportion of participants with complete DST results (i.e. with results entered for each of the drugs included in the DR-TB surveillance system);
- number and proportion of participants with comprehensive DST results (i.e. with results entered for each World Health Organization (WHO)-recommended drug, stratified by Group A, B and C; and
- number and proportion of WHO-recommended drugs for which DST results are included in the DR-TB surveillance system, stratified by Group A, B and C drugs.

The baseline for each indicator is what was observed when the previous laboratory testing algorithm (e.g., phenotypic DST) was used. It is anticipated that the incorporation of NGS-based DST into the DR-TB surveillance system will lead to increases in each of the indicators.

To assess the contribution of NGS-based DST to the outcome indicators, the following process indicators can be evaluated:

- number and proportion of participants with DST results (any method) for which NGS-based DST results were recorded in the DR-TB surveillance system.
- Number and proportion of participants with NGS-based DST results recorded in the DR-TB surveillance system:
  - Stratified by individual drug.
  - Stratified by "complete DST results" (i.e., with results entered for each the targeted drugs included in the DR-TB surveillance system).
  - Stratified by "comprehensive DST results" (i.e., with results entered for each WHO-recommended drug, stratified by Group A, B and C).

- Number and proportion of WHO-recommended drugs included in the surveillance system for which NGS-based DST results were recorded; and
- number and proportion of participants identified as having DR-TB based on NGS-based DST result (stratified by drug).

The table below provides an example of detailed information for an outcome indicator.

Objective: improve completeness of surveillance for drug-resistant TB	
Outcome indicator 1	Number and proportion of participants in a DR-TB surveillance system with DST results reported (stratify by drug)
Purpose	Assess the completeness of DST results in the DR-TB surveillance system. Process indicators will assess the contribution of NGS-based DST to improve completeness of DST results
Target	Target is setting specific; number and proportion should increase over time
Numerator	Number of participants included in the drug-resistance surveillance system for whom DST results (any method) were recorded
Denominator	Number of participants included in the DR-TB surveillance system
Monitoring	Monitored annually by the national programme
Data sources	Database of reported TB cases, DR-TB surveillance system database
Process indicators	1.1 Number and proportion of participants included in the drug-resistance surveillance system for whom NGS-based DST results were recorded. 1.2 Number and proportion of participants included in the drug-resistance surveillance system. 1.3 Number and proportion of participants included in the drug-resistance surveillance system for whom DST results (any method) were recorded
Data elements	• Number of bacteriologically confirmed TB patients reported to the NTP. • Number bacteriologically confirmed TB patients reported to the NTP who were included in the DR-TB surveillance system. • Number of participants s included in the drug-resistance surveillance system for whom DST results (any method) were recorded. • Number of participants included in the drug-resistance surveillance system for whom NGS-based DST results were recorded
Remarks	• Countries may analyse this impact measure based on all WHO-recommended drugs or on the drugs available for use in the country. • Results should be stratified by drug (the impact of NGS-based DST is likely to vary by drug, with the largest impact being seen for drugs that do not have a reliable phenotypic DST method)

DR-TB: drug-resistant TB; DST: drug susceptibility testing; NGS: next-generation sequencing; NTP: national TB programme; TB: tuberculosis; WHO: World Health Organization.

The table below gives an example of detailed information for a process indicator.

Process Indicator 1.1: Number and proportion of participants for whom NGS-based DST results were recorded in the DR-TB surveillance system	
Purpose	Assess the use of NGS-based DST in the DR-TB surveillance system; assess the uptake of NGS testing, identify gaps and assist with planning
Target	This target is setting specific; the number should increase over time
Numerator	Number of participants for whom NGS-based DST results were recorded in the DR-TB surveillance system
Denominator	Number of participants for whom DST results (any method) were recorded in the DR-TB surveillance system
Monitoring	Monitored quarterly
Data source	DR-TB surveillance database
Remarks	• If applicable, disaggregate by NGS testing laboratory. • Disaggregate by region or participant enrolment site; this may identify issues related to implementation of the new testing algorithm or to specimen referral

DR-TB: drug-resistant TB; DST: drug susceptibility testing; NGS: next-generation sequencing; TB: tuberculosis.

For further information, please contact:

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Switzerland

Web site: www.who.int/tb

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