



Deeplex[®] Myc-TB

From clinical sample to drug resistance profile



USER MANUAL

March 2023 - V5

RESEARCH
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GenoScreen
Innovative Genomics

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User manual version history

Version	Date	Description of Change
1	Nov. 2019	Initial Release
2	July 2020	Added "1.8.3 Sequencing" and "1.9 Disclaimer". Updated "4. Characteristics and performances". Update "Appendix II" for NextSeq® 500/550 Mid Output kit reference Corrected language throughout the document Updated GenoScreen contact information.
3	Jan 2022	Updated "1.1 Principle", "1.2", "1.3", "1.4", "1.8 Kit specifications and intended use", "1.9" and "4. Characteristics and performances". Added "2.5.3. Dilution" Corrected : new names and references of Illumina products Corrected language throughout the document
4	April 2022	Updated "1.7. Kit specifications", "2.5.1. Clean-up", "3.2.2. Manual data upload", "3.2.3. Unpaired compressed FASTQ files", "3.4.2.2. Detailed results", 3.4.3. Detailed results - target view", "3.4.6. Saving, printing and sharing results". Updated visuals throughout all the document. Corrected language throughout the document.
5	March 2023	Updated "1.1 Principle", "1.5 Equipment and consumables to be supplied by user», «1.9 Disclaimer», «2.6 Library preparation for sequencing», «7 References», «Appendix II». Updated visuals throughout all the document. Corrected language throughout the document.

1. Product description

1.1. Principle

Deeplex[®] Myc-TB is an all-in-one targeted next generation sequencing (NGS)-based assay of *Mycobacterium tuberculosis* complex (MTBC) strains, directly applicable on clinical samples. The assay relies on deep sequencing of a single 24-plex amplicon mix for simultaneous mycobacterial species identification, genotyping and prediction of drug resistance of MTBC strains (Figure 1). Samples can be pooled and analysed in a single bench top NGS platform run (Table 1).

Table 1. Deeplex[®] Myc-TB specifications for using with different Illumina[®] platforms

Platforms	Specifications	Run time (2x150bp)	Number of samples	Number of tests (including controls)
iSeq [®] 100	1.2Gb	~ 19h	13	16
MiniSeq [®]	Mid Output (2.1Gb)	~ 17h	21	24
	High Output (6.6Gb)	~ 24h	66/69	72
MiSeq [®] v2	Nano kit (300Mb)	~ 17h	1	4
	Micro kit (1.2Gb)	~ 19h	13	16
	Standard kit (4.5Gb)	~ 24h	45	48
NextSeq [®] 500/550	Mid Output (32.5Gb)	~ 26h	372	384
	High Output (100Gb)	~ 29h	Not enough indices available	Not enough indices available

The assay relies on deep sequencing of 18 main MTBC gene targets associated with first- and second-line drug resistance (Table 2). Sequencing of mycobacterial gene targets can be achieved at high read depths. As a result each sequence position can be covered by many reads, enabling highly confident mutation calls. Variant can be detected in mutant/heteroresistant subpopulations representing as low as 3% (even 1% under position-specific read depth conditions) of bacteria in the sample, which is inaccessible to other rapid molecular tests. Extracted DNA representing as low as 100-1000 mycobacterial genomes/bacilli, thus below the limit of detection by classical microscopy, can be characterised. The kit also includes access to a secure web application, the Deeplex[®] Web Application, for fast and easy analysis and interpretation of the sequencing data.

Identification of mycobacterial species is performed by sequence analysis of the well established hsp65 gene as a marker. MTBC strain genotyping is done at (sub-)lineage level, by interrogation of the so-called spoligotyping target (CRISPR/Direct Repeat locus) and of phylogenetic single nucleotide polymorphisms (SNPs) in drug resistance-associated targets. Identification of mutations associated with resistance or susceptibility to anti-tuberculosis drugs is performed by comparison with known variants included in built-in reference databases, including the WHO catalogue of mutations in *Mycobacterium tuberculosis* complex and their association with drug resistance.

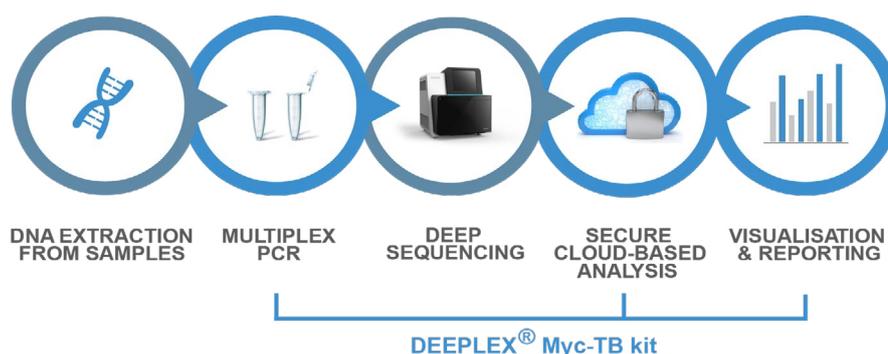


Figure 1. Deeplex[®] Myc-TB workflow.

Table 2. Deeplex® Myc-TB drug resistance-associated targets. (*full genes)

Genomic targets	Drugs	Genomic targets	Drugs
<i>rpoB</i>	rifampicin	<i>rrs*</i>	streptomycin, amikacin, kanamycin, capreomycin
<i>inhA</i>	isoniazid, ethionamide	<i>eis</i>	kanamycin
<i>fabG1</i>	isoniazid, ethionamide	<i>tlyA*</i>	capreomycin
<i>katG</i>	isoniazid	<i>gyrA</i>	fluoroquinolones
<i>ahpC</i>	isoniazid	<i>gyrB</i>	fluoroquinolones
<i>pncA*</i>	pyrazinamide	<i>ethA*</i>	ethionamide
<i>embB</i>	ethambutol	<i>rrl</i>	linezolid
<i>gidB*</i>	streptomycin	<i>rplC</i>	linezolid
<i>rpsL*</i>	streptomycin	<i>rv0678*</i>	bedaquiline, clofazimine

1.2. Context

Members of the MTBC, which are the causative agents of tuberculosis (TB), remain a leading cause of bacterial infectious disease worldwide. According to the World Health Organization, there were 10 million new TB cases and the disease caused 1.5 million deaths in 2020[1]. Moreover, there were more than half a million new cases of rifampicin (RR) or multi-drug (MDR) resistant (i.e., resistant to at least rifampicin and isoniazid) forms, including more than 25,000 pre-extensively drug resistant (pre-XDR: MDR additionally resistant to fluoroquinolones) or extensively drug resistant (XDR: MDR additionally resistant to fluoroquinolones and, at least one additional group A drug [bedaquiline, linezolid]) forms[1].

Early and accurate detection of drug resistance or susceptibility in TB is important to ensure rapid definition of appropriate treatment and avoid transmission of drug resistance.

1.3. 48-Test kit content

Content	48 tests format	Color code/comment
Deeplex® Myc-TB Master Mix	850 µl	Blue
External Positive Control (BCG)	5 µl	Colourless
Internal Amplification Control (non-mycobacterial DNA)*	3 µL	Green
Deeplex® Web application activation code	48 credits	Card included in the kit

*The internal control has to be spiked in with all samples except the negative control. It consists of non-mycobacterial DNA that is used as control for PCR inhibition. Water may evaporate and leave the tube empty, however, DNA still remains. Proceed to the dilution as described in section 2.4.1.

1.4. Storage conditions

Store all components at -20°C. Use consumable before the expiration date mention on the kit's box.

Important : in order to preserve the kit performances, it is strongly recommended to aliquot the components and to avoid repeated freeze-thaw cycles when a kit is to be used over separate test sets.

1.5. Equipment and consumables to be supplied by user

Equipment

- Personal protection equipment (lab coat, gloves, goggles, etc)
- Single channel and multi-channel pipettes
- Centrifuges for 1.5 ml low-binding microtubes
- Vortex mixers
- Heat block for 1.5 ml low-binding microtubes (for DNA extraction)
- PCR amplification systems
- DynaMag™-2 Magnet (Thermo Fisher™, 12321D)
- Qubit™ 4 Fluorometer (Thermo Fisher™, Q33216)

If 96-well plates are used:

- Centrifuge for 96-well plate
- 96-well PCR amplification systems
- DynaMag™-96 Side Skirted Magnet for plate (Thermo Fisher™, 12027)
- Fluorescence microplate reader (e.g. Infinite® 200, TECAN®) and microplate associated
- TruSeq® Index Plate Fixture Kit (Illumina®, FC-130-1005)

Consumables

• Extraction

- 1.5 ml screw cap or safe-lock microtubes
- 1.5 ml low-binding microtubes 2ml microtubes
- PCR-grade 1M Tris-HCl, pH 7.8, molecular grade (e.g. Sigma Aldrich™, T2569-100 ml)
- 0.15 mm zirconium beads (e.g. Next Advance™), to be autoclaved
- Glycogen, molecular grade (e.g. Sigma Aldrich™, 19-22mg/ml, G1767-1VL)
- PCR-grade 3M sodium acetate pH 5.2, molecular grade (e.g. Sigma Aldrich™, S7899)
- Ethanol 100%, molecular grade (e.g. Sigma Aldrich™, 32221)
- Ultra-pure PCR-grade water
- Filter tips

• PCR amplification

- 0.2 ml 96-well plates for PCR amplification (e.g. 0.2 ml Rigid PCR Ultra plate, Sorenson™) or PCR microtubes or strips
- Adhesive PCR plate films (e.g. Adhesive PCR film, ABgene™)
- 1.5 ml low-binding microtubes
- Ultra-pure PCR-grade water
- Filter tips

• PCR clean-up

- 1.5 ml low-binding microtubes or 0.2 ml 96-well plates for PCR amplification (e.g. 0.2 ml Rigid PCR Ultra plate, Sorenson™)
- Agencourt AMPure XP® (Beckman Coulter™) or NucleoMag® NGS clean-up and size select (Macherey Nagel®) magnetic beads
- Ultra-pure PCR-grade water
- Ethanol 100%, molecular grade (e.g. Sigma Aldrich™, 32221)
- PCR-grade 1M Tris-HCl, pH 7.8, molecular grade (e.g. Sigma Aldrich™, T2569-100 ml)
- Filter tips

• Quantification

- Using tubes:
 - Qubit™ dsDNA HS Assay Kit (ThermoFischer™, Q32851 (100 assays) or Q32854 (500 assays))

- 0.5 ml microtubes
- Using 96-well plates:
 - Fluorescence microplate reader (e.g. Infinite® 200, TECAN®) and associated microplate
- Filter tips

• Library preparation

If you are using Nextera® XT:

- Nextera® XT DNA Library Preparation kit (96 samples, FC-131-1096, or 24 samples, FC-131-1024)
- Nextera® XT Index Kits v2. If the number of tests per sequencing run does not exceed 48, a single set of Nextera® XT Index Kit v2 is sufficient (set A, FC-131-2001; B, FC-131-2002; C, FC-131-2003 or D, FC-131-2004). If the number of tests per sequencing exceeds 48, then the alternative use of two sets of Nextera® XT Index Kit v2, namely set A FC-131-2001 and set D FC131-2004, is recommended to avoid risk of spill over between successive sequencing runs.

If you are using Illumina® DNA Prep:

- Illumina® DNA Prep Kit (96 samples: 20060059, or 24 samples: 20060060).
- If the number of tests per sequencing run does not exceed 48, a single set of indexes is sufficient (IDT® for Illumina® DNA/RNA UD Indexes Set A (20027213), Set B (20027214), Set C (20042666) and Set D (20042667)). If the number of tests per sequencing exceeds 48, then the alternative use of two sets of indexes, namely set A and set D, is recommended to avoid risk of spill over between successive sequencing runs.
- Agencourt AMPure XP® (Beckman Coulter™) magnetic beads
- Ultra-pure PCR-grade water
- Ethanol 100%, molecular grade (e.g. Sigma Aldrich™, 32221)
- 1.5 ml low-binding microtubes
- Tween20, molecular grade (e.g. Sigma Aldrich™, P1379 - 500ml)
- Tris-HCl 10 mM, pH 8.5, molecular grade (e.g. Sigma Aldrich™, T2569-100 ml)
- Filter tips

• Sequencing

- Illumina® PhiX Control v3 (FC-110-3001)
- Illumina® sequencing kit (MiSeq® Reagent Kit V2 300 cycles PE, MS-102-2002 or MiSeq® Reagent Micro Kit v2 300 cycles: MS-103-1002 or MiSeq® Reagent Nano Kit v2 300 cycles: MS-103-1001)
- 1.0 N or 10 N NaOH, molecular grade (e.g. Sigma Aldrich™, 72068)
- Tween20, molecular grade (e.g. Sigma Aldrich™, P1379 - 500ml)
- Tris-HCl 10 mM, pH 8.5, molecular grade (e.g. Sigma Aldrich™, T2569-100 ml)
- NaClO (e.g. Fischer Scientific™, 11909012)
- Ultra-pure PCR-grade water
- 1.5 ml low-binding microtubes

 **Note: microtubes, plates and tips must be DNase- and RNase-free.**

If using Illumina® DNA Prep Kit, please refer to appendix II.

If using iSeq®100, MiniSeq® or NextSeq® 500/550 platforms, please refer to appendix II.

1.6. Precautions

Given the potential infectious risk, all steps before completing heat inactivation of biological samples must be performed according to local precautions and prescribed procedural guidelines.

Clinical specimens should be collected following standard procedures and transported in a timely manner to the laboratory at a temperature between 2°C and 8°C before processing.

As Deeplex® Myc-TB uses PCR amplification, adequate procedures should be followed to avoid risks of DNA contamination. All solutions and water used for samples processing must be of PCR-grade (i.e., DNase and contamination-free). DNA extraction from specimens, set-up of solutions and reaction mixtures, PCR amplifications and processing of amplicons should be performed in separate rooms.

Careful reading of the protocol sections of this User Manual is recommended, especially if Deeplex® Myc-TB is used for the first time.

1.7. Kit specifications

1.7.1. Applicability

The Deeplex® Myc-TB kit is designed for prediction of susceptibility or resistance to 13 anti-tuberculosis drugs/drug classes, for genotyping of MTBC strains, and for identification of mycobacterial species (including but not restricted to the MTBC).

1.7.2. Samples

The Deeplex® Myc-TB kit is conceived to be used on DNA extracted from heat- or ethanol-inactivated clinical samples from patients (potentially) suffering from TB, and from heat-inactivated mycobacteria positive cultures. Each kit lot is validated for successful mycobacterial identification, drug susceptibility and drug resistance prediction, and MTBC strain genotyping with ≥ 1000 extracted genomes (see limit of detection in section 4.1), under the conditions described in this user manual. The performances on a user's sample depend on the bacterial load and the efficiency of DNA extraction in the sample. We recommend the kit to be used on microscopy-positive samples. Although results can also be obtained on a number of microscopy-negative samples (see section 4.1), the performances on such samples cannot be ensured. The kit is also applicable on DNA extracted from cultured, heat inactivated isolates. The kit has not been evaluated on samples obtained from TB patients under treatment.

1.7.3. Sequencing

The Deeplex® Myc-TB kit and Web application are configured for paired end sequencing only. Optimal performances are obtained with 2x150 bp read lengths, although 2x250 bp read lengths can also be used.

1.7.4. Quality control

As part of the stringent GenoScreen quality assurance program (ISO 13485:2016), the performance of Deeplex® Myc-TB kit is monitored routinely and on a lot-to-lot basis.

1.8. Disclaimer

Resistance is reported when a documented resistance-conferring mutation is detected in targets of interest^{4,7,9}. **The absence of detected mutations does not exclude the possibility of resistance.** Low-frequency heteroresistance below the limit of detection by sequencing may affect typing results. The interpretation provided is based on the current understanding of genotype-phenotype relationships. All results reference the *M. tuberculosis* mutation numbering system, which differs from *Escherichia coli* numbering system.

1.9. Technical assistance

In order to facilitate technical assistance, please refer to Deeplex® Myc-TB lot number when contacting our support available at (+33)0 362 263 774, or at deeplexsupport@genoscreen.com. You may also contact us via the ticketing system in the Deeplex® Web Application (see section 3.5.1).

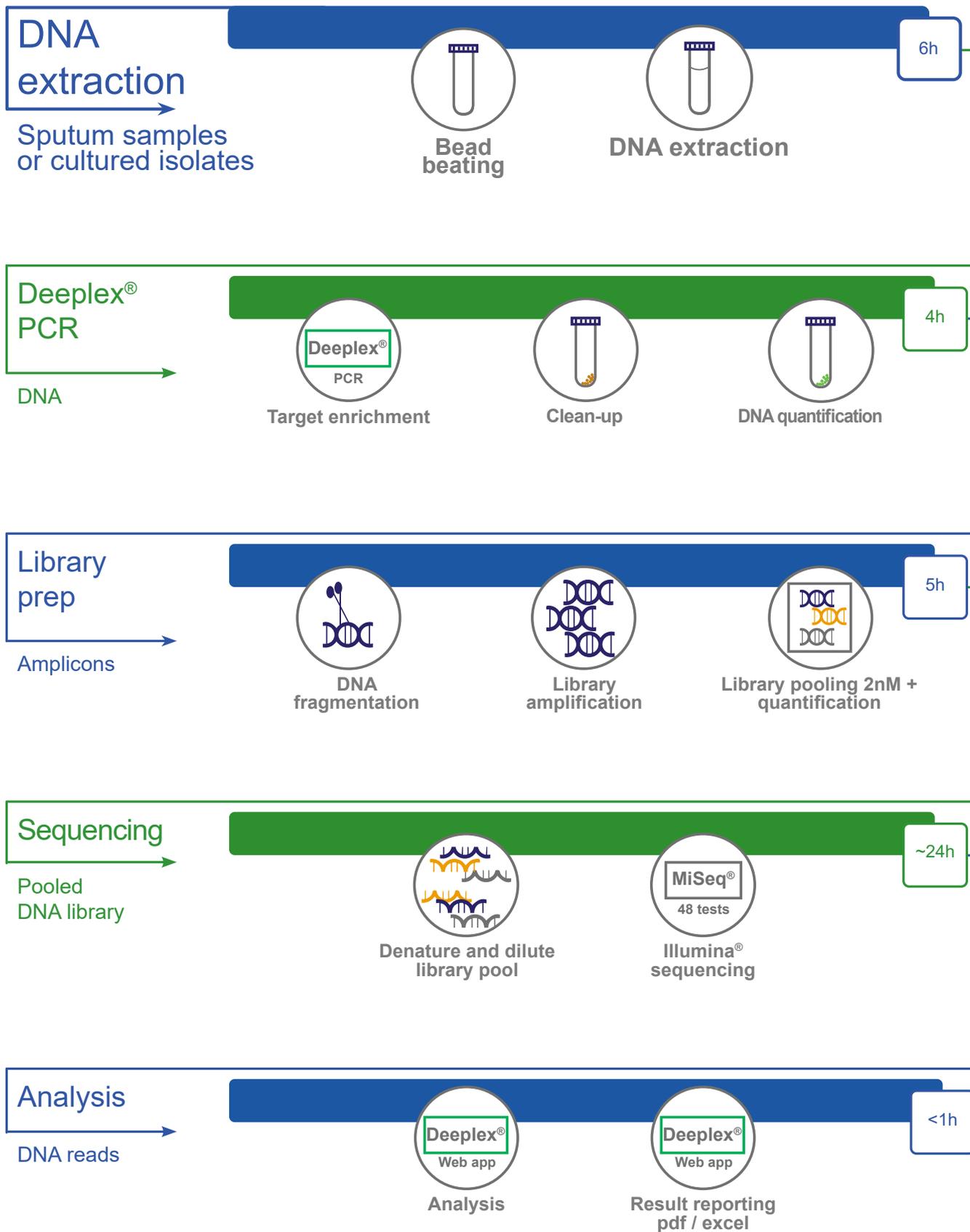


Figure 2. Overview of the Deeplex[®] Myc-TB protocol. Estimated times provided for 48 tests sequenced on a MiSeq.

2. Molecular protocol

Important points before starting

1. Use disposable tips containing hydrophobic filters to minimise cross-contamination.
2. Set up all reaction mixtures in an area separated from that used for PCR product analysis.
3. DNA extraction from specimens, set-up of solutions and reaction mixtures, PCR amplifications and processing of amplicons should be performed in separate rooms.

2.1. Preamble: digestion and decontamination of clinical samples

The following protocol has been validated on clinical samples digested and decontaminated by using N-acetyl-L-cysteine (NALC) combined with sodium hydroxide and sodium citrate procedure, utilising NACPAC™RED kit from AlphaTec™ (USA), followed by treatment by NPC67™ neutralising buffer and resuspension in 0.5 ml – 1 ml of PRB™ pellet resuspension buffer, both from AlphaTec™. These

steps should be performed by using screw cap or safe-lock microtubes.

If no decontamination is performed, after heat inactivation, centrifuge a minimum of 200µL of sample at 20,000 x g for 10 minutes, discard the supernatant and resuspend the pellet in 200µL ultra-pure PCR grade water.

2.2. Heat inactivation of decontaminated clinical samples

1. If part of the suspension is to be used for culturing or other analyses, transfer a minimum of 100 µL, and ideally 500 µL of the suspension into a new screw cap or safe-lock microfuge tube before heat inactivation.
2. Heat the tubes containing the suspensions at 95°C for 30 min using a heat block or an oven.
3. Store at -20°C or proceed to the next step.

2.3. DNA extraction



1. Centrifuge the tubes containing the suspensions at 20,000 g (or maximum speed) for 30 min
2. Remove and discard supernatant
3. Add 250 µl of 10 mM Tris-HCl pH 7.8 and vortex briefly
4. Incubate at 95°C for 15 min and spin down briefly to collect potential droplets in tube cap
5. Transfer the entire volume (avoiding the pellet) in a new tube containing ~ 0.5 g of zirconium beads in 2ml microtubes
6. Vortex each tube at least 30s, using a benchtop vortex mixer at full speed and spin down briefly Incubate at least 30 min at -20°C
7.  **SAFE STOPPING POINT, if you do not plan to proceed immediately to the next step, this incubation time can be longer (~ 1 h)**
7. Place at room temperature for thawing, briefly spin down and transfer the supernatant to a new 1.5 ml microtube, avoiding resuspension of beads
8. (~ 200 µl)
8. A DNA concentration step is performed as follows
 - 8.1 Add 1 µl of glycogen solution and mix
 - 8.2 Add 0.1 volume of 3 M sodium acetate at pH 5.2 and mix
 - 8.3 Add 3 volume 100% pre-cooled ethanol
 - 8.4 Vortex vigorously for 10s
 - 8.5 Incubate at -20°C for 10 min
 - 8.6 Centrifuge at 15,000 g (or maximum speed) for 20 min
 - 8.7 Discard supernatant
 - 8.8 Add 600 µl of freshly prepared, pre-cooled 70% ethanol
 - 8.9 Centrifuge at 15,000 g (or maximum speed) for 5 min
 - 8.10 Discard supernatant
 - 8.11 Air dry for 15 min or by using a vacuum concentrator for 5 min
 - 8.12 Resuspend pellet in 20 µl of sterile water
8.  **SAFE STOPPING POINT, if you do not plan to proceed immediately to the next step, DNA can be stored at -20°C (for few months).**

2.4. PCR amplification



2.4.1. PCR preparation

When DNA concentrations exceed 100ng/μl, we recommend to adjust the extracted DNA concentration to a maximum of 100ng/μl by applying the minimum necessary dilution ratio in molecular biology-grade water.

In a PCR product-free area:

1. Add 35 μl of PCR-grade water to the positive control solution (colourless tube). Vortex well and spin down. To be done only once.
2. Add 897 μl of PCR-grade water to the internal control solution (green tube). Vortex well and spin down. To be done only once.
7. Add 9 μl of the diluted positive control solution in the positive control well (e.g. G6, colourless tube).
8. Add 9 μl of sterile water to the internal and negative control wells (e.g. F6 & H6).
9. Tightly seal the 96-well plate with an adhesive PCR film.
10. Spin down.

Note: water may evaporate and leave the tube of internal control empty, however, DNA still remains. Proceed to the dilution.

3. For each test (samples + controls) mix in a new 1.5 mL microtube :
 - 15.8 μl of Deeplex® Myc-TB Master Mix (blue cap)
 - 0.2 μl of diluted internal control
4. Distribute 16 μl of the above mix in all but the last well which is kept for the negative control (see Figure 3).
5. In the last well (e.g. H6), load 16 μl of Deeplex® Myc-TB Master Mix (without the internal control – blue tube), see Figure 3.
6. Add 9 μl of DNA suspension in each of the 45 sample wells (e.g. A1-E6).

Note: for DNA extracted from cultured isolates, 9 μl of a suspension at minimum 1 pg/μl can be used.

Figure 3. Example of a 48 tests PCR spreadsheet. Wells F6 to H6 have been assigned to the controls.

	1	2	3	4	5	6	7	8
A	Sample 01	Sample 09	Sample 17	Sample 25	Sample 33	Sample 41		
B	Sample 02	Sample 10	Sample 18	Sample 26	Sample 34	Sample 42		
C	Sample 03	Sample 11	Sample 19	Sample 27	Sample 35	Sample 43		
D	Sample 04	Sample 12	Sample 20	Sample 28	Sample 36	Sample 44		
E	Sample 05	Sample 13	Sample 21	Sample 29	Sample 37	Sample 45		
F	Sample 06	Sample 14	Sample 22	Sample 30	Sample 38	internal control		
G	Sample 07	Sample 15	Sample 23	Sample 31	Sample 39	positive control		
H	Sample 08	Sample 16	Sample 24	Sample 32	Sample 40	negative control		

Master mix plus internal control
Master mix only

2.4.2. PCR program

These steps must be done in a post-PCR lab. These conditions have been successfully tested on GeneAmp® PCR System 9700 (Applied Biosystems™) using 100% temperature ramp rate (3.0°C/sec for the sample block or 2.3°C/sec for the sample).

SAFE STOPPING POINT, if you do not plan to proceed immediately to the next step, PCR products can be stored at 4°C during 1 or 2 days, or at -20°C during one week.

Amplification cycles:

2 min	95°C	} 35 cycles
1 min	94°C	
30s	55°C	
1 min 30s	72°C	
10 min	72°C	
∞	4°C	

2.5. Clean-up and quantification of Deeplex® Myc-TB amplicons

2.5.1. Clean-up

Deeplex® Myc-TB amplification products must be purified before resuspension in 10 mM Tris-HCl pH 7.8.

The following protocol is recommended. Agencourt AMPure XP® (Beckman Coulter™) or NucleoMag® NGS clean-up and size select (Macherey Nagel®) magnetic beads can be used.

1. Preliminary steps:
 - a. Bring the Agencourt AMPure XP® or NucleoMag® bead-containing containers to room temperature (at least 30 min)
 - b. Prepare fresh 80% ethanol
 - c. Prepare 10 mM Tris HCl pH 7.8
2. Spin down the 96-well PCR plate containing the amplification products
3. To the PCR reaction,
 - a. Add 75 µl of 10 mM Tris-HCl pH 7.8
 - b. Vortex the magnetic bead suspensions to ensure that the beads are well resuspended, and add 65 µl of bead suspensions to each PCR plate well
 - c. Mix by gently pipetting up and down 10 times
 - d. Incubate at room temperature for 5 min
 - e. Place on magnetic rack for 5 min
 - f. Discard supernatant
 - g. First washing step:
 - i. Add 180 µl (if working with plates) or 500 µl (if working with tubes) freshly prepared 80% ethanol avoiding bead resuspension
 - ii. Incubate on magnetic rack for 30 s
 - iii. Discard supernatant
 - h. Second washing step:
 - i. Add 180 µl (if working with plates) or 500 µl (if working with tubes) freshly prepared 80% ethanol avoiding bead resuspension
 - ii. Incubate on magnetic rack for 30 s
 - iii. Discard supernatant
 - i. Carefully remove residual traces of 80% ethanol using a P10 pipette
 - j. Allow beads to dry for about 5 min (**do not allow the beads to dry over 5 min**)
 - k. Remove the 96-well plate from the magnetic rack
 - l. Add 26 µl 10 mM Tris-HCl pH 7.8
 - m. Mix by gently pipetting up and down 10 times to resuspend beads
 - n. Incubate at room temperature for 2 min
 - o. Incubate on magnetic rack for 5 min
 - p. Transfer 25 µl of purified amplicon DNA to a new plate



SAFE STOPPING POINT, if you do not plan to proceed immediately to the next step, purified PCR products can be stored at -20°C for up to one week.

2.5.2. Quantification

1. Quantify purified amplification products using a Qubit™ fluorometer or SYBR® Green quantification with a microplate reader (e.g., Infinite® 200, TECAN®) following the manufacturer instructions
2. Verify that the negative control is below detection level and the external positive control is well above 20 ng/µl. Concentration of sample tests above 5 ng/µL are expected to generate successful sequencing results. However, successful library preparation and sequencing can also be achieved in a number of cases even with a concentration of amplification products between undetectable level and 5 ng/µL.

2.5.3. Dilution

1. Dilute purified amplification products at 0.2 ng/µl in PCR-grade water (**and not in TE solution**) for library preparation



Important: Successful library preparation using the Nextera® XT DNA Library Preparation kit (see below) is largely dependent upon precise DNA quantification. Therefore, if the concentration of purified amplification products is high (more than 10 ng/µl), it is recommended to first dilute samples at 2 ng/µl, then quantify these dilutions, and perform final dilutions at 0.2 ng/µl based on this intermediate quantification.

2.6. Library preparation with Illumina® DNA Prep Kit

The following steps are intended to use with Illumina® DNA Prep Kit. Please refer to Appendix II if using Nextera® XT DNA Library Preparation Kit. All Illumina® protocols have to be downloaded at support.illumina.com. Follow the manufacturer instructions (Nextera® XT DNA Prep Kit Reference Guide, please used the most current reference guide), with the following adaptations and recommendations.

Consumables

- Illumina® DNA Prep Kit (96 samples: 20060059, or 24 samples: 20060060).
- If the number of tests per sequencing run does not exceed 48, a single set of indexes is sufficient (IDT® for Illumina® DNA/RNA UD Indexes Set A (20027213), Set B (20027214), Set C (20042666) and Set D (20042667)). If the number of tests per sequencing exceeds 48, then the alternative use of two sets of indexes, namely set A and set D, is recommended to avoid risk of spill over between successive sequencing runs.
- At “Pool libraries”, quantify each library as described in step 2.5.2 above.
- **Do not perform the “Check Library Quality”.** In “Dilute Libraries to the Starting Concentration”, steps are replaced by the following procedure:



Note: for all libraries to be included in one equimolar pool, library quantification should be performed in parallel, using the same method.

Protocol

Follow the “Illumina® DNA Prep Reference Guide” (please used the most current reference guide) with the following adaptations and recommendations.

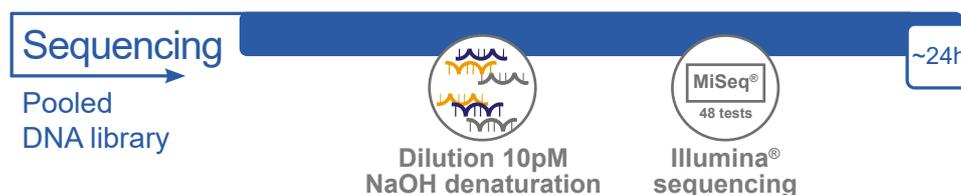
- Use 5 µl of input DNA at 0.2 ng/µl (i.e. a total of 1 ng) for each library.
- Choose a different set of 48 index primers between consecutive sequencing runs to avoid the risk of spill over between them.
- Use the Index adapter pooling guide to verify that the selection of index primers is correct (please use the most current reference guide) to verify that the selection of index primers is correct and create the sample sheet with anonymised sample names (using the Local Run Manager v3, please use the most current reference guide).
- At “Amplify Tagmented DNA” follow the procedure for 1-9 ng of input DNA:
- At “Clean-up libraries”, perform step 5 for standard DNA input. Do not perform step 6 for small PCR amplicon input.
- Multiply the obtained mass concentrations (in ng/µl) by 3 to convert them to molar concentrations (nM).
- For each sample, calculate the volume of library needed to obtain a final concentration of 2 nM in a final solution of 10 µl (but do not adjust the volume to 10 µl).



Note: for libraries with a concentration equal to or lower than 2 nM, use 10 µl of undiluted library.

- Pool calculated volumes for the individual libraries in a single tube.
- To this library pool, add the volume of Tris HCl 10 mM pH 8.5 + 0.1 % Tween 20 required to reach a total volume equal to the number of samples x 10 µl (e.g. 480 µl for 48 tests).
- Store the library pool on ice. If the library won't be run immediately store the pool according to the instructions in the reference guide.

2.7. Sequencing



The following steps are intended to be used when performing sequencing using a MiSeq®. Refer to Appendix II if using iSeq®100, MiniSeq® or NextSeq® 500/550 platforms.



Important: follow the recommended number of tests per run as described in table 1 to ensure optimal data output.



Note: remove the sequencing kit from -20°C and thaw at room temperature in water at least 1h before starting.

1. To prepare libraries prior to sequencing with MiSeq®, follow the “Protocol A: Standard Normalization Method”, “Denature a 2 nM library”, “Dilute Denatured 10 pM Library” and “Denature and Dilute PhiX control”
2. Sequencing can then be performed, by following instructions from the “MiSeq® System Guide” (please used the most current reference guide).
3. Run quality metrics can be visualised using the Sequencing Analysis Viewer (SAV) tool (download at support.illumina.com).

3. Sequencing data analysis



IMPORTANT: the following description applies to the current version of the Deeplex® Web Application. A new version is expected to be available during the course of the Seq & Treat trial. As a result, in addition to bug fixes and improvements in sequencing data analytics, there will be changes in visual presentation, and addition of new functionalities. A short remote presentation session will be proposed for update in accordance.

3.1. Access to the Deeplex® Web Application

Important: the sequencing data files must be in compressed FASTQ format (.fastq.gz ; standard output format generated by Illumina® sequencers).

To analyse the compressed FASTQ files (.fastq.gz) obtained after sequencing, you need to access the Deeplex® Web Application (app.deeplex.fr). For this, you have to log in as a registered user. Registering enables the storage of all Deeplex® results to your own personal account.

You will need to register at first use. Click “Register” and fill in the information required. Passwords need to be at least 6 characters long, mixed case and contain one of the following characters: “.”, “!”, “@”, “#”, “\$”, “%”, “^”, “&”, “*” or “-”. You may provide a mobile phone number (incl. country code) to enable two-factor identification. When enabled, an identification code-containing text message will be sent to your mobile phone each time you want to log in your account.

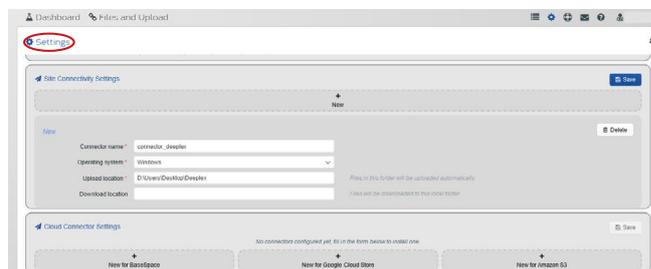


3.2. Upload sequencing data

Important: sequencing data should be pseudonymised before upload.

3.2.1. Automatic data upload

To enable automated upload of your sequencing data onto the Deeplex® Web Application, you first need to install a software called “connector”. This connector will upload any sequencing data, in compressed FASTQ format (.fastq.gz), from a user-defined directory on your computer to the Deeplex® Web Application (please see 3.2.2 for alternative manual data upload).

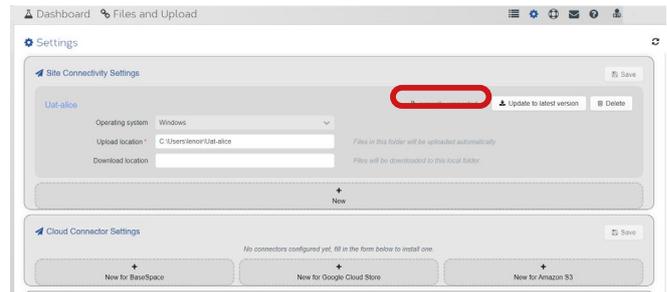


On the Deeplex® Web Application, go to “Settings” > “Site Connectivity Settings”. Provide a name for your connector and the path to your data directory. The directory needs to exist before the connector installation. To get the path to your data directory, right-click on the directory and go to “Properties” on Windows and Linux and “Get info” on macOS.

Finally, select your operating system from the list and click “Save”. Download of the connector should start and an activation key is provided. The procedure that follows depends on your operating system.

In **Windows**, follow the connector install instructions and type activation key when required. Refresh your Deeplex® session by using the refresh button (top right of the web page); the connector should be displayed as connected. You may install one connector only.

- In **macOS**, follow the connector install instructions and type activation key when required. Open the connector from the Launchpad or by logging out and back in your computer session to activate it. Refresh your Deeplex® session by using the refresh button (top right of the web page); the connector should be displayed as connected.
- In **Linux**, you need Java SE 8+ installed to run the installer. Open the Terminal and find your installer. Type in `bash genoscreen-connector.sh`; depending on whether you have an X server running or not, it will display a user interface, or follow a command line installation procedure. You can force a command line installation by adding a `-c` flag to the previous command. When done, you may need to start the connector from within the genoscreen directory by typing `./genoscreenserviceconnector start`. Refresh your Deeplex® Web Application session by using the refresh button (top right of the web page); the connector should be displayed as connected.



You may want to install multiple connectors if you work on more than one machine or if you have data in several folders (not on Windows). To do so, click “New” and reiterate the process.

Note: that connectors should be uniquely named (this includes deleted connectors). Uploaded unanalysed sequencing data remain available for 7 days. After this delay, sequencing data must be re-uploaded.

3.2.2. Manual data upload

To manually upload compressed FASTQ files, go to the “Files and Upload” tab, where you can either drag and drop the files or click on “Choose a file”, select your folder and your files.



3.2.3. Unpaired compressed FASTQ files

Once the files are uploaded to the Deeplex® Web Application, you should see samples appearing in the Dashboard. If you do not, wait for a few more seconds and refresh your Deeplex® Web Application session (top right icon). If nothing appears then, this may mean that file pairing did not succeed. To correct this, go to the “Files and Upload” tab, select your R1 and R2 files for a specific sample and click “Create sample”. You can then choose any name for your sample and confirm.



3.2.4. Consideration on compressed FASTQ file size

If sizes of individual compressed FASTQ files (i.e., of each R1 and R2 files) exceed 100 Mb, they may fail to run on the Deeplex® Web Application. Therefore, we recommend using individual R1 and R2 files of less than 100 Mb per sample.

To avoid sample files that exceed 100 Mb :

- Avoid reducing the numbers of samples per run by more than 30% compared to recommended numbers indicated in **Table 1**.
- If you multiplex with other types of samples (e.g., for whole genome sequencing; they must then be prepared with the same library preparation kit), the proportion of Deeplex® Myc-TB samples should be adjusted in the pool by accounting for target sequence outputs for these other samples.

Note: samples need to be analysed within 6 days after upload or they will be deleted from the Deeplex® Web Application. Results will be stored on the Deeplex® Web Application for 1 year. For long-term storage of the results, see section 3.4.5 “Saving and printing results” or use credits to re-analyse your samples and add another year of storage.

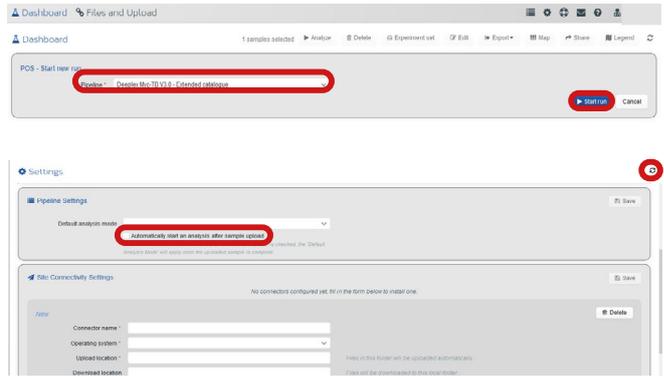
3.3. Data analysis

3.3.1. Starting a run analysis

To start an analysis, select samples in the “Dashboard” and click “Analyze”. Choose a Deeplex® pipeline from the drop-down menu and click “Start run”. Two pipelines are available :

- WHO catalogue - based: interrogation of only the WHO mutation catalogue for drug resistance prediction¹²
- Extended catalogue : Interrogation of the WHO mutation catalogue and other reference publications for drug resistance prediction^{4,7-9,12}

You can also start an analysis automatically right after upload of the data by going to “Settings” > “Pipeline” Settings and by ticking the box next to “Automatically start an analysis after sample upload”. Click “Save”. If data have been uploaded already, the analysis needs to be started manually.



Analysed results remain available for 1 year, starting from the analysis submission date. Check completion of the analysis in the first column in the Dashboard called “Status” by using the “refresh” icon in the Web Application (top right):

 **Note: If for any reason, analysis fails please contact us for assistance. In this case, credits used are automatically restored.**

-  **Unanalyzed:** Sample has not been analysed yet.
-  **Ongoing:** Analysis is still running.

-  **Succeeded:** Analysis has successfully run.
-  **Failure:** Analysis has failed. Please contact our support.

3.3.2. Control validation

Controls are an important part of the Deeplex® Myc-TB assay. They indicate that:

- Variants can be accurately called from samples with enough input DNA (as seen from the positive control result)
- Amplification of mycobacterial targets was not inhibited during PCR (based on the internal control result, in samples and in a separate internal control)
- Sample testing was contamination-free (as seen



from the negative control result)

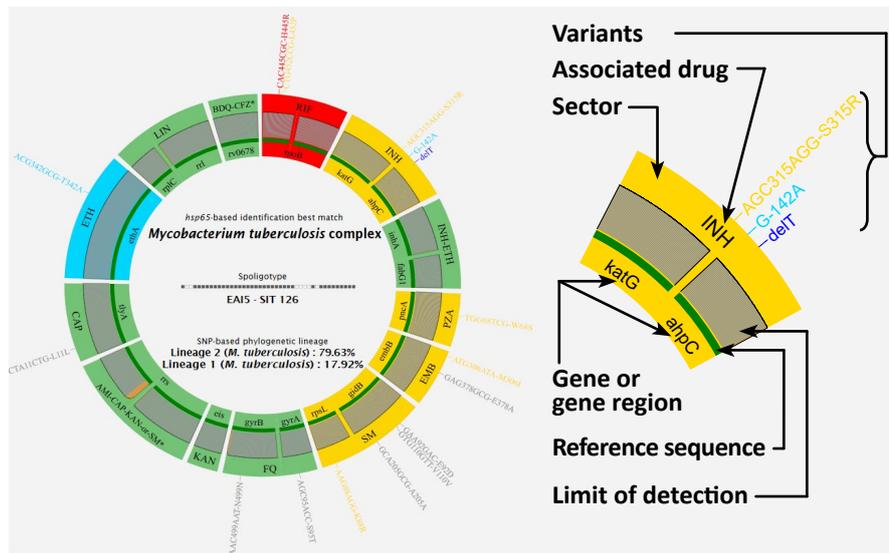
After completion of analyses of all samples and controls from an experiment, select these lines and click “Experiment set”. On the right-hand side of the “Experiment set” screen, select your controls from the sample list (positive, negative and internal). They should all show up as “VALID”. If they do, go to section 3.4. If they are “NOT VALID”, go to 3.3.3. for troubleshooting.

Controls are validated if:

- **Positive control:**
 - o *hsp65*-based species identification is *M. tuberculosis* complex
 - o Spoligotype is BOV_1 _SIT 482
 - o Lineage identification is *M. bovis*
 - o 9 expected BCG variants detected at >99% and no other variants detected at >5%
- **Negative control:**
 - o No composite reference coverage breadth at mycobacterial targets and internal control (but accounting for possible background noise).
- **Internal control:**
 - o Coverage breadth > 95% on control target
 - o No composite reference coverage breadth at mycobacterial targets (but accounting for possible background noise).

3.3.3. Troubleshooting invalid controls

Please refer to the previous section and check whether the control specifications and thresholds are met. If any of these controls are invalid, further troubleshooting can be done via the ticketing system (see section 3.5.1.) or email at deeplexsupport@genoscreen.com.



3.4.2.1. The Deeplex® map

From centre to margin

- **Taxonomic information:**
 - *hsp65*-based identification best match: Mycobacterial species identified by best match analysis versus *hsp65* reference sequences².
 - *Spoligotype*: Spoligotype followed by spoligotype (sub-)family name and SIT number (Spoligotype International Type)³. Only if *M. tuberculosis* complex is identified as best match.
 - *SNP-based lineage identification*: Based on detection of one or more phylogenetic SNPs, according to SNP barcodes and hierarchical classification of MTBC strain lineages⁵. Only if *M. tuberculosis* complex is identified as best match.
- **Target genes or gene regions:**
 - Displayed in *groups*, depending on which antibiotic(s) they may impact
 - The colour code used for the group is identical to that of the resistotype. If different types of variants are identified, priority is given to yellow to red colour, when one or more resistance variants are detected
 - *Reference sequences* are shown as segments above the gene names, the colour of which depends on the amplification and sequencing success:
 - Green: > 95% of the reference target sequence covered
 - Black: < 95% of the reference target sequence covered
 - *Limit of detection (histograms on top of reference sequences)*: Histograms in grey or orange represent the coverage depths obtained over the reference sequences, with the following meanings:
 - Grey: The achieved limit of detection to detect minority variants is 3%
 - Orange: The achieved limit of detection to detect minority variants is >3% and ≤ 80%
- **Variants:**
 - In the *format*: CAC57GAC-H57D where
 - CAC is the 57th reference codon
 - GAC is the 57th variant codon
 - H is the 57th reference amino acid
 - D is the 57th variant amino acid
 - In the *format*: delT or inserT where
 - «del» stands for deletion and «inser» for insertion
 - T is the nucleotide deleted or inserted
 - The *colour code* used for variants is identical to that of the resistotype:
 - *Red to light yellow*: Variant associated with antibiotic resistance, either (almost) fixed (red) or present in low frequency in the reads within a sample (light yellow)
 - *Dark blue to light blue*: Uncharacterised non-synonymous variant, either (almost) fixed (dark blue) or present in low frequency in the reads within a sample (light blue)
 - *Grey*: Variant known not to be associated with resistance. It can be a phylogenetically informative variant or a synonymous variant or a benign (i.e., not associated with drug resistance) non-synonymous variant.

Additional description in “Legend” (bottom left of the Deeplex® map).

3.4.2.2. Detailed results

A more detailed summary of the results is displayed at the right of the Deeplex® map.

- **Sample information:** Sample name, quality and experiment set as well as the date of submission and other custom metadata if any.
- **Resistotype:** See section 3.4.1. for a description of the resistotype, legend of the Deeplex® map for the colour scheme and antibiotics abbreviations.
- **Sample controls and metrics:**
 - *Controls (internal, positive and negative)* can be “valid” or “not valid”. See sections 3.3.2. and 3.3.3. for more information
 - *Composite reference coverage:* Fraction of all reference sequences covered at more than 5x (%)
 - *Median depth of coverage* of reference sequences with minimum and maximum values
 - *Sequencing coverage and depth per gene target:*
 - Sequencing depth (1st bar) ≥134x (grey) or <134x (orange), left y-axis
 - Target coverage (2nd bar) >95% (green) or <95% (black), right y-axis
- **hsp65-based species identification:**
 - *Av coverage depth (x):* Average number of reads per base for *hsp65*
 - *Consensus length:* Length of consensus sequence for *hsp65* from sample aligned vs a *hsp65* reference from the mycobacterial species identified as best match
 - *% Identity:* Percentage of *hsp65* bases identical to the indicated reference *hsp65* sequence.
 - *E-value:* as reported by BLAST v.2.7.1+⁵
 - *Best match:* Mycobacterial species identified by best match analysis versus *hsp65* reference sequences².

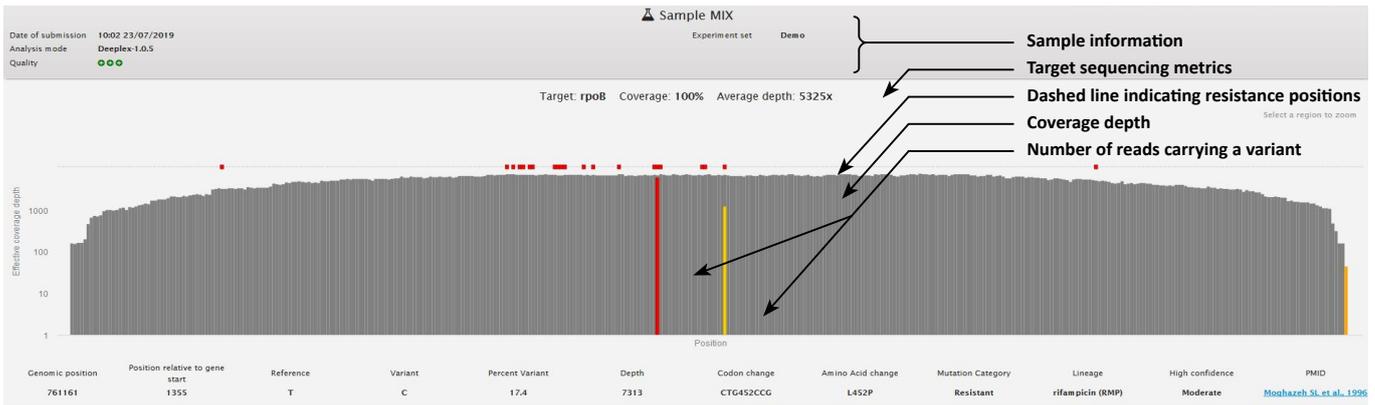
Note: If there is no mycobacterial species detected, coverage depth and coverage breadth are shown for the internal control. If amplified, this indicates no mycobacteria are detectable in the sample (no PCR inhibition) (see section 2.4.).

- **Drug resistance-associated variants**^{*4,7-9,12}:
 - *Gene:* Name of the gene carrying a resistance-associated variant
 - *Genomic position:* Genomic coordinate in the reference *M. tuberculosis* H37Rv strain
 - *Codon change:* For the format, see section 3.4.2.1.
 - *% Variant:* Percent of reads carrying the variant
 - *Dx-score:* Depth excess score. Ratio of the coverage depth at the variant position relative to the minimum needed to call the mutation; 1 meaning that the observed number of reads is the minimum needed to call the variant at the observed percentage and e.g., 1000 meaning that the observed number of reads is 1000 fold higher than this minimum.
 - *AA change:* For the format, see section 3.4.2.1.

- *Drug:* The drug resistance with which the mutation is associated. Abbreviations for antibiotics are detailed in “Legend”, bottom left of the Deeplex® map.
- *Confidence*¹⁰: Confidence levels of association with resistance as can be reported by reference databases.
 - Associated with resistance/High: Strong association of the mutation with phenotypic drug resistance; sufficient evidence that the mutation confers or is strongly associated with drug resistance.
 - Associated with resistance - interim/Moderate: Moderate association of the mutation with phenotypic drug resistance; additional data desirable for improved evidence that the mutation confers or is strongly associated with drug resistance.
 - Low: Weak association of the mutation with phenotypic drug resistance; insufficient evidence to ensure that the mutation confers or is strongly associated with drug resistance. Substantial additional data required.
- *Resistance level: degree of resistance conferred by the variant*
- *Reference:* link to the published work about variant
- **Uncharacterized and uncertain significance variants***:
 - Uncharacterized variants designate sequence variants of as yet unknown association with drug susceptibility or resistance. Uncertain significance variants designate variants that could not be characterized yet as either drug resistant or drug susceptible according to the current WHO confidence grading classification. See “Drug resistance-associated variants” for columns description.
- **Spoligotype***:
 - *Histogram:* Average read depth at 43 reference spacers within the direct repeat (DR) locus of *M. tuberculosis* complex. The detection threshold for spacers is 10x.
 - *Summary table:*
 - *Octal code / Binary code*
 - *Average read depth:* read depth averaged over sequenced spacers
 - *SIT:* SIT number³
 - *SITVIT occurrence:* frequency in SITVIT database. The higher, the more frequent is the spoligotype.
 - *Clade:* (sub-)family name³
- **SNP-based phylogenetic lineage***: Based on phylogenetic SNP barcodes⁵
- **Potential mixed infection***: Mixed infection reported here when at one or more phylogenetically informative sites, heterologous nucleotide calls are detected, with at least two variants identified, each with a read frequency between 3% and 97%.

* Only if *M. tuberculosis* complex is identified as best match using the *hsp65* locus.

3.4.3. Detailed results - target view



For a more detailed view of results for a gene target, double click on the target on the Deeplex® map
From top to bottom:

- **Sample information:** See section 3.4.2.2.
- **Target sequencing metrics:** Gene name, coverage breadth and average depth.
- **Sequencing coverage depth and breadth histogram:**
 - **Dashed line:** Each red square indicates positions known to be associated with drug resistance and present in our database. On mouseover or click, detailed information about each drug resistance associated position can be obtained.
 - **Histogram:**
 - For each covered position, the vertical bar height indicates the coverage depth. The colour code used for vertical bars is identical to that used in the Deeplex® map, i.e., sequencing depth $\geq 134x$ in grey or $< 134x$ in orange. (see section 3.4.2.2). On mouseover, detailed metrics on the position can be obtained
 - By clicking on a vertical bar, detailed information on the position can be obtained (below the histogram), including genomic position, reference nucleotide and depth.
 - If a variant is detected, the vertical bar height reflects the number of reads carrying the variant (please note the logarithmic scale on the y-axis). The colour code is identical to that of the variants in the Deeplex® map (see section 3.4.2.1.).
 - **Red to light yellow:** Variant associated with antibiotic resistance, either (almost) fixed (red) or present in low frequency in the reads within a sample (light yellow)
 - **Dark blue to light blue:** Uncharacterized (non-synonymous) variant, either (almost) fixed (dark

blue) or present in low frequency in the reads within a sample (light blue)

- **Grey:** Variant known not to be associated with resistance. It can be a phylogenetically informative variant or a synonymous variant or a benign non-synonymous variant.
- Additional information is displayed, e.g., about the association with drug resistance with corresponding information of reference publication.

3.4.4. Adding metadata

User-defined columns can be added to pre-defined columns, in order to include possible metadata. To do so, go to “Settings” > “Metadata Settings” and type names of columns to be added. Up to 10 fields can be added.

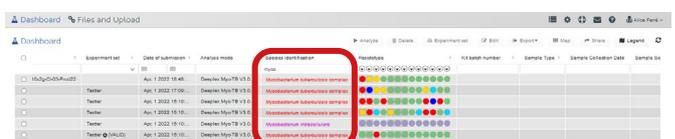


Note: patient information are not allowed.



3.4.5. Filtering samples

To facilitate result visualization, samples can be filtered using the following procedure in the Dashboard. Type a text in a field, or select criterion at the top of the columns. You can also order columns by clicking on the headers.



3.4.6. Saving, printing and sharing results

- **Detailed report in PDF:** Save the Deeplex® map along with detailed results (see section 3.4.2.). Click “Save full results as PDF” from the Dashboard, for a selection of multiple samples or from the sample view to save only the current sample.
- **Summary report in PDF:** Save summary information by selecting a sample from the Dashboard and click on “Save summary results as PDF, or from the sample view , to save only the current sample.
- **XLS:** Save resistotype, identified mutations, genotyping results and sequencing metrics for one or multiple samples in an Excel file. Click “Save as XLS” from the Dashboard, for a selection of multiple samples or from the sample view to save only the current sample. The colour code used for resistance/susceptibility is the same as that used for the resistotype and is described in “Legend” (bottom left of the Deeplex® map).
- **Printing:** Print the Deeplex® map along with detailed results (see section 3.4.2.). Go to the Dashboard or double-click on the sample and then choose “Print”. One sample only.
- **Map:** For easy visualization and comparison of sample results, select at least two samples from the Dashboard and choose “Map”.
- **Share:** Share only the selected samples with a colleague or the Deeplex support by providing the corresponding email address. The recipient will be able to see the results, but will not be able to save, print or share them. To revoke sharing go to the sample view and click “Revoke sharing”.

3.5. Deeplex® Web Application support

3.5.1. Ticketing system

A ticketing system is available if you encounter any issues with the Deeplex® Web Application. To use it, go to Tickets > New, fill in the form and click “Create”. Our support team at GenoScreen will contact you quickly to resolve the issue.

For additional help, email us at deplexsupport@genoscreen.com or contact us via the ticketing system.

3.5.2. Notifications

Notifications from GenoScreen can be found in the “Messages” tab of the Deeplex® Web Application. We use these notifications to pass on information about changes/improvements made on the Deeplex® Myc-TB assay.

4. Characteristics and performances

Performances stated in the following sections were evaluated under a 3% detection threshold for minority populations. On cultured isolates and clinical samples, the background sequencing noise is generally less than 2%. The detection threshold for minority populations is set at 3% for safety. However, this threshold can even be at 1% under position-specific read depth conditions.

4.1. Limit of detection

Although we recommend a Maxwell-based DNA extraction protocol (see section 2.3), the use of the Deeplex® Myc-TB kit does not depend on a specific DNA extraction method. Therefore, the assay's limit of detection was quantitatively estimated as the fraction of detectable sequence variants in 4 replicated analyses using serially diluted purified, pre-quantified genomic DNA from three well-characterized *M. tuberculosis* strains, and a mixture of two strains at a 5-95% ratio. All (near-)fixed of the 36 resistance variants tested were detectable with 10^4 and 10^3 genomes, and 83.3% (30/36) with 10^2 genomes (complete variant profiles obtained for 13/16 tests)^[11]. For 16 resistance variants tested at 5% frequency, the detected fractions were 100% with 10^4 genomes, and 81.3% (13/16; with complete minor variant profiles in 3 out of 4 tests) and 43.8% (7/16; none with a complete minor variant profile) with 10^3 and 10^2 genomes, respectively. Fixed and minority variants were not detected with 10 genomes only. Based on *hsp65* sequence best match analysis, all samples were identified as *M. tuberculosis* at 10^4 and 10^3 genomes, 13/16 samples (81.2%) at 10^2 genomes while none were identified with 10 genomes. Likewise, all three independent replicated analyses of 10^2 , 10^3 and 10^4 genomes of *M. intracellulare* resulted in the expected 100% identification based on *hsp65* best match analysis, while two of three replicates with 10 genomes generated this expected result^[11].

The rate of successful Deeplex® Myc-TB sequence analysis on clinical specimens (sputum) was investigated versus microscopic grade on a set of 1494 direct sputum samples from a nationwide survey conducted in the Democratic Republic of Congo. To note, this clinical specimen set represented especially challenging testing conditions, as yield of culture from CPC-stored sputum largely suffered from transport delays, whereas ethanol-preserved sputum samples were kept at room temperature for subsequent DNA extraction using the recommended Maxwell®-based DNA extraction protocol.

Of the 1143 sputum samples with available microscopic examination data, mean pan-target read depth exceeded 1000x for samples graded 1+, 2+ and 3+ although, as expected, the dispersion towards lower values was inversely correlated with microscopic grading. Broadly similar read depths were also observed for samples that were graded negative (n=16 only, as per the standard survey design normally enrolling smear-positive TB patients only) or without reported grading (n=351). Despite the indeterminate bacterial loads in one third of the 1494 samples, MTBC was identified in 1258 (84.2%) of them. Of the 19,422 expected phenotypes in the 1494 samples, 73.5% (14,277) were automatically predicted (80.7%-82.4% for the four first-line drugs), based on detected resistance mutations, or absence of both resistance and uncharacterized mutations with minimal 5x read depth over $\geq 95\%$ of the reference targets. Uncharacterized mutations were detected for an additional 5.9% (n=1137), and 625 additional susceptible phenotypes (3.2%) could be predicted after verification of effective minimal coverage of all resistance positions in the corresponding target. When distinguished by microscopic grade, the proportion of predicted phenotypes in samples without microscopy results (81.1%) was actually higher than for samples that were graded 0+ (67.8%), 1+ (69.4%), 2+ (69.5%) and 3+ (76.3%).

Importantly, as the above conditions were very challenging, rates of successful Deeplex® Myc-TB sequence analysis tested in routine condition on clinical specimens are even higher. Typically, results can be obtained at least on microscopy positive samples, expected to correspond to an amount of extracted DNA exceeding the limit of detection of 100-1000 mycobacterial genomes/test (with at least 200 μ L of clinical specimen used for extraction). Although results can also be obtained on a number of microscopy-negative samples, the performances on such samples cannot be ensured.



Note: The threshold for minority variant calling is raised to 10% frequency for known, pre-identified noisy positions (Table3).

Loci	Genomic position
<i>rrs1</i>	1472692, 1472707, 1472714, 1472750, 1472751, 1472752, 1472755, 1472790, 1472895, 1473079, 1473094, 1473123, 1473246, 1473247, 1473329, 1473316, 1473384
<i>rrs2</i>	1471848, 1471851, 1471853, 1472150, 1472172, 1472225, 1472251, 1472282, 1472337, 1472338, 1472344, 1472358, 1472359, 1472362, 1472379, 1472382, 1472400, 1472416, 1472422, 1472494, 1472496, 1472498
<i>rrl3</i>	1475956, 1476131, 1476164, 1476179, 1476251, 1476311, 1476357, 1476359, 1476381, 1476466, 1476471, 1476506, 1476529
<i>ethA</i>	4326764
<i>gyrB</i>	6933, 6934
<i>gidB</i>	4407422
<i>tlyA</i>	1917933
<i>rpoB2</i>	760294
<i>rpoB1</i>	761152
<i>fabG1</i>	1673560, 1673561, 1673562

Table 3. List of noisy positions where the threshold is raised to 10%

4.2. Repeatability

Repeatability was measured by using the same experimental conditions (reagent batches, operator, instruments) through different experiments on control samples. On different kit productions, controls were validated for correct and identical *hsp65*-based identification, spoligotypes, SNP profiles, and for target coverage depth and breadth resulting in 3+ grades of sequencing results. Validation is made on the basis of reference samples with 1000 genomes.

4.3. Reproducibility

Reproducibility was measured through experiments realised by three different operators, using the same experimental set-up (reagent batches, instruments). For reference samples, correct and identical *hsp65*-based identification, spoligotypes, SNP profiles were obtained, and target coverage depth and breadth resulted in 3+ grades of sequencing results.

4.4. Analytical specificity

Specificity was evaluated by using mixtures of MTB DNA and a mix of several non-mycobacterial DNAs. Non-mycobacterial DNAs were extracted from:

- *Actinomyces odontolyticus*
- *Bacillus subtilis*
- *Clostridium difficile*
- *Geobacter bemidjiensis*
- *Escherichia coli*
- *Koribacter versatilis*
- *Marinobacter similis*
- *Methanobrevibacter smithii*
- *Haloferax volcanii*
- *Novosphingobium aromaticivorans*
- *Pedobacter heparinus*
- *Propionibacterium acnes*
- *Pseudomonas putida*
- *Roseobacter denitrificans*
- *Staphylococcus argenteus*
- *Streptococcus gallolyticus*
- *Sulfurimonas denitrificans*

Regardless of the ratio of mycobacterial DNA/non-mycobacterial DNA in the sample, the presence of non-mycobacterial DNA in the sample did not change the sensitivity of the kit. Different ratios were tested from 10,000 genomes of the non-mycobacterial DNA mixture and a 10 genomes of MTB DNA to 10 genomes of the non-mycobacterial DNA mixture and a 10,000 genomes of MTB DNA. Non-specific amplification, if occurring, had no impact on the detection and prediction results.

4.5. Stability study

Storage, transport and in-use stability, were evaluated by comparing control validation, *hsp65*-based identification, spoligotypes, target coverage depth and breadth, SNP detection and frequency in different conditions.

The kit is stable at least:

- For 1 year stored at -20°C,
- with two freeze/thaw cycles,
- 3 days in dry ice for transport,
- and at room temperature during the day of use.

4.6. Robustness

The robustness is supported by results of reproducibility of the test and stability of the kit. The robustness was qualitatively approached by risk analysis, including minor modifications of the protocol:

- The PCR program was tested with 40 and 35 cycles. Both programs gave similar results.
- Stability at -20°C and room temperature (see section 4.5.).

4.7. Method comparison

4.7.1. Mycobacterial identification vs phenotypic/molecular methods

For extensive testing of mycobacterial species identification, DNA extracts from 370 nontuberculous mycobacterial (NTM) strains were used^[11]. These represented 73 different NTM species/species complexes, identified based on a combination of phenotypic testing, *rpoB* and/or 16S rDNA Sanger sequencing. Multiple strains from the major clinically relevant taxa were purposely included to represent a potential intraspecies diversity. After application of the assay on DNA extracts, the Deeplex® Myc-TB web application was used for automated best-match analysis using *hsp65* as a primary gene target, complemented by *rrs* and *rrl* sequence analysis for some mycobacterial species.

Out of these 370 strains, 292 strains were identified at (sub)species or species complex levels by both reference and Deeplex Myc-TB testing^[11]. Of these, 194 (66.4%) were best-matched at the same taxonomic level by both methods, while 76 (26%) were matched at the same complex level, with a sublevel identification by Deeplex® Myc-TB (n=20; essentially *M. intracellulare* complex strains), or the reference (n=40; essentially *M. avium* complex strains, relatively overrepresented in this dataset, which are not further identified as (*avium* or *hominisuis*) subspecies by Deeplex® Myc-TB), or showing a different subspecies (n=16) across methods. Four of 292 (1.4%) strains had a partial match either because they were best matched between two possible equidistant species (n=1), or detected in a mixture with another species (n=3).

Only 18 (6.2%) strains had taxonomically discordant results between both methods, i.e. without full or partial match as above. These mostly consist of single discordant cases among otherwise partially/fully concordant strains for a species (e.g. *M. ulcerans*, n=1/13; *M. kansasii*, n=1/14), or species rarely involved in infections (e.g. *M. peregrinum*; n=2). To note, for residual isolates with discrepant subspecies within a matching complex (e.g. *M. intracellulare* vs *M. chimaera*), the correctness of the Deeplex® Myc-TB identification was actually often possible or probable. Indeed, in these cases, conflicting or ambiguous identifications were frequently seen between the *rpoB* and 16S rDNA reference probes, with one or the other partially or fully matching the Deeplex® Myc-TB result. This held also true for some of few individual isolates (of otherwise well identified species) that were discordant even at complex level (e.g. *M. kansasii* vs *M. gastri*).

Of the remaining 78 strains out of the 370 analyzed in total, 5 had no identification by either method while the others were identified to (sub)species/complex level by Deeplex® Myc-TB (n=28) or the reference (n=45) only. The latter mostly consisted of strains from species of rare clinical prevalence (including 5 other *M. peregrinum* or *M. septicum* strains), or reflect a minority of cases among strains otherwise partially/fully concordant for a species or complex (e.g. 6/16 *M. chelonae* complex, including 1/11 *M. chelonae* subsp *chelonae*)^[11].

4.7.2. Resistance variant detection versus WGS *in silico*

We evaluated, *in silico*, the ability of Deeplex® Myc-TB to capture 120 anti-tuberculous drug resistance-determining mutations spread across 14 genes, along with their concurrent first- and second-line resistance phenotypes, algorithmically identified in a WGS study by Walker *et al*^[7]. Of these 120 resistance determinants, 106 (88.3%) are included in the Deeplex® Myc-TB targets and variant catalogue^[11], spread across 13 of the above 14 genes, the exception being *rpsA*, a minor target associated with pyrazinamide resistance. With these 106 variants, 644 of 663 (97.1%) resistant phenotypes predicted by WGS in the Walker's training set of 2099 MTBC isolates were retrieved. Likewise, 53 of 58 resistance-determining mutations from the training set that were retrieved in the Walker's validation set of 1552 isolates were captured by Deeplex® Myc-TB, enabling the prediction of 1199 out of 1207 (99.3%) concurrent resistant phenotypes in this dataset^[11].

4.7.3. Phenotype prediction on isolates versus WGS and phenotypic testing

We compared the ability of both Deeplex® Myc-TB and Illumina-based WGS analysis to detect variants in DNA extracts from 429 MTBC strains. Of the 2403 variants identified in the Deeplex® Myc-TB targets by either method, 2373 (98.8%; 2293 SNPs and all 80 indels), including 797 (99.9%) resistance variants, were detected by both Deeplex® and a WGS pipeline under low frequency mode. The remaining 30 (1.2%) SNPs were all minority variants mostly with frequencies of ~3-10% only identified by targeted deep sequencing, including one mutation associated with resistance (to ethambutol)^[11].

Deeplex® Myc-TB drug-susceptibility predictions based on these 2403 variants were compared to pDST results. In this set, 268 isolates were phenotypically resistant to at least one drug including 156 MDR and 6 extensively drug resistant isolates, resulting in 664 resistant and 1705 susceptible phenotypes. Of these 2369 phenotypes, 2184 (92.1%) were predicted by Deeplex® Myc-TB with a mean sensitivity of 95.6% and a mean specificity of 97.4%. The remaining 185 phenotypes (7.9%) could not be predicted due to the presence of mutations uncharacterized in the variant database^[11].

The proportion of resistant phenotypes accurately predicted as resistant by Deeplex® Myc-TB was above 90% (90.7% for streptomycin-100% for amikacin) for most individual drugs, except for pyrazinamide (85.7%), kanamycin and capreomycin (75%, but reflecting 4 isolates only in both cases). For the MDR-defining compounds rifampicin and isoniazid, 159/160 (99.4%) and 176/179 (98.3%) isolates with resistance variants were correctly classified resistant, accounting for 98.1% and 94.1% of all rifampicin- and isoniazid-resistant isolates, respectively. For pyrazinamide, although all (42/42, 100%) isolates containing resistance variants for this drug were correctly predicted as resistant, these accounted for 79.2% of all isolates with phenotypic resistance to pyrazinamide. Of note, 7 minority resistance variants (with frequencies of 21.1 to 77%), successfully predicted resistance to pyrazinamide (n=3), fluoroquinolones, kanamycin, capreomycin and ethionamide (n=1 each).

Of the 601 resistant phenotypes with Deeplex® Myc-TB predictions (i.e. not uncharacterized), only 28 (4.7%) were predicted susceptible due the absence of resistance-associated mutation in the Deeplex® targets. In these phenotypes, the possible presence of high confidence resistance-associated mutations was searched in 13 secondary resistance-associated gene targets, outside the Deeplex® Myc-TB target regions. WGS analysis detected a high confidence resistance mutation in these regions for only one of these phenotypes (ethionamide resistant; L203L in *fabG1*). Likewise, *fabG1* L203L was the sole established resistance-associated mutation (for an isoniazid resistant phenotype) detected by WGS outside of the assay's targets in 63 phenotypes uncharacterized by Deeplex® Myc-TB^[11].

Of the 1583 susceptible phenotypes with prediction, only 41 (2.6%) were discordantly predicted as resistant. They all involved ethambutol and *embB* mutations, ethionamide and *ethA* frameshift-causing indels (mechanistically expected to cause ethionamide resistance), or known low-level isoniazid (*inhA* S94A, *ahpC* G-48A), rifampicin (*rpoB* L452P, H445N and D435Y) or streptomycin (*gidB* A138V) resistance mutations, all notoriously associated with poor phenotypic reproducibility^[11].

4.7.4. Deeplex[®] Myc-TB on clinical specimens vs WGS on culture

We compared variant detection and phenotype predictions based on available Deeplex Myc-TB sequencing data directly obtained from 109 clinical specimens from a nationwide survey conducted in Djibouti, versus analysis of WGS data obtained after culturing. Overall, 693 of 752 total variants (92.2%) were detected by both Deeplex Myc-TB and our WGS pipeline used under low frequency detection mode. Of 59 (7.8%) discordances, all but one (a fixed *pncA* SNP not detected by Deeplex[®], albeit on a well-covered position) were minority variants undetected by this WGS pipeline, consistent with the average coverage depth of 3921x at Deeplex[®] targets by Deeplex[®] Myc-TB versus 57x by WGS^[11].

Deeplex[®] Myc-TB phenotype predictions were matched to those obtained with WGS data independently analyzed with the WGS-based analysis tools PhyResSE, Mykrobe and MTBSeq (used as a primary reference in the comparison). With the 1150 predicted phenotypes by Deeplex[®] Myc-TB (155 resistant, 995 susceptible), the mean sensitivity and specificity versus available MTBSeq predictions were 93.5% and 98.5%, respectively. One hundred and eight additional phenotypes (8.6%) were not predicted by Deeplex[®] Myc-TB due to the presence of uncharacterized mutations (not accounted for by PhyResSE, Mykrobe and MTBSeq). Agreement on resistance prediction was 100% for all applicable drugs, except for rifampicin (93.5%) and pyrazinamide (71.4%). The rifampicin score resulted from probable differences between primary samples directly tested by Deeplex[®] Myc-TB and cultures used for WGS analysis. Indeed, two resistance predictions by MTBSeq (also by PhyResSE and Mykrobe) reflected detection of two minor resistance-associated variants in *rpoB* that were undetected by Deeplex[®] Myc-TB despite high coverage depths at these positions, suggesting genotypic heterogeneity/contamination introduced/amplified after sputum processing, during subsequent culturing or WGS. For pyrazinamide, the lower sensitivity of Deeplex[®] Myc-TB versus MTBSeq mostly resulted from different interpretation of some sequence variants or different regions interrogated in "*M. canettii*" isolates, which are naturally resistant to pyrazinamide and locally prevalent in and highly restricted to Djibouti. Deeplex[®] Myc-TB did identify 7 *M. canettii*-containing samples based on the phylogenetic SNP *pncA* A46A, but did not (at the time) explicitly predict resistance to pyrazinamide in contrast to MTBSeq based on *panD* M117T (and Mykrobe, based on *pncA* A46A)^[11].

Conversely, 15 of the 995 (1.5%) phenotypes predicted as susceptible by MTBSeq (excluding uncharacterized phenotypes by Deeplex[®] Myc-TB) were identified as resistant by Deeplex[®] Myc-TB. Of these, 10 (66.6%) discordances were due to minority resistance-associated variants at 3.3-12.8% only detected in sputa by Deeplex[®] Myc-TB. Importantly, 8 out of these 10 minority variants co-occurred with one or more minority resistance and/or phylogenetic variants within an individual sputum. This further supports true-positive variant calls reflecting genuine mixed strains and/or combined heteroresistance detected by targeted deep sequencing, missed by WGS analysis due to lower coverage depth or potential culture bias. The five remaining discordant resistance predictions resulted from divergent variant interpretation, involving one *ethA* frameshift-causing indel (mechanistically expected to cause ethionamide resistance, see above), two debated *embB* mutations and a *gidB* mutation (n=2) associated with low-level streptomycin resistance, which were associated with resistance by both Deeplex[®] Myc-TB and PhyResSE and/or Mykrobe, but not by MTBSeq^[12]. Mean sensitivity and specificity of the phenotypes predicted by Deeplex[®] Myc-TB versus available PhyResSE and Mykrobe predictions was 98.5/93.1% and 97.2/95.3%, respectively^[11].

5. Product use restriction/warranty

In their current format, Deeplex® Myc-TB Kit components are designed, distributed and sold as RUO kit and are intended to be used as a research use only.

This GenoScreen product is shipped with documentation stating specifications, performances and other technical information. GenoScreen warrants meeting the stated specifications.

GenoScreen does not warrant against damages or defects arising in shipping and handling (transport insurance for customers excluded), or out of improper or abnormal use of this product; against defects in products or components not manufactured by GenoScreen, or against damages resulting from such non-GenoScreen components or products. In the event products fail to perform as warranted due to any reasons other than these ones GenoScreen will replace them free of charge.

The purchaser must verify the appropriateness of the product for its particular use. We reserve the right to modify any product to improve its performance and design.

If a GenoScreen product does not meet your expectations, or if you have any suggestion or question, please feel free to contact us.

6. Symbols

The device has the following information, in the form of symbols in accordance with the standard ISO EN15223 and ISO EN 980.

Symbols used	Meaning	Symbols used	Meaning	Symbols used	Meaning
	CE marking		Manufacturer		Product reference
	Expiration date		Sufficient for		Batch number
	Storage temperature		Instruction manual		

7. References

1. World Health Organisation, Global tuberculosis report. 2021.
2. Dai J, Chen Y, Lauzardo M. Web-accessible database of hsp65 sequences from Mycobacterium reference strains. *J Clin Microbiol* 2011; **49**: 2296–303.
3. Demay C, Liens B, Burguière T, *et al.* SITVITWEB - A publicly available international multimarker database for studying Mycobacterium tuberculosis genetic diversity and molecular epidemiology. *Infect Genet Evol* 2012; **12**: 755–66.
4. Feuerriegel S, Schleusener V, Beckert P, *et al.* PhyResSE: A web tool delineating Mycobacterium tuberculosis antibiotic resistance and lineage from whole-genome sequencing data. *J Clin Microbiol* 2015; **53**: 1908–14.
5. Coll F, McNerney R, Guerra-Assunção JA, *et al.* A robust SNP barcode for typing Mycobacterium tuberculosis complex strains. *Nat Commun* 2014; **5**: 4–8.
6. Camacho C, Coulouris G, Avagyan V, *et al.* BLAST+: Architecture and applications. *BMC Bioinformatics* 2009; **10**: 1–9.
7. Walker TM, Kohl TA, Omar S V., *et al.* Whole-genome sequencing for prediction of Mycobacterium tuberculosis drug susceptibility and resistance: A retrospective cohort study. *Lancet Infect Dis* 2015; **15**: 1193–202.
8. Starks AM, Aviles E, Cirillo DM, *et al.* Collaborative Effort for a Centralized Worldwide Tuberculosis Relational Sequencing Data Platform. *Clin Infect Dis* 2015; **61**: S141–6.
9. Miotto P, Cabibbe AM, Feuerriegel S, *et al.* Mycobacterium tuberculosis Pyrazinamide Resistance Determinants: a. *MBio* 2014; **5**: 1–10.
10. Miotto P, Tessema B, Tagliani E, *et al.* A standardised method for interpreting the association between mutations and phenotypic drug resistance in Mycobacterium tuberculosis. *Eur Respir J* 2017; **50**(6):1701354
11. Jouet A, Gaudin C, Badalato N, *et al.* Deep amplicon sequencing for culture-free prediction of susceptibility or resistance to 13 anti-tuberculous drugs. *Eur Respir J.* 2021; **57**(3):2002338
12. Catalogue of mutations in Mycobacterium tuberculosis complex and their association with drug resistance” (WHO, 2021)

8. Appendices

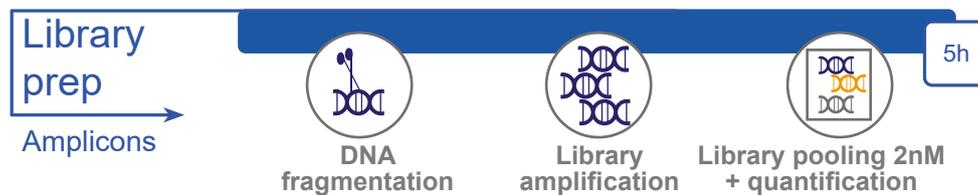
Appendix I:

List of mycobacteria included in the *hsp65* database of Deeplex® Myc-TB. Species identification of highlighted mycobacteria was confirmed by an independent reference.

<i>M. abscessus</i> (subsp <i>abscessus</i> , <i>bolletii</i>)	<i>M. chelonae</i>	<i>M. haemophilum</i>	<i>M. montefiorensis</i>	<i>M. scrofulaceum</i>
<i>M. africanum</i>	<i>M. chimaera</i>	<i>M. hassiacum</i>	<i>M. moriokaense</i>	<i>M. sediminis</i>
<i>M. agri</i>	<i>M. chitae</i>	<i>M. heckeshornense</i>	<i>M. mucogenicum</i>	<i>M. senegalense</i>
<i>M. aichiense</i>	<i>M. chlorophenolicum</i>	<i>M. heidelbergense</i>	<i>M. murale</i>	<i>M. senuense</i>
<i>M. algericum</i>	<i>M. chubuense</i>	<i>M. hiberniae</i>	<i>M. nebraskense</i>	<i>M. seoulense</i>
<i>M. alvei</i>	<i>M. colombiense</i>	<i>M. hodleri</i>	<i>M. neoaurum</i>	<i>M. septicum</i>
<i>M. aromaticivorans</i>	<i>M. conceptionense</i>	<i>M. holsaticum</i>	<i>M. neworleansense</i>	<i>M. setense</i>
<i>M. arosiense</i>	<i>M. confluentis</i>	<i>M. houstonense</i>	<i>M. onchromogenicum</i>	<i>M. sherrisii</i>
<i>M. arupense</i>	<i>M. conspicuum</i>	<i>M. immunogenum</i>	<i>M. noviomagense</i>	<i>M. shimoidei</i>
<i>M. asiaticum</i>	<i>M. cookii</i>	<i>M. insubricum</i>	<i>M. novocastrense</i>	<i>M. shinjukuense</i>
<i>M. aubagnense</i>	<i>M. cosmeticum</i>	<i>M. interjectum</i>	<i>M. obuense</i>	<i>M. shottsii</i>
<i>M. aurum</i>	<i>M. crocinum</i>	<i>M. intermedium</i>	<i>M. pallens</i>	<i>M. simiae</i>
<i>M. austroafricanum</i>	<i>M. diernhoferi</i>	<i>M. intracellulare</i>	<i>M. palustre</i>	<i>M. smegmatis</i>
<i>M. avium</i> (subsp <i>avium</i> , <i>paratuberculosis</i> , <i>silvaticum</i>)	<i>M. doricum</i>	<i>M. kansasii</i>	<i>M. paraffinicum</i>	<i>M. sphagni</i>
<i>M. boenickei</i>	<i>M. duvalii</i>	<i>M. komossense</i>	<i>M. parafortuitum</i>	<i>M. stomatepieae</i>
<i>M. bohemicum</i>	<i>M. elephantis</i>	<i>M. kubicae</i>	<i>M. parascrofulaceum</i>	<i>M. szulgai</i>
<i>M. botniense</i>	<i>M. europaeum</i>	<i>M. kumamotoense</i>	<i>M. paraseoulense</i>	<i>M. terrae</i>
<i>M. bouchedurhonense</i>	<i>M. fallax</i>	<i>M. kyorinense</i>	<i>M. parmense</i>	<i>M. thermoresistibile</i>
<i>M. bourgelatii</i>	<i>M. farcinogenes</i>	<i>M. lacus</i>	<i>M. peregrinum</i>	<i>M. timonense</i>
<i>M. bovis</i>	<i>M. flavescens</i>	<i>M. lentiflavum</i>	<i>M. phlei</i>	<i>M. tokaiense</i>
<i>M. bovis</i> BCG	<i>M. florentinum</i>	<i>M. lepraemurium</i>	<i>M. phocaicum</i>	<i>M. triplex</i>
<i>M. branderi</i>	<i>M. fluoranthenvivorans</i>	<i>M. leprae</i>	<i>M. porcinum</i>	<i>M. triviale</i>
<i>M. brisbanense</i>	<i>M. fortuitum</i> (subsp <i>acetamidolyticum</i> , <i>fortuitum</i>)	<i>M. llatzerense</i>	<i>M. poriferae</i>	<i>M. tuberculosis complex</i>
<i>M. brumae</i>	<i>M. fragae</i>	<i>M. madagascariense</i>	<i>M. pseudoshottsii</i>	<i>M. tusciae</i>
<i>M. canariensis</i>	<i>M. frederiksbergense</i>	<i>M. mageritense</i>	<i>M. psychrotolerans</i>	<i>M. ulcerans</i>
<i>M. canettii</i> (CIPT 140010059, STB-D, E, G, H, I, J, K, L)	<i>M. gadium</i>	<i>M. malmoense</i>	<i>M. pulveris</i>	<i>M. vaccae</i>
<i>M. caprae</i>	<i>M. gastris</i>	<i>M. mantanii</i>	<i>M. pyrenivorans</i>	<i>M. vanbaalenii</i>
<i>M. celatum</i>	<i>M. genavense</i>	<i>M. marinum</i>	<i>M. rhodesiae</i>	<i>M. vulneris</i>
	<i>M. gilvum</i>	<i>M. marseillense</i>	<i>M. riyadhense</i>	<i>M. wolinskyi</i>
	<i>M. goodii</i>	<i>M. massiliense</i>	<i>M. rufum</i>	<i>M. xenopi</i>
	<i>M. gordoniae</i>	<i>M. microti</i>	<i>M. rutilum</i>	<i>M. yongonense</i>
		<i>M. monacense</i>	<i>M. salmoniphilum</i>	
			<i>M. saskatchewanense</i>	

Appendix II:

Library preparation for sequencing



The following steps are intended to use with Nextera® XT DNA Library Preparation kit. Please refer to Appendix II if using Nextera® XT DNA Preparation Kit. All Illumina® protocols have to be downloaded at support.illumina.com. Follow the manufacturer instructions (Nextera® XT DNA Library Prep Kit Reference Guide, please use the most current reference guide), with the following adaptations and recommendations.

1. Use 5 µl of input DNA at 0.2 ng/µl (i.e., a total of 1 ng) for each library.
2. Choose a different set of index primers between consecutive sequencing runs to avoid the risk of spill over between them.
3. Use the Index adapter pooling guide to verify that the selection of index primers is correct (please use the most current reference guide) to verify that the selection of index primers is correct and create the sample sheet with pseudonymised sample names (using the Local Run Manager v3, please use the most current reference guide).
4. At “Clean-Up Libraries”. Pay attention to the following steps:
 - a. Step 4: Use 30 µl Agencourt AMPure XP® (recommended for libraries with largest amplicons > 500 bp).
 - b. Step 13: After removal of the plate from the magnetic stand, add 26.5 µl of RSB (resuspension buffer), instead of 52.5 µl.
 - c. Step 17: Transfer 25 µl of the supernatant to a new plate, instead of 50 µl.
5. **Do not perform the “Check Library Quality” and “Normalize Libraries” steps included in the Illumina® protocol.** In “Dilute Libraries to the Starting Concentration”, steps are replaced by the following procedure:



Note: for all libraries to be included in one equimolar pool, library quantification should be performed in parallel, using the same method.

- a. Quantify each library as described in step 2.5.2 above.
- b. Multiply the obtained mass concentrations (in ng/µl) by 3 to convert them to molar concentrations (nM).
- c. For each sample, calculate the volume of library needed to obtain a final concentration of 2 nM in a final solution of 10 µl (but do not adjust the volume to 10 µl).



Note: for libraries with a concentration equal to or lower than 2 nM, use 10 µl of undiluted library.

- d. Pool calculated volumes for the individual libraries in a single tube
- e. To this library pool, add the volume of Tris HCl 10 mM + 0.1 % Tween 20 required to reach a total volume equal to the number of samples x 10 µl (e.g., 480 µl for 48 tests).
- f. Store the library pool on ice.

II SAFE STOPPING POINT, if you do not plan to proceed immediately to the next step, libraries can be stored at -20°C for up to one week. After thawing, libraries have to be homogenised and quantified as described in step 2.5.2.

Preparation of library pools for sequencing with MiniSeq®.

Consumables

- MiniSeq® High Output Reagent Kit (300-cycles), FC-420-1003 or MiniSeq® Mid Output Kit (300-cycles), FC-420-1004.

Protocol

Follow the “Protocol A: Standard Normalization Method” (choosing a final concentration of 1.5pM of denaturated libraries), “Denature and dilute PhiX Control” (choosing 1% of spike-in PhiX) steps as detailed in the Illumina® protocol entitled “Denature and dilute libraries guide” for MiniSeq® system (please use the most current reference guide). Sequencing run can then be performed by following instructions in the “MiniSeq® System Guide” (please use the most current reference guide).

Preparation of library pools for sequencing with NextSeq® 500/550.

Consumables

- NextSeq® 550 Mid Output Kit (300 cycles), 20024905.

Protocol

Follow the “Protocol A : Standard Normalization Method” (choosing a final concentration of 1.5pM of denaturated libraries), “Denature and dilute PhiX control” (choosing 1% of spike-in PhiX) steps as detailed in the Illumina® protocol entitled “Denature and dilute libraries guide” for NextSeq® 550 systems (please use the most current reference guide). Sequencing run can then be performed, by following instructions in the “NextSeq® 550 System Guide” (please use the most current reference guide).

Preparation of library pools for sequencing with iSeq®100.

Consumables

- iSeq®100 i1 Reagent V2, 20031371.

Protocol

Follow the “Prepare the Flow Cell and Libraries” choosing a final concentration of 100 pM of denaturated libraries for Illumina® DNA Prep libraries or choosing a final concentration of 150 pM of denaturated library if using Nextera® XT DNA preparation kit and then perform the sequencing run by following instructions in the “iSeq®100 System Guide” (please use the most current reference guide).

Appendix III:

Deeplex® Myc-TB glossary

- **Co-infection:** Coexistence in a patient sample of both a Mycobacterium tuberculosis complex strain and a non-tuberculous mycobacteria strain. Such co-existence is indicated by the detection in the sequencing data from a sample of two different hsp65 marker sequences, each of which best match a hsp65 reference sequence from a different mycobacterial species, as per the hsp65 reference database (Dai et al, 2011). The proportion of each detected hsp65 sequence is given. Example: M. tuberculosis complex 90%/M. intracellulare 10%
- **Mixed infection:** Coexistence in a sample of at least two strains of the M. tuberculosis complex (MTBC). Mixed infections with strains from different MTBC (sub-)lineages can be detected by the presence of mixed nucleotide calls at one or more phylogenetic SNP positions that are characteristic of MTBC (sub-)lineages. Phylogenetic (sub-)lineages are named according to a standardized numerical nomenclature (Coll et al, 2014; Merker et al., 2020?). The proportion of each (sub)lineage detected is given, based on the proportion of the different nucleotide variants detected on the phylogenetic SNP positions considered. Example: Lineage 1 (95%), Lineage 2 (5%).
- **Fixed variant:** Variant present at 97-100% frequency in the sequencing data of a sample, indicating that the variant is borne by the (almost) entire bacterial population in the sample.
- **Unfixed variant:** Variant below 97% in the sequencing data of a sample, indicating that the variant is borne by a fraction of the bacterial population in the sample.
- **Minority variant:** Variant below 50% frequency in the sequencing data of a sample, indicating that the variant is borne by a minority of the bacterial population in the sample
- **Heteroresistance:** Simultaneous presence within a patient sample of drug susceptible and drug resistant MTBC strain subpopulations. Heteroresistance is observed when a drug resistance-associated variants is detected at a frequency below 97% (i.e. when it is unfixed), indicating a mixture of a resistance-associated mutation and wild-type sequence at the corresponding target position.
- **Clonal interference:** Presence within a sample of several drug resistant MTBC strain subpopulations, competing for fixation. Clonal interference is observed when multiple resistance-associated variants are detected at different frequencies for a same drug. Example: pncA delG 5%, pncA V139L 35%, pncA A134V 44%.

9. Legal notice

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