LABORATORY MANUAL

CENTRAL MYCOBACTERIOLOGY LABORATORY

Version 3.0: 13 MAY 2021

Protocol Number: NC-007 (B-Pa-L)/ **ZeNix**
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UCL Central Mycobacteriology Laboratory Sign Off

All relevant laboratory staff must sign that they have read and understood this laboratory manual and agree to follow the procedures as written:

Name	Signature	Date read

Version History:

Version Number/ date	Change
Version 1.0/ 27 Sep 2018	Original
Version 2.0/ 21 Jan 2019	3. Introduction – amendment to priority of isolates to ship to UCL. 4.1.2.2: Section A point 1- Sterile saline added; 4.1.2.2: Section B point 1- Step 5 changed to Step 4. 4.2.3.2: Section C point 1- formatting correction. 4.2.3.2: Section B and Section C- distilled water changed with running water. 4.3.1: Table 3- reference to section 4.3.3.4 added. 4.3.4: Negative control- sterile saline added. 4.4.3.1: Step 4- updated to include 1:5 dilution step. 4.4.3.1: Step 9: sub-culture of MGIT cultures added. 4.4.4: Quality Control Section- updated to include procedure for inoculum preparation from liquid culture and Solid culture. Solid culture section updated to specify culture requirements. 4.5.3: Process sections updated to reflect UCL workflow 4.5.3.2: new section added: "Estimation of DNA integrity and concentration using the Agilent 4200 TapeStation System" 4.6.3.7: Expansion on processing DSTs with error (X200 and X400) results. 4.6.3.8: SIRE resistant results clarified for SimpliciTB (NC008) patients. 4.7.3.2: Preparation of Linezolid stock solution DMSO volume corrected from 1.780ml to 3.56ml. 4.7.3.2: Table 9- Pretomanid stock solution calculations updated for 2ml total volume; 4.7.3.6: Inoculation of tubes containing test drugs section- updated to exclude the number of MGIT tubes. 4.7.3.8: Point 1- updated to simplify the process. 4.7.3.8: Point 3- time frame entry added. 4.7.3.9: Point 4- Appendix C: MIC LRF to be completed from the worklist or the interpretation report. 4.8.1 and 4.8.3: updated to reflect UCL workflow 4.8.5: Two references added (point 6 and point 7) Appendix A, B, C, D and E: patient number/patient ID fields updated to Subject ID in-line with the laboratory manual terminology. Appendix A, B, C and D: Subject ID field updated to include the correct number of boxes. Appendix A: Date of test field changed to DNA extraction Date; Appendix A: Results Date field changed to Hybridisation Date; Appendix A: Date Reported field removed, these results are not reported. Appendix F: Acceptable MIC ranges for the H37Rv reference strain quality control updated.
Version 3.0/ 12 May 2021	Title Page: Updated current protocol versions for ZeNix and SimpliciTB, version number for the laboratory manual included on the cover. New TB Alliance Logo in header throughout. Addition of reading log page consistent with the Local Laboratory Manual 2. Contact details: Table removed and contact details only for the CCM laboratory listed. Additional contact details for courier and laboratories performing WGS moved to Table 17 (4.8 SOP 8 Whole Genome Sequencing). Laboratory Manager changed from Julio Canseco to Priya Solanki. 3. Introduction: Table 1, corrections made regarding post baseline isolates sent to UCL

	4.1.1 Purpose: Overview of the processing workflow for received isolates added including reference to UCL Chain of Custody SOP. Table 2 updated with changes to DNA storage requirements– not necessary to store all DNA long term if whole genome sequence (WGS) data available. 4.1.2.2: Information on storage of isolates consolidated and reference made to updated Appendix H. Details added on recording storage of 'working cultures' in updated Appendix I. 4.2.5: Recording of AFB results from resistant results onto LRFs clarified 4.3.1: Table 3 – Hain CM updated to Hain Mycobacterium CM; reference made to rejection log for samples that are not MTBC 4.3.5: Reference added to also complete Appendix I with Hain results 4.4: Use of consistent wording for referred solid cultures (Site LJ slopes) and UCL original MGIT (first MGIT subculture) here and throughout 4.4.2/4.4.3 Step Five: Work up of positive MGIT tube made optional, as this is not applicable to all MGIT subcultures (only if suspicion of contamination). Note added about typical 'breadcrumb' appearance. 4.4.3 Step Three and Step Four: Blood agar result from the organism suspension used for first subculture to be reported on Appendix I. Original MGIT tube number also to be recorded on Appendix I. 4.4.3. Step Six: BAC 'not done' removed from diagram; additional information added to workflow on when to proceed with NaOH decontamination of contaminated cultures. Section of 'false positive' cultures removed as only relevant to sputum MGIT cultures. 4.4.3. Step Seven: additional information included on sample storage 4.4.3. Step Eight: modifications to the removal of negative MGIT tube sections to only include details relevant to MGIT subculture, and add actions required if subcultures are negative. 4.4.3. Step Nine: further options for preparing a uniform bacterial suspension for subculture added 4.4.4. Quality Control – clarification to document BA from a failed QC directly onto the MGIT print out 4.5: SOP 5 DNA extraction. Clarification the DNA extracted on all baselines. Addition of yeast tRNA as a co-precipitant 4.5.3.1: Information added regarding new QC form Appendix G: DNA extraction and quantification reagent worksheet, to record lot numbers and expiry/preparation dates of DNA extraction reagents; clarification to preparation of extraction reagents; additional information added for yeast tRNA, ultrapure water, and Tris-EDTA buffer; Process: additional details added to the DNA extraction procedure to improve yield. Protein Kinase K amount increased from 10 to 20 mg/ml 4.5.3.2 Estimation of DNA concentration: more information added on the purpose of different quantification methods; reference to updated Appendix E (DNA quantification and Storage Worksheet) added; note about solvent used for blank run in Nanodrop added; additional information added about the Qubit – including the use of the Broad Range (BR) kit, Table 4: Qubit quantification ranges, clarifications to the handling of qubit reagents, sample volume, calculations for converting to the final DNA concentration; Tapestation – referenced added to Appendix E which now contains space to record TapeStation data; details on storage of TapeStation electronic data added 4.5.3.3. Storage of DNA. Section updated – no longer a requirement to store DNA from all isolates if WGS is available. DNA stored short term (before sending for WGS), and long term in cases where DNA remaining after WGS. Storage information for DNA added in text and to revised Appendix E. 4.6.3.2. Table 5 and 6: Note added about additional dilution step required for moxifloxacin 4.6.3.5. Removed section on preparing a BA plate for PZA tubes, as already described in section 4.6.3.6 (inoculation of tubes containing drug) 4.6.3.7. Sections on X200 and X400 updated to refer to subculture section in MGIT SOP, flag use of TBeXIST for slow growing cultures, and actions to take if case of contamination 4.6.3.8: Information from NTF16 added clarifying when confirmation of resistant results is required in ZeNix and SimpliciTB trials.

	4.6.4: Blood Agar QC requirements added, including a note added to record BA result from a failed QC on unloaded MGIT report. Note added that drug stocks must be prepared in presence of second person. 4.6.6. BA plate and Kinyoun staining reagent QC forms added to Documentation section (GATB Quality Manual Attachments Eii and Eiv) 4.7 SOP 7 Minimum Inhibitory Concentration: Table 10 clarifications to preparation of stock and working drug solutions; Table 8: Moxifloxacin routine testing range extended to include one further lower dilution (0.06-2µg/ml); addition of Table 12 with details of high and low range testing and Table 13 with drug preparation details for high range testing. Reference to specific high and low range concentrations added as appropriate to other sections. 4.7.4: Table 14 inclusion of Moxifloxacin MIC cut off. Note added about Moxifloxacin MIC values of 0.5 or 1µg/ml. 4.7.5. Quality control: Note added that drug stocks must be prepared in presence of second person. Blood Agar QC requirements added 4.7.6: Updated to include TBeXiST User guides, TB eXiST worklists, interpretation reports and plots, and BA plate and Kinyoun staining reagent QC forms (GATB Quality Manual Attachments Eii and Eiv) 4.8. SOP 8 Whole Genome Sequencing: Addition of UCL Pathogen Genome Unit (PGU) as an acceptable laboratory to perform WGS in addition to Public Health England (PHE). Reasons for using additional laboratory included. 4.8.1: Clarifications to study requirements for testing at or after the end of treatment 4.8.2: Table 16 updates to include DNA requirements for both PHE and PGU; clarification on purposes of the different DNA quantification methods moved to DNA Extraction SOP (SOP 5) 4.8.3.2.1: Increase maximum volume for 50 to 60µl 4.8.3.2 Preparation of samples – section included with details for both laboratories; Figure 7 and 8 added with example of each DNA submission form; information on preferred DNA concentrations required added; details on completion of revised Appendix E (DNA Quantification and Storage Worksheet) and use of WGS plate preparation spreadsheet for DNA dilutions added; updates to DNA submission process at PHE; previous Appendix G (BW0303 Instructions on sample submission and receiving results) now added as a reference to Table 20: PHE SOP list instead of being Appendix. 4.8.3.3 Shipment of DNA samples: Information added for PGU. Table 17 updated with contact details for PGU. 4.8.3.4 WGS at PGU – section added with details of sequencing at PGU, including Figure 9 (example of processing report form), Table 18 PGU SOP list, Table 19 Summary of QC steps 4.8.3.5 WGS at PHE – minor changes to details of data retention at PHE; Table 20: PHE SOP list updated to include new SOPs, and the previous Appendix G (see section 4.8.3.2); clarification as to how WGS data is transferred and stored. 4.8.3.6 Data analysis by study bioinformatician: more details included on data transfer to the study bioinformatician and naming conventions. Use of the TBA Sharepoint Myco Characterisation Sharepoint removed, as data can now be stored on the UCL RDS drive to which the TBA Microbiology Director and the study bioinformatician can access. More detail of file storage added and reference added to the UCL WGS Data Storage and Handling SOP Sequence analysis section: updated to include further details of paired analysis report; inclusion of bedaquiline resistance gene SNPs in the pipeline and details included in resistance gene SNP reports; inclusion of upstream regions; capturing lineage information, updates to Table 21 (Data files generated during sequence analysis) to define file types and frequency generated. Phylogenetic tree generated by study, not on ongoing basis. Transfer of Analysis data: updated with clarifications on data storage. Use of the TBA Sharepoint Myco Characterisation Sharepoint removed, as data can now be stored on the UCL Research Data Services drive

	to which the TBA Microbiology Director and the study bioinformatician can access. Appendices: (GATB changed to TBA in all appendices titles and document names). As per Mycobacteriology Laboratory Memorandum across trials #5 (dated 12 July 2018), all appendices included in the Laboratory Manual document are examples only. This is so that they may be updated separately to the manual if required. Changes made concurrent with the release of v3.0 of the Laboratory Manual are outlined below. Appendix A, B, C, D: update to visit schedule to 'weeks' for post treatment visits instead of months, plus typo in 'withdrawal'. Appendix E: divided into 3 sections (Part A, B and C). Updated to include space to document more Qubit information for repeated dilutions, Tapestation data and DNA storage. Study and UCL ID number added. Page 2 – moved to excel format as a local UCL spreadsheet to capture the same information of preparation of DNA dilutions for WGS. Minor changes to format to make more user friendly. Appendix F: update to acceptable range for moxifloxacin in line with Table 15 Appendix G: this is no longer from PHE Guidance document BW0303, which has now been added to Table of local PHE SOPs (Table 20). Appendix G is a new form DNA Extraction and Quantification Log Appendix H: Isolate Storage Log – title amended to Frozen Isolate Storage Log as now for use for frozen stocks only and separated into two parts. Part A: capturing the same information as in pages 1-3 of word version and moved to Excel format to complete electronically Part B – capturing page 4 in word format to be completed by hand, with minor edits to include UCL No, and tick box for the study (NC-007/NC-008). Appendix I: additional information added on the storage and destruction of the routine MGIT and solid cultures, so it is able to act as an inventory of 'working cultures' in place of using Appendix H. General changes made throughout: reference to UCL Central Mycobacteriology Laboratory made consistent, TB Alliance Microbiology Consultant changed to Microbiology Consultant, GATB changed to TBAlliance (except in reference to the GATB Quality Manual). Patient ID changed to Subject ID to be consistent with study terminology. Reference to ZN staining performed at UCL central mycobacteriology Laboratory amended to Kinyoun, as this is the staining method used for detection of AFB.

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1. Abbreviations

AFB	Acid Fast Bacilli
ART	Aerosol Resistant Tips
AST	Antibiotic Susceptibility Testing
ATCC	American Type Culture Collection
B	Bedaquiline
BA/BAC	Blood Agar/Blood Agar Culture
BD	Becton Dickinson
BSC	Biological Safety Cabinet
CTAB	Cetyl trimethylammonium bromide
CL3/BSL3	Containment Level 3/Biosafety Level 3
CQIF	Continuous Quality Improvement Form
CRF	Case Report Form
DIN	DNA Integrity Number
DMSO	Dimethyl Sulfoxide
DST	Drug Susceptibility Testing
E	Ethambutol
EMS	Early Morning Sputum
FLD	First Line Drugs
FQ	Fluoroquinolones
GATB/TBA	Global Alliance for TB Drug Development/TB Alliance
GC	Growth Control
GU	Growth Units
I	Isoniazid
HYB	Hybridization Buffer
IQC	Internal Quality Control
K	Kanamycin
L	Linezolid
LAN	Laboratory Accession Number
LIMS	Laboratory Information Management System
LJ	Lowenstein Jensen
LM	Laboratory Manual
LRF	Laboratory Report Form
M	Moxifloxacin
MF	McFarland
mg	Milligram
MGIT	Mycobacteria Growth Indicator Tube
MIC	Minimum Inhibitory Concentration
Min	Minute(s)
mL	Millilitre
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
NALC	N-Acetyl L-Cysteine
NaOH	Sodium Hydroxide
ng	Nanogram
NTM	Non-Tuberculous Mycobacteria
OADC	Oleic Acid Albumin Dextrose Catalase

OD	Optical Density
Pa	Pretomanid
PANTA	Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim, Azlocillin
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PPE	Personal Protective Equipment
PZA	Pyrazinamide
QC	Quality Control
R	Rifampicin
RDS	Research Data Services
RT	Room Temperature
S	Streptomycin
SIRE	Streptomycin, Isoniazid, Rifampicin, Ethambutol
SLD	Second Line Drugs
SOP	Standard Operating Procedure
STR	Stringent Wash Solution
T	Temperature
TBeXiST	TB eXtended Susceptibility Testing
TE	Tris-EDTA
TIP	Time in Protocol
TTD	Time to Detection
µg	Microgram
UCL	University College London
WGS	Whole Genome Sequencing
WHO	World Health Organisation
Z	Pyrazinamide
ZN	Ziehl-Neelsen

2. Contact Details

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3. Introduction

This manual will be used by the Central Mycobacteriology Laboratory for studies NC-007 (B-Pa-L) (ZeNix) and NC-008 (B-Pa-M-Z) (SimpliciTB), Centre for Clinical Microbiology, University College London (UCL). The manual describes the characterisation tests and procedures performed at UCL; and is to be used in combination with Appendices A to I, the GATB Laboratory Quality Manual, and local UCL SOPs: TBA Clinical Trials: UCL Chain of Custody SOP and TBA Clinical Trials: Data Reporting SOP for UCL Central Mycobacteriology Laboratory.

Sputum samples collected at key timepoints during ZeNix and SimpliciTB at study sites will be cultured at the study local laboratories and MTB isolates shipped to UCL as detailed in Table 1.

Table 1: MTB Isolates tested at UCL

Study	Isolates shipped to UCL	Tests performed
ZeNix (NC 007)	<u>Baseline isolates</u> The first culture that is found to be positive for MTB and non-contaminated for a given participant during Screening to Week 4 period, is the one to be shipped to UCL. <u>Pre-Screening isolates</u> In case all baseline visits listed above are negative or contaminated; an isolate obtained prior to Screening can be shipped. This pre-Screening isolate will be tested as if it were baseline MTB. <u>From suspected treatment failure/relapse cases ("post-baseline isolates")</u> Preferably the first non-contaminated culture positive for MTB at/after **Week 16**.	- Storage of all shipped pure MTBC cultures, as well as the DNAs extracted from these (4.1- SOP 1) - AFB Microscopy to detect presence of AFB organisms in cultures (4.2-SOP 2) - Hain line probe assay (LPA) to confirm purity of MTBC cultures (4.3- SOP 3) - MGIT cultures to confirm viability of MTBC isolates, to assess culture purity and to propagate isolates for further analysis (DST and MIC) (4.4- SOP 4) - DNA extraction for WGS (4.5- SOP 5) - DST for streptomycin (S), isoniazid (I), rifampicin (R), ethambutol (E), moxifloxacin (M), kanamycin (K) and pyrazinamide (Z) (4.6- SOP 6) - MIC for study drugs: bedaquiline (B), pretomanid (Pa) and linezolid (L) (4.7- SOP 7) - WGS analysis (4.8- SOP 8) of all baseline isolates, and on pairs consisting of MTB isolated at baseline and at/after Week 16
SimpliciTB (NC 008)	<u>Baseline isolates</u> As described above for ZeNix. <u>Post-baseline isolates</u> 1. Treatment failures Preferably the first non-contaminated culture positive for MTB at/after **Week 17 (if 4-month arm)**, or at/after **Week 26 (if 6-month arm)**, from individuals who have not converted. 2. Suspect relapses	**Same as described above except:** - DST not performed for Kanamycin (K) - On isolates found to be resistant to moxifloxacin (M), MIC for this drug instead of L - On all isolates, MIC for B and Pa

	Confirmed culture positive for MTB after the end of treatment (preferably the first non-contaminated isolate) from individuals who have previously converted to culture negative (excluding isolated positives).	

4. Standard Operating Procedures (SOP)

4.1 SOP 1: Receipt of shipment and preparation of isolates for Storage

4.1.1 Purpose

The purpose of this SOP is to outline the storage requirements of *M. tuberculosis* (MTB) isolates and DNA samples from trial sites.

LJ or 7H11 cultures of positive samples at local laboratories are sent to the UCL Central Mycobacteriology Laboratory for further analysis. Upon receipt, the UCL Central Mycobacteriology Laboratory performs sub-culture onto solid (LJ/7H11) and liquid (MGIT) media, followed by confirmation of the MTBC identification and assessment of potential contaminating NTMs using the Hain Mycobacterium CM test. The absence of other contamination is also confirmed by plating onto blood agar (BA) plates. Once a pure *M. tuberculosis complex* culture is confirmed, DST, MIC testing, isolate storage and DNA extraction (for WGS) is performed. The samples to be stored at the UCL Central Mycobacteriology Laboratory in this trial are described in Table 2. The workflow for receiving and initial processing of isolates is described in the TBA Clinical Trials: UCL Chain of Custody SOP and logged on the Received Isolates Worksheet (Appendix I).

Table 2: Storage of Microbiological Samples*

Sample	Stored Samples	Storage Period	Shipped Samples
MTB isolates after mycobacteriological characterisation	For each isolate: 2 culture at -70°C to -80°C in 50% glycerol	Minimum of 5 years after the trial closure or until informed otherwise by the sponsor	N/A
MTB DNA after mycobacteriological characterisation	For each isolate: Extracted DNA at -20°C	DNA is stored in the short term (up to 6 months) prior to sending to the WGS laboratory. Any remaining DNA not required for WGS will be kept for a minimum of 5 years after the trial closure or until informed otherwise by the sponsor	DNAs shipped to WGS laboratory DNAs extracted from paired isolates (baseline/post-baseline) are priorities, but all baseline DNA will also be sent

*See notes below

NOTES:

- More than one baseline isolate for each patient may be shipped to UCL, however, **only one baseline isolate for each patient** will be subjected to mycobacterial characterisation (unless requested by the sponsor).
- In case several post-baseline isolates are available for a given participant, **preferably the isolate from the first positive culture** will be characterised (unless requested by the sponsor).
- UCL may also store isolates for medium term (6 months-1 year on LJ or 7H11 slope and/or the original MGIT subculture) while mycobacterial characterisation testing is ongoing.
- Shipment of DNA samples for WGS is detailed in SOP 8: Whole Genome Sequencing (WGS).

4.1.2 Procedure

Storage of isolates

All procedures are to be carried out inside a biosafety cabinet (BSC), using the appropriate biosafety procedures and personal protective equipment (PPE)

4.1.2.1 Equipment and Reagents

- Biological Safety Cabinet (BSC)
- Discard bucket
- Cotton wool or paper towels soaked in Mycobactericidal disinfectant (as per the local health and safety guidelines)
- 2ml Cryovial (with rubber O-ring seal), and appropriate storage box
- Sterile micropipettes and aerosol resistant tips (ART), or disposal pipettes
- Middlebrook 7H9 medium plus OADC
- Sterile saline with beads
- Falcon tube (if freezing from MGIT tube)
- Sterile glycerol 50% (in PBS or 7H9 medium)
- LJ slope/7H11 slopes

4.1.2.2 Storage of isolates in 50% glycerol solution at -70°C to -80°C

- Prepare 50% glycerol solution in PBS or 7H9 medium
- MGIT cultures should be incubated for at least two additional weeks from the time the instrument flagged positive (to ensure good growth) before the glycerol stocks are prepared for storage.
- For low volume MGIT cultures (<1ml; e.g. if some of the biomass has been used for other tests) and solid cultures more than 6 weeks post positive, prepare a new sub-culture for storage.
- Label 2ml cryovial(s) (screw-capped with rubber 'O'-ring seal) with the study Patient Identifier (Subject ID or screening number if pre-enrolment) and the Laboratory Accession Number (LAN).
- When storing 2 vials of an isolate, the vials should ideally be stored in different freezers, or at a minimum in different storage boxes kept in different sections of the same freezer.
- The exact location of each prepared cryovial(s) should be recorded on the electronic Frozen Isolate Storage Log (Appendix H; Part A) located on the UCL Shared drive (S drive_TBA Clinical Trials_Trackers_Frozen Isolate Storage Log_NC007_NC008). Copies of the electronic storage log are printed (signed and dated) every 6 months and filed in the Laboratory Site File (LSF). If any isolate needs to be regrown from the frozen stocks, it should be replaced at earliest opportunity from the first positive MGIT tube and documented on Frozen Isolate Storage Log (Appendix H; Part B).

A. <u>Storage from solid culture:</u>
   1. Take a loop full of the solid culture (<u>≤</u>6 weeks post culture positive) and re-suspend in a minimum of 0.5ml of sterile saline or 7H9-OADC media.
   2. Using a sterile Pasteur pipette or micropipette with aerosol resistant tip, transfer 0.5ml of the 50% glycerol solution to the labelled 2ml cryovials and add 0.5ml of the culture suspension, giving a 25% glycerol final concentration.
   3. If storing two aliquots repeat step 2.
   4. Place the cryovial(s) in an appropriate cryogenic storage box and freeze at -70°C to -80°C.

B. <u>Storage from a liquid culture:</u>
   1. Decant the MGIT culture (minimum volume for centrifugation is 3ml) into a falcon tube.
      NOTE: For MGIT cultures 1ml-3ml, do not centrifuge and proceed to step 4.
      Using centrifuge buckets with aerosol-resistant lids, centrifuge the falcon at 3000g for 15 minutes.
   2. Taking care not to discard the sediment, tip off all but 1.5ml-2.0ml of the supernatant into a discard bucket containing appropriate disinfectant.
   3. Re-suspend the pellet in the remaining supernatant.
   4. Using a sterile Pasteur pipette transfer 0.5ml of the 50% glycerol solution to the labelled 2ml cryovials and add 0.5ml of the culture suspension, giving a 25% glycerol final concentration. Place the cryovial(s) in an appropriate cryogenic storage box and freeze at -70°C to -80°C.

C. Storage on LJ or 7H11 slope:

1. To inoculate an LJ and 7H11 slope, use a sterile Pasteur pipette to take a few colonies directly from the MGIT sediment (if available). Alternatively, gently mix the positive MGIT culture to re-suspend the clumps of *M. tuberculosis*, and then use a sterile Pasteur pipette to aspirate 0.25ml of culture.
2. Dispense a couple of drops of culture onto an LJ slope which has been labelled with Subject ID (or screening number if pre-enrolment), Laboratory Accession Number (LAN) and date.
3. Ensure the lid is securely fastened and incubate at 37°C.
4. Once confluent growth is obtained, the positive slope should be stored in a rack in a cool dark place.
5. To maintain the isolates, LJ slopes should be sub-cultured every 6 months (unless required earlier due to the slope disintegrating).

   All initial subcultures (LJ/7H11, and MGIT) set up during isolate processing (outlined in the TBA Clinical Trials: UCL Chain of Custody SOP) are logged onto Appendix I. This acts as an inventory for all the UCL 'working' cultures which are maintained while testing is ongoing and present in the laboratory at any given time. When the referred solid culture (site LJ) and/or UCL subcultures (original LJ/7H11 slopes and MGIT cultures) are discarded, this is documented in the discard section of Appendix I. Shipment logs (Local Laboratory Manual Appendix E) also act as an inventory for all site LJ slopes, and any isolates discarded before processing will be documented on this log. Full details of isolate storage are detailed in the TBA Clinical Trials: UCL Chain of Custody SOP

4.1.3 Documentation

Appendix H: Frozen Isolate Storage Log
Appendix I: Received Isolates Worksheet
Appendix E: DNA quantification and Storage Worksheet

4.2 SOP 2: Acid-fast Bacilli (AFB) Microscopy

4.2.1 Purpose

To detect acid-fast bacilli (AFB) by microscopic examination of positive cultures. Both viable and non-viable bacilli will stain.

4.2.2 Principle

Kinyoun is a variant of the Ziehl Neelsen (ZN) method where cold Carbolfuchsin KF stain is used. It is used for confirming the presence of AFB in positive MGIT cultures and on LJ slopes if the colonies do not resemble MTB. The method uses a Carbolfuchsin KF stain, acid alcohol decolouriser and Brilliant Green K counter stain. Acid-fast organisms stain purple and the background and debris stains light green.

4.2.3 Procedure

Kinyoun stain is used to confirm the presence of AFB in cultures with resistance to any drug by the MGIT DST (SOP 6, Section 4.6) or MIC assay (SOP7, Section 4.7), or on MGIT or LJ/7H11 sub-cultures (SOP 4, Section 4.4) set up to prepare these assays.

Each time a batch of smears is prepared, a positive QC smear using MTB (control strain H37Rv) must be stained alongside the samples to ensure the staining quality of the slides.

4.2.3.1 Equipment and Reagents

- Aerosol Resistant Tips (ART)
- Biological Safety Cabinet (category CL3/BSL3 laboratory before heat-fixation of the slide)
- Plastic discard container with absorbent Vernagel sachets
- Mycobactericidal disinfectant (specified in the local health and safety guidelines)
- Distilled water (chlorine free)
- Hot plate (or slide warmer)
- Microscope slides, frosted at one end, new and unscratched
- Fixative (optional)
- Immersion oil
- Lens paper & lens cleaning solution
- Light microscope
- Positive control slide used with each batch (containing *M. tuberculosis* H37Rv)
- Paper towel soaked in appropriate disinfectant (specified in the local health and safety guidelines)
- Pasteur pipette (Pastette)
- Pencil for labelling slide
- Slide drying rack
- Staining rack
- Staining sink
- Slide storage box
- Wash bottle with distilled water
- Waste containers

<u>For Kinyoun stain (BD Kit; cat. No.212522):</u>

- Carbolfuchsin KF, 3% acid alcohol, Brilliant Green K or Methylene Blue

4.2.3.2 Process

A. Step One: Preparation of smears

Prior to heat-fixation, the slides must remain in the BSC inside the CL3/BSL3 Laboratory.

1. Label the frosted end of a clean, dry, new and unscratched slide with the Subject ID (or screening number if pre-enrolment), lab accession number and date using a pencil.
2. Prepare the smears as follows:
    a. Positive MGIT cultures
        i. Vortex MGIT tube well, allow to settle for at least 30 minutes.
        ii. Unscrew cap and sample an aliquot (approx. 50µl or 2 drops) of broth onto the slide using an ART or Pastette.
        iii. Dispose of ART or pipette into the appropriate waste container.
    b. Positive solid cultures (LJ or 7H11)
        i. Dispense approx. 50µl of distilled water on the glass slide with an ART or Pastette.
        ii. Using a sterile loop or disposable applicator stick, transfer 2-3 colonies to the water drop and gently mix to make a smooth, thin suspension.
        iii. Dispose of loop or applicator stick into the appropriate waste container
3. Spread sample on the glass slide, covering a circle approximately 2 cm in diameter. Allow the slides to air dry before heat fixing, to avoid the slides boiling and generating aerosols.
4. Place the slides for at least 30 minutes on a hotplate set to 85-95°C to heat-fix the samples.
5. Once heat-fixed, smears can be stained outside the BSC but within the CL3/BSL3 laboratory.
6. After staining, smears can be examined by microscopy in either the Containment Level 2 (CL2/BSL2) or CL3/BSL3 Laboratory once they are dry.
   NOTE: Heat-fixing the smears does not kill mycobacteria, gloves MUST be worn when handling unstained smears.

B. Step Two: Procedure for Kinyoun staining

1. Place the slides on the staining rack, including a H37Rv positive control slide, and flood with TB Carbolfuchsin KF for 4 minutes. Do not heat.
2. Wash gently with running water.
3. Decolorize with TB decolouriser (3% acid alcohol) for 3-5 seconds.
4. Wash gently with running water.
5. Counterstain with either TB Brilliant Green K or TB Methylene Blue for 30 seconds.
6. Wash gently with running water.
7. Air dry. If using TB Methylene Blue, dry over gentle heat.
8. Apply a drop of immersion oil, prior to examination using a 100x oil objective (if the slides are to be stored add a cover slip).

C. Step Three: Microscopic examination and reading Kinyoun-stained smear

Examine the Kinyoun-stained smears with a light (bright field) microscope and the 40x and 100x oil objective.

1. Examine the smears 100 fields using a regular pattern, such as the one shown in Figure 1. Start with the positive QC slide. If the QC slide is:
    - Positive- continue with reading the test slides and report the results
    - Negative- do not report any smear results obtained from that batch. Document, investigate and resolve the problem. Repeat the smears, as necessary.

2. To confirm positive MGIT or LJ/7H11 sub-cultures or resistant DST/MIC cultures, only record whether smear was positive or negative for the presence of AFB.

Figure 1: Scanning Scheme for Smear Examination

Possible false results:
When atypical rods are seen, they may be other mycobacteria (pathogenic or non-pathogenic) or other partially acid-fast organisms.

The morphology should be broken down and analysed using the following categories to confirm and distinguish mycobacteria from any possible artefacts:

- Size (length and width)
- Colour (shade and intensity of stain)
- Shape (curved, straight, etc.)
- Pattern (beaded, banded, etc.)
- Distribution on smear (e.g. cording)
- Uniformity of appearance

Acid-fast artefacts may be present in the smear; therefore, it is essential to view cell morphology carefully. Most artefacts show considerable variation while mycobacteria are uniform in size, arrangement, and staining patterns within a slide.

A few examples of the causes of artefacts (and possible solutions) are:

- Possible contamination of slides by tap water with saprophytic mycobacteria
- Spots of stain deposit (when the slide is not properly decolorized) can be mistaken for AFB – **review the control slide to ensure slides were decolorized appropriately.**
- Waxes and oils in dirty specimen containers may appear as acid-fast particles or react with non-acid-fast bacteria and make them appear acid-fast.
- Heavy metal ions in staining solutions or high chlorine content in water interfere with the fluorescent staining and may disrupt the fluorescent adhesion to the mycobacteria.
- If the smear is too thick, debris may cover AFB and make it hard to visualize.
- If the smear is too thin, there may not be enough material to see, showing a low number of (or possibly no) AFB.

NOTE: For smears prepared from positive MGIT tubes, these can be discarded once the respective MGIT results have been reported. Discard these slides into a covered sharps container.

4.2.4 Quality Control

The following QC is required:

- Each new shipment or lot number of staining reagents (Carbolfuchsin KF, Brilliant Green K/Methylene Blue or 3% acid alcohol) must be QC tested using a positive QC smear containing MTB (H37Rv) strain and a negative QC smear containing *E. coli*. Both the positive and negative controls must pass for the reagents to be used for staining samples. If the QC fails, repeat the test with new controls.

If the repeat test fails, do not use the reagents and contact the supplier.
Results of this QC are to be reported using GATB Mycobacteriology Quality Manual Attachment Eii.

- Each batch of MGIT samples stained should also include a positive control slide containing MTB (H37Rv), to check the staining procedure has been performed correctly. If the positive control fails, then the results from the cultures cannot be relied on and should be repeated with a new positive QC slide.
  Results of this QC are to be reported using GATB Mycobacteriology Quality Manual Attachment H.

4.2.5 Documentation

GATB Mycobacteriology Quality Manual Attachment Eii: Ziehl-Neelsen or Kinyoun Stain Reagents
GATB Mycobacteriology Quality Manual Attachment H: Daily AFB Microscopy
Appendix B: DST LRF
Appendix C: MIC LRF

For confirmation of AFB in resistant DST or MIC MGIT tubes, report the date the slide was read, the person reading and the result (AFB+/AFB-) in the spaces provided on the reverse of the appropriate LRF (Appendix B: DST LRF and Appendix C: MIC LRF).

4.3 SOP 3: HAIN Line Probe Assay (LPA)

4.3.1 Purpose

This SOP is used to confirm the speciation result obtained by the local laboratory prior to any additional characterization work. Table 3 below details which specific LPAs can be used.

Table 3: Use of LPAs in TB Alliance studies at UCL

Sample	LPA (GenoType) Test	Comment
All isolates sent to UCL Laboratory - baseline and post-baseline Any subsequent sub-cultures (MGIT or solid) from the local laboratory slants, or MGIT tubes used in DST/MIC where there is a doubt about the morphology or a discrepant result which may be the result of NTM contamination.	Hain Mycobacterium CM/AS Hain Mycobacterium MTBC	LJ or 7H11 cultures that are received at UCL should be sub-cultured into MGIT tubes. Hain Mycobacterium CM can be performed on the original LJ received or the sub-cultured MGIT broth when it flags positive (see Section 4.3.3.4 for detailed procedure). This is to rule out contamination with potential non-tuberculous mycobacteria (NTM), which may not be detected on the primary LJ (only once grown up in liquid media). Since this MGIT culture is used to seed all subsequent DST/MICs it is important to check the purity of the isolate. If MTBC is detected using the Hain Mycobacterium CM and further speciation is required, then a Hain Mycobacterium MTBC will be performed on the culture. If Hain Mycobacterium CM identifies MTBC but there remains a suspicion of NTM contamination - Perform the Hain Mycobacterium AS on the MGIT culture - Repeat the testing from the original LJ using Hain Mycobacterium CM or AS (if applicable). If Hain Mycobacterium CM identifies an NTM only - request a new culture from the local laboratory If Hain Mycobacterium CM identifies a mixture of NTM and MTBC – review the colony morphology on the original site LJ and assess if MTBC colonies can be picked to obtain a pure culture and sub-cultured onto fresh solid media. Once growth is visible, colony morphology should be assessed, and a Hain Mycobacterium CM performed to confirm the culture identification. If a pure MTBC culture cannot be isolated, a new culture should be requested from the local laboratory. Details for logging 'rejected' samples and requests for new samples are included in the TBA Clinical Trials: UCL Chain of Custody SOP. If a local laboratory has repeated cultures that are NTMs or contaminated with NTMs, the local laboratory manager and laboratory co-ordinator should be informed.

4.3.2 Principle

Line probe assay technology involves the following steps: First, DNA is extracted from a culture of mycobacteria or directly from decontaminated and concentrated clinical specimens. Next, polymerase chain reaction (PCR) amplification of the resistance-determining region of the gene under question and/or a genomic region, allowing for speciation, is performed using biotinylated primers. Following amplification, labelled PCR products are hybridized with specific oligonucleotide probes immobilized on a strip. Captured labelled hybrids are detected by colorimetric development, enabling detection of the presence of MTBC or the species within the complex, as well as the presence of wild-type and mutation probes for resistance. If a mutation is present in one of the target regions, the amplicon will not hybridize with the relevant probe. Mutations are therefore detected by lack of binding to wild-type probes, as well as by binding to specific probes for the most commonly occurring mutations. The post-hybridization reaction leads to the development of coloured bands on the strip at the site of probe binding and is observed by eye or using an automated reader (GenoScan).

In this study, DNA will be isolated at the local laboratories from sputum samples or cultures using the GenoLyse method, which is described below (see Section 4.3.3.4 for more details). UCL Central Mycobacteriology Laboratory will extract DNA using a boiling/sonicating method, which is also described below. For a description of the LPA method, consult the instructions provided by Hain Lifesciences for the selected LPA – **taking into consideration the available test version.**

4.3.3 Procedure

4.3.3.1 Equipment and reagents required for DNA extraction

- Absorbent paper
- Calibrated thermometer
- Microfuge with fixed rotor angle
- Graduated cylinders
- Biological safety cabinet (BSC)
- 95°C water bath
- Micropipettors, 20-200µl, 100-1000µl
- Aerosol resistant tips (ART)
- 3 ml Pasteur Pipettes (Pastettes)
- 1.5 ml screw-cap Eppendorf tubes
- 2 ml screw-capped Microfuge tubes
- Appropriate Disinfectant (as specified in the local health and safety guidelines)
- Genolyse Lysis Buffer (A-LYS)
- Genolyse Neutralisation Buffer (A-NB)
- Plastic discard container with absorbent Vernagel sachets

4.3.3.2 Equipment and reagents not provided in the kits required for Amplification

- PCR tubes (DNase and RNase free)
- Dedicated Laboratory Gowns + hooks to hang the gowns on
- Nitrile disposable gloves (Small, Medium, Large)
- DNAway
- PCR Hood with UV-lamp
- Bacterial DNA extracts
- Aliquoted master mix
- Thermal Cycler (heating rate: 3°C/sec, cooling rate: 2°C/sec, precision: +/- 0.2°C)
- Micropipettes (P20, P200, P1000)
- Pipette stand
- Aerosol-resistant tips (ART)

- Plastic discard container with absorbent Vernagel sachets
- Marker Pen

4.3.3.3 Equipment and reagents not provided in the kits required for Detection

- Shaking water bath or TwinCubator
- Sterile saline / sterile water (molecular biology grade)
- Graduated cylinder
- Timer
- Tweezers
- Vortex
- Plastic discard container with absorbent Vernagel sachets
- Water bath or heating block (set to 95°C)
- Sterile universal container (15 ml)

4.3.3.4 Process

A) Step One: DNA isolation from positive cultures for speciation by Hain Mycobacterium CM/AS and Genotype MTBC

DNA isolation is to be carried out inside a BSC, using the appropriate biosafety procedures and PPE

Genolyse DNA Extraction Method*:*

If using bacteria grown in liquid media:

1. For each sample, label the sides of two 1.5 ml screw-capped Eppendorf tube(s) with the laboratory number. The first tube will be used for the DNA extraction process and the second will receive the isolated DNA.
2. Transfer 1mL of bacteria growing in liquid media into a labelled 1.5mL screw cap tube
3. Centrifuge for 10 minutes at 10,000 x g in a standard table top centrifuge with aerosol tight rotor.
4. Carefully remove the supernatant. If the pellet has been aspirated, place the contents back into the same tube and centrifuge again.
5. Discard supernatant into the plastic discard container with Vernagel sachets.
6. Resuspend pellet in 100µl Lysis Buffer (A-LYS) by vortexing. No clumps should be visible.
7. Incubate for 5 minutes at 95°C in a water bath. Briefly spin down.
8. Add 100µl Neutralisation Buffer (A-NB) and vortex sample for 5 seconds.
9. Spin down for 5 minutes at full speed in a standard table top centrifuge with an aerosol tight rotor.
10. Using a P200 micropipette and ART, immediately pipette 100µl of the supernatant (which now contains the DNA) slowly and carefully into the second labelled Eppendorf tube. Directly use 5µl of the supernatant for PCR. The remainder of the sample should be stored at - 20°C, until hybridisation is complete and a test result reported.

If using bacteria grown on solid media:

1. Collect bacteria with an inoculation loop and suspend in 100µl Lysis Buffer (A-LYS), vortex, and continue to steps 7-10 above.

Sonication DNA Extraction Method*:*

1. When using bacteria grown on solid medium (LJ slopes), collect bacteria with an inoculation loop and suspend in approximately 300µl of sterile saline or water (molecular biology grade or sterile distilled/deionised water) in 2 ml screw-capped tubes.
2. When using bacteria grown in liquid media, directly apply 300µl into the tubes.
3. Incubate the tubes for 30 min at 95°C (heat block).
4. Incubate for 15 minutes in an ultrasonic bath at room temperature.

5. Spin down for 5 minutes at full speed and directly use 5µl of the supernatant for PCR. In case DNA solution is to be stored for an extended time period, transfer supernatant to a new tube. If debris is taken into the PCR it may inhibit the reaction.

NOTE: For Hain Mycobacterium CM, an internal control (IC) DNA must be added to all test samples (including the positive and negative controls) before DNA extraction. Add 2µl IC DNA to each tube of bacterial suspension before proceeding with DNA extraction.

B) Step Two: Amplification and Hybridisation

Observe the usual precautions for amplification set-up (see 'Good laboratory practice when performing molecular amplification assays' at the end of this SOP). It is essential that all reagents and materials used in the set-up for DNA isolation and amplifications are free from DNases.

C) Step Three: Hybridisation

From this point on strictly follow the instructions for use (IFU) provided by Hain Lifesciences for the selected LPA. These are batch dependant and must be downloaded from the Hain website (https://www.hain-lifescience.de/en/instructions-for-use.html) for each new batch.

4.3.4 Quality Control

Each time the test is performed a positive and negative control must be run alongside samples:

- Positive control: A suspension (0.5 McFarland) of a MTB control strain (H37Rv) subjected to the 3 steps mentioned above – DNA isolation (GenoLyse or boiling/sonication), PCR amplification, and hybridization.
- Negative control: sterile saline or molecular grade water subjected to the same 3 steps mentioned above.
- For Hain Mycobacterium CM an additional positive control may be included using control DNA (C+) DNA included in the kit, for the amplification and hybridisation. This is *M. kansasii* DNA and should show the *M. kansasii* banding pattern without the IC (as IC is not included in this control).

Results of this QC are to be reported using GATB Mycobacteriology Quality Manual Attachments L.

4.3.5 Documentation

Report test results on Appendix A (Speciation LRF) and also Appendix I. The UCL Hain result is for UCL workflow purposes only, to confirm the culture received is a pure MTBC isolate before proceeding with further characterisation tests (MGIT DSTs and MICs). This result is not entered into RAVE.

A scanned copy of the Evaluation Sheet provided with the kit, with the developed strips (covered with a clear adhesive tape) should also be kept. This will be stored on the department shared drive (S Drive) in the TBA_Clinical Trials and saved as HAIN (kit name) Worksheet_Hybridisation date (dd/mmm/yyyy) (i.e. HAIN CM Worksheet_23Aug2018).

4.3.6 Good Laboratory Practice When Performing Molecular Amplification Assays

4.3.6.1 Introduction

This SOP describes key elements of how to organise facilities for polymerase chain reaction (PCR) testing including workflow, reagents, consumables and staff within a molecular diagnostic laboratory.

The ability of PCR to produce large numbers of copies of a target sequence from minute quantities - sometimes single copies - of DNA has provided the exquisite sensitivity that makes PCR a powerful diagnostic tool. However, this ability also necessitates that extreme care be taken to avoid the generation of false-positive results.

False-positive results can result from sample-to-sample contamination and, perhaps more commonly, from the carry-over of DNA from a previous amplification of the same target.

Careful consideration should be given to facility design and operation within clinical laboratories in which nucleic acid amplification-based assays are performed. This document describes procedures that will help to minimise the carry-over of amplified DNA.

4.3.6.2 General Considerations

Organisation of Work

Practise good housekeeping policy at all times. Do not keep tubes or reagents any longer than necessary. All reagents, reaction tubes etc. should be clearly labelled. Records of batch numbers of all reagent batches used in individual assays should be kept.

Avoid entering pre-amplification rooms immediately after working in rooms where products, cloned materials and cultures are handled. If working with these materials is inconvenient or unavoidable, use of clean lab coats, gloves and hand washing is necessary. Change gloves frequently.

Ensure that all equipment, including paper, pens and lab coats are dedicated for use only in that particular laboratory (i.e. laboratory coat) for each of the PCR rooms. Workbooks and sheets that have been in contaminated areas shall not be taken into clean PCR areas.

PCR reagents should be aliquoted to avoid excessive freeze-thawing and to protect stock reagents if contamination occurs.

Pulse centrifuge tubes before opening the reagents. Uncap and close tubes carefully to prevent aerosols.
Bench areas in PCR laboratories should be wiped daily with hypochlorite solution or 70% ethanol following use.
All new members of staff, visitors and students must be trained in use of the PCR facilities.

4.3.6.3 Specimen Processing

Avoid molecular contamination problems of PCR through care (Good Laboratory Practice), being tidy and following the unidirectional workflow (see below).

Physical Separation of Pre-PCR and Post-PCR Assay Stages

To prevent carry-over of amplified DNA sequences, PCR reactions should be set up in a separate room or containment area from that used for post-PCR manipulations.

A complete separate set of necessary laboratory equipment, consumables, and laboratory coats should be dedicated for the specific use of pre- or post-PCR manipulations according to the area designation. Care must be taken to ensure that amplified DNA, virus cultures or DNA clones other than low copy number control material do not enter the 'Pre-PCR area'.

Reagents and supplies should be taken directly from storage into the pre-PCR area and should never be taken or shared with areas in which post-PCR analyses are being performed. Similarly, equipment such as pipettes should never be taken into the containment area after use with amplified material.

The Unidirectional Workflow

Workflow between these rooms/areas must be unidirectional i.e. from clean areas to contaminated areas, but not from contaminated areas to clean labs. Dedicated laboratory coats should be supplied for each area and gloves shall be changed between areas.

Reagent Preparation Clean Room (DNA –Free Room)

It is very important to keep this room/area free of any biological material (this includes DNA/RNA extracts, samples, cloned materials and PCR products).

Procedures carried out in this area include preparation and aliquoting of reagent stocks and preparation of reaction mixes prior to the addition of the clinical nucleic acid. Aliquoting of primers and other reagents is recommended to minimise any consequence of contamination and reduce assay downtime.

The Nucleic Acid Extraction Room

Extraction of nucleic acid from clinical samples must be performed in areas where PCR products and stocks of cloned materials have not been handled. A second clean area is thus required for this purpose. The second area is where the samples are processed, where the reverse transcriptase step of RT-PCRs is performed and where the extracted DNA or cDNA and positive control is added to the PCR reaction mixes (previously prepared in the reagent preparation room).

Specimens for PCR should come directly from the clean specimen receipt room into the extraction laboratory; the samples should never enter rooms where PCR products and cloned DNA are present.

The Amplification Room

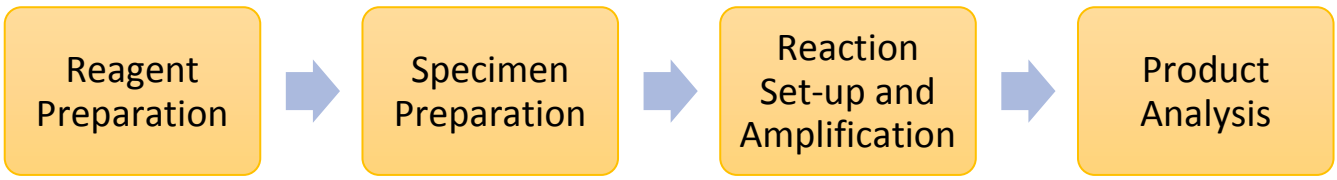
The amplification room is the area in which the PCR machines are housed. It may also contain a containment area in which, for nested PCRs, the second-round reaction mixes are inoculated with the primary reaction product. Cloned DNAs should not be brought into this area.

Where PCR machines are shared, a clear booking system is recommended to provide a cohesive system for the assays. Individual users' PCR programs in the thermocyclers should not be edited by other users (even temporarily) without notification to the program owner.

The Product Analysis Room

This is the room in which post-PCR manipulations are performed (e.g. agarose gel electrophoresis of products, PCR-ELISA detection systems). This is a contaminated area and therefore no reagents, equipment, laboratory coats etc. from this room should be used in any of the other PCR areas.

Figure 2: Diagram Showing Work Flow in a PCR Laboratory



NOTE: Although four rooms are ideal, many laboratories only have two rooms available. Pre-PCR and extraction can therefore be carried out within defined areas of a larger laboratory and amplification and product analysis are in a second laboratory

4.3.6.4 References

PHE UK Standards for Microbiology Investigation 'Good Practice when Performing Molecular Amplification Assays', Quality Guidance, Q4, Issue no. 5, Issue Date 19.02.2018

4.3.7 Documentation

GATB Mycobacteriology Quality Manual Attachment Lii: GenoType® MTBC

GATB Mycobacteriology Quality Manual Attachment Liv: GenoType® Mycobacterium CM/AS
Appendix A: Speciation LRF

4.4 SOP 4: Sub-culture of referred cultures using liquid (MGIT) and solid (LJ/7H11) growth media

4.4.1 Purpose

Used for the sub-culture of **referred solid cultures from local laboratories (site LJ slopes)** for:

- Confirmation of viability of MTBC isolates.
- Assessment of culture purity.
- Propagation of MTBC isolates for further analysis (DST and MIC).

4.4.2 Principle

MGIT tubes contain a fluorescent compound embedded in silicone on the base of the tube. This complex is sensitive to the presence of oxygen dissolved in the liquid medium during continuous incubation at 37°C. The instrument monitors the tubes every hour for increasing fluorescence. The presence of fluorescence beyond a threshold identifies a tube as positive. An instrument positive tube contains approximately 10^5 to 10^6 CFU/ml.

The usual 'MGIT protocol', and the one adopted in this study, lasts 42 days. A MGIT culture remaining negative for 42 days and showing no other visible sign of growth is considered negative.

LJ and 7H11 are used to gain a higher bacterial load and for assessment of colonial morphology.

4.4.3 Procedure

Refer to the manufacturer's instructions for the overview of the MGIT instrument as well as detailed procedures (e.g. MGIT tubes preparation, incubation conditions, uploading and unloading of the tubes in the instrument).

NOTES:

- MGIT tubes can be prepared for inoculation in a BSC outside of the Containment Level 3 (CL3/BSL3) laboratory.
- Inoculation of the MGIT tubes with the referred cultures and any confirmatory testing of positive tubes must be carried out in the BSC in a CL3/BSL3 lab.
- Prior to use, examine all tubes and vials for evidence of contamination or damage - in particular, cracks on the tubes or lid; dropped tubes must be examined carefully for damage. Unsuitable or damaged tubes MUST be discarded.
- In the unlikely event of a broken tube in the machine – close the drawer and turn off the machine, evacuate the room. Local Health and Safety Guidelines should be followed for actions following a spill.

4.4.3.1 Equipment and Reagents

- Biological Safety Cabinet (BSC)
- Appropriate PPE
- Mycobactericidal disinfectant (specified in local Health and Safety guidelines)
- Ethanol 70%
- BACTEC MGIT 960 7 ml MGIT tubes
- MGIT PANTA and MGIT Growth Supplement
- Graduated Plastic Pasteur Pipettes
- Discard bucket
- Biohazard bags
- 1000µl pipette and aerosol resistant tips
- Sterile, disposable 10 ml pipettes
- Marker Pen

- Sterile saline with beads
- LJ media slopes
- 7H11 media slopes
- Blood agar (BA) plate
- 10µl loop
- Plastic discard container with absorbent Vernagel sachets

4.4.3.2 Process

Step One: Preparation of BSC and MGIT components

1. Using absorbent cotton wool or paper towels, decontaminate the BSC with an appropriate mycobactericidal agent, followed by 70% ethanol.
2. Place the necessary equipment, consumables and waste containers inside the BSC. Then, decontaminate these as well, starting with the mycobactericidal agent, followed by 70% ethanol.
3. Place a clean Benchguard/ absorbent paper sheet (or alternative work surface protectant) inside the BSC.
4. Always ensure that the daily QC and maintenance of the instrument (see Section 4.4.4) has been done and that the incubation protocol is set for 42 days prior to placing the inoculated MGIT tubes into the BACTEC MGIT 960 instrument.
5. Reconstitute MGIT PANTA (Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim, Azlocillin) with 15 ml of MGIT OADC (Oleic Acid Albumin Dextrose Catalase) Growth Supplement. Mix completely until dissolved. This supplement mixture is stable for 5 days if stored at 2-8°C (write the date of reconstitution on the bottle to document this).

Step Two: Preparation of Inoculum from the referred culture (Site LJ slope)

1. Visually inspect the culture for contamination.
2. Touch various colonies of the solid culture received with a sterile loop. Add this to the bijoux containing sterile saline with beads and vortex to mix.
3. Allow the organism suspension to settle for at least 30 minutes.

Step Three: Inoculation onto solid media

1. For each culture, label two LJ slopes (or one LJ slope and one 7H11 slope) with the Subject ID (or screening number if pre-enrolment) and the laboratory accession number label.
2. Inoculate each slope with 0.25ml of the organism suspension.
3. Inoculate the organism suspension onto a BA plate using a 10µl disposable loop and incubate in a 37°C incubator for 48 hours. Check for growth or contamination on the BA plate daily and record the result on Appendix I after 48 hours.

Step Four: Inoculation and incubation of the MGIT tubes (culture of the UCL original MGIT)

1. For each culture, label a MGIT tube with the Subject ID (or screening number if pre-enrolment) and the laboratory accession number (LAN) label. Record the MGIT tube number in the Received Isolates Worksheet (Appendix I).
2. Add 0.8 ml of the PANTA supplement (mixed above in Step one) to each MGIT tube using a sterile pipette. Be careful to avoid contamination. The mixed PANTA supplement should be added to the MGIT medium prior to inoculation of the culture in the MGIT tube. Do not add the mixed PANTA supplement after the inoculation of specimen.
3. Dilute the culture suspension (prepared in Step Two) to make a 1:5 culture suspension.
4. Add 0.5 ml of the diluted culture suspension (prepared in point 3 above) to the appropriately labelled MGIT tube. Use a separate graduated Pasteur pipette or micropipette for each specimen. Immediately recap the MGIT tube tightly and mix well by inverting several times.

5. Inoculate the diluted culture suspension (used in point 4 above) onto a BA plate using a 10µl disposable loop and incubate in a 37°C incubator for 48 hours. Check for growth or contamination on the BA plate daily and record the result on Appendix I after 48 hours.

Note: If both solid (LJ/7H11 slopes) and liquid (MGIT) cultures are prepared at the same time, the organism suspension is inoculated onto one BA plate.

6. Dispose of waste pipette tips and loops into the appropriate discard container.
7. Wipe the MGIT tube with a paper towel soaked in disinfectant (Tristel) before removing it from the BSC.
8. Record the MGIT tube barcode for each specimen on Appendix I.
9. Enter the tubes in the machine following procedures provided by the manufacturer (always scan the MGIT barcode first and assign station through the <Tube entry> function).
10. Store the referred culture in an appropriate rack, in the designated storage cupboard, at room temperature until characterisation of the isolate is complete.

NOTES:

- Do not place tubes without the instrument assigning a station.
- Do not remove tubes unless they are positive or out-of-protocol negatives (negative at 42 days)
- Do not re-assign tubes to a new station

Step Five: Removal and work-up of positive MGIT tubes

NOTE: Only discard positive MGIT tubes after autoclaving.

Equipment and Reagents

- BSC
- Plastic discard container with absorbent Vernagel sachets
- Mycobactericidal disinfectant (specified in local Health and Safety guidelines)
- Blood agar (BA) plate
- 10µl loop
- Plastic tube for centrifugation of sample
- Glass microscope slide
- Sterile Pasteur pipette and aerosol resistant tips (ART).
- LJ media slope
- 37°C incubator
- Centrifuge/microfuge

1. Remove the MGIT tubes from the machine according to manufacturer's instructions.
   NOTE: A positive tube can be re-entered in the machine for further incubation, but within 5 hours of removal. If the tube is returned via the 'tube entry' operation (see manufacturer's instructions), positive routines are reset, the start of incubation is retained, and monitoring of the tube resumes.
2. For positive MGIT tubes, print out the 'Unloaded Positives Report' which records the date the tube flagged positive and the number of days and hours taken to reach positivity (TTD).
3. Record the Subject ID (or screening number if pre-enrolment) and laboratory accession numbers of all unloaded tubes next to their corresponding results on the printouts. The printouts must be **signed and dated** by the staff member unloading the tubes. Result sheets must be kept in an unloaded positives folder or with the corresponding patient's worksheets/LRFs.
4. For positive tubes, the following must be performed:
   Visually inspect for signs of contamination:
   a. If the tube is clear and has the typical 'breadcrumb' appearance proceed with the required testing.

b. If the tube looks contaminated (turbid/cloudy) it may be necessary to perform Kinyoun stain microscopy (SOP 2; Section 4.2) and set up a Blood Agar Culture (BAC) as follows:
    1. Label one BA plate with the laboratory accession number and the Subject ID (or screening number if pre-enrolment) .
    2. Vortex the MGIT tube well, allow the tube to settle for a minimum of 30 minutes.
    3. Inoculate the BA plate with liquid from the MGIT tube using a 10µl disposable loop and incubate in a 37°C incubator for 48 hours.
    4. In the meantime, the MGIT tube must also be re-incubated. Return the tube to the MGIT machine or place it in an incubator at 37°C.
    5. Check for growth or contamination on the BA plate daily.

5. Once the results of the Kinyoun stain and BAC become available, refer to the algorithm shown on Step Six below.

NOTE: MGIT sub-culture tubes must have a TTD **of at least 4 days** to be used for DST and MIC testing. If a tube is positive in less than 4 days a new MGIT sub-culture must be prepared before proceeding with DST and MIC testing. Check for the presence of contamination, as described above, as this may contribute to a short TTD. If no contamination is present, further dilute the starting culture used to seed the new MGIT subculture.

Step Six: Interpretation of positive MGIT results

BAC		**AFB (Kinyoun smear)**		**Result**
Negative	and	Present (positive)	this is	True Positive

BAC		**AFB (Kinyoun smear)**		**Result**
Positive or Other*	and	Present or Absent	this is	Contaminated

* A MGIT may also be considered contaminated in the following situations (in which cases the BAC conclusion should be marked as 'Other' and the reason provided in the source documents):
a. BAC is negative, but MGIT tube is visibly contaminated with fungal or other microbial growth.
b. BAC is negative, but evidence of fungal hyphae seen on Kinyoun smear.

1. If the sample is confirmed as **contaminated** or **contaminated** (BAC positive or other) **with AFB** (Kinyoun stain positive) examine the original culture received from the site laboratory:
    a. If no visible signs of contamination, perform a new sub-culture from the original isolate.
    b. If visible signs of contamination present:
        i. If the presence of an NTM is suspected - confirm by performing a Hain Mycobacterium CM (SOP 3, Section 4.3). If an NTM is detected – request a new/alternative isolate from the site laboratory following the rejection procedure outlined in the TBA Clinical Trials: UCL Chain of Custody SOP. It may be necessary to perform the Hain CM from the contaminated MGIT in cases where the NTM was not detected on the original site LJ, but has grown in the liquid culture.
        ii. if contamination is caused by another organism AND is a critical or priority isolate (e.g. no other available positives, alternative samples have been contaminated, urgent follow up sample, time restraints related to end of study or key analysis timelines. To be discussed and agreed with the TB Alliance Microbiology Director), re-decontaminate the MGIT subculture with 4% NaOH as follows:
            - Inside a BSC, aseptically transfer the entire volume of the MGIT tube into a labelled 50ml disposable centrifuge tube.
            - Add an equal quantity of 4% NaOH solution, for a final concentration of 2% NaOH.
            - Mix well, and leave to stand for 20 minutes, mixing and inverting the tube periodically.
            - Add phosphate buffered saline (PBS; pH 6.8) to the tube, up to the 40ml mark, and mix well.

- Using centrifuge bucket with aerosol-resistant lids, bring the buckets and lids into the BSC, place the tube into the bucket and tighten the lid. Centrifuge the tube at 3000g for 15 minutes. After spinning, open the centrifuge bucket inside the BSC.
- Carefully pour off the supernatant fluid, taking care not to dislodge the pellet.
- Re-suspend the sediment in 0.5 ml of PBS and mix well.
- Aseptically add 0.8 ml of PANTA-OADC supplement mixture (see Section 4.4.3) to a fresh, appropriately labelled MGIT tube.
- Using a sterile pipette, inoculate 0.5 ml of the sediment suspension into the MGIT tube. Tighten the lid and mix well by inverting several times.
- Wipe the MGIT tube with a paper towel soaked in disinfectant before removing it from the BSC.
- Load the tube into the BD BACTEC as per the manufacturer's instructions and incubate.

iii. if contamination is caused by another organism AND is NOT a critical or priority isolate as defined above, request a new/alternative isolate from the site laboratory following the rejection procedure outlined in the TBA Clinical Trials: UCL Chain of Custody SOP.

Step Seven: Storage for further investigations

Two samples must be stored for each isolate in 50% glycerol at -70°C to -80°C for long term storage. The first is prepared from the site LJ when the sample is processed for initial subculture (Step Two above), and the second prepared within 12 weeks of the initial subculture onto LJ or 7H11 (Step Three above) when heavy growth is observed (see the TBA Clinical Trials: UCL Chain of Custody SOP for more details). For the preparation of cultures for storage, follow SOP 1 (Section 4.1). For medium term storage, the first MGIT subculture will be maintained and stored at room temperature, until all testing for the sample is complete. A record must be kept of all isolates in storage (Appendix I for working cultures and Appendix H for frozen stocks).

Step Eight: Removal of negative MGIT tubes

The threshold for tubes to be declared as negative in BACTEC MGIT 960 is 42 days. Any tube that has not flagged positive prior to or at day 42 should be considered negative.

1. Remove the MGIT negative tubes from the machine according to manufacturer's instructions.
2. If it is the original MGIT subculture prepared from the referred culture that is negative, go back to the original referred isolate (site LJ slope) and repeat the inoculation outlined in Step 2 and Step 4, using a more concentrated inocula (i.e. add 0.5 ml of culture suspension without dilution). This culture will have been confirmed MTB positive by Hain CM (refer to Appendix I).
3. If the MGIT is a subsequent subculture (as described in Step Nine) or a repeated negative from the referred culture, troubleshoot for potential issues (e.g. user error with inoculation, old seed culture used, issues with reagents), and repeat inoculation from either the referred culture, original UCL MGIT culture or frozen stocks as appropriate. If any frozen stocks are used, they MUST be replenished from the first positive MGIT culture and documented on Frozen Isolate Storage log (Appendix H).
4. If there is repeatedly no growth in the MGIT, it will be necessary to request a new/alternative isolate from the local laboratory, following the rejection procedure outlined in the TBA Clinical Trials: UCL Chain of Custody SOP.
5. Print the 'Unloaded Negatives Report', record the Subject ID (or screening number if pre-enrolment) and laboratory accession number of all unloaded tubes next to the results on the print outs. The print outs must be **signed and dated** by the staff member unloading the tubes and must be kept in an unloaded negatives folder.
6. Autoclave negative tubes prior to discarding.

Step Nine: Sub-culture of MGIT cultures

Any sub-cultures required for further/follow up testing should be performed on the original UCL MGIT culture set up from the referred LJ slope, as described above (Step Four), or the original referred LJ slope if not possible (Step Two and Three above).

In instances where the sub-culture is to be performed from a MGIT culture, proceed as follows:

1. Prepare a fresh MGIT tube as described above (Step One: points 1-5 and Step Two: points 1-2).
2. Ensure the original MGIT culture is well mixed to re-suspend the pellet prior to sampling (e.g. pipetting up and down or inverting the tube). If necessary, the culture can be vortexed to further break up clumps (with or without glass beads) and left for 30 mins to settle.
3. Using a sterile pipette, transfer 50µl from the original MGIT culture into the appropriately labelled, fresh MGIT tube.
4. Immediately recap the fresh MGIT tube tightly and mix well by inverting several times.
5. If contamination is suspected, inoculate the MGIT culture onto a BA plate using a 10µl disposable loop, and incubate in a 37°C incubator for 48 hours. Check for growth or contamination on the BA plate daily.
9. Dispose of waste pipette tips and loops into the appropriate discard container.
10. Wipe the MGIT tube with a paper towel soaked in disinfectant (Tristel) before removing it from the BSC.
11. Enter the tube in the machine following procedures provided by the manufacturer (always scan the MGIT barcode first and assign station through the <Tube entry> function). Store the MGIT culture in an appropriate designated rack, until characterisation of the isolate is complete.

4.4.4 Quality Control

New media and supplements

An MTB reference strain (H37Rv) is tested to ensure that the medium supports growth of mycobacteria. A solid (LJ/7H11) or a liquid (MGIT) culture can be used to prepare the QC inoculum.

Procedure using Liquid Culture

1. A fresh MGIT culture of the MTB reference strain (H37Rv) should be prepared and used within 5 days of flagging positive by the BACTEC.
2. The inoculum preparation will vary depending on the number of days since the culture flagged positive (See Section 4.6.3.3 for full details):
   a) If the culture used is between 1-2 days of positivity: Dilute the 1:100 using sterile saline;

   OR

   b) If the culture used is between 3-5 days of positivity: Dilute 1:500 using sterile saline (i.e. Dilute 1:5 then dilute 1:100)
3. Prepare the QC MGIT tube following the normal MGIT culture procedure (see Section 4.4.3.1, Step One).
4. Label each tube with the inoculation date, expiry date and mark as 'Control'.
5. Inoculate one MGIT tube from each new batch number with 0.5 mL of the inoculum prepared in point 2.
6. The control tube should become positive within 4-13 days. If the QC tubes do not give the expected results, do not use the remaining tubes of the batch. Repeat the QC test and if it fails, contact the manufacturer for troubleshooting.

Procedure using Solid Culture

1. Prepare a fresh culture of the MTB reference strain (H37Rv), cultures should be positive within 10-15 days of the sub-culture flagging positive and should be used within this period as aged cultures can give unreliable results.
2. Prepare a suspension in Middlebrook 7H9 media and adjust the turbidity to 0.5 McFarland.
3. Dilute the 0.5 McFarland suspension as follows to obtain a 1:500 dilution:
   - Add 1 ml of the suspension to 4 mL of sterile saline – Dilution 1 (1:5).
   - Add 100µl of Dilution 1 to 900µl of sterile saline – Dilution 2 (1:50).
   - Add 100µl of Dilution 2 to 900µl of sterile saline – Dilution 3 (1:500).
4. Prepare the MGIT tube following the normal MGIT culture procedure (see Section 4.4.3.1, Step One).
5. Label each tube with the inoculation date, expiry date and mark as 'Control'.
6. Inoculate one MGIT tube from each new batch number with 0.5 mL Dilution 3.

7. The control tube should become positive within 6-10 days. If the QC tubes do not give the expected results do not use the remaining tubes of the batch. Repeat the QC test and if it fails, contact the manufacturer for troubleshooting.

NOTE: If the QC fails and contamination is expected, a BAP should be set up a read after 48 hours. The results of the BAP should be recorded directly onto the unloaded positives MGIT printout with the read date and laboratory staff initials.

Results of this QC are to be reported using GATB Mycobacteriology Quality Manual Attachment Eiii
MGIT maintenance must be performed daily and monthly, preferably before unloading or loading of tubes, and **recorded on GATB Mycobacteriology Quality Manual Attachment J.**

MGIT failure/breakdown lasting more than 24 hours:
Investigate the issue and document on a continuous Quality Improvement (CQI) Form (**GATB Mycobacteriology Quality Manual Attachment M**). Perform a repeat sub-culture from the original LJ received.

4.4.5 Documentation

GATB Mycobacteriology Quality Manual Attachment Eii: Ziehl-Neelsen or Kinyoun Stain Reagents
GATB Mycobacteriology Quality Manual Attachment Eiv: Blood Agar Plates
GATB Mycobacteriology Quality Manual Attachment Exiii: MGIT TUBES/ PANTA with Growth Supplement and OADC
GATB Mycobacteriology Quality Manual Attachment Exii: 7H11 and LJ Media
GATB Mycobacteriology Quality Manual Attachment J: MGIT 960 Daily Maintenance Log
GATB Mycobacteriology Quality Manual Attachment K: MGIT Calibration Tube Log
GATB Mycobacteriology Quality Manual Attachment M: Continuous Quality Improvement
Appendix A: Speciation LRF
Appendix I: Received Isolates Worksheet

4.5 SOP 5: DNA Extraction

4.5.1 Purpose

Used to extract DNA from all baseline isolates, and from post baseline isolates at/after Week 16 (as defined in Table 1) for subsequent WGS analysis as described in SOP 8, Section 4.8.

The method described here is designed to yield microgram quantities of high molecular weight DNA suitable for genotyping.

4.5.2 Principle

Although DNA can be extracted from *M. tuberculosis* (MTB) bacilli by a variety of methods, with a range of complexity, the method described here is designed to yield high quality large fragment DNA from a colony pick. Using a combination of enzymatic digestion and organic partition, colonies picked from the LJ slope yield nanogram to microgram quantities of DNA. Following heat killing of the colonies, bacteria are digested first with lysozyme to breakdown the cell wall then with proteinase K, which has further action on the cell wall but, importantly, digests any enzymes released by the lysed bacterium, including DNases. MTB is lipid rich and so two rounds of detergent are used, first SDS and then CTAB. These detergents have action on molecules with different charges thus affecting different cell wall components. EDTA is used to chelate Mg^{+2} and Ca^{+2} ions, inhibiting DNase activity; similarly, high salt concentrations inhibit DNA-enzyme binding. Finally, organic solvents are used to partition the DNA to an aqueous phase, leaving lipids and proteins in the organic phase. The aqueous phase is then concentrated using isopropanol, this concentrates the DNA and removes excess salt. Isopropanol is used in preference to ethanol as a lower volume for precipitation can be used (1:1 rather than 2:1). Yeast tRNA is used during the alcohol precipitation step as it is an effective co-precipitant to aid in recovery of small amounts of nucleic acids and improve the DNA yield.

4.5.3 Procedure

4.5.3.1 Isolation of genomic DNA from *M. tuberculosis*

Equipment/Reagents

- Biological Safety Cabinet
- Ventilated Cabinet
- Waterbath or heating blocks (80-95°C, 60-65°C and 37°C)
- 10µl loops
- 1.5 ml screw-capped Eppendorf tubes with rubber 'O' ring seal
- 1000µl pipette and aerosol resistant tips
- 200µl pipette and aerosol resistant tips
- Tris-EDTA (TE) buffer
- Microfuge
- 10 mg/ml lysozyme
- 10% Sodium dodecyl sulphate (SDS)
- 10 mg/ml Proteinase K
- 5M NaCl
- Cetyl trimethylammonium bromide (CTAB)
- Chloroform
- Isoamylalcohol
- Yeast tRNA (10 mg/mL) (Catalog number: AM7119)
- Sterile DNAase-free 1.5 ml eppendorf tubes
- 70% ethanol
- Isopropanol

- -20°C freezer
- 4°C refrigerator
- Molecular grade water or sterile distilled water
- Plastic discard container with absorbent Vernagel sachets

Preparation of extraction reagents

For each batch of DNA extractions, Appendix G: DNA Extraction and Quantification Reagent Worksheet must be completed to document reagent log numbers, expiry dates and preparation dates, and to ensure reagents are all used within the acceptable timelines outlined below. Each batch of extraction will also be assigned a sequential batch number (TBA001; TBA002, etc.) completed on the header of this log, and subsequently on the DNA Quantification and Storage Worksheet (Appendix E). This will assist with the tracking of DNA extraction data and samples.

Lysozyme solution: 10 mg/ml.

Store in small aliquots at -20°C. Use a new aliquot each time, do not refreeze. Any remaining reagent should be discarded.

10% SDS

Add distilled water to 10g of SDS to make up 100ml of total solution. Dissolve by heating at 65°C for 20 minutes or let stand overnight. Do not autoclave. Store at room temperature for no longer than 1 month from the preparation date.

Proteinase K: 20 mg/ml.

Store in small aliquots at -20 °C or as otherwise specified by the provider. Use a new aliquot each time, do not refreeze. Any remaining reagent should be discarded.

5M NaCl

29.2g NaCl/100 ml distilled water. Heat to 65°C and mix until dissolved (this may take hours). Autoclave on a media cycle to sterilise. Store at room temperature for no longer than 1 year from preparation date. This can be purchased commercially (SIGMA, S5150), in which case store at room temperature and use within a 1 year of opening. Aliquot sufficient for the sample numbers in the batch and use a new aliquot each time.

CTAB/NaCl (10% CTAB in 0.7 M NaCl)

Dissolve 4.1g NaCl in 80ml distilled water. While stirring, add 10g CTAB. If necessary, heat solution to 65 °C. Adjust the volume to 100 ml with distilled water and autoclave on a media cycle to sterilise. Store at room temperature for no longer than 6 months from the preparation date.

Chloroform/isoamylalcohol (24:1)

Mix 1 part of isoamylalcohol with 24 parts of chloroform. Mix thoroughly by shaking vigorously for 5 seconds. Store in cool, dark, ventilated place, use within 6 months from preparation date or by expiry date, if indicated. This can be purchased commercially (SIGMA, C0549); if so, mix well before removing aliquots.

70% Ethanol

70ml 100% ethanol in 30ml distilled water, store at -20°C. For the ethanol stock solution, use within 5 years of opening

Isopropanol

Store at room temperature for no longer than 1 year after opening. Ready-to-use aliquots may be stored at -20°C, as needed.

Yeast tRNA (10mg/ml)
Store in small aliquots at -20°C. Use a new aliquot each time, do not refreeze. Any remaining reagent should be discarded.

Ultrapure molecular grade DNase/RNase free water
Prepare 50ml aliquots (or sufficient for the sample number in the batch) inside a Class 2 BSC to avoid frequent opening of stock bottle. Record the date the aliquot was prepared in the tube and use a new aliquot for each batch

Tris-EDTA (TE) buffer
100x TE buffer are purchased commercially (SIGMA, T9285) and diluted to 1x in molecular grade DNase/RNase free water (e.g. 500ul of 100x TE buffer and 49.5ml of water).

Process
All steps, including the heat killing of MTB culture (step 7) and addition of chloroform/isoamylalcohol (step 15) must be carried out in an appropriate BSC inside a BSL/CL3 Laboratory.

1. Switch on the heating block (set at 80°C).
2. Label sufficient 2.0ml clear screw-capped tubes containing 'O' rings with Subject ID (or screening number if pre-enrolment) and laboratory accession number. It is important to use clear tubes for ease of collection of aqueous phase after chloroform/isoamylalcohol (Step 18). Label both lid and side, as some of the solvents could delete the writing.
3. Aliquot 400µl volumes of 1x Tris-EDTA (TE) buffer into the tubes using aerosol resistant tips.
4. From solid media (LJ/7H11 slopes) with good growth, take all organisms using a 10µl loop and emulsify them in the appropriate tubes containing the TE buffer taking care not to create splashes or aerosols.
5. Pulse down the tubes in the microfuge using the aerosol-containing rotor for 5 seconds to ensure that all organisms are at the bottom of the tube and unload the rotor in the BSC.
6. Place the tubes in a suitable rack and heat-kill in the heating block at 80°C for 30 minutes.
7. Sonicate in a water bath for 15 minutes at room temperature.
8. Pulse down the tubes, as above.
9. Place the tubes in a suitable rack and add 50µl 10mg/ml lysozyme and vortex briefly. Incubate at 37°C in the incubator or heating block overnight (if overnight is not possible at least one hour is required).
10. Switch on 60°C heating block. Pre-warm the CTAB/NaCL to 60°C. Ensure a sufficient volume of Isopropanol (450µl per sample) and 70% Ethanol (1ml per sample) is at -20°C. Take aliquot of tRNA out of the -20°C freezer, vortex for a few seconds when fully thawed, and place on ice.
11. Add 70µL 10% SDS and 5µl 20mg/ml proteinase K. Vortex briefly and incubate at 60°C for 10 minutes.
12. Add 100µl 5M NaCl to each tube.
13. Add 100µl CTAB/NaCl (pre-warmed to 60°C). Vortex briefly and incubate at 60°C for 10 minutes.
14. Ensure that the chloroform/isoamylalcohol (24:1 v/v) is well mixed and add 750µl to each tube inside a ventilated cabinet. Vortex.
15. Wipe tubes externally with Tristel disinfectant and place inside a zip bag.

***Tubes can now be removed from the Containment Level 3 Laboratory**.*

16. Set the heating block temperature to 30°C.
17. Microfuge at 10,000g for 5 minutes.
18. Label lid and side of sterile DNAase-free 1.5ml microfuge tubes and transfer the aqueous supernatants into these. Take care not to disturb the interface.
19. Add 3µl tRNA to each tube
20. Aliquot 450µl of ice-cold isopropanol to each tube. Vortex briefly and place at -20°C for at least 30 minutes. Keep isopropanol and ethanol (step 22) on ice while in use.

21. Microfuge at 10 000 g for 15 minutes at room temperature.
22. Remove the supernatants and wash the pellets with 1ml 70% ethanol at -20°C. Invert gently.
23. Microfuge at 10,000g for 5 minutes at room temperature. Remove and discard as much of the ethanol as possible. Place the tubes with open lids in a heating block at 30°C to allow the pellets to air-dry (at least 15 min).
24. Rehydrate the pellets in approx. 100µl (depending on pellet size) molecular grade water (preferred) or TE buffer with a maximum concentration of 0.1mM EDTA overnight at 4°C (or 1 hour at 65 °C). Ensure the rehydration date is added on all the DNA tubes – this will be captured as the **DNA extraction date** in all associated documentation.

4.5.3.2 Estimation of DNA concentration

DNA concentration will be estimated using the methods detailed below to ensure the quantity and quality is sufficient for WGS analysis. All DNA quantification results will be recorded on the DNA Quantification and Storage Worksheet (Appendix E). The analyses are:

Nanodrop – used to determine the 260/280 ratio, which gives a measure of the purity of the preparation. This is only required when DNA is sent to the Public Health England (PHE) laboratory for sequencing. Note: although the Nanodrop also gives a readout of concentration this must not be used to quantitate DNA. As there is no RNase step most of the nucleic acid will be RNA, and readings are likely to be much higher (10x or more) than the actual DNA concentration. This will also be affected by the addition of tRNA used in the extraction process.

Qubit – used to measure dsDNA concentration. The read out from the Qubit must be used to determine the DNA concentration to be sent.

TapeStation – used as an additional measure of concentration and quality, providing information that can be used for QC and troubleshooting of the DNA extraction process if the sequence quality is low, rather than monitoring individual samples. DNA integrity is assessed using the TapeStation giving a DNA Integrity Number (DIN). The DIN is an automated numerical software assessment which allows integrity determination of genomic DNA samples from the distribution of signal across the size range. Normally, TapeStation will be carried out on a subset of samples with each DNA extraction batch, it is not a requirement to perform on every DNA sample.

Estimation of DNA Concentration Using Nanodrop

Equipment/Reagents

- NanoDrop Spectrophotometer
- BM compatible PC (see NanoDrop user's manual for computer requirements, software installation and set-up)
- 2µl Pipette with appropriate tips
- Soft laboratory wipe/tissue
- Molecular grade water
- Tris-EDTA (TE)
- Plastic discard container with absorbent Vernagel sachets

Process

1. Install software onto your computer and attach USB cable between the NanoDrop and PC (as described in the User's Manual).
2. To measure nucleic acid concentration and quality select the 'Nucleic Acid' application module.
3. Follow instructions to initialise the instrument. Wipe pedestals clean (using lint-free tissue).

4. Select sample type 'DNA-50' for double stranded DNA (default). Enter sample ID if appropriate.
5. Always perform a blank run before testing DNA samples (this will ensure the instrument is working properly and the pedestal is clean).
6. With the sampling arm open, pipette 2µl molecular grade water (or TE buffer with a maximum concentration of 0.1mM EDTA if this was used to rehydrate the DNA) onto the lower measurement pedestal. Make sure the blank run is performed with the same solvent the DNA is re-suspended in (i.e. water, TE buffer), ideally taken from same source used to dissolve the DNA.
7. Close the sampling arm and click on the 'Blank' button.
8. When the measurement is complete, wipe the blanking buffer from both pedestals using a laboratory wipe (lint-free tissue).
9. Analyse an aliquot of the blanking solution as though it were a sample. This is done by using the 'Measure' button (F1). The result should be a spectrum with a relatively flat baseline. Wipe the blank from both the upper and lower pedestal surfaces and repeat the process until the spectrum is flat.
10. Clean the pedestals by wiping with a laboratory wipe.
11. Pipette 2µl of sample DNA onto the lower measurement pedestal (if you are unsure about your sample or your pipettor accuracy, a 1.5 – 2µl sample is recommended to ensure the liquid sample column is formed and the light path is completely covered by sample).
12. Make sure the sample type is DNA-50 and enter any sample ID details.
13. Click 'Measure'.
14. Repeat for any other samples, wiping the pedestals in between samples.
15. The results will save automatically and at the end of the set of samples click 'Show Report'. Record the DNA concentration and the 260/280 ratio in Appendix E: Part B DNA Quantification: Nanodrop and TapeStation.
16. Clean after use by wiping with 70% ethanol followed by distilled water.

Estimation of DNA Concentration Using the Qubit Fluorometer

The Qubit dsDNA Assay Kits are designed for accurate DNA quantification and are highly selective for double-stranded DNA (dsDNA) over RNA.

Qubit® dsDNA Assay is available as 2 kits- Broad Range (BR) and High Specificity (HS); and either can be used as appropriate to ensure the DNA concentration reading is within the quantification range of the kit (See Table 4). Normally, the BR kit is used first and the HS kit is then used only if the concentration of the sample is too low for the quantification range of the BR kit to be reliably measured.

Table 4: Qubit kit quantification ranges

Qubit Kit	Initial Sample Concentration (overall testable range)	Qubit Quantification Range	Quantification range using 2ul of DNA sample
Qubit dsDNA **BR** Assay kit	100pg/µl -1000ng/µl	2 - 1000ng	1-500ng/µl
Qubit dsDNA **HS** Assay Kit	10pg/µl-100ng/µl	0.2-100ng	0.1-50ng/µl

Equipment/Reagents

- Qubit® dsDNA Reagent (Component A)
- Qubit® dsDNA Buffer (Component B)
- Qubit® dsDNA Standard #1 (Component C)
- Qubit® dsDNA Standard #2 (Component D)
- Disposable plastic container for mixing the Qubit® working solution
- Thin-wall, clear, 0.5ml PCR tubes. Acceptable tubes include Qubit® assay tubes (Life Technologies Cat No. Q32856) or Axygen® PCR-05-C tubes (VWR Cat No. 10011-830).

- Qubit Fluorometer

Storage of reagents: The Qubit® dsDNA Reagent and Buffer must be stored at room temperature (22-28°C) and the Qubit® DNA standards must be stored at 4°C. The Qubit Reagent must be protected from light. When stored as directed kits are stable for 6 months after opening.

Note: The Qubit assay delivers optimal performance at room temperature (22-28°C). Do not hold tubes in hand before reading as this raises the temperature and lowers the Qubit reading.

Process

1. Set up the required number of 0.5ml tubes for the samples and standards (2 tubes) and label the lids with the sample Laboratory Accession Number (LAN) or UCL ID number. Do not label the sides of the tube as this can interfere with the reading.
2. Prepare Qubit® working solution by diluting the Qubit® dsDNA Reagent 1:200 in the Qubit® dsDNA Buffer in a clean plastic tube. The final volume in each tube is 200µl, so prepare enough working solution for all the samples and the two standards. For example, for 8 samples and 2 standards make 2ml of working solution by diluting 10µl of reagent into 1990µl of buffer.
3. Add 190µl of Qubit® working solution to each of the standard tubes, and then add 10ul of each Qubit® standard to the appropriate tube and mix by vortexing for 2-3 seconds. Check there are no bubbles; if seen, tap the tube to release them.
4. Add Qubit® working solution to individual sample tubes so that the final volume including the sample is 200µl. Routinely, add 2µl of the DNA sample and 198µl of working solution. The Qubit method allows up to 20µl of sample to be used if needed, i.e. in cases with low DNA concentration, in which case the volume of working solution should be adjusted accordingly. If a volume **other than 2µl** is used this should be noted in the comments on Appendix E, as it must be accounted for in the calculations.
5. Add each sample to the appropriate assay tubes and mix by vortexing for 2-3 seconds. Check there is no bubbles or air gap at the bottom of the tube, if seen, tap the tube to release them.
6. Allow all tubes to incubate at room temperature for 2 minutes - the samples are now ready to be read on the fluorometer. After this incubation period the fluorescence is stable for 3 hours at room temperature.
7. On the home screen of the Qubit® Fluorometer, press **DNA** and then select the kit used (**dsDNA High Sensitivity or dsDNA Broad Range**) as the assay type. The standards screen is displayed.
8. Press **Yes** to read the standards. Insert the tube containing Standard #1 into the sample chamber, close the lid and press **Read**. When the reading is complete (~3 seconds) remove the standard. Insert the tube containing Standard #2 into the sample chamber, close the lid and press **Read**. When the reading is completed remove the standard. When the calibration is complete the instrument displays the Sample screen.
9. Insert a sample tube into the sample chamber, close the lid and press **Read**. When the reading is complete (~3 seconds) remove the tube. The instrument displays the results on the screen. The first value displayed is the concentration in the assay tube. The value displayed is in ng/ml. To calculate the concentration of the original sample:
    - Multiply the value shown by 100 to account for the dilution factor (2µl of DNA in 200µl of working solution). To convert units to ng/µl (units needed for sending DNA for WGS) divide by 1000 (therefore overall divide original reading by 10). NOTE: ng/µl is the SAME as µg/ml.

NOTE: If needed, the final concentration in the original sample can be calculated by the Qubit instrument. Press **Calculate Stock Conc** and the **Dilution Calculator Screen** is displayed. Select the volume of the sample that you added to the assay tube, once selected the Qubit® Fluorometer calculates the original sample concentration using the volume and the measured assay concentration. If using the dilution calculator, the units displayed are **ng/ml** and will also need to be converted to ng/µl.

10. Record the concentration in the table provided in the Appendix E: DNA Quantification and Storage Worksheet
11. Press **Read Next Sample**, and repeat steps 9 and 10 for all remaining samples.

NOTE: If the concentration is too low using the BR kit (Qubit will give a <0.010 error), repeat using the HS kit.

Record the DNA concentration values in Appendix E.
If any further dilutions or Qubit measurements are required, these should also be recorded on Appendix E in the space provided.

Estimation of DNA integrity and Concentration Using the Agilent 4200 TapeStation System

The Agilent 4200 TapeStation system (G2991AA) is an automated platform for scalable, flexible, faster and more reliable electrophoresis. The Genomic DNA ScreenTape assay is designed for analysing genomic DNA in the sizing range from 200 to >60000 bp.

Equipment/Reagents

- Agilent 4200 TapeStation with attached computer and software, as described in the user manual
- Agilent Loading tips (5067- 5598, 1pk or 5067- 5599, 10pk)
- Agilent 96-well Plates (5042- 8502) and 96-well Plate Foil Seal (5067- 5154)
- Agilent Optical Tube 8x Strip (401428) and Optical Cap 8x Strip (401425)
- Vortex mixer IKA MS3 with adapter (or equivalent that can vortex a strip or plate)
- Volumetric pipette
- Centrifuges for tube strips and well plates
- Agilent 5067-5365 Genomic DNA ScreenTape 7 ScreenTape devices
- Agilent 5067-5366 Genomic DNA Reagents
- Agilent Genomic DNA Ladder
- Agilent Genomic DNA Sample Buffer

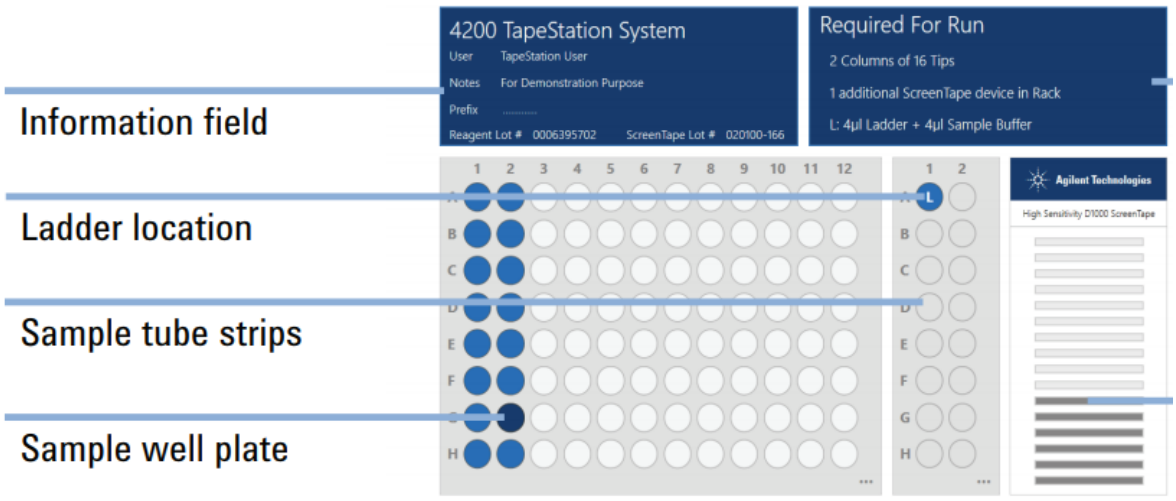
Process:

1. Allow Genomic DNA Reagents (5067- 5366) to equilibrate at room temperature for 30 minutes.
2. Launch the Agilent 4200 TapeStation Controller Software.
3. Check there are no bubbles in the capillaries of the Genomic DNA ScreenTape device (5067- 5365), and tap to remove any that are visible, and load it into the 4200 TapeStation instrument.
4. Place loading tips (5067- 5598) into the Agilent 4200 TapeStation instrument.
5. Vortex reagents and spin down before use. Take care to not over mix ladder vial.
6. Prepare ladder:
   - For 1 – 15 samples: pipette 10µl Genomic DNA Sample Buffer (green) and 1µl Genomic DNA Ladder (yellow) at position A1 in a tube strip (401428).
   - For 16 or more samples: pipette 20µl Genomic DNA Sample Buffer (green) and 2µl Genomic DNA Ladder (yellow) at position A1 in a tube strip.
7. For each sample, pipette 10µl Genomic DNA Sample Buffer (green) and 1µl DNA sample in a well plate (5042-8502) or a tube strip (401428).
8. Apply foil seal (5067- 5154) to sample well plate and caps (401425) to tube strips with ladder or sample.
9. Mix liquids in sample and ladder vials using the IKA vortex (or equivalent) at 2000 rpm for 1 min.
10. Spin down to position the sample and ladder at the bottom of the well plate and tube strip. Check to ensure no bubbles and tap to remove any.

Sample Analysis

1. Load samples into the Agilent 4200 TapeStation instrument. Carefully remove caps of tube strips.

2. Place ladder in position A1 on tube strip holder. This must be done with the ladder in a tube or strip, even if a plate is being used.



3. Select required sample positions on the 4200 TapeStation Controller Software.
4. Click Start.
5. The Agilent Tapestation Analysis Software opens after the run and displays results.
6. For each sample, the mean DNA fragment size, the quantity of DNA, and the DNA Integrity Number (DIN) should be recorded in the output.
7. A DIN is calculated on a scale from 1 to 10. A high DIN indicates highly intact gDNA, whereas a low DIN corresponds to a strongly degraded gDNA sample. DNA with a low DIN (<3) is more likely to fail in a sequencing run, and also indicates that there may be a problem with the DNA extraction process or reagents, if samples consistently have a low DIN. It is also useful to view the trace as this provides more information than the DIN alone, e.g. a sample with plenty of high molecular weight DNA, but a smear, might have a low DIN. The most important factor for sequencing is that the bulk of the DNA is not too small (<1kb).
8. Record the TapeStation data (DNA concentration and DIN) in Appendix E: DNA Quantification and Storage Worksheet. At the end of the procedure a pdf report can be exported. A copy of this report must be saved in the S drive (CCM_Clinical_Trials/TBA_Clinical_Trials /WGS/Tapestation /NC007 and NC008).
9. If, after completion of all quantification methods, there is concern about either the quality (e.g. low DIN) or the concentration of the DNA (e.g. Qubit or TapeStation results), this should be flagged for further review on Appendix E. A decision will be made (if necessary, and often in conjunction with WGS service facility as outlined in SOP 8: Section 4.8.3.1), as to whether the DNA sample is acceptable for to be sent for sequencing

4.5.3.3 Storage of DNA

Extracted DNA is stored in the short term (up to 6 months) prior to sending to the WGS laboratory for sequencing (SOP8: Whole Genome Sequencing). Any remaining DNA not required for WGS will be kept for a minimum of 5 years after the trial closure or until informed otherwise by the sponsor, as detailed in Section 4.1 SOP1: Receipt of shipments and preparation of isolates for storage; Table 1.

DNA storage details (Freezer/Box/Position) will be documented on Appendix E: DNA Quantification and Storage Worksheet at the time the extracts are prepared. This will also capture details of additional diluted aliquots that are prepared for quantification purposes and retained. This storage information will also be captured in the TB Alliance DNA and WGS tracker (Excel sheet) which is stored on the UCL Shared Drive (S: TBA Clinical Trials/Trackers), to track DNA sent for WGS and to relate WGS coverage (mean read depth) to the DNA sample.

4.5.4 Documentation

GATB Mycobacteriology Quality Manual Attachment M: Continuous Quality Improvement
Appendix E: TB Alliance DNA Quantification and Storage Worksheet
Appendix G: TB Alliance DNA Extraction and Quantification Reagent Worksheet
TapeStation pdf report
TB Alliance DNA and WGS tracker

4.6 SOP 6: Drug Susceptibility Testing (DST) by Mycobacteria Growth Indicator Tube (MGIT)

4.6.1 Purpose

Used for Drug Susceptibility Testing (DST), isolates will be set up for DST based on the study:

- **ZeNix:** streptomycin (S), isoniazid (I), rifampicin (R), ethambutol (E), moxifloxacin (M), kanamycin (K) and pyrazinamide (Z)
- **SimpliciTB:** streptomycin (S), isoniazid (I), rifampicin (R), ethambutol (E), moxifloxacin (M), and pyrazinamide (Z)

DST to S, I, R, E, M and K is performed using the BACTEC MGIT 960 SIRE kit. Whereas DST to Z is performed using the BACTEC MGIT 960 PZA kit. Critical concentrations tested are the ones recommended by the World Health Organization (WHO) Technical Report on Critical Concentrations for Drug Susceptibility testing of Medicines Used in the Treatment of Drug-resistant Tuberculosis (see Table 6).

Susceptibility testing will be performed on baseline and post-baseline isolates.

NOTE: Prior to any drug susceptibility testing (DST), a HAIN assay (SOP 3; Section 4.3) is performed at the UCL Central Mycobacteriology Laboratory to confirm the presence of MTB complex (MTBC) only. If the HAIN test does not confirm MTBC or is a mixed population of MTBC and NTMs, and the MTBC bacteria cannot be isolated, the local laboratory will be contacted, and a new culture will be sent.

4.6.2 Principle

Susceptibility testing in the MGIT 960 system is based on the same principles as isolation from sputum (detection of growth). DST is performed using an AST (antibiotic susceptibility testing) set, which consists of a Growth Control tube and one tube for each drug, as well as a bar-coded tube carrier that holds the set. A known concentration of drug is added to a MGIT tube, along with the specimen, and growth is compared with a drug-free control of the same specimen. If the drug is active against the mycobacterial isolate (isolate susceptible), growth will be inhibited and fluorescence will be suppressed in the drug-containing tube; meanwhile, the drug-free control will grow and show increasing fluorescence. If the isolate is resistant, growth and its corresponding increase in fluorescence will be evident in both the drug-containing and the drug-free tube. The growth rate of the test isolate is compared in the presence and in the absence of antibiotics. An isolate is determined resistant if 1% or more of the test population grows in the presence of the critical concentration of the drug.

4.6.3 Procedure

Equipment/Reagents

- Biological Safety Cabinet
- Plastic discard container with absorbent Vernagel sachets
- Mycobactericidal disinfectant (specified in local Health and Safety guidelines)
- 7ml MGIT tubes
- BD SIRE MGIT kit reagents
- BD Moxifloxacin HCl lyophilised powder
- BD Kanamycin sulphate lyophilised powder
- BD BACTEC MGIT supplement (for SIRE and PZA drug kits)

- McFarland standards
- p1000, p200 and p20 pipettes and aerosol resistant tips
- Sterile saline
- Blood agar plates
- Glass slide

For DST from LJ slopes:

- Middlebrook 7H9 broth
- Capped sterile tube containing glass beads
- Vortex

4.6.3.1 Preparation of drug stocks for susceptibility testing

Preparation of drug stocks and MGIT tubes can be carried out outside of the CL3/BSL3 laboratory.

Table 5: Preparation of DST Drug Stock Solutions

TASK	INSTRUCTIONS
NOTE – the following may be reconstituted with different volumes. Failure to use the appropriate volume of sterile distilled/deionised water for reconstitution of the drugs will invalidate these tests	
Prepare BACTEC™ MGIT™ 960 SIRE Drug Kit	• Reconstitute each BACTEC™ MGIT™ 960 SIRE Kit Streptomycin lyophilised drug vial with **4 ml** of sterile distilled/deionised water to make a stock solution of 83µg/ml. • Reconstitute each BACTEC™ MGIT™ 960 SIRE Kit Isoniazid lyophilised drug vial with **4 ml** of sterile distilled/deionised water to make a stock solution of 8.3µg/ml. • Reconstitute each BACTEC™ MGIT™ 960 SIRE Kit Rifampicin lyophilised drug vial with **4 ml** of sterile distilled/deionised water to make a stock solution of 83µg/ml. • Reconstitute each BACTEC™ MGIT™ 960 SIRE Kit Ethambutol lyophilised drug vial with **4 ml** of sterile distilled/deionised water to make a stock solution of 415µg/ml.
Prepare BACTEC MGIT 960 Pyrazinamide Kit	• Reconstitute each BACTEC™ MGIT™ 960 PZA drug vial with **2.5 ml** of sterile distilled/deionised water to make a stock solution of 8000µg/ml.
Prepare BD Moxifloxacin HCl	• Reconstitute each BD Moxifloxacin Hydrochloride drug vial with **3 ml** of sterile distilled/deionised water to make a solution of 166µg/ml. This is the stock solution that is frozen as per the note at the bottom of this table. Before use, **this must then be diluted 1:8 in sterile distilled/deionised water to reach a stock solution of 20.75µg/ml.** This extra dilution step is required because the Moxifloxacin drug stock will be used for both DST, and MIC (SOP 7) for NC-008 isolates
Prepare BD Kanamycin Sulphate	• Reconstitute the BACTEC™ MGIT™ 960 Kanamycin lyophilised drug vial with **4 ml** of sterile distilled/deionised water to make a stock solution of 207.5µg/ml.

NOTE: On receipt of SIRE and PZA kit reagents and moxifloxacin and kanamycin powder, store the lyophilised drug vials at 2 - 8°C. Once reconstituted, the antibiotic solutions should be aliquoted out and may subsequently be frozen and stored at -20°C or colder for up to six months, but must not exceed the original expiry date of the kit/vial. Once thawed, use immediately. Discard any unused portions.

4.6.3.2 Preparation of MGIT tubes for DST testing

For the MGIT DST, the drugs can be set up as 4 individual sets each with a growth control (SIRE, M, K, and Z) using the 5- and 2- tube carrier sets as outlined below. MGIT DSTs may also be performed via the EpiCenter software (a user interface for the MGIT instrument) including the TBeXiST (TB eXtended Susceptibility Testing) module, as is done for the MIC testing (see Section 4.7; SOP 7: Minimum Inhibitory Concentration (MIC)). Using TBeXiST it is possible to extend susceptibility testing to all MTB isolates against primary drugs. For example, some MDR-TB and XDR strains are slow growing and therefore may not reach the completed threshold within the 13 days required for the growth control (GU>400) for automatic DST interpretation in the MGIT using the carrier sets, giving an x200 readout. TBeXiST can be used for SIRE, K and M as one set with a single growth control, and if required can be combined with the MIC testing so that these DSTs and MICs are performed in a single experiment with one growth control (up to 50 drug tubes can be set up with a single growth control). For pyrazinamide, this requires different tubes and supplement, so needs to be set up as an independent experiment with a separate growth control. This should be done routinely using the carrier sets, but it is acceptable to use the TBeXiST if the standard test fails due to slow growth outside the acceptable window (>21 days). For information about labelling and loading MGIT tubes into MGIT using the EpiCenter and TBeXiST software, refer to Section 4.7, and associated user guides – 'Registering TBeXiST MGIT tube for MIC-DST Users Guide' and 'Interpretation and Reporting of MIC-DST in TBeXiST Users Guide'. The drug concentrations selected must be those detailed in Table 6.

Preparation of the drug sets using AST carrier sets:

<u>For preparation of SIRE set:</u>

1. Label five 7 mL MGIT tubes for each test isolate with the appropriate laboratory accession label and the Subject ID (or screening number if pre-enrolment). In addition, label tubes with one of each of the following: GC (Growth Control), STR (streptomycin), INH (isoniazid), RIF (rifampicin), EMB (ethambutol).
2. Place the tubes in the correct sequence in the 5 tube AST set carrier (see BACTEC MGIT 960 User's Manual, AST Instructions).
3. Aseptically add 0.8 ml of BACTEC MGIT SIRE Supplement to each SIRE tube. It is important to use the supplement supplied with the kit.
4. Aseptically pipette 100µl of 83 µg/mL MGIT STR solution to the appropriately labelled MGIT tube.
5. Aseptically pipette 100µl of 8.3 µg/mL MGIT INH solution to the appropriately MGIT tube.
6. Aseptically pipette 100µl of 83 µg/mL MGIT RIF solution to the appropriately MGIT tube.
7. Aseptically pipette 100µl of 415 µg/mL MGIT EMB solution to the appropriately labelled MGIT tube.
8. It is important to add the correct drug to the corresponding tube. No antibiotics should be added to the MGIT GC tube.

<u>For preparation of the moxifloxacin set:</u>

1. Label two 7mL MGIT tubes for each test isolate with the appropriate laboratory accession label and the Subject ID (or screening number if pre-enrolment). In addition, label tubes with one of each of the

following: GC (Growth Control) and MOX
2. Place tubes in the correct sequence for the 2 tube AST set carrier (see BACTEC MGIT 960 User's manual, AST instructions)
3. For moxifloxacin set, the supplement from the BD SIRE set can be used. Aseptically add 0.8ml of BACTEC MGIT SIRE supplement to each MGIT tube.
4. Aseptically pipette 100µl of 20.75µg/ml MGIT MOX solution (as per Table 5 and Table 6) to the appropriately labelled MGIT tube.
5. No antibiotics should be added to the MGIT GC tube.

For preparation of the kanamycin set:
1. Label two 7mL MGIT tubes for each test isolate with the appropriate laboratory accession label and the Subject ID (or screening number if pre-enrolment). In addition, label tubes with one of each of the following: GC (Growth Control) and K (kanamycin)
2. Place tubes in the correct sequence for the 2 tube AST set carrier (see BACTEC MGIT 960 User's manual, AST instructions)
3. For kanamycin set, the supplement from the BD SIRE set can be used. Aseptically add 0.8ml of BACTEC MGIT SIRE supplement to each tube.
4. Aseptically pipette 100µl of 207.5ug/ml MGIT K solution to the appropriately labelled MGIT tube.
5. No antibiotics should be added to the MGIT GC tube.

For preparation of the pyrazinamide set:
1. Label two 7mL PZA MGIT tubes for each test isolate with the appropriate laboratory accession label and the Subject ID (or screening number if pre-enrolment). In addition, label tubes with one of each of the following: GC (Growth Control) and PZA (pyrazinamide)
2. Place tubes in the correct sequence for the 2 tube AST set carrier (see BACTEC MGIT 960 User's manual, AST instructions)
3. Aseptically add 0.8mL of BACTEC MGIT PZA supplement to each PZA tube. It is important to use PZA tubes and supplement as the pH of the medium is lower (pH 5.9)
4. Aseptically pipette 100µL of 8000µg/ml MGIT PZA solution to the appropriately labelled MGIT tube.
5. No antibiotics should be added to the MGIT GC tube.

Table 6: Working concentrations of DST Drugs

Drug	Concentration of drug after reconstitution	Volume added to MGIT tubes for test	Final concentration in MGIT tubes
MGIT STR	83µg/ml	100µl	**1.0µg/ml**
MGIT INH	8.3µg/ml	100µl	**0.1µg/ml**
MGIT RIF	83µg/ml	100µl	**1.0µg/ml**
MGIT EMB	415µg/ml	100µl	**5.0µg/ml**
MOX	20.75 µg/ml NOTE: this includes addition 1:8 dilution step after reconstitution	100µl	**0.25 µg/ml**
KAN	207.5µg/ml	100µl	**2.5µg/ml**
PZA	8000µg/ml	100µl	**100µg/ml**

4.6.3.3 Using inoculum from positive MGIT – carried out in BSC in CL3/BSL3 laboratory

Once a MGIT tube has become positive it must be used for DSTs within the appropriate timeframe (1-5 days). The concentration of the inoculum is critical to the correct performance of susceptibility testing and the following instructions must be adhered to strictly.
On the day the MGIT flags positive (day 0), the culture should be identified as a PURE growth of *M. tuberculosis* and tube should be re-incubated for a minimum of one day (day 1). This can be in the MGIT machine or in a separate 37°C incubator.

Day 1 and Day 2 – the growth in the tube can be used directly. Glass beads should be used to break up the clumps in the MGIT culture and obtain a uniform bacterial suspension. Pre-prepared sterile glass beads (minimum 4 beads, 5 mm diameter) in saline (3 ml) are used. Remove the saline by pipetting and pour all the beads into the MGIT tube. Mix well by vortexing to break up clumps as much as possible (between 2 and 10 minutes) and allow settling for at least 30 minutes. Use the supernatant undiluted to set up the GC and DST drug tubes.

Days 3, 4 and 5 – the growth in the tube should be diluted before use. Vortex the MGIT culture with beads, as described above, and allow to settle for at least 30 minutes. Dilute 1 ml of supernatant in 4 ml of sterile saline (1:5 dilution). Use this diluted culture for the GC and DST drug tubes.

>5 Days – sub-culture into a new MGIT tube and wait for this to flag positive. Treat as above and use within 5 days to set up the DST.

NOTE: Cultures grown in liquid or solid media can be used to prepare a seed MGIT tube. When positive, the seed MGIT can then be used to prepare the inoculum as described above. After incubation, the TTD of the seed MGIT tube must be 4 days or more for use as a DST inoculum. If the seed tube becomes positive in 4 days or less, a new seed tube should be prepared as described in section 4.4.3.

4.6.3.4 Using an inoculum from LJ slope – carried out in BSC in CL3/BSL3 laboratory

1. All preparations must be made from the pure cultures of MTB. The isolate must be confirmed, by appropriate identification techniques.
2. Add 4 ml of Middlebrook 7H9 Broth (or BBL MGIT broth) to a suitable sterile tube with cap containing 4 – 5 glass beads.
3. Scrape with a sterile loop as many colonies as possible from growth no more than 14 days old, trying not to remove any solid medium. Suspend the colonies in the Middlebrook 7H9 Broth.
4. Vortex the suspension for 2 – 3 min to break up the larger clumps. The suspension should exceed a 1.0 McFarland standard in turbidity.
5. Let the suspension sit for 20 min without disturbing.
6. Transfer the supernatant fluid to another suitable sterile tube with cap (avoid transferring any of the sediment) and let sit for another 15 min.
7. Transfer the supernatant fluid (it should be smooth, free of any clumps) to a third suitable sterile tube. NOTE: The organism suspension should be greater than a 0.5 McFarland standard at this step.
8. Adjust suspension to a 0.5 McFarland standard by a visual comparison with a 0.5 McFarland turbidity standard. Do not adjust below a 0.5 McFarland Standard.
9. Dilute 1 ml of the adjusted suspension in 4 ml of sterile saline (1:5 dilutions).

4.6.3.5 Growth Control tube preparation and inoculation – carried out in a BSC in CL3/BSL3 laboratory

For SIRE, MOX and KAN Growth Control Tubes:

1. Dilute the organism suspension (used to inoculate drug tubes) to achieve a 1:100 suspension to be used as the GC suspension (1% growth control), as follows:
    a. Aseptically pipette 0.1 ml of the organism suspension into 0.9 ml of sterile saline to prepare a 1:10 dilution. Tightly recap the tube, and mix thoroughly by inversion.
    b. Aseptically pipette 0.1 ml of the 1:10 dilution into 0.9 ml of sterile saline to prepare the 1:100 dilution to be used for the GC inoculation. Tightly recap the tube, and mix thoroughly by inversion.

2. Inoculate 0.5 ml of the 1:100 GC suspension into the MGIT tubes labelled "GC", using a micropipette and aerosol resistant tips. Dispose of pipette into discard pot of liquid disinfectant

For PZA Growth Control Tubes:

1. Aseptically pipette 0.1 ml of the organism suspension (used to inoculate drug tubes) into a total of 0.9 ml of sterile saline to prepare the 1:10 GC suspension (1% growth control).
2. Mix the GC suspension thoroughly.
3. Inoculate 0.5 ml of the 1:10 GC suspension into the MGIT tubes labelled "GC", using a micropipette and aerosol resistant tips. Dispose of pipette into discard pot of liquid disinfectant.

4.6.3.6 Inoculation of tubes containing test drugs – carried out in BSC in CL3/BSL3 laboratory

1. Aseptically pipette 0.5 ml of the organism suspension into each of the seven remaining drug tubes (STR, INH, RIF, EMB, PZA, MOX and KAN), using a micropipette and aerosol resistant tips. Dispose of pipette into discard pot of liquid disinfectant.
2. Tightly recap the tubes.
3. Mix tubes thoroughly by gentle inversion 3 to 4 times.
4. For entry into the MGIT instrument, **without** the TBeXiST software, use the carrier sets and enter AST set into the BACTEC MGIT 960 using the AST set entry feature (refer to the BACTEC MGIT 960 User's Manual, AST Instructions). Ensure that the order of the tubes in the AST set carrier conforms to the set carrier definitions selected when performing the AST set entry feature (from left to right):
    - SIRE – 5 tube carrier set (GC, S, I, R, E)
    - PZA – 2 tube carrier set (GC, Z)
    - MOX – 2 tube carrier set – load as 'undefined drug' (GC, M)
    - KAN - 2 tube carrier set – load as 'undefined drug' (GC, K)
5. After inoculation of MGIT tubes, spread 10µl of the organism suspension to a BA plate.
6. Enclose the BA plate in a plastic bag.
7. Incubate at 35-37°C.
8. Check the blood agar plate at 48 hours for bacterial contamination. If the BA plate shows no growth, then allow AST testing to proceed. If the BA plate shows growth, discard the AST set (refer to the BACTEC MGIT 960 User's Manual, AST Instructions) and repeat testing with pure culture.

4.6.3.7 How to interpret DST results

The BACTEC MGIT 960 instrument continually monitors all tubes for increased fluorescence. Analysis of fluorescence in the drug-containing tubes compared to the fluorescence in the Growth Control tube is used to determine susceptibility results. Using the carrier sets, the BACTEC MGIT 960 automatically

interprets these results and reports a susceptible (S) or resistant (R) result for the SIRE and PZA tests on the AST print outs

For moxifloxacin and kanamycin, because the AST has been loaded as 'undefined drug' the results need to be interpreted manually. The growth unit of the GC tube should be 400 GU. For the drug tube, if the **growth units are more than 100 the isolate is resistant (R)**, whereas if the **growth units are less than 100 the isolate is sensitive (S).** It is also important to check the time in protocol (TIP) is within the acceptable timeframe of 4-13 days. If outside this range the test should be considered invalid because the growth control failed to reach 400 GU in the required time (see Error messages below). This result [Susceptible (S)/Resistant (R)/Invalid (X)] and the drug name (moxifloxacin or kanamycin) should be documented on the AST print out.

All AST print outs should be labelled with the Subject ID (or screening number if pre-enrolment) and laboratory accession numbers of the samples and signed and dated by the member of staff unloading the tubes.

Error messages – If the AST print out shows an 'X' (X200 and X400) – this means the run has failed because the growth control tube reached 400 GU outside of the acceptable time frame:
SIRE, moxifloxacin and kanamycin- 4 to 13 days, PZA- 4 to 21 days.

In this case the result is invalid and no interpretation (S/R) will be shown. This could be caused by contamination with rapid growing microorganisms (including NTMs), or the result of the inoculum being prepared incorrectly (adding too many or too few mycobacteria). These samples will need to be repeated as follows depending on the error code:

X200: System cannot detect sufficient indication of growth in the Growth Control tube in the specified protocol time and does not provide an interpretation of the AST set results. Often a result of too little inoculum, non-viable organisms, or a slow growing drug-resistant strain. Perform repeat DST as follows:

- Prepare a new MGIT culture from primary MGIT culture tube as per the instructions in SOP4: Section 4.4.3; Step Nine. When the tube turns positive, **re-incubate for 3-5 days and use this culture (DO NOT DILUTE)** to set up the DST as described above (Section 4.6.3.5).
- If necessary, use the TBeXiST software for the repeat DST.

X400: System detects indications of possible contaminated or over-inoculated tube and does not provide an interpretation of the AST set results. Check the tube for turbidity and sub-culture to a blood agar plate to rule out contamination of the specimen. It may also be necessary to perform an AFB stain (SOP2) to further assess for contamination.

If there is no indication of contamination in the culture (i.e. no growth on BA plate or visible contamination in the tube or in AFB+ smear) perform a repeat the DST as follows:

- Prepare a new MGIT culture from primary MGIT culture tube as per the instructions in SOP4: Section 4.4.3; Step Nine.
- When the tube turns positive, **use the culture at 1-2 days** old to set up the DST as described above (Section 4.6.3.5).

If the culture is confirmed contaminated, it is important to identify if this contamination was present in the seed culture (Site LJ or original UCL MGIT) or introduced during assay set up. It may be necessary to perform additional BA culture or AFB+ staining to identify a clean culture from which to restart the subculture (See SOP 4; Section 4.4.3). If a clean culture cannot be identified for a given isolate, follow process outlined in SOP 4: Section 4.4.3; Step Six.

4.6.3.8 Confirming resistant isolates

All resistant isolates should be verified by preparing a blood agar culture (BAC) to check for growth of contaminating bacteria (SOP 4, Section 4.4). All resistant tubes must also be visually inspected to check for typical 'breadcrumb' morphology of MTB. This visual inspection and the blood agar result should be documented on the reverse of Appendix B (DST LRF). If there is anything unusual about the growth seen in the MGIT tube (turbidity suggestive of contamination, or atypical growth), or the resistance profile is not as expected (i.e. inconsistent with previous results), a smear should be performed to confirm the presence of AFB. Staining of smears for AFB from resistant cultures will be performed using the Kinyoun (SOP 2; Section 4.2). The smear result will also be recorded on the reverse of Appendix B (DST LRF). Together these additional tests will confirm the culture was pure and the resistant result not caused by growth of contaminating bacteria.

- If the BAC shows no growth, the colony morphology is typical **and** the smear shows no concomitant flora (if performed), you can accept the resistant result.
- If the BAC shows growth and / or the smear shows concomitant flora, you cannot use the resistant result, repeat the susceptibility testing with a pure MTB culture.

As mentioned above, AFB smear is not performed routinely on the resistant cultures, but only if there is any ambiguity or sign of contamination not seen on BAC. This is because:

- The culture used to prepare the inoculum/or the sub-culture for the inoculum has a Hain, which confirms the presence of MTB, before DSTs are set up.
- All resistant results are routinely repeated.
- BAC confirms lack of contamination which could be causing false resistance.

MGIT DST results must be confirmed by repeat testing based on the study as outlined below. Local laboratory data can be reviewed either in RAVE or using the Big Query Report (BQR) for the relevant study.

ZeNix (NC-007):

- SIRE: Repeat SIRE panel if I and/or R result is discrepant from historical or Screening data (local laboratory data); If there is no reported historical or local screening data and the I and/or R is resistant the SIRE panel should also be repeated to confirm the resistant result.
  If S and/or E are resistant do not repeat to confirm.
- M, K and Z: If the first DST result is resistant, repeat to confirm.

SimpliciTB (NC-008):

- SIRE: Repeat SIRE panel if the I and/or R MGIT DST result is discrepant from Screening data (local laboratory data);
  If S and/or E are resistant do not repeat to confirm.

- Z: If the first DST result is resistant, repeat to confirm.
- M: If the first DST is resistant confirm via Moxifloxacin MIC testing.

Similarly, if the results for one of these drugs are inconsistent with previous results for the same patient – e.g. baseline MGIT DST result was resistant and follow-up result was susceptible – review the QC and repeat the test. If the results of the confirmatory test match the initial MGIT DST, then the results can be accepted. If the repeat testing shows a susceptible result, the data is discrepant, and the DST must be repeated a third time to confirm which result is correct. It is not acceptable to automatically assume the susceptible result is the valid result. Likewise, in NC-008, if the Moxifloxacin DST and MIC results are discrepant (Moxi MIC result is NOT ≥ 0.25µg/ml), the test/s should be repeated to resolve the discrepancy.

In addition, when sub-culturing isolates for repeat testing it is important, as far as possible, to go back to the original positive culture (the original UCL MGIT, or the referred LJ slope from the local laboratory) rather than performing multiple subcultures. This will help to minimise the risk of cross contamination or modification of drug resistance profile, through selection.

4.6.4 Quality Control

It is extremely important to perform quality control on the drug sensitivity testing procedure. This must be carried out for each new batch of reagents (drug kits and tubes), using the pan-susceptible MTB strain H37Rv (ATCC 27294/NCTC 7416), which is susceptible to all of the test drugs. If the QC fails, all results for the batch should be reviewed, new reagents purchased and testing of clinical samples repeated.

These QC results should be recorded in the GATB Quality Manual Attachment Eviii, Eix & Ex.

NOTE: If an H37Rv QC fails because the DST result is resistant, a BA culture must be prepared to check for possible contamination. This should be read after 48 hours and the results recorded directly on the MGIT Unloaded AST report, next to the appropriate DST results, and the entry signed and dated by the member of laboratory staff

In addition, for each new batch of drug stocks prepared from the drug powder, a second person must be present to ensure that the drug preparation is performed correctly. This must be documented on the appropriate GATB Quality Manual Attachment (Exiii, Eix, Ex).

QC must also be carried out on each new batch of BA plates and **recorded on GATB Quality Manual Attachment Eiv: Blood Agar Plates.**

4.6.5 References

World Health Organization. Technical Report on Critical Concentrations for Drug Susceptibility testing of Medicines Used in the Treatment of Drug-resistant Tuberculosis. Geneva, WHO, 2018 (WHO-CDS-TB-2018.5)

4.6.6 Documentation

GATB Quality Manual Attachment Eii: Ziehl-Neelsen or Kinyoun Stain Reagents
GATB Quality Manual Attachment Eiv: Blood Agar Plates

GATB Quality Manual Attachment Eviii: MGIT SIRE Drug Susceptibility Testing Kit
GATB Quality Manual Attachment Eix: MGIT PZA Drug Susceptibility Testing Kit
GATB Quality Manual Attachment Ex: Moxifloxacin and Kanamycin Drug Susceptibility Testing
GATB Mycobacteriology Quality Manual Attachment J: MGIT 960 Daily Maintenance Log
GATB Mycobacteriology Quality Manual Attachment K: MGIT Calibration Tube Log
GATB Mycobacteriology Quality Manual Attachment M: Continuous Quality Improvement
MGIT Unloaded AST Report
Appendix B: DST LRF

4.7 SOP 7: Minimum Inhibitory Concentration (MIC)

4.7.1 Purpose

This SOP is for use by the UCL Central Mycobacteriology Laboratory to determine the minimum concentration of each of the study drugs that inhibits the growth of *Mycobacterium tuberculosis* (MTB) in liquid medium. Minimum Inhibitory Concentration (MIC) testing will be performed on baseline and post-baseline isolates. Depending upon the study the following drugs will be tested:

- **ZeNix:** Bedaquiline (B), pretomanid (Pa) and linezolid (L)
- **SimpliciTB:** Bedaquiline (B), pretomanid (Pa) and moxifloxacin (M)

NOTE:

- Prior to any MIC testing, a Hain MTBC assay (SOP 3, Section 4.3) is performed at the UCL Central Mycobacteriology Laboratory to confirm the presence of MTB complex (MTBC). If the Hain test does not confirm MTBC, the local laboratory will be contacted, and a new culture will be sent.
- Only SimpliciTB isolates first found resistant to 0.25 µg/ml moxifloxacin by MGIT DST will be subjected to MIC for the drug.

4.7.2 Principle

MIC testing is based on the same principle as the MGIT DST (see SOP 6; Section 4.6). For MIC, a range of concentrations of the test drugs (Pa, B, L, M) are added to a panel of MGIT tubes and inoculated with the test culture. A growth control (GC) tube with 1/100 the inoculum of the test isolate is included with each test and serves as a comparison for growth. If the test drug concentration is active against the mycobacterial isolate (susceptible), growth will be inhibited and fluorescence will be suppressed in the drug-containing tube (and hence growth units (GU) suppressed). The growth control (GC) and any drug concentrations at which the mycobacterial isolate is resistant will grow and will have increasing fluorescence and GU detected by the MGIT instrument.

To interpret this growth, when the GC tube reaches 400 GU the test is considered finished and the growth units are checked for every drug concentration. In the drug containing tubes, results above 100 GU are considered resistant to that particular concentration, while anything lower than 100 GU is considered susceptible. The MIC is defined as the lowest concentration where the GU are less than 100 GU.

For standard MGIT-based breakpoint DSTs (SOP 6, Section 4.6), the interpretation can be performed by the instrument and included on the antimicrobial susceptibility testing (AST) reports. For MIC tests and breakpoint DSTs (outside of the standard BD sets), the EpiCentre software (a user interface for the MGIT instrument) including the TBeXiST (TB eXtended Susceptibility Testing) module must be used. With this eXtended Susceptibility Testing module, it is possible to:

- Extend susceptibility testing to all TB isolates against primary drugs. For example, some MDR-TB and XDR strains are dysgenic and therefore may not reach a completed signal within the 13 days required for the growth control (GU>400) for automatic DST interpretation of SIRE results in MGIT instruments.
- Freely test a drug belonging to the currently described 2nd line classes, and potential new drugs.
- MIC testing of any chosen drugs at a range of freely chosen concentrations. This is the scope of using the EpiCentre and TBeXiST covered in this SOP.

4.7.3 Procedure

This MIC protocol will be carried out with all MTB isolates received at the UCL Central Mycobacteriology Laboratory eligible for DST testing as described above.

MIC testing will be performed using pretomanid (Pa), bedaquiline (B), and linezolid (L) or moxifloxacin (M), see Table 7 for manufacturer and storage details. The standard concentrations tested for these drugs are 2-fold serial dilutions, as detailed in Table 8 for the isolates and Table 9 for the control strain H37Rv which is included in every run. If MIC values obtained for any drug are outside the routine range, the test will be repeated at a higher (Pa, B, L) or lower ranges (Pa only), as defined in Table 12.

4.7.3.1 Equipment/Reagents

- Biological Safety Cabinet (BSC)
- Plastic discard container with absorbent Vernagel sachets
- Mycobactericidal disinfectant (specified in local Health and Safety guidelines)
- Vortex mixer
- Micropipettes (p1000, p200 and p20)
- Filtered pipette tips (100-1000µl tips, 10-200µl tips and 1-20µl tips)
- Appropriate racks to contain all the necessary tubes
- 7ml MGIT tubes
- BD BACTEC MGIT supplement (OADC)
- Sterile saline
- Sterile 5mm glass beads
- Blood agar plates (BAP)
- Drug stocks – see below for preparation details
- Sterile DMSO for drug stock serial dilutions
- 1.5ml Eppendorf tubes

4.7.3.2 Preparation of drug stocks and working solutions

Table 7: Manufacturer and storage instructions for the study drugs

Drug	Code	Solubility	Storage (Powder)	Manufacturer
Pretomanid	Pa	DMSO	4°C	Metrics Inc
Bedaquiline	B	DMSO	RT	Janssen
Linezolid	L	DMSO	-20°C	Generon
Moxifloxacin	M	Water	4°C	Becton Dickinson (BD)

Table 8: Routine drug concentrations used for MGIT MIC testing of clinical isolates

Drug		Drug concentration (µg/ml)				
Pa	1	0.5	0.25	0.125	0.063	0.032

B			1	0.5	0.25	0.125
L				1	0.5	0.25
M	2	1	0.5	0.25	0.125	0.063

Table 9: Drug concentrations used for MGIT MIC testing of H37Rv

Drug	Drug concentration (µg/ml)			
Pa	0.25	0.125	0.063	
B	0.5	0.25	0.125	
L	1	0.5	0.25	
M	0.5	0.25	0.125	0.063

Drug manufacturer details and storage conditions for each of the study drugs are detailed in Table 7.

For preparation of the **stock solutions** see directions below (and Table 10 and Table 11):

Pretomanid (Pa): Dissolve 0.04g (40mg) of Pa in 10ml of sterile DMSO (4mg/ml stock solution). Aliquot into sterile cryotube vials; volumes of 50µl, or as required.

Bedaquiline (B): Dissolve 0.04g (40mg) of B in 10ml of sterile DMSO (4mg/ml stock solution). Aliquot into sterile cryotube vials; volumes of 50µl, or as required.

Linezolid (L): Dissolve 0.025g (25mg) of L in 3.56ml of sterile DMSO (14mg/ml stock solution). Aliquot into sterile cryotube vials; volumes of 50µl, or as required.

Moxifloxacin (M): Reconstitute each vial of BD Moxifloxacin Hydrochloride drug (498µg) with 3ml of sterile distilled/deionised water to make a solution of 166µg/ml. Aliquot into sterile cryotube vials; volumes of 500µl, or as required.

Once reconstituted, the aliquoted antibiotic solutions should be stored frozen at -20°C or colder for up to six months, or up to the original expiry date of the drug powder vial, whichever comes first. Once thawed, use immediately. Discard any unused portions.

Table 10: Pretomanid, Bedaquiline and Linezolid concentrations used for routine MGIT MIC Testing

	Stock Solution preparation			Working Solution preparation for Routine Testing		
Drug	Dry Weight (mg)	Reconstitution volume (DMSO, ml)	Stock Concentration (mg/ml)	Dilution Factor (DF) and volume (in DMSO)	Volume added to MGIT	Final Concentration (in MGIT tube of 8.4ml total)
Pretomanid (Pa)	40	10	4	**DF 1:47.6** 42µl to 1958µl	100µl	1 µg/ml
Bedaquiline (B)	40	10	4	**DF 1:47.6** 42µl to 1958µl	100µl	1 µg/ml
Linezolid (L)	25	3.560	7	**DF 1:83.3** 24µl to 1976µl	100µl	1 µg/ml

Table 11: Moxifloxacin concentration used for MIC Testing

Drug	Dry Weight (µg)	Diluent	Reconstitution Volume (H2O, ml)	Working Concentration (µg/ml)	Volume added to MGIT	Final Concentration (in MGIT tube of 8.4ml total)
Moxifloxacin (M)	498 µg	Sterile Distilled/ Deionised Water	3.0	166	100µl	2 µg/ml

The concentrations described in Table 10 and Table 11 correspond to the highest of the range in Table 8. Starting from this concentration, serial 2-fold dilutions in DMSO (Pa, B and L) or sterile distilled/deionised water (M) will be repeated to achieve the lowest concentration to be tested. The exact volumes used to prepare the 2-fold dilutions will depend on the number of samples being tested in a batch (See Step 2: Prepare and Add Drugs).

In cases where MIC testing gives a result out of the routine testing range, this must be repeated at higher (Pa, B, L) or lower (Pa only) concentration ranges as defined in Table 12. Higher and lower ranges must overlap with the routine testing concentrations by two dilutions (as shown by the hashed boxes in Table 12) to ensure the MIC is not missed in the repeat run because of inter-assay variation.

For low-range testing of pretomanid, working drug solutions should be prepared as for the routine concentration range (Table 10), but making two further 2-fold dilutions in the series (W7 and W8) as defined in Step Two: Prepare and Add Drugs.

Table 12: High, Routine and Low MIC drug testing concentrations

Drug	Drug concentration (µg/ml)											
Pa	16	8	4	2	1	0.5	0.25	0.125	0.063	0.032	0.016	0.008
L				8	4	2	1	0.5	0.25			
B		8	4	2	1	0.5	0.25	0.125				

High MIC Drug Testing Concentrations

Routine MIC Drug Testing Concentrations

Low MIC Drug Testing Concentrations

Included in Routine and low MIC Drug Testing

Included in Routine and high MIC Drug Testing

For high-range testing, working solutions should be prepared from the same stock solutions as defined in Table 10, but using the dilution steps outlined in Table 13. Serial dilutions will be prepared as defined in Step Two: Prepare and Add Drugs.

NOTE: Testing of higher or lower concentration ranges must always be accompanied by the reference strain H37Rv tested at the same concentration range as for routine testing, as defined in Table 9. This means additional 2-fold dilutions (as defined in Step Two: Prepare and Add Drugs) will need to be prepared in the series when for Higher concentration ranges, to ensure the required H37Rv range is covered. These lower drug concentrations only need to be used for the H37Rv drug tubes.

Table 13: Preparation of working solutions for high range concentration MIC testing

Drug	Stock Concentration (mg/ml)	Dilution Factor (DF) and volume (in DMSO)	Volume added to MGIT	Final Concentration (in MGIT tube of 8.4ml total)
Pretomanid (Pa)	4	**DF 1:2.97** 672µl to 1328µl	100µl	16 µg/ml
Bedaquiline (B)	4	**DF 1:5.95** 336µl to 1664µl	100µl	8 µg/ml
Linezolid (L)	7	**DF 1:10.4** 192µl to 1808µl	100µl	8 µg/ml

If required in exceptional circumstances (i.e. to resolve a discrepancy) or as requested by the sponsor, testing may be required at different concentration ranges to those outlined in Table 12, details of the preparation of the working drug solution must be detailed on the MIC LRF (Appendix C) in the Comments section.

4.7.3.3 TBeXiST Workflow Description Overview

An overview of the TBeXiST workflow is as follows:

1. Request and register the MIC tests in TBeXiST, print barcodes and assign to MGIT tubes
2. Add MGIT supplement and diluted drugs to the MGIT tubes (this can be done outside the CL3 laboratory)
3. Prepare test inocula and add to the MGIT tubes (this should be carried out inside a BSC in the CL3/BSL3 laboratory)
4. Load tubes into the MGIT instrument
5. Once test complete, interpret and finalise the test
6. Remove completed tubes from the MGIT instrument
7. Print the Interpretation Report and complete MIC LRF (Appendix C) with MIC values

Step 1: Specimen Registration, order TBeXiST tests, print barcodes

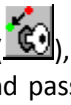
For routine MIC testing in the TB Alliance studies, all drugs will be tested together at the concentrations outlined in Table 8 and compared against a single growth control. As required, individual drugs can be repeated in isolation or the concentration range adjusted if the MIC value for a given sample is out of the routine range. Higher and lower concentration ranges are defined in Table 12, and working stock calculations for high range testing is outlined in Table 13.

One BACTEC MGIT 7ml tube is required per drug concentration tested and one per growth control per test group (i.e. for each test run with a given MTB isolate/Lab accession number). Hence for routine testing, 14 tubes are required for each ZeNix sample and 17 tubes for each SimpliciTB sample, if moxifloxacin is tested, and 11 tubes, if moxifloxacin is not tested:

- 1 tube - Growth Control
- 6 tubes - Pretomanid concentrations
- 4 tubes - Bedaquiline concentrations
- 3 tubes - Linezolid concentrations (ZeNix samples only)
- 6 tubes - Moxifloxacin concentrations (only for SimpliciTB samples which are resistant to Moxifloxacin MGIT DST at 0.25µg/ml)

Prior to use, examine all MGIT tubes for evidence of damage. Do not use any tube that is cracked or has other defects. Do not use a tube if the medium is discoloured, cloudy or appears to be contaminated.

Create Patient/Specimen - for further stepwise instructions on the using the EpiCenter software for specimen registration including annotated screenshots, refer to the **Registering TBeXiST MGIT tube for MIC-DST Users Guide**.

1. Logon to the EpiCenter by clicking the Logon icon (), and enter the assigned user name and password. Each operator has a unique username and password which allows an audit trail to see which user performed different activities/tests.

2. Click the specimen registration icon () and go to the Rapid Login tab. In the Rapid Login tab, fill in the Patient ID and the Patient Name fields with the study patient identifier preceded by the prefix 'Z' for the ZeNix-TB study (NC-007) and 'S' for the SimpliciTB study (NC-008) (format Z-00-0000-000 or S-00-0000-000). Assign an accession number – this will be the ZeNix-TB or SimpliciTB laboratory accession number and the date in the following format, e.g. P1234567_20Nov2018.

   NOTE: It is essential that the accession number field is unique to every test run (each set of tests samples and the corresponding growth control), so the date must be included to allow for repetition of testing on a single accession number. If multiple independent tests are set up on a given sample on the same day (unlikely), then run numbers will also need to be added to the accession number field e.g. P1234567_20Nov2018_run1.

3. Order the Growth Control by selecting the "TBeX Growth Control" Test Group from the Available Tests frame. In the pop-up window, the 'Isolate Number' must be completed as '1' and the 'organism ID' as '*Mycobacterium tuberculosis*'. Scan the first MGIT tube using the barcode reader – the test and the MGIT tube number will appear in the 'Ordered Tests' frame.

4. Order the Antimicrobial test groups. These are pre-programmed test groups for each drug at the concentrations specified in Table 8. Select 'TBeX-Pretomanid' to add the concentrations for pretomanid to the 'Ordered Tests' frame. As above complete Isolate number as '1' and scan each of the MGIT tube barcodes in order. Repeat for bedaquiline by selecting 'TBeX-Bedaquiline'.

5. For ZeNix TB (NC007) samples select the test group 'TBeX-Linezolid' and for SimpliciTB (NC008) samples select 'TBeX-Moxifloxacin', if required.

6. The 'Ordered tests' frame will now list 14 test drug concentrations and a growth control per sample for Zenix TB samples. For SimpliciTB samples the frame will list either 17 (if moxifloxacin is tested) or 11 (if moxifloxacin is NOT tested) test drug concentrations and a growth control per sample. Each tube will be listed with its associated MGIT tube number (in sequence # column); press save.

7. Individual drug concentrations can also be selected for any drug, as needed, if testing higher or lower concentration ranges.

8. Once saved, all the new barcode labels will be printed automatically. These should then be affixed to each corresponding MGIT tube at the same height as the pre-existing MGIT tube barcode (sequence #) and without obscuring it. Cross-reference the sequence # on the MGIT tube with that on the 'Ordered Tests' frame to ensure each label is attached to the correct tube.

Figure 3: Example of printed barcodes

Step 2: Prepare and add drug dilutions to MGIT tubes

For preparation of the drug sets:
Aseptically add 0.8 mL of BACTEC MGIT Supplement in all tubes. BBL MGIT OADC 15 ml (cat# 245116), BBL Middlebrook OADC Enrichment (cat# 212240) or BACTEC MGIT SIRE supplement can be used.

NOTE: It is recommended that not more than 4 samples plus the H37Rv control are tested at once, so MGIT tubes numbers are manageable. The below dilution series is an example for preparing drug tubes in this scenario, making sufficient to allow for pipetting. However, drug serial dilutions volumes should be adjusted if smaller or larger (e.g. testing only one drug with more samples) sample numbers are being tested.

Pretomanid

Prior to use, thaw one vial of stock solution, ensure the drug is fully dissolved and follow the steps below:

1. Take one vial of stock solution (4mg/ml) and prepare the working solution (1.0µg/ml final concentration in the MGIT), as per the details in Table 10. This 2ml working solution (W1) is the highest drug concentration and the volume is sufficient for 6 isolates.
2. Add 800µl of DMSO to 5 screw-cap Eppendorf tubes (labelled W2-W6) for the serial dilutions for each drug.
3. For each drug, take 800µl of W1 and add to the DMSO in the W2 tube, mix by pipetting. This will create a new, two-fold diluted working solution (0.5µg/ml final concentration in the MGIT).
4. Take 800µl of W2 and add to the DMSO in the WS3 tube, mix by pipetting. This will create a new, two-fold diluted working solution (0.25 µg/ml final concentration in the MGIT)
5. Take 800µl of W3 and add to the DMSO in the W4 tube, mix by pipetting. This will create a new, two-fold diluted working solution (0.12 µg/ml final concentration in the MGIT)
6. Take 800µl of W4 and add to the DMSO in the W5 tube, mix by pipetting. This will create a new, two-fold diluted working solution (0.06 µg/ml final concentration in the MGIT)
7. Take 800µl of W5 and add to the DMSO in the W6 tube, mix by pipetting. This will create a new, two-fold diluted working solution (0.03 µg/ml final concentration in the MGIT)
8. Add 100µl of each working solution to the corresponding MGIT tubes. The drug and drug concentration are clearly marked on the TBeXiST barcode label.
   NOTE: for the H37Rv control panel, only W3-5 are required as only 3 concentrations are tested (see Table 9)

Bedaquiline

Prior to use, thaw one vial of stock solution, ensure the drug is fully dissolved and follow the steps below:

1. Take one vial of stock solution (4mg/ml) and prepare the working solution (1.0µg/ml final concentration in the MGIT), as per the details in Table 10. This 2ml working solution (W1) is the highest drug concentration and the volume is sufficient for 6 isolates.
2. Add 800µl of DMSO to 3 screw-cap Eppendorf tubes (labelled W2-W4) for the serial dilutions for each drug.

3. For each drug, take 800µl of W1 and add to the DMSO in the W2 tube, mix by pipetting. This will create a new, two-fold diluted working solution (0.5µg/ml final concentration in the MGIT).
4. Take 800µl of W2 and add to the DMSO in the W3 tube, mix by pipetting. This will create a new, two-fold diluted working solution (0.25 µg/ml final concentration in the MGIT)
5. Take 800µl of W3 and add to the DMSO in the W4 tube, mix by pipetting. This will create a new, two-fold diluted working solution (0.12 µg/ml final concentration in the MGIT)
6. Add 100µl of each working solution to the corresponding MGIT tubes. The drug and drug concentration are clearly marked on the TBeXiST barcode label.
   NOTE: for the H37Rv control panel, only W2-4 are required as only 3 concentrations are tested (see Table 9)

Linezolid

Prior to use, thaw one vial of stock solution, ensure the drug is fully dissolved and follow the steps below:

1. Take one vial of stock solution (7mg/ml) and prepare the working solution (1ug/ml final concentration in the MGIT) as per the details in Table 10. This 1ml working solution (W1) is the highest drug concentration and the volume is sufficient for 4 test isolates.
2. Add 800µl of DMSO to 2 screw-cap Eppendorf tubes (labelled W2-W3) for the serial dilutions.
3. Take 800µl of W1 and add to the DMSO in the W2 tube, mix by pipetting. This will create a new, two-fold diluted working solution (0.5µg/ml final concentration in the MGIT)
4. Take 800µl of W2 and add to the DMSO in the W3 tube, mix by pipetting. This will create a new, two-fold diluted working solution (0.25µg/ml final concentration in the MGIT).
7. Add 100µl of each Working solution to the corresponding MGIT tubes. The drug and drug concentration are clearly marked on the TBeXiST barcode label.

NOTE: for the H37Rv control panel all 3 concentrations are tested (see Table 9)

Moxifloxacin

Prior to use, thaw one of the moxifloxacin working solution (166 µg/ml), ensure the drug is fully dissolved and follow the steps below:

1. Take one vial of working solution (166 µg/ml), this 0.5 ml working solution (W1) is the highest drug concentration and the volume is sufficient for 2 isolates.
2. Add 250µl of Sterile Distilled/ Deionised water to 5 screw-cap Eppendorf tubes (labelled W2-W6) for the serial dilutions for each drug.
3. For each drug, take 250µl of W1 and add to the water in the W2 tube, mix by pipetting. This will create a new, two-fold diluted working solution (1.0 µg/ml final concentration in the MGIT).
4. Take 250µl of W2 and add to the water in the W3 tube, mix by pipetting. This will create a new, two-fold diluted working solution (0.5 µg/ml final concentration in the MGIT)
5. Take 250µl of W3 and add to the water in the W4 tube, mix by pipetting. This will create a new, two-fold diluted working solution (0.25 µg/ml final concentration in the MGIT)
6. Take 250µl of W4 and add to the water in the W5 tube, mix by pipetting. This will create a new, two-fold diluted working solution (0.12 µg/ml final concentration in the MGIT)

7. Take 250µl of W5 and add to the water in the W6 tube, mix by pipetting. This will create a new, two-fold diluted working solution (0.06 µg/ml final concentration in the MGIT)
8. Add 100µl of each Working solution to the corresponding MGIT tubes. The drug and drug concentration are clearly marked on the TBeXiST barcode label.
   NOTE for the H37Rv control panel, only W3-6 are required as only 4 concentrations are tested (see Table 9)

Step 3: Preparation of test inoculum and inoculation of MGIT tubes - carried out in BSC in CL3/BSL3 laboratory

Preparing inoculum from positive MGIT (preferred)

Once a MGIT tube has become positive it must be used for MIC testing within 1-5 days. The concentration of the inoculum is critical to the correct performance of susceptibility testing and the following instructions must be adhered to strictly. On the day the MGIT flags positive (day 0), the culture should be re-incubated for a minimum of one extra day (day 1). This can be in the MGIT machine or in a separate 37°C incubator.

Day 1 and Day 2 – the growth in the tube can be used directly. Glass beads should be used to break up the clumps in the MGIT culture and obtain a uniform bacterial suspension. Pre-prepared sterile glass beads (minimum 4 beads, 5 mm diameter) in saline (3 ml) are used. Remove the saline by pipetting and pour all the beads into the MGIT tube. Mix well by vortexing to break up clumps as much as possible (between 2 and 10 minutes) and leave to settle for at least 30 minutes. Use the supernatant undiluted.

Days 3, 4 and 5 – the growth in the tube should be diluted before use. Vortex the MGIT culture with beads as described above and allow to settle for at least 30 minutes. Dilute 2 ml of supernatant in 8 mL of sterile saline (1:5 dilution). Use this diluted culture for the MIC drug tubes.

NOTE: to be able to run a complete set of all three drugs at once, the MGIT culture must be incubated until days 3-5 and diluted as described above in order to have sufficient inocula for the number of MGIT tubes per sample, as specified above in section 4.7.3.4.

>5 Days – sub-culture (as detailed below) into a new MGIT tube and wait for this to flag positive. Treat as above and use within 5 days to set up the MIC test.

NOTE: Cultures grown in liquid or solid media can be used to prepare a seed MGIT tube. When positive, the seed MGIT can then be used to prepare the inoculum as described above. From a liquid culture, a 1:100 dilution should be made of the broth, and 500µl added to the seed MGIT tube. For solid media, a loop of growth scraped from the slope or plate should be added to the seed MGIT tube. After incubation, the TTD of the seed MGIT tube must be 4 days or more for use as a MIC test inoculum. If the seed tube becomes positive in less than 4 days, a new seed MGIT tube should be prepared.

Preparing an inoculum from an LJ slope

1. Add 4ml of Middlebrook 7H9 Broth (or BBL MGIT broth) to a 16.5 x 128 mm sterile tube with a cap containing 8 – 10 glass beads.
2. Scrape with a sterile loop as many colonies as possible from growth no more than 14 days old, trying not to remove any solid medium. Suspend the colonies in the Middlebrook 7H9 Broth.
3. Vortex the suspension for 2 – 3 min to break up the larger clumps. The suspension should exceed a 1.0 McFarland (MF) standard in turbidity.

4. Let the suspension sit for 20 min without disturbing.
5. Transfer the supernatant fluid to another 16.5 x 128mm sterile tube with cap (avoid transferring any of the sediment) and let sit for another 15 min.
6. Transfer the supernatant fluid (it should be smooth, free of any clumps) to a third 16.5 x 128mm sterile tube.

   NOTE: The organism suspension should be greater than a 0.5MF standard at this step.

7. Adjust suspension to a 0.5MF standard by a visual comparison with a 0.5MF turbidity standard. Do not adjust below a 0.5MF Standard.
8. Dilute 2ml of the adjusted suspension in 8mL of sterile saline (1:5 dilutions).

Growth Control tube preparation and inoculation

1. Dilute the organism suspension (used to inoculate drug tubes) to achieve a 1:100 suspension to be used as the GC suspension (1% growth control), as follows:
   a. Aseptically pipette 0.1 ml of the organism suspension into 0.9 ml of sterile saline to prepare a 1:10 dilution. Tightly recap the tube, and mix thoroughly by inversion.
   b. Aseptically pipette 0.1 ml of the 1:10 dilution into 0.9 ml of sterile saline to prepare the 1:100 dilution to be used for the GC inoculation. Tightly recap the tube, and mix thoroughly by inversion.
2. Inoculate 0.5ml of the 1:100 GC suspension into the labelled Growth Control (GC) MGIT tube, using a micropipettor and aerosol resistant tips. The Lab Accession Number and Subject ID (or screening number if pre-enrolment) will be clearly marked on the TBeXiST barcode label.
3. Tightly recap the tube.
4. Mix tubes thoroughly by gentle inversion 3 to 4 times.

Inoculation of tubes containing test drugs

1. Aseptically pipette 0.5 ml of the organism suspension into each of the remaining drug tubes, using a micropipettor and aerosol resistant tips.
2. Tightly recap the tubes.
3. Mix tubes thoroughly by gentle inversion 3 to 4 times.

Purity check

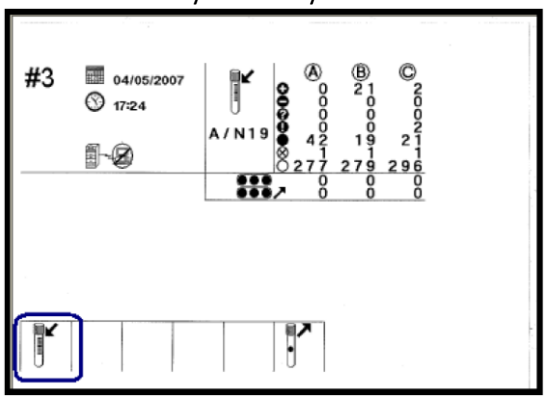
1. To ensure the inoculum that was used to set up the MIC test is not contaminated, spread 10µl of the organism suspension to a blood agar plate (BAP).
2. Seal the BAP in a plastic zip-lock bag.
3. Incubate at 35 - 37°C.
4. Check the BAP at 48 hours for bacterial contamination. If it shows no growth, then allow MIC test to proceed. If the BAP shows growth, discard the MIC tubes and repeat testing with pure culture.

Step 4 - Tube Loading

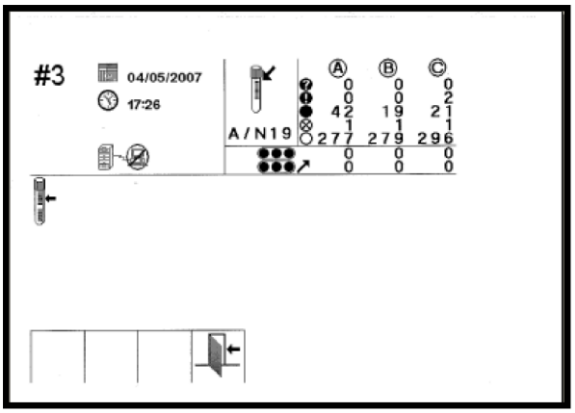
In the BD BACTEC™ MGIT™ software, the option “Accession Barcoding” must be enabled. This will allow the MGIT Instrument to scan both the MGIT tube barcode (sequence #) and the Accession barcode (on the TBeXiST generated barcode label)

NOTE: As all tubes belong to the same TB eXiST set (drug containing tubes and growth control for a given sample), they must have the same start-of-test date and time. **THEY MUST ALL BE LOADED SIMULTANEOUSLY IN THE SAME DRAWER** of the BD BACTEC MGIT instrument by scanning their sequence barcode number and accession barcode number. DO NOT CLOSE THE DRAWER UNTIL ALL TUBES HAVE BEEN ADDED. It is also strongly recommended not to open any drawer during the reading window [hour - 2 minutes (:58) till hour + 5 minutes (:05)] so no tube readings are missed.

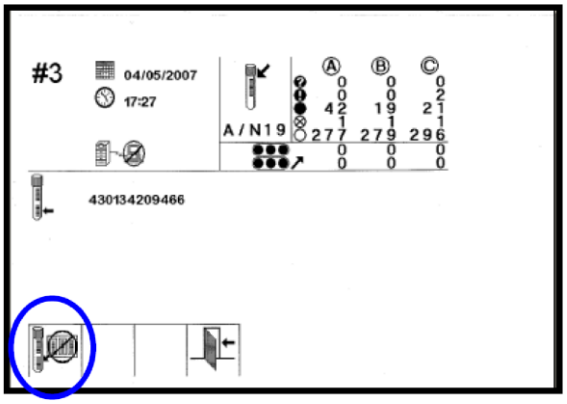
1. Open a drawer and press the "Tube Entry" Soft Key



2. Scan the Tube Sequence #



3. Scan the Accession # (TBeXiST barcode)



4. Load the tube into the instrument - Insert the tube in the station with the green indicator.
5. Repeat steps 2-4 for all remaining tubes and close the drawer

An Accession # associated to TBeXiST tests MUST NOT BE MODIFIED AT ANY TIME. Once inserted into the instrument, a tube MUST NOT BE MOVED to another position in the same drawer or another drawer.

Step 5: Interpreting and Finalising the results of completed test runs

For further stepwise instructions on using the EpiCenter software for interpreting and finalising results including annotated screenshots, refer to the Interpretation and Reporting of MIC-DST in TBeXiST Users Guide.

1. Login to the EpiCenter as described above. Click on the Reports icon, and in the 'Filter Reports' tab, select the TBeXiST Worklist. Click on 'Print Preview', select the 'Enter Accession Number' Prompt (number 4) and enter the Lab Accession Number_Date information for the sample of interest.
2. Print the TBeXiST Worklist, staple together the pages and sign and date. This worklist shows the growth units in all the drug containing tubes at the point the growth control reached 400 GU (the first MGIT instrument reading where the GC ≥ 400 GU). It also shows the GU at the time of generation of the worklist (if different). It is the column at the time point that the GC ≥ 400 GU that must be used for the interpretation (see Figure 4 for an example TBeXiST worklist).

   NOTE: This signed and dated worklist must be kept in the associated TBA LRF folders as it is the source data for the GU in each tube at the time of interpretation.

Figure 4: Example of TBeXiST worklist

TB eXiST Worklist

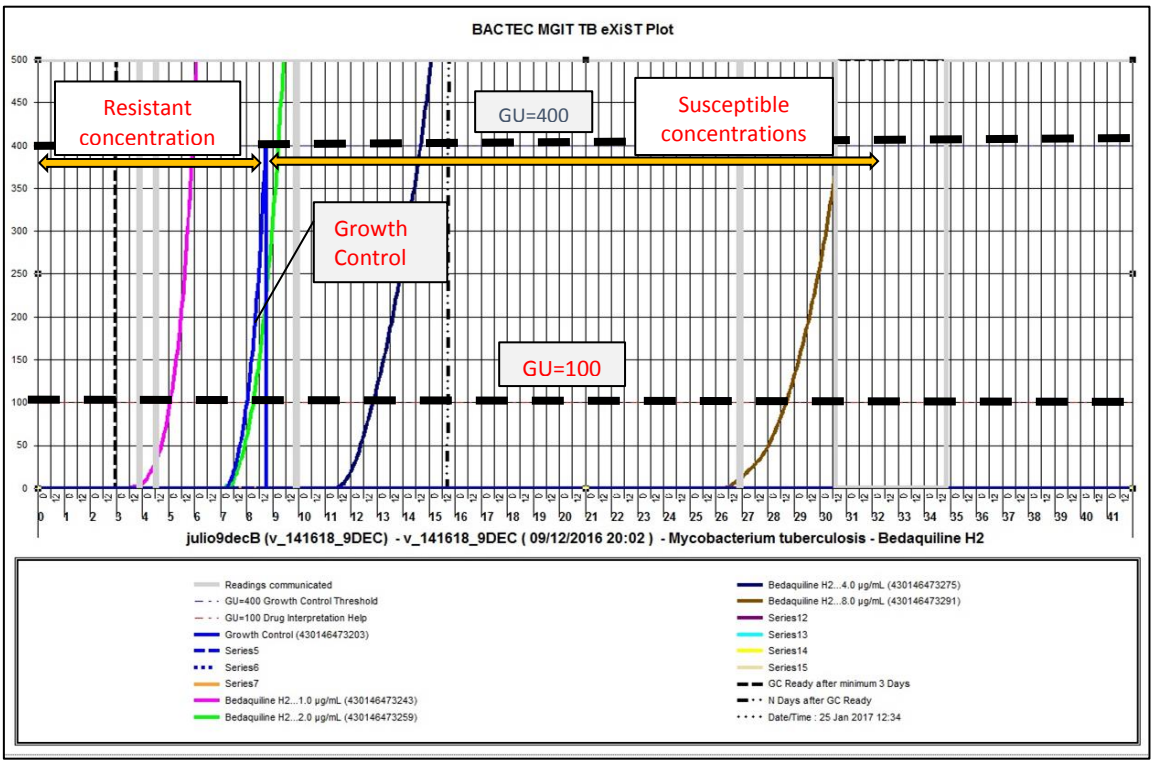
Filter Name: TB eXiST Worklist.flt
Enter Accession Number = Example_DDMMYYYY
Sorted By: None

DD/MM/YYYY
Page 1

		GU when GC reached 400	Actual or Last GU (GC on board)	Test Status --- TBeXiST/MGIT Tube	Extended GU (GC removed)
Patient number: Example_exampleYYYYMMDD					
Accesion #: Example_YYYYMMDD					
Growth Control (days:hours):		406 (15; 2)	15116 (**;20)	---In Progress/Positive	
Start DT: DD/MM/YYYY hh:mm					
Bedaquiline	...0.06 µg/mL	14016 (15; 2)	15478 (**;20)	--- In Progress	
Bedaquiline	...0.12 µg/mL	13885 (15; 2)	16742 (**;20)	--- In Progress	
Bedaquiline	...0.25 µg/mL	3497 (15; 2)	14759 (**;20)	--- In Progress	
Bedaquiline	...0.5 µg/mL	0 (15; 2)	10014 (**;20)	--- In Progress	
Bedaquiline	...1.0 µg/mL	0 (15; 2)	0 (**;20)	--- In Progress	
Bedaquiline	...2.0 µg/mL	0 (15; 2)	0 (**;20)	--- In Progress	
Linezolid	...0.25 µg/mL	3367 (15; 2)	14271 (**;20)	--- In Progress	
Linezolid	...0.5 µg/mL	0 (15; 2)	9211 (**;20)	--- In Progress	
Linezolid	...1.0 µg/mL	0 (15; 2)	26 (**;20)	--- In Progress	
Linezolid	...2.0 µg/mL	0 (15; 2)	0 (**;20)	--- In Progress	
Linezolid	...4.0 µg/mL	0 (15; 2)	0 (**;20)	--- In Progress	
Linezolid	...8.0 µg/mL	0 (15; 2)	0 (**;20)	--- In Progress	
Pretomanid	...0.06 µg/mL	2701 (15; 2)	14271 (**;20)	--- In Progress	
Pretomanid	...0.12 µg/mL	0 (15; 2)	6020 (**;20)	--- In Progress	
Pretomanid	...0.25 µg/mL	0 (15; 2)	1588 (**;20)	--- In Progress	
Pretomanid	...0.5 µg/mL	0 (15; 2)	952 (**;20)	--- In Progress	
Pretomanid	...1.0 µg/mL	0 (15; 2)	0 (**;20)	--- In Progress	
Pretomanid	...2.0 µg/mL	0 (15; 2)	31 (**;20)	--- In Progress	

3. Generate TBeXiST plots for all of the test drugs – this is a graphical representation of the GU seen in the worklist, with a growth curve shown for each drug concentration. See Figure 5 for an annotated example. To do this, click on the 'Reports' icon, and in the 'Filter Reports' tab, select 'BACTEC MGIT TBeXiST plot'. Click on 'Print Preview', select 'Enter Accession Number' from the table and enter the accession number of interest, then select 'Enter Antimicrobial Name' and enter the drug name. Select 'Start plot at day' and enter 0 and 'End plot with day' and enter the last day with a reading result or the date the plot is being generated. Click 'Run'. A separate plot has to be created for each drug. To print these plots in colour, they need to be saved to file and transferred out of the laboratory using a memory stick. To do this, select 'Export', select a JPEG file type, and save in the ZeNix-TB eXiST Plots folder using the filename format of Subject ID_Lab Accession No_Date_Drug. Next, carry out the interpretation and finalising of result. Click on the 'Data View' icon, and select 'TBeXiST Specimens in Progress'. From the sample listing table select the first Lab Accession Number on the TBeXiST worklist. Right click and select 'Specimen Registration' option. This is the window where each drug concentration is assigned a susceptible or resistant interpretation based on the GC noted on the worklist.
4. Select the Growth Control tube from the 'Ordered Tests' frame; choose 'Threshold 400 Reached' from the 'Test Status' window and tick the 'Finalised' box.
5. Working through each drug containing tube – select the tube from the 'Ordered Tests' frame and refer to the GU for the selected drug on the printed worklist. If the GU are ≥100, choose 'Complete-R' from the 'Test Status' window, 'R' from the Interpretation drop down menu, and tick the 'Finalised' box. If the GU are <100, choose 'Complete-S' from the 'Test Status' window, 'S' from the Interpretation drop down menu, and tick the 'Finalised' box.
6. When this is complete for all 12 drug containing tubes, click 'Save'.

Figure 5: Example of a TBeXiST plot, annotated to show the 'resistant' and 'Susceptible' growth curves relative to the Growth Control



To print these plots in colour, they need to be saved to file and transferred out of the laboratory using a memory stick. To do this, select 'Export', select a JPEG file type, and save in the ZeNix-TB eXiST Plots folder using the filename format of Subject ID_Lab Accession No_Date_Drug.

Step 6: Generate Interpretation Report and complete Appendix C (MIC LRF) with MIC values

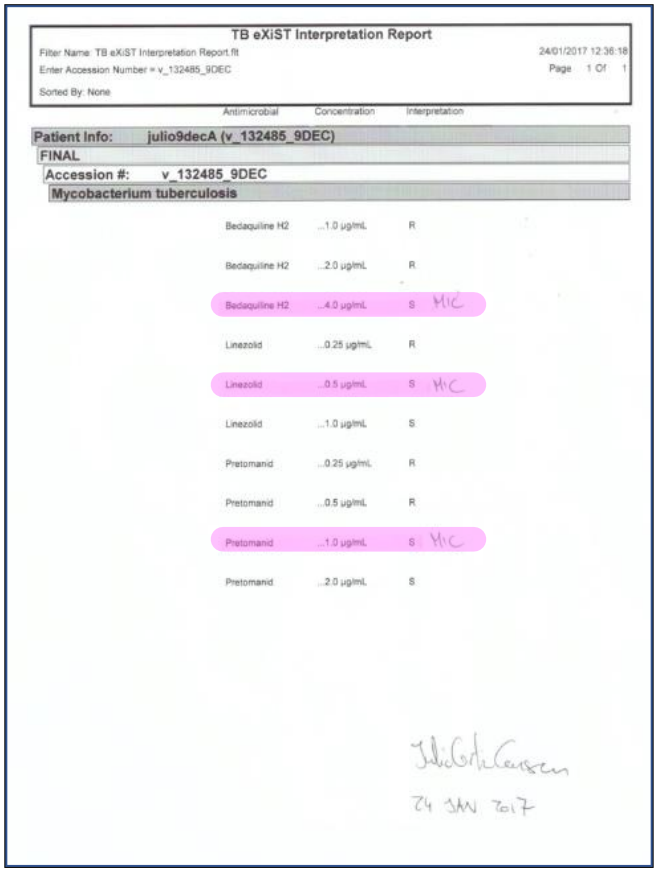
1. To generate an Interpretation Report of the finalised results, click on the Reports icon, and in the 'Filter Reports' tab, select 'TBeXiST Interpretation Report'. Click on 'Print Preview', select 'Enter Accession Number' from the table, enter the Accession Number of the report to be generated, and click 'Run'.
2. Print the TBeXiST Interpretation Report for each sample. One report will include all the drugs and concentrations tested. An example report is shown in Figure 6.
3. Annotate the printed report with the MIC concentration for each drug. **The MIC is the lowest concentration of a given drug that has been assigned a 'Susceptible' status (and therefore has growth units <100).** As the assigning of 'Susceptible' and 'Resistant' status is done manually, the Interpretation report should be cross checked with the corresponding Worklist print out (showing the GU) to double check the MIC is correct. The Interpretation report should then be signed and dated.
4. MIC values for each drug are then entered from the Interpretation report or the worklist into Appendix C: MIC LRF. If the MIC is out of the range of concentrations tested, it should be reported as >1.0µg/ml or ≤0.031µg/ml for Pa, >1µg/ml or ≤0.25µg/ml for L and >1µg/ml or ≤0.125µg/ml for B, >2 µg/ml or ≤0.063µg/ml for M.
5. A further round of testing is required at higher or lower concentration ranges as described in Table 12 and this should be noted in the comments section of the LRF. Details for the preparation of the working drug solutions for the high range testing are shown in Table 13.
6. Checks for transcription accuracy are performed as part of the verification of the LRF, comparing the LRF to the Worklist print out (showing the GU), and the LRF is signed off by the Laboratory Manager or delegate.

4.7.3.10 Step 7: Remove completed MGIT tubes from the instrument

1. The next step is to remove the completed tubes (those that have been finalised in the above process). Click on the 'Data View' icon and select '3. Remove and Finalise'; '1. TBeXiST Completed tubes'.
2. This shows the list of all tubes that have now been assigned a S/R interpretation and finalised. The table shows the drawer and tube position. Print this list and take print out into the CL3/BSL3 laboratory to identify and unload all the required tubes through the normal process of 'Unloaded Ongoing', 'Unloaded Positives' and 'Unloaded Negatives'. After unloading, click 'Refresh' and all samples in this list will disappear. Keep the unloaded tubes in case further processing is required – i.e. performing blood agar culture or Kinyoun stain (SOP2; Section 4.2) in the case of suspected contamination or a putative 'resistant' result (see section 4.6.3.8: Confirming resistant isolates).
3. Next click on the 'Data View' icon and select '3. Remove and Finalise'; '3. TBeXiST Removed Positives'. This is the list of all positive tubes that have now been assigned a S/R interpretation, finalised and removed from the MGIT machine. Select all records, right click and select 'Assign Test Status' from the pop-up menu. Select 'TB-eXiST- Complete' and click 'OK'.
4. Next click on the 'Data View' icon and select '3. Remove and Finalise'; '2. TB-eXiST – Removed Ongoings' and repeat step 3 to assign them a completed test status.

5. To Finalise tubes with a Negative Status that have been removed from the MGIT instrument (this will be infrequent as it will only occur if the MGIT tubes are left in the instrument for the standard 42 day protocol required for growth and detection), click on the 'Data View' icon, and select 'TBeXiST Specimens in Progress'. From the sample listing table select the Lab Accession Number of the sample to be finalised. Select the tube(s) with negative status and choose TBeXiST complete in the 'Test Status' window, and 'Save'.

Figure 6: Example of TBeXiST Interpretation Report



TB eXiST Interpretation Report

Filter Name: TB eXiST Interpretation Report.flt
Enter Accession Number = v_132485_9DEC
Sorted By: None

24/01/2017 12:36:18
Page 1 Of 1

Antimicrobial	Concentration	Interpretation
Patient Info:	**julio9decA (v_132485_9DEC)**	
FINAL		
Accession #:	**v_132485_9DEC**	
Mycobacterium tuberculosis		
Bedaquiline H2	...1.0 µg/mL	R
Bedaquiline H2	...2.0 µg/mL	R
Bedaquiline H2	...4.0 µg/mL	S MIC
Linezolid	...0.25 µg/mL	R
Linezolid	...0.5 µg/mL	S MIC
Linezolid	...1.0 µg/mL	S
Pretomanid	...0.25 µg/mL	R
Pretomanid	...0.5 µg/mL	R
Pretomanid	...1.0 µg/mL	S MIC
Pretomanid	...2.0 µg/mL	S

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4.7.4 Confirming resistant results

1. For any samples where the MIC is greater than the suggested 'resistant' threshold concentrations outlined in Table 14, a BAC and AFB smear should be prepared to confirm presence of AFB and rule out contamination. This should be done from the highest tube where growth was observed (the one before the MIC), and if necessary, the growth control. The blood agar and AFB results should be recorded in the table provided on the reverse of Appendix C: MIC LRF. If the MIC result is valid (AFB positive and no contamination present), the MIC test should be repeated to confirm the MIC value in a second test. Variability of one dilution either side of the first MIC value is acceptable for the repeat to be valid. If the two MIC values vary by more than one 2-fold dilution the test should be repeated again (from the earliest possible sub-culture – first UCL LJ or MGIT) to confirm which result is valid.

Table 14: MIC cut-off values for each drug, for samples considered to be resistant and requiring further testing

Drug	Pa	L	B	M
MIC (µg/ml) 'Resistant' Threshold Concentration	>1 µg/ml	>1 µg/ml	>1 µg/ml	>1 µg/ml

NOTE: If a Moxifloxacin MIC is performed (because the routine DST at cut off of 0.25µg/ml was resistant) and the results of the MIC are either 0.5 or 1µg/ml, it is not necessary to perform BA and Kinyoun stain (unless there is suspicion of contamination or unusual morphology). They should be treated as susceptible. The WHO cut-off for Moxifloxacin resistance by MGIT DST has been revised to 1µg/ml, but DST testing will remain at 0.25µg/ml for consistency across all trial testing.

4.7.5 Quality Control

It is important to perform quality control on the MIC testing procedure. This must be carried out for each new batch of drugs (Pa, L, B and M), MGIT tube and supplement lots, using the MTB reference strain H37Rv (ATCC 27294) which is susceptible to all of the test drugs. This should be recorded on GATB Quality Manual Attachment Eiii and Exi. In addition, for each new batch of drug stocks prepared from the drug powder, a second person must be present to ensure that the drug preparation is performed correctly. This must be documented on the appropriate GATB Quality Manual Attachment (Exi).

In addition, the H37Rv reference strain should be included on each run to check for MIC performance and control for any variability. The concentration range for the H37Rv control included with each batch of test samples is shown in Table 9 (for most drugs this is a narrower concentration range than used for test samples). The same drug preparation tubes/dilutions should be used for the control sample, omitting the top concentration as needed and using the subsequent serial dilutions. The results of a given test run should only be accepted if the H37Rv passes the QC giving an acceptable result (see Table 15). The results of this QC are recorded on the Interpretation Report for the H37Rv sample (signed and dated by the laboratory staff member to confirm the result was acceptable). If the QC fails, all results for the batch should be reviewed and, if necessary, new reagents purchased and prepared, and testing of clinical isolates repeated.

Table 15: Acceptable MIC range for the MTB H37Rv reference strain quality control

Drug	Pa	L	B	M
MIC (µg/ml)	0.125 and 0.25	0.5 and 1.0	0.25 and 0.5	0.125 to 0.5

If contamination is suspected in any samples, visually inspect the tubes, plate onto blood agar and prepare a smear for AFB staining (SOP 4). The blood agar and Kinyoun stain results should be recorded on the reverse of LRF5.

For all samples where contamination is detected, the H37Rv QC from the run is out of the acceptable range (see Table 15), or the results are not clear for another reason (clearly state the reason in the comments section of LRF5), the test must be repeated.

QC must also be carried out on each new batch of BA plates and **recorded on GATB Quality Manual Attachment Eiv: Blood Agar Plates.**

4.7.6 Documentation

GATB Quality Manual Attachment Eii: Ziehl-Neelsen or Kinyoun Stain Reagents
GATB Quality Manual Attachment Eiv: Blood Agar Plates
GATB Quality Manual Attachment Exi: MIC Testing: Drug
GATB Quality Manual Attachment Eiii: MGIT TUBES and PANTA/Growth Supplement
GATB Mycobacteriology Quality Manual Attachment J: MGIT 960 Daily Maintenance Log
GATB Mycobacteriology Quality Manual Attachment K: MGIT Calibration Tube Log
GATB Mycobacteriology Quality Manual Attachment M: Continuous Quality Improvement
Appendix C: MIC LRF
Appendix F: MIC Quality Control H37Rv Worksheet
TBeXiST Interpretation Report
TBeXiST Worklist
TBeXiST plots
Interpretation and Reporting of MIC-DST in TBeXiST Users Guide
Registering TBeXiST MGIT tube for MIC-DST Users Guide

4.8 SOP 8: Whole Genome Sequencing (WGS)

4.8.1 Purpose

DNA samples are to be sent by **UCL Central Mycobacteriology Laboratory (UCL)** for molecular typing by Whole Genome Sequencing (WGS) to either of the following laboratories (See Table 17 for full address and contact details):

1. Central Sequencing Laboratory, National Infection Service, Public Health England (PHE)
2. UCL Pathogen Genomics Unit (PGU)

WGS is performed on paired DNA extracts from the isolates at baseline (Day 1 or positive sample from Screening to Week 4) and at or after the end of treatment as defined in the study requirements below. This data will be used to determine if the paired isolates are the same strain (relapse) or different strains (re-infection), the outcome of which is important for assigning study endpoints.

Study requirements for testing at or after the end of treatment:
• SimpliciTB: First MTB confirmed positive culture in the follow up period after a patient has converted to culture negative during the treatment period, or first MTB confirmed positive culture at the last treatment visit (Week 17 for 4-month arm or 26 for 6-month arm) if the patient has not converted during treatment.
• ZeNix: First MTB confirmed positive culture at/after Week 16 if patient not responding to therapy and/or first MTB confirmed positive during follow-up to check for potential re-infection in a patient that has previously converted.

WGS is also performed on all baseline isolates. In the context of SimpliciTB, this analysis is important in case discrepancies are found between Screening molecular DST and MGIT DST. WGS may be used to resolve these discrepancies. WGS from baseline and/or post treatment isolates will also provide data on the potential mechanisms of resistance to the trial drugs in cases where MICs have increased.

This SOP details the procedure for referring DNA to both PHE WGS service and to UCL PGU, as both centres are able to perform WGS.

4.8.2 Principle

DNA will be extracted and quantified as per **SOP 5: DNA Extraction at UCL** and sent to either PHE or PGU (to be decided each time depending on availability, sample numbers and DNA concentrations). The use of the two facilities provides a contingency and the most flexibility to ensure that the WGS can be performed within the trial timelines. DNA will be sent in batches for the baseline samples and for cases of suspected re-infection or relapse (as defined above), these will be prioritized with the corresponding baseline (if not already sequenced).

WGS will be performed on Illumina sequencer instruments and data will be analysed using the assembly and mapping pipelines outlined in detail below. The WGS data from the paired isolates will be compared and the number of single nucleotide polymorphisms (SNPs) separating the isolates determined. WGS data will also be used to identify mutations in known genes associated with resistance to anti-TB drugs (first and second line drugs and study drugs) and to determine the MTB lineage. Isolates may also be sent for WGS to resolve discrepancies in laboratory data.

4.8.3 Procedure

4.8.3.1 DNA requirements for WGS analysis

DNA must be extracted and quantified as per **SOP 5 (Section 4.5**). Details of the quantification (Qubit, Nanodrop and TapeStation) must be recorded onto the DNA Quantification and Storage Worksheet (Appendix E). The extracted DNA is regarded acceptable for referral for WGS analysis if it meets the criteria as outlined in Table 16.

Table 16: DNA requirements for shipment of DNA for WGS

PHE	PGU
Nanodrop: 260/280 ratio between 1.8 and 2.0	Nanodrop: Not required
Qubit®: dsDNA concentration between 6 – 100ng/µl	Qubit®: dsDNA concentration between 5 – 30 ng/µl, unless otherwise agreed – see section 4.8.3.2.1
Minimum volume of 60µl	Minimum volume of 35µl; Maximum volume of 60µl, unless otherwise agreed. – see section 4.8.3.2.1

DNA requirements not met

If the above DNA requirements criteria for either laboratory are not met and the DNA sample cannot be sent for WGS, the DNA extraction must be repeated from a fresh culture at UCL. If multiple samples in a batch of DNA extractions do not meet those criteria then the extraction reagents should be reviewed (expiry dates, storage conditions – captured on Appendix X: DNA Extraction and Quantification Reagent Worksheet) and new lots prepared as required and a CQIF (Quality Manual Attachment M) completed.

4.8.3.2 Preparation of samples

4.8.3.2.1 Pathogen Genome Unit (PGU)

The DNA concentration/volume in Table 16 allows sufficient DNA for the optimal total input of 101-200ng per reaction. If this concentration is not achieved, as long as there is a minimum of 5ng of DNA, samples can be provided, if a larger volume is sent. This should be flagged to PGU emailing ich.genomics.pgu@ucl.ac.uk before the sample shipment and PGU will advise accordingly on which samples are sufficient for processing and if any changes are needed to plate layout. For critical or urgent samples, it is possible to send at lower concentrations and obtain successful sequencing results, but the risk of failure is higher.

Routinely, samples are prepared as 60µl volumes at a maximum of 30ng/ul DNA. If the DNA concentration is:

- **>30ng/ml** - dilute to 30ng/ul by pipetting an appropriate volume of molecular grade water into the sequencing plate and adding the DNA up to 60ul.
- **7-29ng/ul** - add 60ul of undiluted DNA to the sequencing plate.
- **<7ng/ul** – add 80ul of the undiluted DNA to the sequencing plate

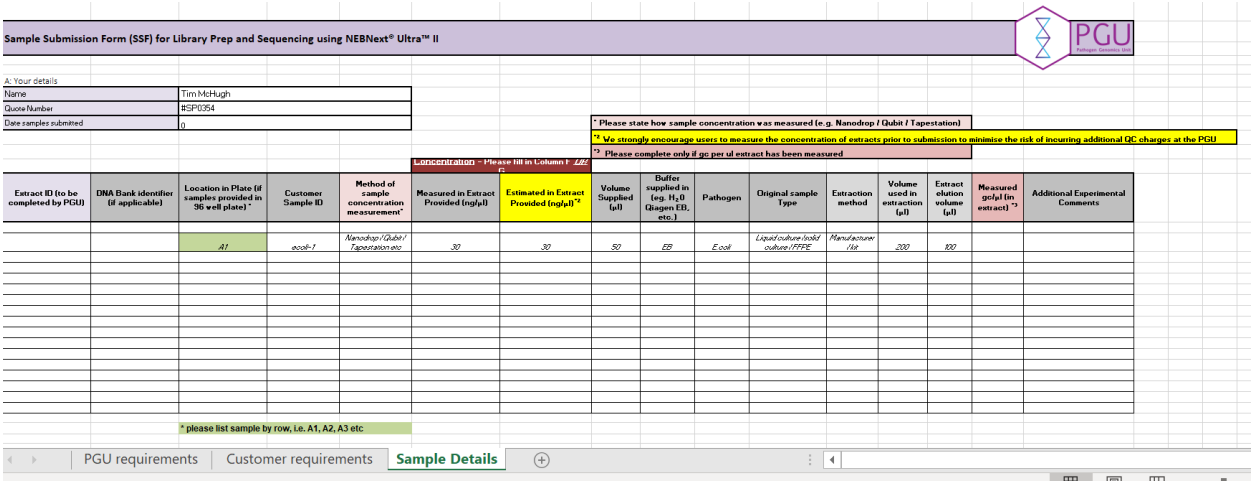
Dilution details to achieve the concentrations outlined above will be documented in the 'WGS plate preparation spreadsheet' proforma, which is created for each batch of DNA sent for sequencing and is stored on the UCL Shared Drive (S: TBA Clinical Trials).

All DNA samples should be provided to the PGU in batches of 48 or 96 samples (unless pre-arranged with PGU) in a V-bottom 96-well plate (preferably Eppendorf Twin-tec skirted plate. Catalogue No: 732-0108. Supplier VWR). The layout of the samples within the plate is by DNA concentration of the samples (A1 high to H12 low by column order, unless requested otherwise by PGU). The Sample Details section of the PGU Submission Form spreadsheet (see example in Figure 7), should be completed with the location in the plate and sample ID (see note below) before transferring the DNA samples into the plate, and then used as a guide to load DNA into the plate correctly. The plate should be labelled as follows; UCL_CCM_BATCHXXX_DDMMMYYYY' where 'XXX' denotes the sequencing batch number e.g. 001, 002 etc. and the date is provided in date, month and year format e.g. 01JAN2020. Plates are to be sealed with an adhesive PCR Film for sealing PCR plates.

NOTE: the sample ID is a combination of the Subject ID (or screening number if pre-enrolment), LAN and visit and **must** be completed in the following format as this will become the filename of the resulting sequence files. Filenames must not include spaces or the following characters \ / - .

Study_WGS_Subject ID_LAN_visit e.g. NC007_WGS_1207006_12070090_D1

Figure 7: Example of PGU Submission Form


Sample Submission Form (SSF) for Library Prep and Sequencing using NEBNext® Ultra™ II

PGU

A: Your details

Name	Tim McHugh
Quote Number	#SP0354
Date samples submitted	0

* Please state how sample concentration was measured (e.g. Nanodrop / Qubit / Tapestation)
** We strongly encourage users to measure the concentration of extracts prior to submission to minimise the risk of incurring additional QC charges at the PGU
*** Please complete only if gc per ul extract has been measured

Concentration – Please fill in Column F and/or G

Extract ID (to be completed by PGU)	DNA Bank identifier (if applicable)	Location in Plate (if samples provided in 96 well plate) *	Customer Sample ID	Method of sample concentration measurement*	Measured in Extract Provided (ng/µl)	Estimated in Extract Provided (ng/µl)**	Volume Supplied (µl)	Buffer supplied in (eg. H_2O Qiagen EB, etc.)	Pathogen	Original sample Type	Extraction method	Volume used in extraction (µl)	Extract elution volume (µl)	Measured gc/µl (in extract) ***	Additional Experimental Comments
		A1	ecoli-1	Nanodrop/Qubit/Tapestation etc	30	30	50	EB	E.coli	Liquid culture/solid culture/FFPE	Manufacturer kit	200	100		

* please list sample by row, i.e. A1, A2, A3 etc

PGU requirements | Customer requirements | Sample Details

One Submission Form must be completed for each DNA-containing plate (for shipments of >96 samples, each plate will have a different form). For the 'Customer requirements' page, only the sections in yellow need to be completed by a member of the team, the remainder will be completed by PGU and should be checked by UCL staff to ensure the stated requirements are correct. On the second page, Section A (Sender details) and Section B (Sample details) must be completed. This includes the number of samples and the shipment date, together with the sample location within the 96 well plate, sample ID, DNA concentration (with method of measurement) sample volume, and pathogen.

The completed form will be emailed by the UCL team to the PGU (ich.genomics.pgu@ucl.ac.uk) before the samples are shipped. The original form, signed by the sender (Section A), will remain at UCL and a copy will be sent with the samples in a 96 well plate to the PGU.

4.8.3.2.2 Central Sequencing Laboratory, PHE

Although PHE will accept concentrations in the range of 6-100 ng/µl, the PHE protocol is to remeasure the DNA at receipt and amend the concentration to 6 ng/µl. However, it is recommended to send samples between 12 and 25 ng/µl to ensure there is sufficient DNA.

If the concentration or volume specified in Table 16 is not achieved, samples with lower concentrations may be able to be processed but this needs to be agreed with PHE beforehand. A copy of Appendix E; Parts A and B should be emailed to the address stated in Table 17 before the sample shipment, flagging any samples that are below the submission range stated above, and PHE can advise accordingly which samples can be accepted and any additional information required.

If required, DNA is to be diluted using molecular grade water (or TE buffer with EDTA concentration below 0.1 mM) to the required concentration range in Table 16. Dilution details will be documented on Appendix E, Part D: DNA dilutions for WGS. Storage locations (for neat and diluted DNA samples) will also be documented on Appendix E, Part C: DNA Storage. Information on DNA concentration and storage location is also captured on the TB Alliance DNA and WGS tracker (Excel sheet) which is stored on the UCL Shared Drive (S: TBA Clinical Trials/Trackers), allowing samples to be suitably organised and tracked. If DNA is required to be diluted prior to sending, the Qubit (preferably using the Qubit dsDNA HS kit) should be repeated (as per SOP 4.5; Section 4.5.3.2) on the diluted sample to confirm that the final solution is within the required DNA concentration range, and this information captured in Appendix E, Part D: DNA dilutions for WGS.

All DNA samples should be provided to PHE in clear 4titude PCR full skirted plates with unique PHE barcode on the left plate edge, A1-H1 side. These must be purchased from 4titude, UK (catalogue number SP-0238) or obtained from PHE directly. Up to 94 samples can be submitted per plate and wells G12 and H12 should be left empty for controls. There is no minimum sample number. The layout of the samples in the plate must match the order submission sheet.

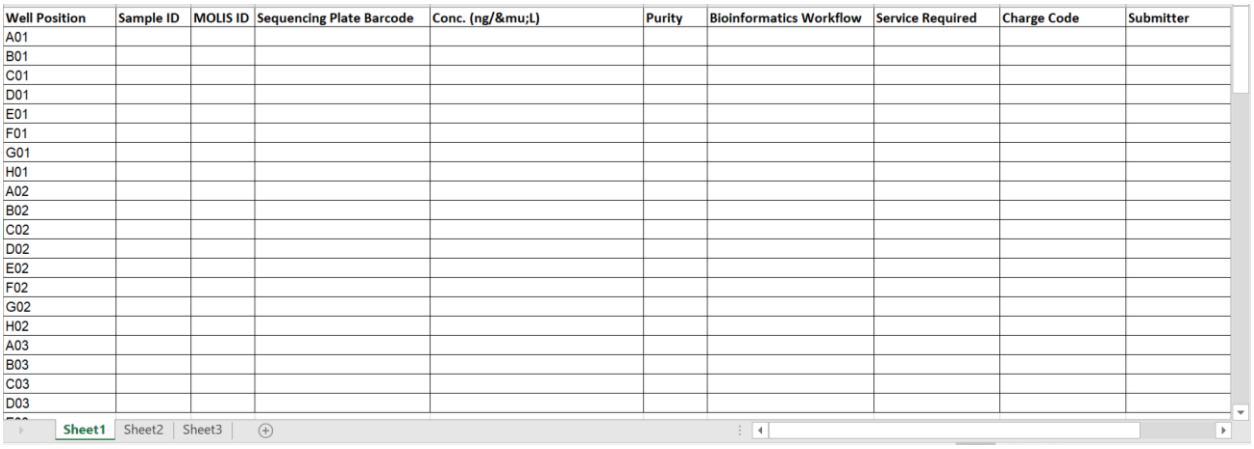
The order submission sheet must be completed (See Figure 8) and must include a unique sample ID which is a combination of the Subject ID, LAN and visit and **must** be completed in the following format as this

will become the filename of the resulting sequence files. (Note: filenames must not include the following characters: spaces \ / : * ? " < >).

Study_WGS_Subject ID_LAN_visit e.g. NC-007_WGS_1207006_12070090_D1

The sample ID together with the plate barcode will be used to track samples at all times during processing.

Figure 8: Example of PHE order submission sheet



Well Position	Sample ID	MOLIS ID	Sequencing Plate Barcode	Conc. (ng/μL)	Purity	Bioinformatics Workflow	Service Required	Charge Code	Submitter
A01									
B01									
C01									
D01									
E01									
F01									
G01									
H01									
A02									
B02									
C02									
D02									
E02									
F02									
G02									
H02									
A03									
B03									
C03									
D03									

Sheet1 Sheet2 Sheet3

Sample information is submitted using the NGS LIMS (Laboratory Information Management System) also known as Genesifter, and results are accessible using an FTP client (e.g. FileZilla software). As UCL is an external client, the completed order submission sheet must be emailed to the address in Table 17 and will be uploaded onto the LIMS by a member of PHE staff. When the order submission is complete, the UCL laboratory manager will receive email confirmation, and will be notified if there are any issues that need to be addressed before the samples are sent. This email will be printed and kept in the UCL Laboratory Site File. Additional details of the online submission process are outlined in guidance document 'BW0303 Instructions on sample submission and receiving results' (See Table 20).

4.8.3.3 Shipment of DNA samples from UCL to PHE and PGU

DNA should be transported under cold conditions (~4°C). A polystyrene box with sufficient ice packs (1-2 depending on size of box and ice packs) to maintain ~4°C temperature for the duration of shipment, including delays, is acceptable. This shipment is non-hazardous and does not require temperature monitoring.

If a courier is used, shipments should be arranged through the courier City Sprint – courier and recipient details are included in the Table 17. City Sprint will provide the required packaging. If necessary, shipments may be delivered by a member of CCM staff in person. UCL will liaise with the receiving laboratory to arrange a suitable day for shipment and receipt of samples, notifying at least one day in advance. Suitable delivery times are shown in Table 17.

Table 17:Contact Details for Shipment of DNA from UCL to PGU or PHE

Courier De tails Arranging delivery of packaging materials and collection of DNA	City Sprint Contact details: General - LondonEastHealthcareCT@citysprint.co.uk Rebecca Allen - RAllen@citysprint.co.uk
Recipient Details for PGU	Zayed Centre for Research into Rare Disease in Children Main reception UCL Genomics 20 Guilford Street London WC1N 1DZ FAO: Helena Tutill/Charlotte Williams/Pat Dyal Bynoe Contact telephone numbers: 0203978 3829 (Helena) / 3826 (Charlotte) / 3828 (Pat) Email: ich.genomics.pgu@ucl.ac.uk Cc email: rachel.williams@ucl.ac.uk Delivery should be between 10:00 and 16:00 Monday - Thursday. If delivering in person, then any day/time is acceptable with prior arrangement.
Recipient Details For PHE	GSDU/Central Stores National Infection Service Public Health England 61 Colindale Avenue London, NW9 5EQ Contact telephone numbers: 020 83277898 NGS.service@phe.gov.uk Delivery should be between 9:00 and 16:00 Monday - Thursday.

4.8.3.4 WGS at PGU

Sample Receipt

Shipments of isolates will be received by delegated members of the PGU team. A paper copy of the Submission Form, signed by a UCL Central Mycobacteriology Laboratory staff member, will accompany the samples. Samples will arrive to the main reception of the Zayed Center for Research. PGU staff will sign for the delivery and take the samples to Genomics Lab B X-1008 or the Equipment Room X-1010. The receiver will check the DNA samples with those listed on the Submission Form. If all the DNA samples are present, in good conditions (not damaged or spilled), correctly labelled and documentation is complete, the receiver will sign (Section C) to indicate this. A scanned copy of the signed Submission Form will be sent back by email to the UCL team (email addresses listed in Section 2: Contact Details) for their records. The plate will be labelled with the following information: pathogen, user details, study and date arrived.

If any DNA samples are missing or there is incorrect or incomplete documentation, the receiver will describe the issue(s) in the Comments section of the Submission Form, which will then be returned to the UCL laboratory by email. If any changes or additions are required, these should be made on the original Submission Form, following GCLP practices, at the UCL laboratory and the revised copy sent by email to the PGU laboratory team. A copy of the email correspondence and the revised form should be attached to the original and filed in the DNA/WGS section of the UCL laboratory Site File, which will contain copies of all documentation relevant to the WGS testing. Copies of the completed sample form and a signed version of this SOP will be stored by the PGU.

If it is not possible to rectify the incomplete documentation, the DNA sample/s will be rejected. The PGU laboratory staff will notify UCL staff by email, documenting the affected sample/s, the reason for rejection and requesting a new sample. This email correspondence will be printed and filed in the DNA/WGS section of the UCL laboratory Site File.

Upon receipt of DNA samples, PGU will repeat the DNA quantification by Qubit. If this shows the DNA is not sufficient then the PGU will liaise with the UCL team to ascertain if the sample should still be processed. In addition, after performing the sequencing if any sample(s) yield poor quality sequence data, UCL will be contacted by email to request a repeat sample and documenting possible reasons for the failed run after a review of the run data by PGU staff. As above, this email correspondence will be printed and filed in the UCL laboratory Site File.

Before processing, PGU will assign their own unique identifier to each DNA sample (Extract ID), completing the first column of the Submission Form (Section B). This will be the identifier for each sample used throughout sample processing at PGU. This can be directly linked to the Sample ID (which includes both the Subject ID and Laboratory Accession Number) at any time by cross referencing the Submission Form. The Extract ID and Sample ID will also be included in the Processing Report Form (See Figure 9), and both the fully completed Submission Form (including the Extract ID) and the Processing Form will be sent back with the results, as outlined below.

At the PGU, samples will be stored in a -20°C freezer located in Genomics Freezer Room X-1072 or the Equipment Room X-1010, until processing. Pooled libraries for sequencing will be stored in -20°C freezers in the Equipment Room X-1010. Any remaining DNA samples or prepared libraries will be temporarily stored in -20°C freezers in the Genomics Freezer Room X-1072. After 3 months any leftover samples will be disposed of at PGU unless UCL staff have requested by email to ich.genomics.pgu@ucl.ac.uk to facilitate return of the samples. In the latter, UCL staff will be responsible for organising for the samples to be returned.

Figure 9: Example of the PGU Processing Report form.

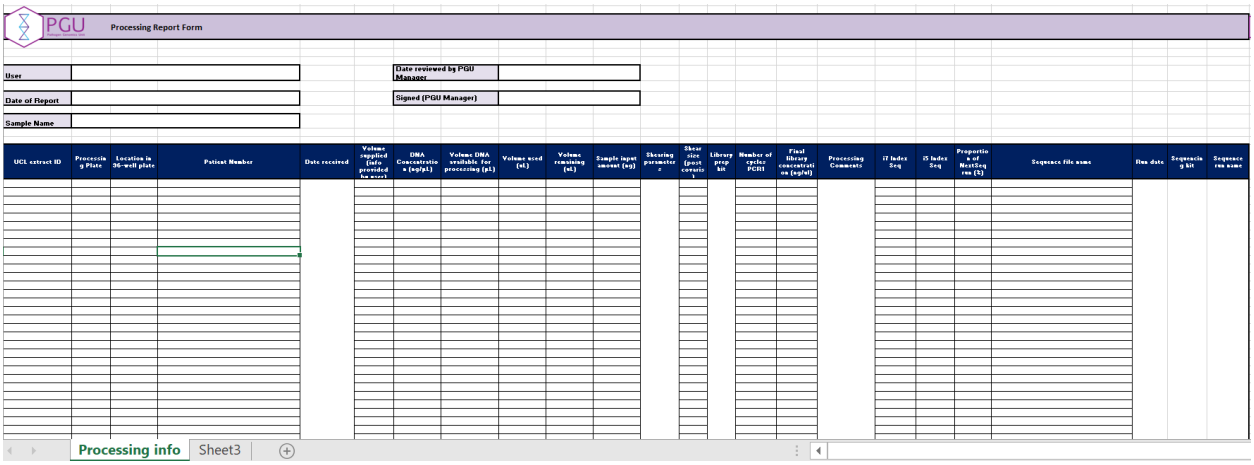


Table 18: PGU SOP and related document list

SOP No.	Title	Process used for
PGU_SOP_001	SampleQC_Qubit_DNA_CCM	Quantification of dsDNA
PGU_SOP_002	Covaris_operation_CCM	Main Processing
PGU_SOP_003	SampleQC_Tapestation_DNA_CCM	Quantification of dsDNA
PGU_SOP_004	Library prep using NEB Ultra II protocol on Bravo_CCM	Main Processing
PGU_SOP_005	Library_pooling_CCM	Main Processing
PGU_SOP_006	NextSeq_sequencing_washing_CCM	Main Processing
PGU_SOP_007	NextSeq_data storage_CCM	Data Storage
PGU_SOP_009	Sample receipt_storage_return_disposal_CCM	Sample handling
PGU_SOP_010	Recording Bravo processing runs_CCM	Data processing
PGU_SOP_011	Storage of reagents_NEBII DNA_NextSeq_CCM	Reagent Storage
PGU_SOP_012	Equipment Inspection (including temperature monitoring)	General
Other Documents	**Title**	**Process used for**
	Submission Form	Sample transfer and chain of custody
	Processing Report Form	Reporting results and Quality Controls

WGS

DNA processing and sequencing will be performed in accordance with the SOPs outlined in Table 18. DNA will be sheared using the Covaris E220 Focussed UltraSonicator. Libraries will be prepared from the MTB DNA extracts using the NEB Next Ultra II DNA protocol adapted for automated processing on Agilent's NGS Workstation, also called 'Bravo platform', for subsequent sequencing on Illumina MiSeq or NextSeq sequencers. The Bravo Platform is a liquid handling platform which allows library preparation of 96 samples simultaneously using a variety of library preparation kits. Bravo processing runs and reagents used (lot numbers/expiry dates) will be recorded according to SOP Recording Bravo processing runs.

Sequencing of the prepared libraries will be performed using a NextSeq sequencer (Illumina), with 300 cycles of 151bp paired end reads and minimum Q30 score of >75. Libraries which have been produced by the PGU from samples received from UCL require a (pooled) final library with a molarity of 4nM (but if this is not achievable, 2nM is sufficient).

The QC carried out pre-processing, during library preparation, post library preparation and for pooling the libraries for the NextSeq run is detailed in Table 19.

Table 19: Summary of QC steps and acceptable outputs during DNA processing

Processing Stage	No. of samples	QC method	Acceptable output
Pre-processing	All samples	Qubit High Sensitivity DNA	5 – 30 ng/ul (unless previously discussed with the PGU)
Post-shearing	15 out of 48 samples	D1000 High Sensitivity Tapestation	Fragment length >200bp
Post-library preparation	All samples	Qubit High Sensitivity DNA	Concentration of final library > 2ng/ul
	15 out of 48 samples	D1000 Tapestation	Fragment length >260 bp
Pooled library for sequencing	N/A	Qubit High Sensitivity DNA	Within 20% of expected pooled library concentration

Transfer of WGS data from PGU to UCL Central Myco Lab

The NextSeq WGS data can be shared between users using Illumina's BaseSpace platform. Data (in a compressed FASTQ format) will be transferred from the PGU account to the UCL Central Mycobacteriology account as a sequencing project. UCL staff can then access the data files directly via the designated BaseSpace account. PGU will only retain a copy of the sequencing data for 6 months. UCL Central Mycobacteriology Laboratory team will be notified by email when the run is available in BaseSpace and will also be sent a copy of the Processing Report Form (See Table 18 and Figure 9). A copy of this email will be printed and stored in the UCL laboratory Site File. The Processing Report Form contains all the processing information and will indicate if the sequencing failed for any samples and provide a reason.

If failed sequences are more than occasional, the DNA extraction pipeline and quality measures should be investigated, and a CQI form completed to document the issue.

All FASTQ files and the Processing Report Forms are saved on the UCL Shared drive (S: CCM Clinical Trials/TB Alliance Trials/TBA Sequence data/PGU) or in a UCL Research Data Storage (RDS) drive. These are both UCL shared drives with restricted access to staff delegated to be working on the study and is backed up daily in accordance with Central UCL IT policy, see UCL WGS Data Storage and Handling SOP for more details Non-UCL staff such as the TB Alliance Microbiology Director and study bioinformatician also have access to the data via the RDS drive.

4.8.3.5 WGS at PHE

Sample Receipt

On arrival, samples will be stored at 2-8°C until ready for sample receipt. During sample receipt the online order and plate will be checked to confirm sample numbers match and the plate will be checked for sample volume. The plate will then be set to "received" through Genesifter and the customer (the UCL laboratory manager) will receive an email. If there is a discrepancy, UCL will be contacted by email to agree the way forward. This communication is logged through Genesifter. The email correspondence related to discrepancies/sample rejection will be printed and kept in UCL Laboratory Site File.

Samples will be rejected for the following reasons:

- Sample concentration is not within the specified range
- Sample volume is different from specified volume
- Samples submitted are not in the designated 96-well plate
- Online submission form has not been completed
- Plate layout does not match the online submission form

At PHE, customer submission plates will be stored in designated storage boxes at +2-8°C for a maximum of 12 weeks from the initial reception date as indicated on Genesifter. After this time the plates will be discarded at PHE and the disposal logged onto the disposal log. External customers will have 12 weeks from the initial reception date to arrange appropriate collection of the plate, if required. All returned plates will be logged on Genesifter with the date of return. Customer sequencing data is stored on the individual instrument hard drive for a maximum of two months, after which it is deleted.

WGS

Libraries will be prepared from the MTB DNA extracts using the Illumina Nextera XT DNA sample preparation kit.

Sequencing of the prepared libraries will be performed using Illumina sequencers and PHE aims to provide a yield per sample of approximately 150 Megabases (Mb) or higher of high quality (Q30 and above), measured by the yield of the control DNA included in each run (this is approximately equivalent to 30-fold coverage for a 5 Mbp size genome).

All reagent batch numbers, instruments used and other processing details are recorded on PHE paperwork. On receipt of the samples and the online order, PHE quantifies the submitted DNA using the Quant-iT ds BR assay kit (Life Technologies, UK). If the DNA submitted is not in the required or pre-arranged concentration it may be rejected.

All PHE SOPs used for processing and sequencing MTB DNA extracts are outlined in Table 20.

Table 20: PHE SOP list

SOP No.	Title	Process used for
B13132	NGS Service Sample Receipt of Customer Submissions	Main Processing
B13141	NGS Service Quantification of Customer Submissions	Main Processing
B13142	NGS Service Consolidation of Customer Submissions	Main Processing
B13133	NGS Tracking and Automation of Sample Preparation	Main Processing
B13134	NGS Service Nextera XT Library Preparation	Main Processing
B13135	NGS Service Fragment Sizing of Nextera XT Library Preparation	Main Processing
B13102	NGS PAL creation and Real-Time PCR Quantification	Main Processing
B13136	NGS Service loading of cBot and HiSeq	Main Processing
B13143	NGS Service loading of MiSeq	Main Processing
B13122	Quantification of dsDNA using Quant-iT HS kit on a microplate fluorometer	Quantification of dsDNA
B13140	Quantification of dsDNA using Quant-iT Assay Kit on Qubit Fluorometer	Quantification of dsDNA: Qubit
B13124	Illumina Nextera XT DNA Library Preparation	Library Prep
B13123	Preparation & Dilution of Samples for NGS	Sample Prep
B13138	Use & Maintenance of Perkin Elmer Robots	Perkin Elmer Robot
B13139	Use & Maintenance of BeckMan BioMek NXP Robotics	Biomek Robot

B13125	Preparation and use of HT DNA High Sensitivity LabChip Kit and LabChip GX Instrument	LabChip
B13126	qPCR for Illumina Sequencing Platforms using the KAPA library Quantification Kit	qPCR/KAPA
B13127	Preparing DNA Libraries for Loading onto Illumina Sequencing Platforms	Library Prep for loading
B13130	Use & Maintenance of HiSeq	HiSeq
B13129	Use & Maintenance of cBot	cBot
B13128	Use & Maintenance of MiSeq	MiSeq
B13131	Final NGS Work Flow (Phase 1)	NGS Workflow
B13146	Use and Maintenance of NextSeq500 for NGS sequencing.	NextSeq
BW0303	Instructions for sample submission and receiving results	Sample receipt and results reporting

On completion of a sequencing run PHE Central Sequencing Laboratory staff check the quality of the run by assessing the negative control and the positive *E. coli* K12 control. A minimum of 150 Mb of Q30 and above should be obtained for the positive control which is equivalent to 30-fold coverage for a 5 Mbp size genome.

Transfer of WGS data from PHE to UCL Central Mycobacteriology Laboratory

UCL will receive an automated email through GeneSifter to notify when the run is completed and the results are available. This email will be printed and kept in the UCL Laboratory Site File. The original data output is in the format of a compressed FASTQ file (fastq.gz -two files per sample) which are used for all downstream analysis.

FASTQ files will be downloaded from the FTP server as described in document BW0303 (see Table 20). It is recommended that the FTP server is accessed using an FTP client software (e.g. FileZilla) which can be downloaded online. All FASTQ files and the Quality Report for a given run should be downloaded and saved on the UCL Shared drive: (S:CCM Clinical Trials/TB Alliance Trials/TBA Sequence data/PHE or in a UCL Research Data Storage (RDS) drive. These are bothis a UCL shared drives with restricted access to staff delegated to be working on the study, and is backed up daily in accordance with Central UCL IT policy. The PHE data are received in folders that are named starting with a date, allowing for easy filing by date. See UCL WGS Data Storage and Handling SOP for more details.

Data from PHE sequence runs are accompanied by a DNA quality evaluation, which includes a 'pass/fail' marker for each sample. Previous data suggests that samples marked as 'fail' also fail to achieve adequate coverage with further analyses, however these should still be sent to the bioinformatician for analysis. If

failed sequences are more than occasional, the DNA extraction pipeline and quality measures should be investigated and a CQI form completed to document the issue.

4.8.3.6 Data analysis by the designated study bioinformatician/s

Downloaded WGS data are stored within a folder named by date and sequencing batch number – these are the considered the original files and remain untouched thereafter. These are not used directly for analysis, but can be compared to any other copies made at any time using process outlined in the UCL WGS Data Storage and Handling SOP. All WGS data files for the run are then copied, and FASTQ file names will be checked and amended, if needed, to the agreed filename nomenclature (See Section 4.8.3). This will ensure that all downstream analysis files have the correct names. All files must also include the processing report date (the result date) as a suffix to easily relate the sequence results to the sequence run information. The visit abbreviations in the filename should be as per the naming convention for the LRFs (see the TBA Clinical Trials: Data Reporting SOP for UCL Central Mycobacteriology Laboratory for full details).

Study_WGS_Subject ID_LAN_visit_result date e.g. NC-007_WGS_1207006_12070090_D1_08Oct2020

UCL will contact, by email, the bioinformatician responsible for analysing the sequence data, and they will access the files, as required, from the S drive or RDS (See UCL WGS Data Storage and Handling SOP). UCL will send a table to the bioinformatician listing the Trial ID, Subject ID and laboratory accession numbers for the samples that require analysis, including if they are for a paired isolate analysis or baseline only analysis. The email correspondence with the bioinformatician will be printed and kept in UCL Laboratory Site File.

Analysis will be performed as described in Witney *et al.* (2017) and outlined in full below and in the UCL WGS Data Storage and Handling SOP:

1. Sequence Quality Control
   - Count reads
   - Align genome with reference strain H37Rv (RefSeq accession: NC_000962.3) using bwa mem (Li, 2013). This generates the BAM file (see Table 15).
   - Sort alignments, and remove duplicates with SAMtools (Li et al, 2009).
   - Reject if Coverage <30x for the aligned sequence or there is significant sequence contamination and do not proceed with further analysis. If the read count is high and the coverage is low, Kraken2, a sequence classification tool, will be used to speciate the reads.

   NOTE: Coverage for each DNA sample is recorded in the TB Alliance DNA and WGS tracker. If coverage is < 30x, t UCL will discuss with PHE/PGU (as appropriate) possible reasons for the low coverage and agree if this can be resolved by re-doing the WGS run (from existing DNA), or if DNA extraction should be repeated and WGS run again from the new sample. If sequence is confirmed non-MTBC (or significantly contaminated), the DNA extraction must be repeated from a confirmed clean MTB isolate at UCL, or if this is not possible, request a new alternative isolate from the local laboratory. These email correspondences will be printed and kept in UCL Laboratory Site file.

2. Sequence analysis pipeline

Call all genome site positions (this generates a Variant Call Format file (VCF) for all sites – see Table 21). Site statistics are generated using SAMtools mpileup

- For phylogenetic analysis, first filter sites on the following criteria:
  - mapping quality (MQ) above 30
  - site quality score (QUAL) above 30
  - at least four reads covering each site with at least two reads mapping to each strand (DP4)
  - at least 75% of reads supporting site (DP4) and an allelic frequency of 1 (AF).

Sites that failed these criteria in any isolate are removed from the phylogenetic analysis.

- INDELS are identified using SAM tools mpileup as above, so that they are detected for analysis of resistance SNPs. INDELS are filtered out from phylogenetics analysis (including in the count of SNPs different in the paired isolate comparison - relapse/reinfection), but details will be included in the full list of variants in the paired analysis report.

Call all variants from H37Rv (this generates a variant call only VCF – see Table 21).

Gene annotation is generated for all variant calls using snpEff software.

Identify SNP variants between the baseline and follow up samples from the same patient to determine relapse/reinfection using above criteria. For all paired isolate analysis, the following will be reported in the 'Annotated SNP list for Paired Analysis' (see Table 21):

  - Depth of coverage (mean read depth) of baseline and follow up samples
  - number of SNPs different
  - the genome position (nucleotide position and codon position (where applicable))
  - the gene (if applicable) – otherwise that it is intergenic and if so any further information generated by snpEff – i.e. proximity to upstream or downstream genes that might indicate SNPs in promoter or other regulatory regions
  - the variant call (e.g. A->G)
  - variant type (e.g. synonymous/mis-sense)
  - the amino acid change (if applicable)
  - Score - generated by snpEff to report putative variant impact on protein function (High; Moderate; Low; Modifier)
  - Evidence for the SNP (Quality and depth – VCF fields DP and DP4)

- All sequences will be used to reconstruct a study-wide phylogenetic tree. Phylogenetic reconstruction will be performed using RAxML (Stamatakis, 2014), with a General Time Reversible (GTR) model of nucleotide substitution and a Gamma model of rate heterogeneity; branch support values are determined using 1000 bootstrap replicates.
- Assess for the presence of SNPs in the following resistance genes:
  - the 6 genes currently known to be associated with resistance to pretomanid (Pa) (*fbiA*, *fbiB*, *fbiC*, *ddn*, *fgd1* and *cofC*).

- the 5 genes currently known to be associated with resistance to bedaquiline (B) (*Rv0678, atpE, pepQ, Rv1979* and *mmpL5*)
- For the selected resistance genes the following will be reported in a standardised output that includes (see Table 21):
  - the genome position (nucleotide position and codon position (where applicable))
  - the variant call (e.g. A->G)
  - variant type (e.g. synonymous/mis-sense)
  - the amino acid change (if applicable)
  - Score - generated by snpEff to report putative variant impact on protein function (High; Moderate; Low; Modifier)
  - Evidence for the SNP (Quality and depth – VCF field DP and DP4)
- Key resistance determining genes for first and second-line drugs – this will be done using an MTB resistance genotype database and analysis platform such as TB-profiler (Coll *et al.* 2015) or MTBseq (Kohl et al. 2018).
- Upstream regions of relevant genes (e.g. 100 bp) must be included in order to detect promoter mutations.
- It may be necessary, at the request of the sponsor, to analyse other genes of interest on a case by case basis.

Lineage – the lineage/sub-lineage will be assigned according to the classification described in Coll *et al.* (2014), this will be reported as part of the output from analysis platform used for determining key resistance genes for FLD/SLD (e.g. MTBseq or TBProfiler). See Table 21.

NOTES:

1. For baseline isolates not analysed along with a follow-up counterpart, only the lists of SNPs in resistance genes will be determined (Pa and B – defined genes as defined above; and the FLD/SLD resistance standard outputs).
2. The pipeline used, including software versions and scripts, will be recorded and the logfiles saved see UCL WGS Data Storage and Handling SOP.

Table 21: Data files generated during the sequence analysis

File Type*	**Details**
FASTQ.gz (compressed FASTQ) *2 files per sample*	Raw sequence data file/s generated by PHE
BAM *1 file per sample*	Sequence data after alignment with H37Rv (reference strain NC_000962.3)
VCF (all sites) *1 file per sample*	All site calls
VCF (variant calls only, including additional gene annotation generated using snpEff software) *1 file per sample*	A subset of the above which includes only the variant calls (SNPs compared to reference strain)

Annotated SNP list– all paired analysis (relapse and reinfection). Excel file *1 file per pair*	Study specific proforma, generated from comparison of the two VCF files (baseline and follow up) to show annotation of all variants identified between the two strains.
Annotated SNP list (Pa and B resistant genes) – all samples. Excel file *1 file per batch analysed per drug*	Standardised outputs generated from the VCF file to show details of all variants in the Pa and B resistant genes
MTBSeq analysis – all samples. *Text/Excel file of SNPs marking those known to confer resistance to FLD/SLD (1 file per sample)* *Text/Excel file of Quality (1 file per batch analysed)* *Text/Excel file of Lineage (1 file per batch analysed)* TB profiler – all samples. Txt file. *1 file per sample*	Standard outputs from these MTB WGS analysis platforms to SNPs that are known to be associated with resistance to standard first and second line drugs
Phylogenetic Tree - initial output as a newick file, and also as png image file. *1 set of files per study*	A phylogenetic tree showing the relatedness of all samples from the study. This will be generated at the end of the study to include all available isolates. It may be required at interim time points to investigate relatedness between isolates or possible cross contamination, at the request of the sponsor.
Analysis software version *1 file per study* (unless necessary updates are required, in which case this will be documented)	Documents the version of all software used for the sample analysis for all samples in the study

***All files per sample will be named to include the sample ID which includes the patient identifier and the lab accession number and the WGS date (**Study_WGS_Subject ID_LAN_visit_result date)

NOTE: The VCF files (all sites and variant calls) will be available for all samples allowing the annotated SNPs to be interrogated further at a later date, should this be required.

Transfer of Analysis Data to UCL

UCL will be informed by email when data is ready and all data files listed in Table 21 (excluding the FASTQ which is transferred as described above) will be uploaded to the S drive or RDS (See UCL WGS Data Storage and Handling SOP), within a folder named by date. . Details of the analysis folder location are included on the TB Alliance DNA and WGS tracker for each sample analysed.

4.8.4 Reporting

The required paired analysis data (No. of SNPs different between the baseline and follow up samples) will be entered on toAppendix D: Paired WGS LRF from the appropriate annotated SNP list file (see Table 20; and also the TBA Clinical Trials: Data Reporting SOP for UCL Central Mycobacteriology Laboratory). For paired isolates where the number of SNPs different is <20, the appropriate annotated

SNP list must also be printed and attached to the LRF for ease of reference. For pairs with >20 SNPs different, the full annotated list will be available as electronic copy only as indicated in Table 20.

4.8.5 References

1. Witney AA, Bateson AL, Jindani A, Phillips PP, Coleman D, Stoker NG, Butcher PD, McHugh TD; RIFAQUIN Study Team. Use of whole-genome sequencing to distinguish relapse from reinfection in a completed tuberculosis clinical trial. BMC Med. 2017 Mar 29;15(1):71.
2. Li H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. ArXiv13033997 Q-Bio. 2013. http://arxiv.org/abs/1303.3997.
3. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25(16):2078–9.
4. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 2014;30(9):1312–3.
5. Coll F, McNerney R, Guerra-Assunção JA, Glynn JR, Perdigão J, Viveiros M, et al. A robust SNP barcode for typing Mycobacterium tuberculosis complex strains. Nat Commun. 2014;5:4812.
6. Coll F, McNerney R, Preston MD, Guerra-Assuncao JA, Warry A, Hill-Cawthorne G, et al. Rapid determination of anti-tuberculosis drug resistance from whole-genome sequences. Genome Med. 2015;7(1):51. https://doi.org/10.1186/s13073-015-0164-0
7. Kohl, TA, Utpatel, C, Schleusener, V, De Filippo, MR, Beckert, P, Cirillo, DM, Niemann, S. MTBseq: a comprehensive pipeline for whole genome sequence analysis of Mycobacterium tuberculosis complex isolates. PeerJ 6:e5895 https://doi.org/10.7717/peerj.5895

4.8.6 Documents

Appendix D: Paired WGS LRF
PHE Guidance document 'BW0303 Instructions on sample submission and receiving results'
PGU Submission Form
PGU Processing Report Form

APPENDICES:

Appendices are provided as separate documents. The UCL Central Mycobacteriology Laboratory should not print copies of the appendices directly from the manual, the appendices shown below should be regarded as examples only. The purpose of the separate Appendices is firstly to allow the sponsor to amend the appendices without need to update the Central Mycobacteriology Manual if no changes are required to the manual itself, and secondly to ensure the Appendices are always version controlled and available in an easy to print/use format for the laboratory.

Appendix A: Speciation LRF (EXAMPLE)

APPENDIX A
TBA Speciation LRF

Version No.			
Date			
Initial			

Laboratory Accession Number			
Subject ID	☐☐☐☐☐☐☐ - ☐	Study No.	☐☐ - ☐☐☐
Visit specification	☐ Screening ☐ Treatment, ☐☐ Week ☐ Unscheduled	☐ Day 1 ☐ Post-treatment, ☐☐ Week ☐ Early withdrawal	
	RESULT SECTION – SPECIATION ☐ **Not Done (add comment)**		
DNA Extraction Date	☐☐ ☐☐☐ ☐☐☐☐ day month year	Tech initials _____	
Test type	Hain Mycobacterium CM ☐	Hain MTBC ☐	Hain Mycobacterium AS ☐
MTB complex confirmed	yes ☐	no ☐	Missing ☐ (add comment)
Comments			
Hybridisation Date	☐☐ ☐☐☐ ☐☐☐☐ day month year	Tech initials _____	
Lab Supervisor (Signature)*: _____		Date: __ / ___ / ____	
Lab Manager (Signature): _____		Date: __ / ___ / ____	

* Verified that the results are transcribed to the LRF correctly

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Appendix B: Drug Susceptibility Testing (DST) LRF (EXAMPLE)

APPENDIX B
TBA Drug Susceptibility Testing LRF

Version No.			
Date			
Initial			

Laboratory Accession Number			
Subject ID	☐☐☐☐☐☐☐ - ☐	Study No.	☐☐ - ☐☐☐
Visit specification	☐ Day 1 ☐ Treatment, ☐☐ Week ☐ Post-treatment, ☐☐ Week ☐ Screening ☐ Unscheduled ☐ Early withdrawal		

RESULT SECTION

Blood Agar Plate Date of evaluation	☐☐ day ☐☐☐ month ☐☐☐☐ year	Result ☐ pos. ☐ neg	Initial ____	
SIRE Date of test / started	☐☐ day ☐☐☐ month ☐☐☐☐ year	Initial ____	Used culture ☐ MGIT ☐ LJ	
Streptomycin	☐ sensitive ☐ resistant ☐ missing (add comment)			Initial ____
Isoniazid	☐ sensitive ☐ resistant ☐ missing (add comment)			
Rifampicin	☐ sensitive ☐ resistant ☐ missing (add comment)			
Ethambutol	☐ sensitive ☐ resistant ☐ missing (add comment)			
Date SIRE removed, or EpiCenter Report generated	day ☐☐ month ☐☐☐ year ☐☐☐☐ ☐ Epicenter used			
PZA Date of test / started	☐☐ day ☐☐☐ month ☐☐☐☐ year	Initial ____	Used culture ☐ MGIT ☐ LJ	
Pyrazinamide	☐ sensitive ☐ resistant ☐ missing (add comment)			Initial ____
Date PZA removed, or EpiCenter Report generated	day ☐☐ month ☐☐☐ year ☐☐☐☐ ☐ Epicenter used			
MOX Date of test / started	☐☐ day ☐☐☐ month ☐☐☐☐ year	Initial ____	Used culture ☐ MGIT ☐ LJ	
Moxifloxacin	☐ sensitive ☐ resistant ☐ missing (add comment)			Initial ____
Date MOX removed, or EpiCenter Report generated	day ☐☐ month ☐☐☐ year ☐☐☐☐ ☐ Epicenter used			
KAN DST for NC007 (ZeNix) samples only				
KAN Date of test / started	☐☐ day ☐☐☐ month ☐☐☐☐ year	Initial ____	Used culture ☐ MGIT ☐ LJ	
Kanamycin	☐ sensitive ☐ resistant ☐ missing (add comment)			Initial ____
Date KAN removed, or EpiCenter Report generated	day ☐☐ month ☐☐☐ year ☐☐☐☐ ☐ Epicenter used			
Comments				

Lab Supervisor (Signature)*: _____ Date: __ / ___ / ____

Lab Manager (Signature): _____ Date: __ / ___ / ____

Date Reported	☐☐ day ☐☐☐ month ☐☐☐☐ year	_____ Signature

* Verified that the results are transcribed to the LRF correctly

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APPENDIX B
TBA Drug Susceptibility Testing LRF

Version No.			
Date			
Initial			

Fill out in case of resistant result:

Drug: ______________ Start Date: ___________ Initials: _____________	Blood agar plate sterile	☐ yes ☐ contaminated	Initials/Date Read
	Visible inspection of MGIT tube – Normal TB Morphology seen	☐ yes ☐ no	Initials/Date Read
	Confirmation by ZN-stain (if applicable)	☐ AFB+ ☐ contamination ☐ No AFB	Initials/Date Read
Drug: ______________ Start Date: ___________ Initials: _____________	Blood agar plate sterile	☐ yes ☐ contaminated	Initials/Date Read
	Visible inspection of MGIT tube – Normal TB Morphology seen	☐ yes ☐ no	Initials/Date Read
	Confirmation by ZN-stain (if applicable)	☐ AFB+ ☐ contamination ☐ No AFB	Initials/Date Read
Drug: ______________ Start Date: ___________ Initials: _____________	Blood agar plate sterile	☐ yes ☐ contaminated	Initials/Date Read
	Visible inspection of MGIT tube – Normal TB Morphology seen	☐ yes ☐ no	Initials/Date Read
	Confirmation by ZN-stain (if applicable)	☐ AFB+ ☐ contamination ☐ No AFB	Initials/Date Read
Drug: ______________ Start Date: ___________ Initials: _____________	Blood agar plate sterile	☐ yes ☐ contaminated	Initials/Date Read
	Visible inspection of MGIT tube – Normal TB Morphology seen	☐ yes ☐ no	Initials/Date Read
	Confirmation by ZN-stain (if applicable)	☐ AFB+ ☐ contamination ☐ No AFB	Initials/Date Read
Drug: ______________ Start Date: ___________ Initials: _____________	Blood agar plate sterile	☐ yes ☐ contaminated	Initials/Date Read
	Visible inspection of MGIT tube – Normal TB Morphology seen	☐ yes ☐ no	Initials/Date Read
	Confirmation by ZN-stain (if applicable)	☐ AFB+ ☐ contamination ☐ No AFB	Initials/Date Read
Drug: ______________ Start Date: ___________ Initials: _____________	Blood agar plate sterile	☐ yes ☐ contaminated	Initials/Date Read
	Visible inspection of MGIT tube – Normal TB Morphology seen	☐ yes ☐ no	Initials/Date Read
	Confirmation by ZN-stain (if applicable)	☐ AFB+ ☐ contamination ☐ No AFB	Initials/Date Read
Drug: ______________ Start Date: ___________ Initials: _____________	Blood agar plate sterile	☐ yes ☐ contaminated	Initials/Date Read
	Visible inspection of MGIT tube – Normal TB Morphology seen	☐ yes ☐ no	Initials/Date Read
	Confirmation by ZN-stain (if applicable)	☐ AFB+ ☐ contamination ☐ No AFB	Initials/Date Read
Lab. Manager (Signature): ____________________ Date: ____________________			

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Appendix C: Minimum Inhibitory Concentration (MIC) LRF (EXAMPLE)

APPENDIX C

TBA Minimal Inhibitory Concentration (MIC) LRF

Version No.			
Date			
Initial			

Fill out in case of resistant result:

Drug: ____ Conc (µg/ml): ____ Start Date: ____ Initials: ____	Blood agar plate sterile	☐ yes ☐ contaminated	____ Initials/Date Read
	Confirmation by ZN-stain	☐ AFB+ ☐ contaminated ☐ No AFB	____ Initials/Date Read
Drug: ____ Conc (µg/ml): ____ Start Date: ____ Initials: ____	Blood agar plate sterile	☐ yes ☐ contaminated	____ Initials/Date Read
	Confirmation by ZN-stain	☐ AFB+ ☐ contaminated ☐ No AFB	____ Initials/Date Read
Drug: ____ Conc (µg/ml): ____ Start Date: ____ Initials: ____	Blood agar plate sterile	☐ yes ☐ contaminated	____ Initials/Date Read
	Confirmation by ZN-stain	☐ AFB+ ☐ contaminated ☐ No AFB	____ Initials/Date Read
Comments:			
Lab. Manager (Signature): ____		Date: ____	

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APPENDIX C

TBA Minimal Inhibitory Concentration (MIC) LRF

Version No.			
Date			
Initial			

Laboratory Accession Number			
Subject ID	☐☐☐☐☐☐☐ – ☐	Study No.	☐☐ – ☐☐☐
Visit specification (Tick and complete as appropriate)	☐ Day 1 ☐ Screening ☐ Treatment, ☐☐ Week ☐ Unscheduled ☐ Early withdrawal ☐ Post-treatment, ☐☐ Week		

RESULT SECTION

Blood agar plate Date of evaluation	☐☐ day ☐☐☐ month ☐☐☐☐ year	Result: ☐ pos. ☐ neg	Initials _____

BDQ	Test Start Date	☐☐ day ☐☐☐ month ☐☐☐☐ year	Initials _____
	MIC value	_____ µg/ml or Missing ☐ Provide reason.....	
	Result Date	☐☐ day ☐☐☐ month ☐☐☐☐ year	Initials _____
PRE	Test Start Date	☐☐ day ☐☐☐ month ☐☐☐☐ year	Initials _____
	MIC value	_____ µg/ml or Missing ☐ Provide reason.....	
	Result Date	☐☐ day ☐☐☐ month ☐☐☐☐ year	Initials _____
LZD MIC for NC007 (ZeNix) samples only			
LZD	Test Start Date	☐☐ day ☐☐☐ month ☐☐☐☐ year	Initials _____
	MIC value	_____ µg/ml or Missing ☐ Provide reason.....	
	Result Date	☐☐ day ☐☐☐ month ☐☐☐☐ year	Initials _____
MOX MIC for NC008 (SimpliciTB) samples only			
MOX	Test Start Date	☐☐ day ☐☐☐ month ☐☐☐☐ year	Initials _____
	MIC value	_____ µg/ml or Missing ☐ Provide reason.....	
	Result Date	☐☐ day ☐☐☐ month ☐☐☐☐ year	Initials _____

QC passed for this run Y / N (highlight if NO)	Comments	

Lab Supervisor (Signature)*: _____ Date: __ /___ /____

Lab Manager (Signature): _____ Date: __ /___ /____

Date Reported	☐☐ day ☐☐☐ month ☐☐☐☐ year	_____ Signature

* Verified that the results are transcribed to the LRF correctly

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Appendix D: Paired WGS LRF (EXAMPLE)

APPENDIX D

TBA Paired WGS LRF

Version No.			
Date			
Initial			

Subject ID	☐☐☐☐☐☐☐ - ☐	Study No. ☐☐ - ☐☐☐

Isolate 1	Visit specification (tick and complete as appropriate)	☐ Screening ☐ Day 1 ☐ Treatment, ☐☐ Week ☐ Unscheduled
	Laboratory Accession Number	
	Collection date	☐☐ day ☐☐☐ month ☐☐☐☐ year
Isolate 2	Visit specification (tick and complete as appropriate)	☐ Treatment, ☐☐ Week ☐ Post-Treatment, ☐☐ Week ☐ Unscheduled ☐ Early withdrawal
	Laboratory Accession Number	
	Collection date	☐☐ day ☐☐☐ month ☐☐☐☐ year

PAIRED WHOLE GENOME SEQUENCING

Was paired whole genome sequencing performed?	☐ Yes ☐ No (add comment)
Sequence Run date*	**Isolate 1** ☐☐ day ☐☐☐ month ☐☐☐☐ year **Isolate 2** ☐☐ day ☐☐☐ month ☐☐☐☐ year
Number of Single Nucleotide Polymorphisms (SNP) differences between isolate 1 and isolate 2* Note: For pairs with <20 SNPs different, please print the associated annotated SNP table and attach to this LRF	
Comments	
Result date (Note: this is the date of the Annotated SNP List Report comparing the two isolates)	☐☐ day ☐☐☐ month ☐☐☐☐ year

Lab Supervisor (Signature)*: _____ Date: __ /___ /____

Lab Manager (Signature): _____ Date: __ /___ /____

* Verified that the results are transcribed to the LRF correctly

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Appendix E: DNA Quantification and Storage Worksheet (EXAMPLE)

APPENDIX E: TBA DNA Quantification and Storage Worksheet

DNA Extraction Batch Name: TBA ___ ___ ___	DNA extraction date:

Part A: DNA Quantification by Qubit

Study NC007/ NC008	UCL Lab No.	Subject ID	Lab accession number	Volume DNA resuspended	BR Qubit® of original tube	HS Qubit® (if required)	Notes (add details of extra tests or dilutions required to give a reading in the kit quantification range, and if any diluted samples are to be stored)
					dsDNA conc in sample (ng/µl)	dsDNA conc in sample (ng/µl)	
Additional Comments					Read Date	Read Date	
					Initials	Initials	

APPENDIX E: TBA DNA Quantification and Storage Worksheet

DNA Extraction Batch Name: TBA ___ ___ ___	DNA extraction date:

Part B: DNA quantification – Nanodrop and TapeStation

Study NC007/ NC008	UCL Lab No.	Subject ID	Lab accession number	Nanodrop (if required)		TapeStation (if required)		Additional Notes	Flagged for further review^
				Conc.(ng/µl)	260/280 Ratio	Conc. (ng/µl)	DIN		
Additional Comments				Read Date:		Read Date:			
				Initials:		Initials:			

^Mark this column is a sample requires further review based on the quantification data and add reason in the 'Additional Notes' section.

APPENDIX E: TBA DNA Quantification and Storage Worksheet

DNA Extraction Batch Name: TBA ___ ___ ___	DNA extraction date:

Part C: DNA Storage

Study NC007/ NC008	UCL Lab No.	Subject ID	Lab accession number	Storage of Original DNA (if applicable*)		Details of any other stored aliquots (e.g. further dilutions) Sample identifier (e.g. D1, D2), Box Name, Position, Volume
				Box Name.	Box Position (if not stored by UCL number)	
Additional Comments				Freezer Number:		Freezer Number:
				Date frozen:		Date frozen:
				Initials:		Initials:

*if all DNA is required to be sent for WGS please indicate that sample was not stored

Appendix F: Minimum Inhibitory Concentration (MIC) H37Rv Worksheet (EXAMPLE)

Appendix F

TBA MIC Quality Control – H37Rv worksheet

Version No.			
Date			
Initial			

	Quality Control	H37Rv
	RESULT SECTION *(Completed by the operator)*	
Blood agar plate Date of evaluation	☐☐ day ☐☐☐ month ☐☐☐☐ year	Result: ☐ pos. ☐ neg Initials _____
BED	Test Start Date	☐☐ day ☐☐☐ month ☐☐☐☐ year Initials .....
	MIC value	..... µg/ml or Missing ☐ Provide reason.....
	Result Date	☐☐ day ☐☐☐ month ☐☐☐☐ year Initials .....
PRE	Test Start Date	☐☐ day ☐☐☐ month ☐☐☐☐ year Initials .....
	MIC value	..... µg/ml or Missing ☐ Provide reason.....
	Result Date	☐☐ day ☐☐☐ month ☐☐☐☐ year Initials .....
LIN	Test Start Date	☐☐ day ☐☐☐ month ☐☐☐☐ year Initials .....
	MIC value	..... µg/ml or Missing ☐ Provide reason.....
	Result Date	☐☐ day ☐☐☐ month ☐☐☐☐ year Initials .....
MOX	Test Start Date	☐☐ day ☐☐☐ month ☐☐☐☐ year Initials .....
	MIC value	..... µg/ml or Missing ☐ Provide reason.....
	Result Date	☐☐ day ☐☐☐ month ☐☐☐☐ year Initials .....
	QC Passed	☐ Yes ☐ No If No, add comment below
	Comments	

Lab Supervisor (Signature)*: _____ Date: __ /___ /____

Lab Manager (Signature): _____ Date: __ /___ /____

Acceptable MIC range for the *M TB* H37Rv reference strain quality control:

Drug	BDQ	PRE	LZD	MOX
MIC (µg/ml)	≤0.5 and ≥0.25 **(0.25 and 0.5)**	≤0.25 and ≥0.12 **(0.12 and 0.25)**	≤1 and ≥0.5 **(0.5 and 1.0)**	≤0.5 and ≥0.125 **(0.125 to 0.5)**

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* Verified that the results are transcribed to the LRF correctly

Appendix F

TBA MIC Quality Control – H37Rv worksheet

Version No.			
Date			
Initial			

Fill out in case of resistant result:

Drug: ____ Conc (µg/ml): ____ Start Date: ____ Initials: ____	Blood agar plate sterile	☐ yes ☐ contaminated	____ Initials/Date Read
	Confirmation by ZN-stain	☐ AFB+ ☐ contaminated ☐ No AFB	____ Initials/Date Read
Drug: ____ Conc (µg/ml): ____ Start Date: ____ Initials: ____	Blood agar plate sterile	☐ yes ☐ contaminated	____ Initials/Date Read
	Confirmation by ZN-stain	☐ AFB+ ☐ contaminated ☐ No AFB	____ Initials/Date Read
Drug: ____ Conc (µg/ml): ____ Start Date: ____ Initials: ____	Blood agar plate sterile	☐ yes ☐ contaminated	____ Initials/Date Read
	Confirmation by ZN-stain	☐ AFB+ ☐ contaminated ☐ No AFB	____ Initials/Date Read
Drug: ____ Conc (µg/ml): ____ Start Date: ____ Initials: ____	Blood agar plate sterile	☐ yes ☐ contaminated	____ Initials/Date Read
	Confirmation by ZN-stain	☐ AFB+ ☐ contaminated ☐ No AFB	____ Initials/Date Read
Drug: ____ Conc (µg/ml): ____ Start Date: ____ Initials: ____	Blood agar plate sterile	☐ yes ☐ contaminated	____ Initials/Date Read
	Confirmation by ZN-stain	☐ AFB+ ☐ contaminated ☐ No AFB	____ Initials/Date Read

Comments:

Lab. Manager (Signature): ____________________ Date: ____________________

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2 / 2 * Verified that the results are transcribed to the LRF correctly

Appendix G: DNA Extraction and Quantification Reagent Log (EXAMPLE)

APPENDIX G: TBA DNA Extraction and Quantification Reagent Log

DNA Extraction Batch Name: TBA___ ___ ___

Complete for each batch of DNA extraction

Reagent	Lot number	Reagent Expiry date (if applicable)	Preparation/ Opened/Frozen Date	Date of use	Expiry Date of prepared solution	Valid for use (Y/N)	Initials	Comments
Lysozyme Solution								
SDS solution					*1 month from prep date*			
Proteinase K								
5M NaCl					*1 year from prep/open date*			
CTAB					*5 years of open date*			
CTAB/NaCl solution					*6 months from prep date*			
Chloroform/isoamylalcohol					*6 months from prep date (if prepared).*			
Ethanol					*5 years of open date*			
Isopropanol					*1 year from open date*			
tRNA								
Ultra-Pure distilled water								

APPENDIX G: TBA DNA Extraction and Quantification Reagent Log

DNA Extraction Batch Name: TBA___ ___ ___

TE buffer								
Reagent	**Lot number**	**Reagent Expiry date** (if applicable)	**Preparation/ Opened/Frozen Date**	**Date of use**	**Expiry Date of prepared solution**	**Valid for use (Y/N)**	**Initials**	**Comments**
Qubit® Kit HS Kit BR Kit (delete as appropriate)					*6 months from opening*			
Agilent Genomic DNA Ladder								
Agilent Genomic DNA Sample Buffer								
TapeStation cartridge								

Appendix H: Frozen Isolate Storage Log (EXAMPLE)

Part A: Frozen Isolate Storage Log

TBA MycoLabMan: Appendix H: Part A Frozen Isolate Storage Log v2.0 10 May 2021				Position of Cryovial No 1 (BOX 1) Box name and location in the box	Date Frozen	Initials	Position of Cryovial No 2 (BOX 2) Box name and location in the box	Date Frozen	Initials	Retention Date
UCL No.	Subject ID	LAN	Visit Schedule							
										Until being informed otherwise by sponsor- to be discussed with sponsor at the end of NC-007 trial

Appendix H: Part B

TBA Frozen Isolate Storage Log

This log is used to document frozen isolate storage. Please tick TBA Study: NC-007 ☐ NC-008 ☐

Part A: Electronic Frozen Isolate Storage Log – see TBA_MycoLabMan_AppH_Frozen Isolate Storage Log excel sheet. This must be printed, signed and dated every 6 months and stored in the laboratory site file

Part B: Frozen isolate removal Log - If any isolates are removed, they must be replenished/replaced and returned to storage. Please document by completing the table below.

UCL ID No.	Subject ID	Lab Accession Number	Current Isolate Location Number	Date Removed (dd/mmm/yyyy)	Removal details – reason, volume taken	Initials	Date Returned (dd/mmm/yyyy)	New Isolate Location Number	Initials	Comments

Appendix I: Received Isolates Worksheet (EXAMPLE)

APPENDIX I

TBA Received Isolates: Processing and Discard Worksheet

LJ cultures of positive samples from local laboratories are sent to the UCL Mycobacteriology Central Laboratory for further analysis. Upon receipt at the UCL Central Laboratory the isolates and their initial processing (subculture (S/C) onto solid media and MGIT, Blood Agar (BA) culture and HAIN CM) are logged using this worksheet.

Please tick TBA Study as appropriate: NC-007 ☐ NC-008 ☐

ISOLATE RECEIPT					INITIAL PROCESSING					DISCARD*		
UCL Number	Date Received (dd/mmm/yyyy)	Subject ID	Original LAN	Initials	Date Processed (dd/mmm/yyyy)	Solid S/C (LJ/7H11) & initials	MGIT S/C Tube Number (–/–/––––)	BA result, Date Read (dd/mmm/yyyy) & initials	HAIN CM result & initials	Date & initials (dd/mmm/yyyy)		
										Site LJ	Solid S/C	MGIT S/C
										Comments:		
										Comments:		
										Comments:		
										Comments:		
										Comments:		

*For rejected samples include the rejection item number (TBA rejection Log) in the discard comments field.

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APPENDIX I

TBA Received Isolates: Processing and Discard Worksheet

ISOLATE RECEIPT					INITIAL PROCESSING					DISCARD*		
UCL Number	Date Received (dd/mmm/yyyy)	Subject ID	Original LAN	Initials	Date Processed (dd/mmm/yyyy)	Solid S/C (LJ/7H11) & initials	MGIT S/C Tube Number (–/–/–––)	BA result, Date Read (dd/mmm/yyyy) & initials	HAIN CM result & initials	Date & initials (dd/mmm/yyyy)		
										Site LJ	Solid S/C	MGIT S/C
										Comments:		
										Comments:		
										Comments:		
										Comments:		
										Comments:		
										Comments:		
										Comments:		

*For rejected samples include the rejection item number (TBA rejection Log) in the discard comments field.