

# LABORATORY MANUAL

## MYCOBACTERIOLOGY LABORATORIES

### Protocol Title:

A Phase 3 open-label trial assessing the safety and efficacy of bedaquiline plus pretomanid plus linezolid in Subjects with pulmonary infection of either extensively drug-resistant tuberculosis (XDR-TB) or treatment intolerant / non-responsive multi-drug resistant tuberculosis (MDR-TB)

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## TABLE OF CONTENTS

2.	ABBREVIATIONS .....	5
3.	CONTACT DETAILS .....	6
4.	INTRODUCTION .....	7
5.	MYCOBACTERIOLOGY LABORATORY TESTING .....	9
6.	STANDARD OPERATING PROCEDURES (SOP) .....	13
6.1.	SOP 1: Sputum Handling.....	14
6.2.	SOP 2: Preparation of Samples for Storage and/or Shipment.....	17
6.3.	SOP 3: Sputum Decontamination .....	20
6.4.	SOP 4: Acid-fast Bacilli (AFB) Microscopy.....	25
6.5.	SOP 5: Hain Line Probe Assays (LPA) .....	31
6.6.	SOP 6: GeneXpert MTB/RIF (or Xpert MTB /RIF) .....	37
6.7.	SOP 7: Liquid Culture by Mycobacteria Growth Indicator Tube (MGIT) .....	42
6.8.	SOP 8: MPT64 Antigen Test .....	49
6.9.	SOP 9: DNA Extraction.....	52
6.10.	SOP 10: Drug Susceptibility Testing (DST) by Mycobacteria Growth Indicator Tube (MGIT).....	57
6.11.	SOP 11: Minimum Inhibitory Concentration (MIC).....	64
6.12.	SOP 12: Whole Genome Sequencing (WGS) .....	77

## Figures

Figure 1: Schematic Microbiology Testing for NiX-TB.....	9
Figure 2: Flowchart for Processing Screening Sputum Samples .....	11
Figure 3: Flowchart for Processing of Sputum Samples During Treatment and Follow-up Phases (Day 1 - Month 24) .....	12
Figure 4: Scanning Scheme for Smear Examination .....	28
Figure 5: Diagram Showing Work Flow in a PCR Laboratory .....	36
Figure 6: Example of GeneXpert View .....	39
Figure 7: Algorithm for Interpretation of Positive MGIT Cultures .....	45
Figure 8: Example of printed barcodes .....	68
Figure 9: Example of TBExiST worklist .....	72
Figure 10: Example of a TBExiST plot, annotated to show the 'resistant' and 'Susceptible' growth curves relative to the Growth Control .....	73
Figure 11: Example of TBExiST Interpretation Report .....	74

## Tables

Table 1: Schedule of Visits with Microbiological Assessments .....	10
Table 2: SOPs Applicable to Each Laboratory Type .....	13
Table 3: Sputum Samples Collected During the Trial .....	14
Table 4: Storage of Microbiological Samples .....	17
Table 5: WHO/IUTLD Grading Scale for 200x (Auramine Staining) .....	28
Table 6: WHO/IUATLD Grading System for ZN Smears .....	29
Table 7: Uses of LPAs in NiX study .....	31
Table 8: Interpretation of the MPT64 Antigen Assays .....	50
Table 9: Preparation of DST Drug Stock Solutions .....	58
Table 10: Working concentrations of DST Drugs .....	60
Table 11: Manufacturer and storage instructions for the study drugs .....	65
Table 12: Drug concentrations used for MGIT MIC testing .....	65
Table 13: Drug concentrations used for MGIT Drug Sensitivity Testing .....	66
Table 14: MIC cut-off values for each drug, for a sample to be considered resistant and therefore require further testing .....	75
Table 15: Acceptable MIC range for the MTB H37Rv reference strain quality control .....	75
Table 16: Contact Details for Shipment of DNA from UCL to PHE .....	78
Table 17: PHE SOP list .....	80
Table 18: Data files generated during the sequence analysis .....	83

## Version History:

Master Version Number/Date	Change
1.0/11 March 2015	Initial version
2.0/08 Jul 2015	Re-organized sections and procedures/content to align with NC-006 lab manual where appropriate
3.0/10 Nov 2016	<ul style="list-style-type: none"> <li>• Updated central laboratories' contact details.</li> <li>• Added suggested Laboratory Reporting Forms (LRF), as well as new sample transport forms.</li> <li>• Reorganized summary figures.</li> <li>• Changed storage/shipment of positive isolates, including where they are shipped and which assessments are performed on isolates in each location.</li> <li>• Added additional smear and speciation assessments to ensure proper clinical care and follow up of subjects.</li> <li>• Consolidated all SOPs related to AFB microscopy into one.</li> <li>• Consolidated all SOPs related to Hain LPA into one.</li> <li>• Updated SOP on MGIT culture (SOP 8).</li> <li>• Introduced a new SOP (SOP 9) on MPT64 antigen testing.</li> <li>• Updated SOP on DST by MGIT (SOP 11).</li> </ul>
4.0/15Aug2017	<ul style="list-style-type: none"> <li>• Updated UCL Central Laboratory contact details and deleted information on TASK central laboratory.</li> <li>• Deleted all references to <i>pncA</i> sequence analysis, including corresponding SOP and forms.</li> <li>• Added SOPs for Minimum Inhibitory Concentration (MIC) and Whole Genome Sequencing (WGS).</li> <li>• Updated Laboratory Reporting Forms (LRF) and sample transport form.</li> <li>• Updated Drug Susceptibility Testing (DST) SOP.</li> <li>• Deleted any reference to St. Andrews Central Laboratory</li> </ul>
5.0/15May2018	<ul style="list-style-type: none"> <li>• Included Appendices 1 – 7 in the list of supportive documents on pages 7 &amp; 8</li> <li>• Updated Whole Genome Sequencing (WGS) SOP and related forms (Appendix H &amp; Appendix I)</li> <li>• Added full headers to Appendices A - I</li> </ul>

## 2. ABBREVIATIONS

AFB .....	Acid Fast Bacilli
ATCC.....	American Type Culture Collection
B .....	Bedaquiline
BA .....	Blood Agar
BSC .....	Biological Safety Cabinet
CL3/BSL3 .....	Containment Level 3
CQIF.....	Continuous Quality Improvement Form
CRF .....	Case Report Form
DMSO .....	Dimethyl Sulfoxide
DST .....	Drug Susceptibility Testing
E .....	Ethambutol
EMS .....	Early Morning Sputum
FQ .....	Fluoroquinolones
GC .....	Growth Control
I .....	Isoniazid
HYB .....	Hybridization Buffer
IQC .....	Internal Quality Control
K .....	Kanamycin
L.....	Linezolid
LIMS .....	Laboratory Information Management System
LM .....	Laboratory manual
LRF.....	Laboratory Report Form
MGIT .....	Mycobacteria Growth Indicator Tube
MIC.....	Minimum Inhibitory Concentration
MIN .....	Minute(s)
M.....	Moxifloxacin
MOTT .....	Mycobacteria other than <i>M. tuberculosis</i>
MTB.....	<i>Mycobacterium tuberculosis</i>
MTBC.....	Mycobacterium tuberculosis complex
NALC.....	N-Acetyl L-Cysteine
NaOH.....	Sodium Hydroxide
OADC.....	Oleic Acid Albumin Dextrose Complex
OD .....	Optical Density
Pa .....	Pretomanid
PANTA .....	Polymyxin B, Amphotericin B, Nalaxidic acid, Trimethoprim, Azlocillin
PBS .....	Phosphate Buffered Saline
PCR.....	Polymerase Chain Reaction
PPE .....	Personal protective equipment
PCR.....	Polymerase Chain reaction
QC .....	Quality Control
R .....	Rifampicin
S .....	Streptomycin
SIRE .....	Streptomycin, Isoniazid, Rifampicin, Ethambutol
SOP .....	Standard Operating Procedure
STR .....	Stringent Wash Solution
T .....	Temperature
TTP .....	Time to Positivity
UCL.....	University College London
Z .....	Pyrazinamide
ZN.....	Ziehl-Neelsen

### 3. CONTACT DETAILS

Isolates from baseline and at/after end of treatment (if applicable), Local Laboratory to send to:					
		Contact Person	Contact Details		
UCL Central Laboratory	To discuss routine shipment receipt and courier requests	Anna Bateson	E-mail	<a href="mailto:a.bateson@ucl.ac.uk">a.bateson@ucl.ac.uk</a>	DEPARTMENT OF CLINICAL MICROBIOLOGY ROYAL FREE HOSPITAL CAMPUS ROWLAND HILL STREET LONDON NW3 2PF UNITED KINGDOM
		Julio Ortiz Canseco	E-mail	<a href="mailto:julio.canseco@ucl.ac.uk">julio.canseco@ucl.ac.uk</a>	
	As permit consignee for shipments	Prof Timothy McHugh	Tel	+44 20 7472 6402	
			Fax	+44 207 794 0433	
			E-mail	<a href="mailto:t.mchugh@ucl.ac.uk">t.mchugh@ucl.ac.uk</a>	

## 4. INTRODUCTION

The NiX-TB clinical trial is a phase 3 open-label trial assessing the safety and efficacy of bedaquiline (B) plus pretomanid (Pa) plus linezolid (L) in subjects with pulmonary infection of either extensively drug-resistant tuberculosis (XDR-TB) or treatment intolerant / non-responsive multi-drug resistant tuberculosis (MDR-TB).

Throughout the study, basic microbiological assays will be conducted at a number of study laboratories receiving samples directly from the sites, whereas characterization assays will be conducted at the central laboratory, University College London (UCL) Central Mycobacteriology Laboratory – as follows:

- Study Laboratories:
  - On screening sputum samples: perform acid-fast bacilli (AFB) smear microscopy, a molecular test – GeneXpert MTB/RIF or HAIN MTBDR<sub>plus</sub> (Version 2) – and MGIT culture to detect *Mycobacterium tuberculosis* (MTB) and obtain time-to-positivity (TTP).
  - On all sputum samples collected post-screening: perform MGIT culture to detect MTB and obtain TTP. In addition, at one baseline visit [preferably the early morning sputum (EMS) at Day 1 or Screening to Week 4 if Day 1 cultures are contaminated or negative) and all visits Week 16 and beyond, speciate the AFB positive MGIT cultures to confirm presence of MTB complex (MTBC) bacteria.
  - Store all non-contaminated, AFB positive MGIT cultures until the end of the trial.
  - Ship isolates obtained at baseline and at/after end of treatment (only first positive visit) to UCL for further characterisation.
- UCL Laboratory:
  - Performs drug susceptibility testing (DST) to multiple antibiotics in liquid culture, and minimum inhibitory concentration (MIC) determinations for the study drugs, on isolates obtained at baseline and at/after end of treatment.
  - Extracts DNA from these isolates and co-ordinates whole genome sequencing (WGS) – shipment of the DNA to a designated reference laboratory, data analysis and reporting of results generated
  - Stores the isolates and DNA for a maximum of 5 years after the trial closure.

The diagrams and tables that follow before the start of the SOPs give an overall orientation to the individual assays, when and where they are performed.

This manual is used in combination with the following documents:

- Appendix A – Sputum Specimen Transport Form
- Appendix B – LRF1: Screening Samples
- Appendix C – LRF2: Treatment and Follow-up Samples
- Appendix D – LRF3: Speciation
- Appendix E – Isolate Shipment to UCL Laboratory  
(Study Lab - complete one per shipment to UCL)
- Appendix F – LRF4: Drug Susceptibility Testing (used by central lab)
- Appendix G – LRF5: Minimum Inhibitory Concentration (used by central lab)
- Appendix H – LRF6: Paired Whole Genome Sequencing (used by central lab)
- Appendix I – DNA Quantification Worksheet (used by central lab)
- Appendix 1 – Hain GenoLyse Instructions for Use
- Appendix 2 – Xpert *M.tb*/Rif System Operator Manual
- Appendix 3 – Hain GenoType MTBDR<sub>plus</sub> Instructions for Use
- Appendix 4 – Hain GenoType MTBDRs/ Instructions for Use

- Appendix 5 – Hain GenoType MTBC Instructions for Use
- Appendix 6 – Hain GenoType Mycobacterium CM Instructions for Use
- Appendix 7 – BW0303: Instructions on sample submission and receiving results
- NiX-TB (B-L-Pa) Protocol
- NiX-TB Laboratory Quality Control Manual



## 5. MYCOBACTERIOLOGY LABORATORY TESTING

The Mycobacteriology laboratory testing that occurs at each laboratory (study and central laboratories) is summarized in Figure 1

Figure 1: Schematic Microbiology Testing for NiX-TB

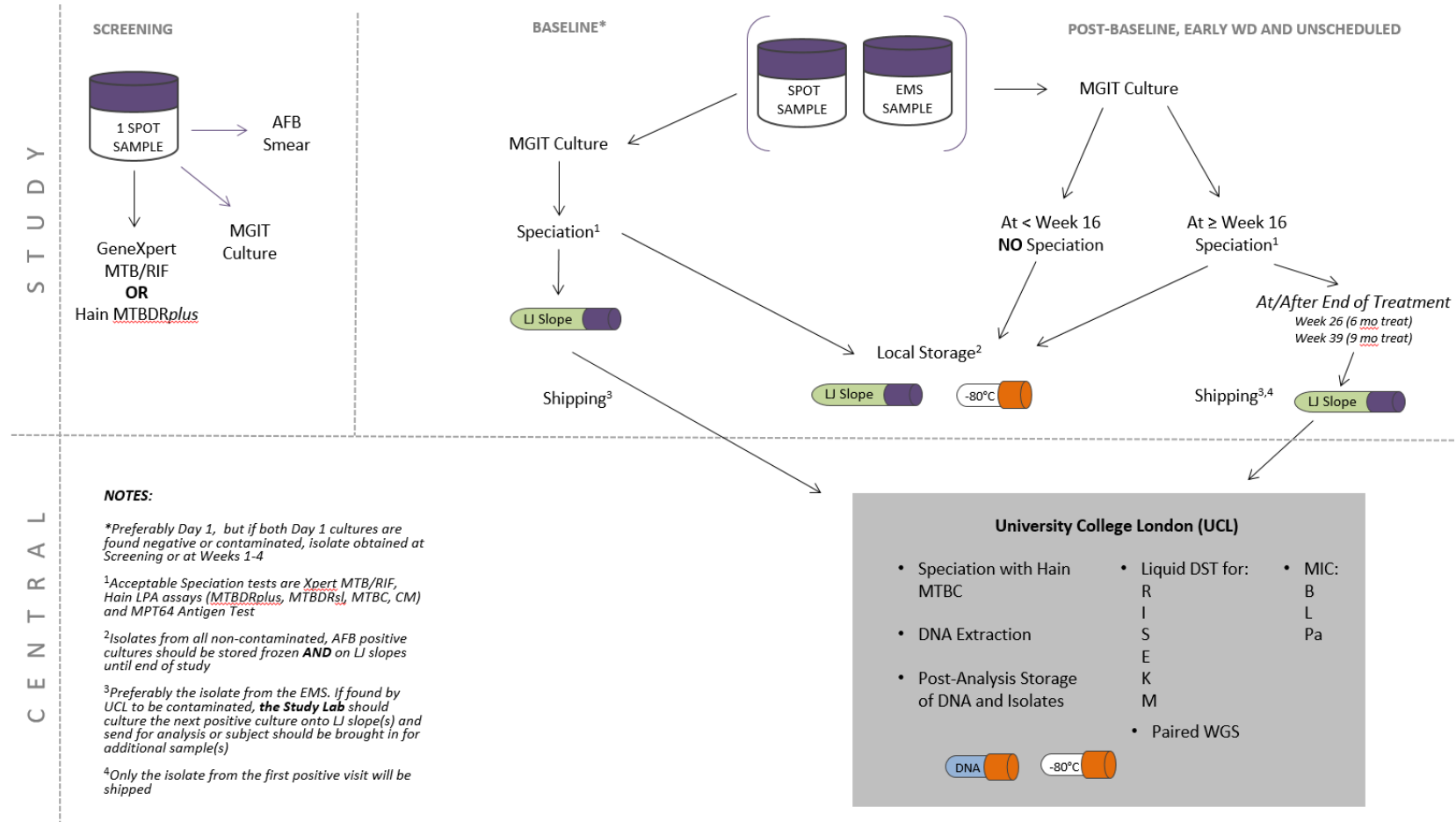


Table 1: Schedule of Visits with Microbiological Assessments

Screening	Treatment	Follow Up Post End of Treatment
Day -9 to Day -1	Day 1	1 month
	Week 1	2 month
	Week 2	3 month
	Week 4	6 month
	Week 6	9 month
	Week 8	12 month
	Week 12	15 month
	Week 16	18 month
	Week 20	21 month
	Week 26	24 month
	Week 30 (9 months treatment)	
	Week 34 (9 months treatment)	
	Week 39 (9 months treatment)	
	Unscheduled	
	Early Withdrawal	

Figure 2: Flowchart for Processing Screening Sputum Samples

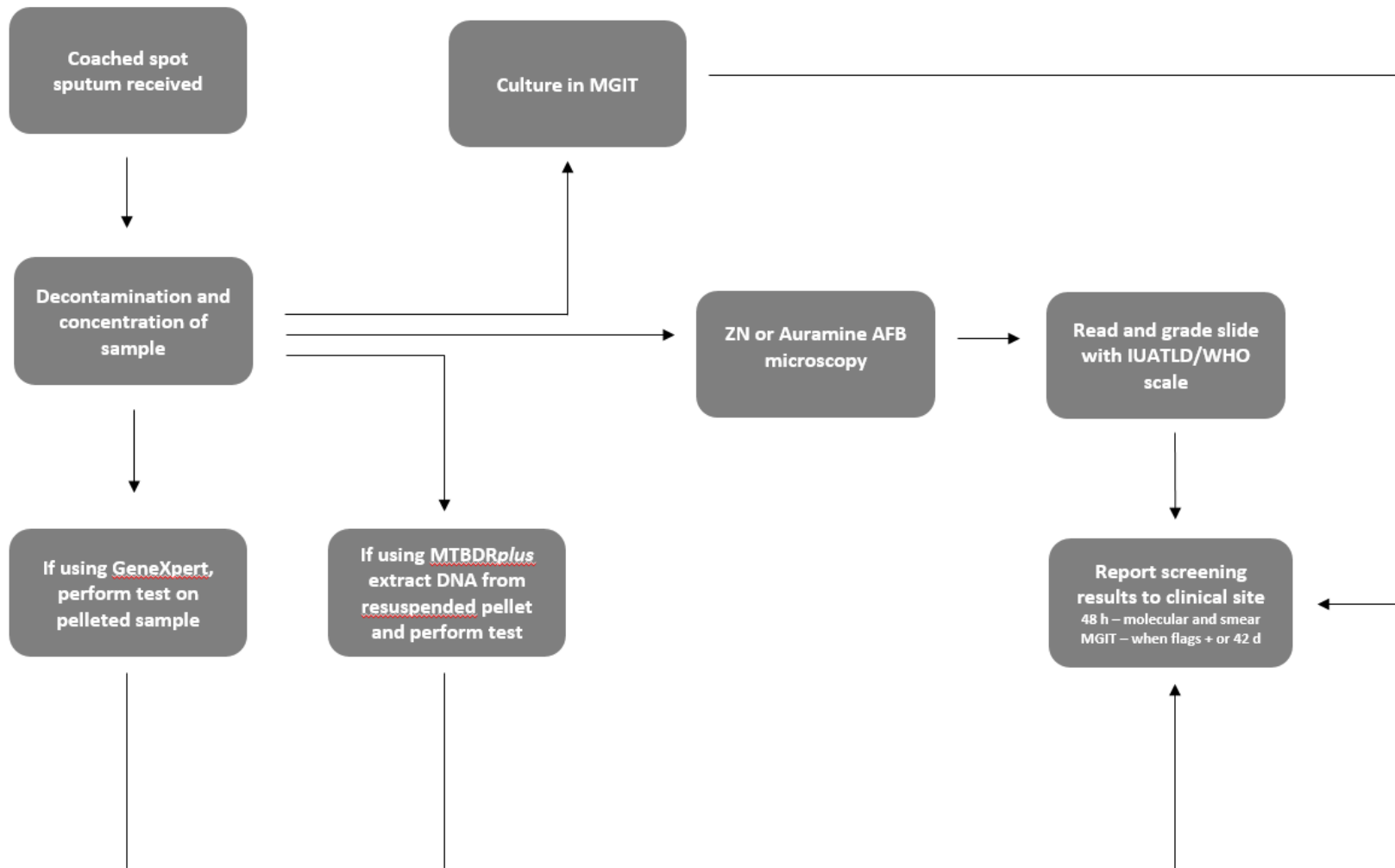
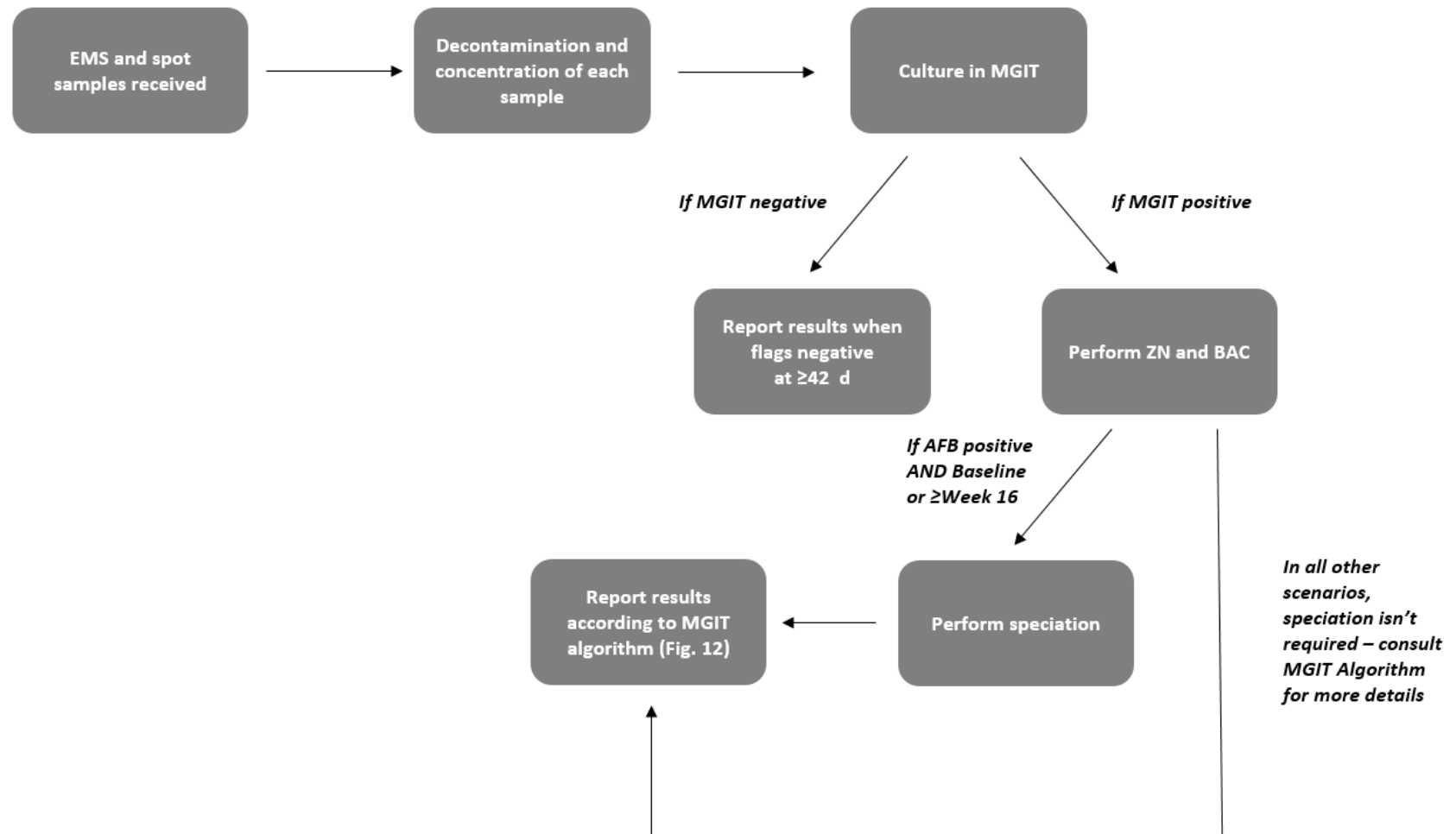


Figure 3: Flowchart for Processing of Sputum Samples During Treatment and Follow-up Phases (Day 1 - Month 24)



## 6. STANDARD OPERATING PROCEDURES (SOP)

The SOPs which apply to each mycobacteriology laboratory type are described below.

Table 2: SOPs Applicable to Each Laboratory Type

Laboratory	Role	SOPs
Study Laboratories	Mycobacteriology assessments during Screening, Treatment and Post-Treatment phases: <ul style="list-style-type: none"> <li>• AFB smear microscopy to confirm presence of AFB in sputum samples or cultures</li> <li>• Molecular test to detect MTB in Screening sputum samples</li> <li>• MGIT culture to detect MTB and obtain TTP</li> <li>• Speciation for confirmation of presence of MTBC bacteria</li> <li>• Storage and shipping of MTB isolates</li> </ul>	1, 3, 4  1, 3, 5, 6 1, 3, 7 5, 6, 8 2
UCL Central Mycobacteriology Laboratory	Mycobacteriology Characterisation: <ul style="list-style-type: none"> <li>• Speciation for confirmation of presence of MTBC bacteria</li> <li>• DST in the MGIT System</li> <li>• MIC determination</li> <li>• Storage of MTB isolates</li> <li>• Whole Genome Sequencing</li> </ul>	5 10 11 2 9, 12

## 6.1. SOP 1: Sputum Handling

### 6.1.1. Purpose

Proper collection and transport of sputum specimens is required to ensure quality laboratory results. Upon receipt of the specimens, proper labelling must be verified before processing the specimens. Prior to and during shipping of samples correct handling procedures must also be followed.

### 6.1.2. Principle

Sputum specimens in this study are collected in the early morning at home or while in the hospital (“early morning” samples), and as spot samples at the research site. These are delivered to the study microbiology laboratory where they are received, checked and logged-in prior to any tests being performed.

### 6.1.3. Procedure

#### 6.1.3.1. Receipt of specimen at the study laboratory

The samples described in Table 3 will be received at the study laboratory.

Table 3: Sputum Samples Collected During the Trial

Timing	Specimens Collected
Days -9 to -1 (Screening)	One spot sputum collected at the research site under the coaching and observation of the trial staff.
<ul style="list-style-type: none"> <li>All visits from Day 1 up to and including Month 24 follow-up visit.</li> <li>If both sputum samples given at Weeks 16, 26 (if 6 mo treatment), 39 (if 9 mo treatment) or Month 24 (follow up) are contaminated, subject should be brought back in to give additional samples.</li> <li>Unscheduled visits: subject should be brought back for an unscheduled visit to collect sputum to ensure that the following conditions are met at end of treatment/end of follow-up or at early withdrawal: <ul style="list-style-type: none"> <li>Two sequential negative sputum results.</li> <li>Two sequential positive sputum results.</li> <li>Unable to produce sputum after documentation of two negative sputum cultures with no intervening positive and are clinically asymptomatic</li> </ul> </li> <li>Early Withdrawal Visit</li> </ul>	<p><u>Two sputum samples:</u></p> <ul style="list-style-type: none"> <li>One early morning sputum (EMS) collected and brought by subject from home or collected in hospital.</li> <li>One spot sputum collected at the site under the coaching and observation of the trial staff.</li> <li><b>Alternatively</b>, two spot sputa collected at the site, in case the patient did not bring an EMS sample.</li> </ul>

### 6.1.3.2. Logging-in of sputum specimen at the study laboratory

1. On receipt of samples at the laboratory:
  - Measure the temperature of the transport box and ensure the temperature is not below 2° C or above 8° C.
  - Assign a unique laboratory accession number to the specimen. The laboratory accession number is used to label tubes for all subsequent downstream processing of this specimen (e.g. cryotubes, MGIT tubes, agar plates, microscope slides, etc.) and for the reporting of data on the approved laboratory source documentation, case report form (CRF) and results.
2. Complete the Study Lab Specific Specimen Transfer Form (the form used by lab that receives samples directly from the site; Appendix A or equivalent)
  - Place a laboratory accession number label on the form for each specimen;
  - Complete the date and time the samples are received; the temperature from the maximum/minimum thermometer inside the transport container; and whether the specimen has been received within 24 hours of collection.
  - Perform a visual check of the specimens to confirm they are in good condition (i.e. the sample does not contain only saliva, or excessive blood quantity and is of appropriate volume) and complete this information onto the form.
3. Check if the specimen label details match those on the Specimen Transport Form.
4. If the specimens are not processed within 30 min of receipt at the laboratory, place in the designated sputum refrigerator (2-8 °C) and record the time and fridge identifier on the Specimen Transfer Form.
5. The specimen register should be completed to link the specimen details with the accession number.
6. The Specimen Transfer Form must be stored at the laboratory in the study laboratory file. A copy of the Specimen Transfer Form may be sent back to the clinic and/or data office as appropriate

### 6.1.3.3. Timing of specimen receipt

1. The laboratory must process all specimens as soon as possible **and no later than 48 hours after the specimen is collected.**
2. Logistical issues related to the personnel shift or appropriate arrangements for specimens shipping must be pre-arranged to meet this timeframe.
3. If laboratories are closed on weekends or for public holidays, short-term storage for no more than 48 hours is acceptable provided that:
  - appropriate refrigerated conditions (2-8 °C) are maintained,
  - the sputum is in good condition (i.e. the sample does not contain only saliva, or excessive blood quantity and is of appropriate volume),
  - the Specimen Transfer Form is completed as described above,

If the sample is processed out of the window period (more than 48 hours after collection), contact TB Alliance in order to assess the validity of the data and the specimens.

### 6.1.3.4. Handling of specimen receipt issues

If the delegated laboratory staff finds leaking specimen containers, mis-labelling, incomplete labelling, incomplete or mismatching specimen labels and accompanying forms, follow the procedures listed below.

#### Leaking container

Carefully scrutinize the specimen containers to determine any sample leakage. Liquid seen could be due to condensation inside the pathoseal bag only. Do not open the pathoseal bag if the specimen container leaked. Instead, after marking the

specimen as “LEAKED” in the Specimen Transfer Form, seal the leaked specimens in a biohazard autoclave bag and discard following the biowaste lab procedures.

Immediately notify the clinical site, who will decide if another specimen(s) could be collected.

### **Mis-labelled specimens**

The Specimen Transfer Form and specimen study specific labels must be fully completed and match. If they do not match, the laboratory must contact the clinical site to obtain any outstanding/missing information before the specimen is processed. The contact with the clinical site must be documented in writing and signed and dated.

Specimens without the matching forms are **not** to be processed until a fully completed Specimen Transfer Form or clarification is received. If attempts to correct the forms cannot ensure proper identification of the specimen, the collection should be repeated.

### **Unlabelled specimens**

Unlabelled specimens will **not** be processed unless the shipment contains multiple specimens from a single visit for a subject. In this case, the clinical site will be contacted and asked to resolve the discrepancies and complete the labelling process. Pending this correction, the specimen will be stored in the laboratory refrigerator.

If there are more than one subject’s samples in a shipment (or the information on samples from a single subject’s visit cannot be obtained within the appropriate time period), the specimen(s) will be discarded and new sample(s) will be requested.

### **Specimens arriving outside the designated temperature range**

Every effort should be made to maintain the sputum samples within the specified temperature range (2-8°C) during all the steps prior to sample processing (i.e. soon after the collection of the sample at the clinical site and during transportation to the laboratory). This is essential to minimize the growth of any contaminating bacteria that may be present in the sputum sample. If a sputum sample arrives at the laboratory outside of the temperature range, a repeat sample should be requested as soon as possible. If it is not possible to obtain another sample (i.e. the patient has left the clinic), the original sample must be processed to avoid losing the time-point.

### **Small volume samples**

Every effort should be made by the site clinical team to ensure a good quality sputum sample of sufficient volume (>2 ml) is collected. Sputum samples cannot be pooled to increase volume. If the sputum sample is less than 2 ml and a good quality specimen, it should still be processed. Although sputum processing is less accurate when the specimen volume is less than 2 ml (because the sputum pellet is re-suspended in 1.5 – 2.0 ml of PBS after centrifugation), it is still valuable to determine whether acid fast bacilli can be detected. The volume must be noted as less than 2.0 ml in the approved laboratory source documentation so these samples can be excluded from the quantitative culture analyses [e.g. Mycobacteria Growth Indicator Tube (MGIT) and Time-To-Positivity (TTP)].

## **6.1.4. Documentation**

Suggested Specimen Requisition Form and Laboratory Report Form (LRF) 1 (if the specimen is from Screening) or LRF2 (for all other visits) are found in Appendices A to C. Study Laboratories may use their own forms, or a Laboratory Information Management System (LIMS), as long as they capture all the required information related to sputum collection, transport and receipt for completion of the electronic CRF (highlighted fields in the LRFs). The use of these alternate forms will have to be approved by TB Alliance.



## 6.2. SOP 2: Preparation of Samples for Storage and/or Shipment

### 6.2.1. Purpose

The samples described in Table 4 are stored and/or shipped in this trial.

Table 4: Storage of Microbiological Samples

Sample	Prepared and Stored by	Stored Samples	Storage Period	Shipped Samples
MTB isolates from all AFB+/BAC- cultures	Study Laboratory	<u>For each isolate:</u> <ul style="list-style-type: none"> <li>1 LJ culture stored at room temp.</li> <li>1 culture at -70-80°C in 50% glycerol stored at study lab</li> </ul>	Until trial closure	<u>If from baseline* or the first positive* at/after end of treatment (after confirmed positive for MTBC):</u> <ul style="list-style-type: none"> <li>1 LJ to UCL</li> </ul>
MTB DNA and isolates after mycobacteriological characterisation	UCL Laboratory	<ul style="list-style-type: none"> <li>Isolates at -70-80°C in 50% glycerol</li> <li>DNA at -20°C</li> </ul>	A maximum of 5 years after trial closure	<u>DNAs shipped to WGS laboratory</u> <ul style="list-style-type: none"> <li>from all baseline isolates</li> <li>from MTB isolated at the first positive sample at/after end of treatment, for which there is a matching baseline counterpart</li> </ul>

\*See notes below

#### NOTES:

- Only one baseline isolate for each patient** will be shipped to UCL Central Laboratory. In order of preference: 1) the isolate obtained from the EMS (or spot sputum) collected at Day 1; or 2) the first positive culture from Screening up to Week 4, in this order of predilection: Screening > Week 1 > Week 2 > Week 4. For all visits, whenever both EMS and spot cultures are positive, the isolate from the former is to be shipped.
- Only isolates confirmed to be MTBC** by any speciation test (SOPs 5, 6 or 9) will be shipped to UCL.

The purpose of this SOP is to describe the methodologies for preparation and required storage conditions of these samples.

### 6.2.2. Procedures

#### Storage of positive MGIT cultures

The **Study Laboratory** will store all MGIT positive cultures found to contain AFB (ZN positive) and no contamination (BAC negative). Two samples should be stored for each isolate, one on an LJ slope and the other in 50% glycerol at -70°C to -80°C, for the duration of the trial. A logbook must be kept of all isolates in storage.

The **UCL Central Laboratory** will store all isolates after mycobacteriology characterisation testing is complete, in 50% glycerol at -70°C to -80°C, as well as their DNAs at -20°C, for a maximum of 5 years after trial closure

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

### **Equipment and Reagents**

- Biological Safety Cabinet (BSC)
- Discard bucket containing appropriate mycobacteriocidal disinfectant (specified in local Health and Safety documentation)
- Cotton wool or paper towels soaked in mycobacteriocidal disinfectant
- Cryovial (with rubber o-ring seal), and appropriate storage box
- Sterile micropipettes and aerosol resistant tips (ART), or disposal pipettes
- Sterile PBS/Middlebrook 7H9 medium
- Sterile glycerol 50% (in PBS or 7H9 medium)
- LJ slope

### **Storage on LJ slope**

To inoculate a LJ slope, vortex well the MGIT tube, open the lid, and using a micropipette or disposal pipette, take 100 - 200µl of the culture and pipette onto the slope. Securely fasten and label with both the patient number, the lab accession number, and the date. Once growth is obtained these positive slopes will be stored in a rack in a cool dark place. To maintain the isolates, LJ slopes should be sub-cultured every 6 months (unless required earlier because the slope is disintegrating).

### **Storage at -70-80°C**

Spin down the MGIT culture and resuspend the deposit with 1 ml of 50% glycerol (in PBS or 7H9 medium) and transfer into a cryovial (with rubber o-ring seal in lid). Securely fasten and label with both patient number, the lab accession number (also handwrite this number in permanent marker in case sticker is removed during freezing) and date. Place in an appropriate storage box and freeze at -70°C to -80°C.

If more than one positive culture is generated from a visit (e.g, early morning and spot); the resulting cryovials should be stored in separate freezers (if possible).

### **LJ slopes for shipment to UCL Mycobacteriology Laboratory**

**Study Laboratory** will also inoculate a LJ slope for shipment to UCL Central Laboratory, at baseline (it is acceptable to use Screening to Week 4 isolates if both Day 1 cultures are contaminated or negative) **and** at or after the end of the treatment period. **Only one isolate**, preferably the one obtained from the EMS, will be shipped in either case.

The LJ slopes will be shipped to the UCL on a 6-weekly basis. LJ slopes should be sealed with parafilm, wrapped in absorbent tissue in bubble wrap, placed securely inside sealable plastic hazard container, inside sealed cardboard hazard box. Packaging should be conforming to IATA ICAO P1620 or as required by qualified courier company. This box should be labelled with appropriate hazard handling labels for IATA class 6.2, category A UN2814 Infectious Substance.

Complete the isolate shipment log (Attachment Q) and send a copy with the shipment and keep the original for records.

The UCL Central Laboratory should be contacted (see Contacts Table on page 7 for full details) to agree on a suitable shipment date and be informed when the package is collected (providing the airway bill number). The Study lab is expected to make its own arrangements with the courier.

### **6.2.3. Documentation**

The suggested LRF2 (Appendix C) captures the information for completing the eCRF fields (highlighted) related to isolate storage and shipment. Study Laboratories may use their own forms or LIMS as long as these pieces of information are included, and they are approved by the Sponsor.

In addition, when shipping samples to UCL Central Laboratory, Local Laboratories must complete Appendix E: Isolate Shipment to UCL (when shipping isolate for DST, MIC and/or WGS analysis), send the signed original along the shipment, and keep a copy in the Study File.

### 6.3. SOP 3: Sputum Decontamination

#### 6.3.1. Purpose

Sputum processing has two major functions: liquefy the specimen and decontaminate of bacteria other than mycobacteria. Although there are several techniques available, none are ideal, i.e., none of them will selectively kill only contaminating flora and achieve complete liquefaction of the specimen. A reasonable compromise is to kill as much of the contaminating bacteria as possible while harming as few mycobacteria as possible.

At screening, only one spot sample is collected, decontaminated and used for the assays detailed in Figure 3. At all other visits, an EMS and a spot sputum are collected, decontaminated and cultured in the MGIT System.

#### 6.3.2. Principle

The decontamination process is carried out using N-Acetyl-L-Cysteine – Sodium Hydroxide (NALC-NaOH), (Equivalent commercially available reagents, e.g. Mycoprep or Alpha Tec NAC-PAC, can also be used as approved by TB Alliance). NALC, a mucolytic agent, is used for rapid digestion, enabling NaOH, the decontaminating agent, to be used at a lower final concentration than that required in the absence of NALC – in this study, the range 1% - 1.5% is acceptable. Sodium citrate is also included in the decontamination solution to chelate heavy metals ions, which if present in the specimen may inactivate the NALC. Phosphate buffered saline is used to neutralise the NaOH and dilute the homogenate to lessen the viscosity and specific gravity prior to centrifugation.

**NOTE:** If the specimen has a significant quantity of blood mixed with it (not just blood tinged), do not use NaOH-NALC method because NALC does not work in the presence of blood. Use NaOH method (4% NaOH only; 1:1 (v/v) with sputum sample).

#### 6.3.3. Procedure

**Decontamination of sputum is to be carried out inside a BSC, using the appropriate biosafety procedures and PPE**

##### Equipment and Reagents

- 2.9% sodium citrate
- 4% NaOH
- NALC powder
- Sterile, break-resistant glass bottle
- 50 ml conical, graduated polypropylene centrifuge tubes with tight screw cap and appropriate rack
- Biohazard bags
- Biological safety cabinet (BSC)
- Benchguard (or alternative work surface protection)
- Appropriate PPE
- Ethanol 70%
- Pipette and aerosol resistant tips (ART)
- 3 ml Pasteur pipette
- New microscope slides, frosted one side and one end, clean and dry
- Paper towel soaked in mycobacteriocidal disinfectant, in case of spills
- Pencil or grease pen for labelling slides
- Permanent marker
- Phosphate buffered saline (pH 6.8)
- Plastic box (if available)
- Refrigerated centrifuge with sealed buckets and inserts suitable for 50 mL tubes

- Sterile (6 ml) graduated pipette
- Test tube rack for 50 ml centrifuge tubes
- Timer
- Vortex mixer
- Waste containers (including splash proof receptacle for liquids containing mycobacteriocidal disinfectant)

### **Specimen Registration**

1. Sputum samples should be processed as soon as possible and **no longer than 48 hours** after the sample is produced to reduce the risk of contamination and maximize the recovery of viable mycobacteria. Logistics for specimen transportation should be pre-arranged so samples are processed within the specific timeframe. **Samples should be refrigerated if they are not processed within 30 min of receipt in the laboratory.**
2. Double check patient data and laboratory accession numbers. Laboratory accession labels should have been attached to the specimen container and the accompanying laboratory source documentation on receipt of the sample.
  - In addition, attach laboratory accession labels to:
    - 50 ml centrifuge tube for NaOH/NALC decontamination process
    - Plastic box for storage of decontaminated specimen
    - MGIT tube used to culture the specimen.
  - The patient screening number or patient number (patient identifier - post enrolment) are also written in permanent marker on all tubes and containers that will subsequently contain the patient specimen.
  - For screening samples only, a microscope slide is labelled with the laboratory accession number and the patient screening number using a pencil or grease pen.

The specimens and all of the labelled bottles and slides are then ready to be processed.

3. The patient details and laboratory accession number are entered into specimen log book or study register. The study visit for which the specimen has been collected (e.g. Screening, Baseline, Week 1 etc.) is also recorded.

### **Preparation of BSC and Good Microbiology Practices**

1. If not used previously that day, the BSC should be allowed to purge for 20 minutes prior to working inside it, and a smoke test done or anemometer used to ensure the functionality thereof.
2. Using absorbent cotton wool or paper towels, decontaminate the BSC interior with the appropriate mycobactericidal agent, followed by 70% ethanol, and dry with a paper towel.
3. Place the necessary equipment, consumables and waste containers inside the BSC. Decontaminate these as well with the appropriate mycobactericidal agent, followed by 70% ethanol.
4. Place a clean Benchguard/ absorbent paper sheet (or alternative work surface protection) inside the BSC.
5. Place a discard bucket with a biohazard bag containing mycobactericidal agent inside BSC for disposal of contaminated materials.
6. Do not work with more specimens than can be placed in the centrifuge at one time. When processing multiple batches on the same day, clean the BSC with disinfectant, turn on UV lights (if available) for 20 minutes, and allow air to circulate in the BSC for 20 minutes between batches, prior to repeating steps 1-5.
7. Place specimen tubes at least one space apart in rack to aid in preventing contamination.
8. Work methodically with the tubes on one side and discard buckets close to the specimens, to avoid spillage/confusion of samples.
9. To reduce the risk of cross-contamination, ensure that reagent containers do not come in contact with the edge of the specimen container.
10. Remove only one cap at a time from the tubes (specimen collection tubes, culture tubes) to avoid cross-contamination and misplacing the caps on the wrong tubes.
11. Ensure that tubes, bottles, racks, pipette aid, etc. that are removed from the safety cabinet are free from any droplets/potential contaminants. If necessary, wipe the rack or tube with a paper towel soaked in mycobactericidal agent prior to removal from the BSC. In addition, if any suspicion of droplets from a specimen is seen on gloves, wipe gloves with a disinfectant-soaked towel and change gloves prior to processing additional specimens.

12. Upon completion of work, place paper towels in discard bucket. Wipe all BSC surfaces with mycobactericidal disinfectant, followed by 70% ethanol, let stand 3 minutes and wipe dry. If BSC is turned off in the evening, be sure to leave on for 1 hour before turning off. If available, turn on UV light inside BSC for at least 1 hour.

#### **Preparation of decontamination mixture (NaOH/NALC/sodium citrate)**

1. Add 500 ml 4% NaOH to 10 g NALC and mix gently to dissolve (do NOT shake vigorously).
2. Pour into a sterile, break-resistant glass bottle.
3. Add 500 ml 2.9% Sodium Citrate to the 500 ml of 4% NaOH/NALC solution. Mix gently. This is the working solution of the decontamination mixture (2% NaOH; 1% NALC; 1.45% sodium citrate) and is stable for 24 hours if stored at 2-8°C.
4. If a smaller volume is required, adjust accordingly e.g. add 200 ml 4% NaOH to 4 g NALC, mix gently and pour into an appropriately sized sterile, break-resistant glass bottle. Add 200 mL sodium citrate to the NaOH/NALC mix to give 400 mL working solution.
5. Transfer some of the working solution into a sterile tube and use this to add to the specimens. This avoids contaminating the stock bottle.

If an equivalent commercially available option has been approved by TB Alliance (e.g. Mycoprep or NAC-PAC) refer to the manufacturer's instructions. Mycoprep contains a concentration of NALC of 0.5% whereas for this study 1% is required – as for the in-house preparation of NaOH-NALC. Therefore, additional NALC powder should be added to the MycoPrep to obtain a similar concentration (i.e. to obtain 1%, 0.75 g of NALC powder must be added to 150 ml MycoPrep).

**NOTE:** TB Alliance must agree **before** any change is made to the concentration of the decontamination solution

#### **Process of decontamination using NALC/NaOH/sodium citrate**

1. Before processing specimens, prepare a waste container with disinfectant at the appropriate concentration and place a paper towel soaked in disinfectant (according to the Local Health and Safety Guidelines) on the work surface inside the BSC.
2. Ensure refrigerated specimens and reagents have been brought to room temperature before processing.
3. Switch on the centrifuge so that it can start to cool to 4-12°C.
4. A batch should **not** consist of more than 7 patient specimens in total.
5. Include a negative control with each batch of specimens (maximum total of 8 tubes per batch, see 'Quality Control' section below).
6. Transfer specimen into a 50 ml centrifuge tube with a screw cap. Make a note of the volume on the approved laboratory source documentation. For samples more than 10ml, see instructions at the end of this section. DNA stored at -80°C
7. Immediately add the NaOH-NALC sodium citrate solution in a volume equal to the quantity of specimen. Tighten the cap.
8. Start the timer.
9. Vortex for 15-30 seconds. Invert the tube so all contents are exposed to NaOH-NALC solution
10. Repeat steps 7, 8 and 10 for the subsequent specimens at 30 seconds or 1 min intervals.
11. It is important to mix well during the decontamination period to expose all the sputum to the digestion solution. It is preferable to use a shaker platform to improve homogenization. However, if a shaker is unavailable, vortex the tubes gently (to mimic the shaking action) 2-3 more times during the incubation period.
12. Make sure the specimen is completely liquefied. If still mucoid, add a further small quantity of NaOH-NALC sodium citrate solution. Mix well with the vortex again.
13. After 20 minutes, add phosphate buffered saline (PBS, pH 6.8) up to 50 ml. **Addition of sterile water is not a suitable alternative for phosphate buffer.** Mix well (lightly vortex or invert a few times). Continue to add the PBS to all specimens at 30 seconds or 1 min intervals (as above), so each specimen is **only** exposed to decontamination solution for 20 minutes; mycobacteria will be killed off if exposed to NaOH for longer than the stipulated time.  
**NOTE:** if using the Alpha Tec NAC-PAC Red Kit, add buffer until the red colour disappears. It might not be up to the 50 ml mark.
14. Transfer tubes in a 50 ml tube rack to the centrifuge.

15. Place the tubes in the centrifuge bucket, ensuring that they are equally balanced, and that the biosafety covers have been put in place for each centrifuge bucket. The centrifuge should be pre-cooled.
16. Centrifuge the specimen at a speed of 3,000 g (**NOT** 3,000 rpm, the centrifuge must be calibrated) for 15 min at 4°C (see Notes).
17. After centrifugation, remove centrifuge buckets and place in the BSC before opening. Do not open the buckets on the open bench in case there has been a spillage or breakage during centrifugation.
18. Carefully decant as much of the supernatant as possible into a suitable splash proof container (discard container) containing a mycobactericidal disinfectant (according to the Local Health and Safety Guidelines). **Make sure the sediment is not lost during decanting of the supernatant fluid.** The discard container must contain an appropriate starting concentration of disinfectant such that the final concentration of the disinfectant after addition of all the supernatants is still sufficient to kill *M. tuberculosis*.
19. Add 2 ml of phosphate buffered saline (pH 6.8) to the sediment using a sterile pipette/3mL Pasteur pipette and re-suspend it using a pipette or vortex mixer if required. Use the re-suspended pellet to prepare smears for acid-fast bacteria (AFB) microscopy (screening samples only; SOP 4) and for inoculation of MGIT tube (SOP 8).
20. Store any leftover sediment in the bijoux container (or, if not available, in the original 50 ml tube) at 4°C, for 10 days until it is confirmed the inoculated media are not contaminated.
21. If contamination is detected in the MGIT culture within 10 days, the decontamination procedure should be repeated with this remaining sediment following exactly the same procedure and new culture inoculated.

**NOTE:** For large volume samples (greater than 10 ml) there is not sufficient space in the 50 ml tube to complete the process. For these samples, note the total volume on the approved laboratory source document, then split the sample into two approximately equal volumes. Process the samples separately and combine back into a single tube when adding PBS (add 1 ml to each sample) to re-suspend the sediment (step 19). Use the combined sample for microscopy and Hain MTBDR*plus*/GeneXpert (where applicable) and to inoculate a MGIT tube.

### 6.3.4. Quality Control

A negative control tube is added in the middle of each batch of specimens processed in order to ensure that there is not contamination present in stock solutions and no carry-over of MTB from one specimen to another. The negative control must be treated the same way as the patient samples. The negative control is included in microscopy, Hain MTBDR*plus* (where applicable) and MGIT culture. If there is only one specimen in the batch a negative control is not required.

If a positive signal – presence of AFB or other microorganism on the slide, or MTB confirmed by MTBDR*plus*, or growth on the MGIT culture is detected from the negative control, **notify the TBA Microbiology Consultant immediately.** In addition,

- Observe the technician who processed these specimens for issues known to contribute to contamination; e.g., poor organization of tubes in the BSC, splashing when adding reagents, opening caps too soon after vortexing etc.
- Visually check all reagents used, and subculture to blood agar (BA) if contamination is suspected.
- Monitor closely the patient samples processed in that batch closely for expected results.

### NOTES:

- The NaOH-NALC reagent contains strong alkali and causes severe burns. NaOH is irritating to the eyes and skin. Gloves and eye/face protection must be worn when working with NaOH. In the event of eye or skin contact, rinse immediately with an eye wash system or tap water for at least 15 min and seek medical advice. If ingested, seek medical advice.
- All sample processing related to sputum culture must be done in a class I BSC in a CL3/BSL3 Laboratory unless BSC class I is not available. A class 2A BSC laboratory is acceptable if it is under negative pressure.
- 
- NALC loses activity rapidly in solution, so it **must** be made fresh daily.
- The final pH of the specimen concentrate greatly affects the recovery and time-to-detection of mycobacteria.
- High pH will lower the positivity rate and increases the time-to-detection of positive culture and may also cause

transient false fluorescence.

- With NaOH-NALC digestion, do not agitate the tube vigorously. Extensive aeration causes oxidation of NALC and makes it ineffective.
- Always use a refrigerated and calibrated centrifuge. Temperature build up during centrifugation increases the killing effect on mycobacteria and adversely affect the positivity rate and time-to-positivity in cultures.

### **6.3.5. Documentation**

Study Laboratories must report the date and time of specimen processing on LRF1 or LRF2, or sponsor-approved lab-specific forms or LIMS. In addition, record the order samples were processed and decontamination times using Quality Manual Attachment F.



## 6.4. SOP 4: Acid-fast Bacilli (AFB) Microscopy

### 6.4.1. Purpose

To detect acid-fast bacilli (AFB) by microscopic examination of clinical specimens and cultures. Both viable and non-viable bacilli will stain. A quantitative grading system (WHO/IUATLD scaling system) is used to report the number of AFB observed in stained smears from direct or concentrated sputum specimens. Results of sputum smears must be reported to the study staff within 48 hours of receipt of the sputum specimen.

### 6.4.2. Principle

In the NiX-TB study, fluorescent microscopy is the preferred method for examining screening samples. Like Ziehl-Neelsen (ZN), fluorescence staining includes a stain, decolouriser and counter stain. With auramine-O stain, organisms fluoresce bright yellow, non-specific debris stains pale yellow, and the background is almost black. With auramine-rhodamine stain organisms fluoresce yellow-red in an almost black background.

Brightfield microscopy, ZN staining, is used for confirming the presence of AFB in positive MGIT cultures and on LJ slopes if the colonies do not resemble MTB. The ZN method uses a carbol fuchsin stain, acid alcohol decolouriser and methylene blue counter stain. Acid-fast organisms stain purple and the background and debris stains blue. ZN can also be used for examining screening samples.

Kinyoun is a variant of the ZN method where cold carbol fuchsin stain is used.

### 6.4.3. Procedure

- Auramine-O smear microscopy is the preferred method for screening sputum specimens.
- ZN smear microscopy is performed:
  - As part of the MGIT Culture work-up process (SOP 8) to confirm the presence of AFB in the MGIT positive cultures.
  - To confirm the absence of AFB in decontamination negative controls.
  - On LJ slopes prepared for storage or to ship to UCL if the colonies do not resemble MTB.
  - As requested by the investigator when considering permanently withholding linezolid for the remainder of treatment.
- Kinyoun is used at **UCL Mycobacteriology Laboratory** to confirm the presence of AFB in cultures found resistance to any drug by the MGIT DST assay, or on MGIT/ LJ subcultures set up to prepare these assays.

Prior to the preparation of the smear, all sputum specimens are decontaminated and concentrated as described in SOP 3. Smears must be prepared, air dried and heat-fixed on the same day they are decontaminated.

Smear results should be reported as soon as possible, **no later than 48 hours of sample receipt**. Each time a batch of patient smears is prepared, a positive QC smear using MTB (control strains H37Rv/H37Ra) must be stained alongside the samples to ensure the quality staining of the slides.

### Equipment and Reagents

- Aerosol Resistant Tips (ART)
- Biological Safety Cabinet (category CL3/BSL3 laboratory before heat-fixation of the slide)
- 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
- Positive control slide used with each batch (containing *M. tuberculosis* H37Rv)
- Paper towel soaked in appropriate disinfectant
- Pasteur pipette (Pastette)
- Pencil for labelling slide
- Slide drying rack
- Staining rack
- Staining sink
- Slide storage box
- Wash bottle with distilled water
- Waste containers

For auramine-O Stain or auramine-rhodamine stain:

- Auramine-O or auramine-rhodamine staining kit
  - o Or auramine/auramine-rhodamine staining solution, 0.5-1% acid alcohol, 0.5% potassium permanganate prepared in-house (see laboratory's procedure for preparation)
- Fluorescence or LED microscope
- Dark room

For Ziehl-Neelsen stain:

- Ziehl-Neelsen staining kit
  - o Or carbol fuchsin solution, 3% acid alcohol, methylene blue solution (see laboratory's procedure for preparation)
- Bunsen burner or electric heating block
- Light microscope
- Immersion oil
- Lens paper & lens cleaning solution

For Kinyoun stain:

- Carbol fuchsin KF, 3% acid alcohol, brilliant green or methylene blue (see laboratory's procedure for preparation)

**Process**

Step One: Preparation of smears

Decontamination, culture inoculation and smear preparation must be performed before the screening sputum sample is removed from the BSC in the Containment Level 3 (CL3/BSL3) Laboratory. 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 patient screening number, lab accession number and date using a pencil.
2. Vortex the decontaminated deposit to resuspend and mix thoroughly.
3. Transfer 50 µl of well-mixed resuspended pellet from the decontaminated sputum specimen onto the slide, using a pipette with sterile ART (or an appropriate loop or Pastette).
4. Spread sample, covering a circle approximately 2 cm in diameter. Allow the slides to air dry before heat fixing.
5. Place the slides for at least 15 minutes on a hotplate set between 65°C to 75°C to heat-fix the samples (or as specified in the local Health and Safety guidelines).
  - o Once heat-fixed, smears can be stained outside the BSC in the CL3/BSL3 laboratory and can be examined

by microscopy in either the Containment Level 2 (CL2/BSL2) or CL3/BSL3 Laboratory once it is dry. Heat-fixing does not kill mycobacteria, so be careful when handling smears.

**NOTES:** if preparing smears from positive cultures, only step 2 will differ, as follows:

- From positive MGIT cultures, vortex MGIT tube well, unscrew cap and sample, using an ART or Pastette, an aliquot (approx. 50 µl or 2 drops) of broth onto the slide. Dispose of ART or pipette into the appropriate waste container.
- When examining colonies on solid medium, dispense approx. 50 µl of distilled water on the glass slide with an ART or Pastette. 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. Dispose of loop or applicator stick into the appropriate waste container.

#### Step Two: Procedure for fluorescent staining (Auramine-O or Auramine-rhodamine)

1. Place slides on a staining rack so that they are at least 1 cm apart and flood with auramine / Auramine-rhodamine stain and let sit for 15 minutes.
2. Rinse the auramine / auramine-rhodamine away with distilled water and drain the slides.
3. Decolourise with 0.5% acid alcohol for 2 minutes.
4. Rinse again with distilled water and drain.
5. Flood slides with 0.5% potassium permanganate for 2 min.
6. Rinse potassium permanganate away with distilled water. Do not allow potassium permanganate to act longer than 2 min., or it might quench the fluorescence of AFB.
7. Air-dry the slides. **Do not blot!**
8. Protect smears from light by placing in a storage box and examine as soon as possible since the fluorescence diminishes quickly. **Examine smear within 24 hours of staining.**

**NOTE:** Include an AFB positive and AFB negative slide in each staining batch

#### Alternative Step Two: Procedure for ZN staining

1. Place the slides on the staining rack so that they are at least 1cm apart and flood with carbol fuchsin.
2. Heat the slide to steaming with the flame from a Bunsen burner. An electric heating block may also be used. Apply only enough additional heat to keep the slide steaming for 5 minutes. Do not let the stain boil or dry.
3. Re-flood the slide with fresh carbol fuchsin and heat again until steaming, then let stand for 5 minutes.
4. Wash away the carbol fuchsin with distilled water.
5. Flood slides with 3% acid alcohol.
6. Let stand for 2-3 minutes (more acid alcohol should be used if the liquid becomes heavily stained).
7. Wash away the acid alcohol with distilled water and drain the slides.
8. Flood the slides with malachite green (or methylene blue) and leave to stand for 1-2 min.
9. Wash away the malachite green (or methylene blue) with distilled water and tilt the slides to drain.
10. Allow slides to air dry in the slide rack. **Do not blot!**
11. Once air dry and immediately prior to examination of the slide, apply a drop of immersion oil.

#### Alternative Step Two: Procedure for Kinyoun staining

1. Place the slides on the staining rack and flood with TB Carbol fuchsin KF for 4 minutes. Do not heat.
2. Wash gently with distilled water.
3. Decolorize with TB decolorizer (3% acid alcohol) for 3-5 seconds.
4. Wash gently with distilled water.
5. Counterstain with either TB Brilliant Green K or TB Methylene Blue for 30 seconds.
6. Wash gently with distilled water.
7. Air dry. If using TB Methylene Blue, dry over gentle heat.
8. Apply a drop of immersion oil and a cover slip if needed to keep slides stored.

#### Step Three: Microscopic examination and reading of fluorescent stained smear

Examine the auramine-stained smears with a fluorescent source microscope.

1. Scan the entire smear with the 20x or 15x objective, with 10x eye piece (this is a 200X or 250X magnification).
2. Use a scanning pattern of rows either up and down the slide or across and back. Using 200 or 250X magnification, one 2 mm length is equivalent to 30 fields, which is sufficient to report a negative result.
3. Occasionally use the 40x or 45x objective for closer examination of the bacterial morphology.
4. Confirming morphology at higher magnification avoids false positive results due to fluorescing debris.
5. Examine the control slides first. If expected results are not observed, do not proceed to examine the slides from the screening spot sample.
6. The mycobacteria can appear as rod, coccoid, and filamentous shapes. In sputum smears, individual rods of MTB may be aggregated side-by-side or end-to-end to form “cords”. Count bacilli in the number of fields appropriate for degree of positivity, e.g., the higher the smear positivity, the fewer fields need to be counted. Average the count for the number of fields and record on the lab’s microscopy worksheet according to the WHO/IUTLD grading scale below.

Table 5: WHO/IUTLD Grading Scale for 200x (Auramine Staining)

No of AFB per length/field (200x)	WHO/IUTLD Grading 200x Auramine
No AFB per one length	No AFB seen/negative
1-4 AFB/one length	Confirmation required*
5-49 AFB/one length	Scanty
3-24 AFB/one field	1+
25-250 AFB/one field	2+
>250 AFB/one field	3+

Ref: <http://www.stoptb.org/wg/gli/documents.asp?xpanse=2>

7. All slides must be kept for the duration of the study.

\*Have another microscopist reexamine the smear and if still scanty, prepare another smear from the same specimen and examine before reporting results. If 1-4 AFB/one length are seen on repeat, report this as “Scanty”.

#### Alternative Step Three: Microscopic examination and reading of ZN- or Kinyoun-stained smear

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

1. Examine the smears 100 fields using a regular pattern, such as the one shown in Figure 4. Start with the positive QC slide. If the QC slide is negative, do not report smear results obtained from that batch. Report and resolve the problem.
2. If smear is being done at investigator’s request to possibly remove subject from linezolid treatment, record both the average number of AFBs and assign the corresponding grading as shown in Table 6. To confirm positive MGIT or LJ subcultures, only record whether smear was positive or negative for the presence of AFB.

Figure 4: Scanning Scheme for Smear Examination



Table 6: WHO/IUATLD Grading System for ZN Smears

No. of AFBs (average over 100 fields)	WHO/IUATLD Reporting
None	No AFB seen (NS)
1-9 per 100 fields	Scanty
1-9 per 10 fields	+
1-9 per field	++
>9 per field	+++

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:

- Contamination of slides by tap water with saprophytic mycobacteria – **always use distilled water.**
- Spots of stain deposit (when 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:** Sputum smears from screening samples and investigator queries should be kept for the duration of the study in case any smear needs to be rechecked. Gently wipe the slide clean with a tissue and store in a labelled box in the BSL2 or BSL3 laboratory. 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 bin inside the BSC in the BSL3 laboratory

**6.4.4. Quality Control**

The following QC is required:

- Each new shipment or lot number of staining reagents (for ZN/Kinyoun: carbol fuschin, malachite green/methylene blue or 3% acid alcohol; for fluorescent: auramine / auramine-rhodamine, 0.5% acid alcohol, 0.5% potassium permanganate) must be QC tested using a positive QC smear containing MTB (H37Rv/H37Ra) 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 NiX-TB Mycobacteriology Quality Manual Attachment Ei.**

- Each time a batch of patient sputum smears is carried out, include a negative control (decontamination mixture only) and a positive QC smear containing MTB (H37Rv/H37Ra strain) to check that each stage of the procedure is working correctly. The positive control slide must pass for the microscopy results to be reported. If the positive QC fails, new smears from all samples in the batch must be prepared and re-stained.

**Results of this QC are to be reported using Quality Manual Attachment F and H.**

- Each batch of MGIT samples stained should also include a positive control slide containing MTB (H37Rv/H37Ra), 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 Quality Manual Attachment H.**

For every ten screening slides examined:

1. A review by a second person (i.e. Delegated Laboratory Staff, DLS) is required and the results recorded independently using the **NiX-TB Mycobacteriology Quality Manual Attachment I.**
2. The results for the WHO/IUATLD scaling system should be the same for both counts. If the results do not match:
  - Inform the Laboratory Manager.
  - A third person will count and confirm results. The two equivalent accounts from the three are the final result.
  - Re-train staff member who got the results wrong.

#### **6.4.5. Documentation**

For screening specimens:

Report the date slide was read and the result of reading, following the WHO/IUATLD scaling system, in LRF1, or sponsor-approved lab-specific form or LIMS.

For AFB presence confirmation in cultures:

Report the date slide was read and the result of reading in LRF2, or sponsor-approved lab-specific form or LIMS.

## 6.5. SOP 5: Hain Line Probe Assays (LPA)

### 6.5.1. Purpose

Used by the **Study Laboratories** as a rapid test to confirm the presence of MTB in screening sputum, as well as in AFB positive MGIT cultures from Baseline and at/after Week 16.

It will also be used by **UCL Mycobacteriology Laboratory** to confirm the speciation result obtained by the Study Laboratory prior to any additional characterization work.

Table 7 below details which specific LPAs can be used on each situation.

Table 7: Uses of LPAs in NiX study

Sample / Laboratory	LPA (GenoType) Test	Comment
Screening sputum at Study Laboratories	MTBDR <i>plus</i> ( <b>Version 2 only</b> )	<b>Only</b> if GeneXpert was not used
AFB pos. MGIT culture at baseline and at/after Week 16, at Study Laboratories	MTBC, Mycobacterium CM, MTBDR <i>plus</i> , MTBDR <i>s</i> /	<b>Only</b> if MTP64 Ag test was not used, or was used but the test result was “invalid” or was “negative” and presence of MTBC is strongly suspected
Baseline and first MTBC pos. MGIT culture at or after end of treatment, at UCL Laboratory	MTBC	

### 6.5.2. 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, labeled PCR products are hybridized with specific oligonucleotide probes immobilized on a strip. Captured labeled 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 a specialized scanner (GenoScan).

In this study, DNA will be isolated at the study labs from sputum samples or cultures using the GenoLyse method, which is described below (see Appendix 1 for more details). UCL central lab will extract DNA by 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**.

### 6.5.3. Procedures

#### 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, 20-200 µL, 100-1000 µL
- Aerosol resistant tips (ART)
- 3 ml Pasteur Pipettes (Pastettes)
- 1.5 ml screw-cap Eppendorf tubes
- Appropriate Disinfectant
- Genolyse Lysis Buffer (A-LYS)
- Genolyse Neutralisation Buffer (A-NB)

#### **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)
- Cover Shoes
- Spray bottle for 1% freshly prepared sodium hypochlorite
- Spray bottle for 70% alcohol
- 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)
- Pipette stand
- Aerosol-resistant tips (ART)
- Discard container with plastic bag and 1% freshly prepared for sodium hypochlorite
- Medical waste box for contaminated material
- Marker Pen

#### **Equipment and reagents not provided in the kits required for Detection**

- Shaking water bath or TwinCubator
- Sterile water (molecular biology grade)
- Graduate cylinder
- Timer
- Tweezers
- Vortex
- Waste receptacles (including splash proof receptacle for liquids containing appropriate liquid disinfectant)
- Water bath or heating block (set to 95°C)

#### **Process**

##### **Preparation of BSC**

1. The BSC should be allowed to purge for 20 minutes prior to beginning work. Whilst the BSC is purging, the heating block / water bath (filled with tap water) should be switched on and have reached the desired temperature of 95°C before commencing the DNA extraction procedure. The temperature must be verified by placing a suitable thermometer (up to 110°C), into the appropriate hole in the water bath.
2. Disinfect the BSC with the appropriate mycobactericidal disinfectant, followed by 70% ethanol.
3. Clean all surfaces, pipettes and tip boxes, with 1% sodium hypochlorite, followed by 70% ethanol.

##### **Step One: DNA isolation from decontaminated sputum and from positive cultures**



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

*If using bacteria from sputum or grown in liquid media:*

1. For each sample, label the sides of two 1.5 ml screw-capped Eppendorf tube(s) with the laboratory no. The first tube will be used for the DNA extraction process and the second will receive the isolated DNA.
2. Using a 1-3 mL sterile, disposable Pasteur pipette, transfer 500µl of the decontaminated sputum into a labelled 1.5mL screw cap tube; when using bacteria grown in liquid media, transfer 1mL.
3. Centrifuge for 10 minutes at 10,000 x g in a standard table top centrifuge with aerosol tight rotor.
4. Using a Pasteur pipette, 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 a 50 mL tube filled with 10 mL of 3.5-5% sodium hypochlorite.
6. Resuspend pellet in 100 µl 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 100 µl 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. Switch off the heating block / water bath. If a water bath was used, empty the contents thereof. Clean all surfaces, pipettes, tip boxes, storage boxes, etc. with 1% sodium hypochlorite followed by 70% ethanol. Disinfect the BSC with the appropriate mycobactericidal disinfectant, followed by 70% ethanol.

*If using bacteria grown on solid media:*

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

**Alternative Step One: DNA isolation from positive cultures for speciation by GenoType MTBC (UCL Only)**

1. When using bacteria grown on solid medium (LJ slopes), collect bacteria with an inoculation loop and suspend in approximately 300 µl of water (molecular biology grade)
2. When using bacteria grown in liquid media, directly apply 1 ml into a suitable tube and spin down for 15 minutes in a standard centrifuge tube with an aerosol tight rotor at approx 10000 x g. Discard supernatant and re-suspend the bacteria in 100-300 µl of water (molecular biology grade) by vortexing.
3. Microfuge briefly after vortexing to ensure all liquid is in the bottom of the tube before heat killing (step 4).
4. Incubate bacteria from 1 or 2 for 30 min at 95°C (water bath).
5. Incubate for 15 minutes in an ultrasonic bath
6. Spin down for 5 minutes at full speed and directly use 5 µl of the supernatant for PCR. In case DNA solution is to be stored for an extended time period, transfer supernatant to a new tube.

**Step Two: Amplification**

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.

**From this point on strictly follow the instructions provided by Hain Lifesciences for the selected LPA (PDF files of the Instructions for Use provided by Hain with each kit are available as Appendices 2-6.**

**6.5.4. 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/H37Ra) subjected to the 3 steps mentioned above – DNA isolation (GenoLyse or boiling/sonication), PCR amplification, and hybridization.

- Negative control: molecular grade water subjected to the same 3 steps mentioned above.

**Results of this QC are to be reported using NiX-TB Mycobacteriology Quality Manual Attachments L.**

### **6.5.5. Documentation**

Report test results either using LRF1, if it is a Screening specimen, or LRF3, if the visit required speciation (baseline or Week 16 or beyond). Alternatively, lab-specific forms or LIMS may be used as long as the required fields (highlighted in LRF1 and LRF3) are present and approved by the Sponsor.

A photocopy of Evaluation Sheet provided with the kit or local equivalent, with the developed strips (covered with a clear adhesive tape) should also be kept along the result report form.

### **6.5.6. Good Laboratory Practice When Performing Molecular Amplification Assays**

#### **6.5.6.1. 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.

#### **6.5.6.2. 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.

### 6.5.6.3. 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 pipettors 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**

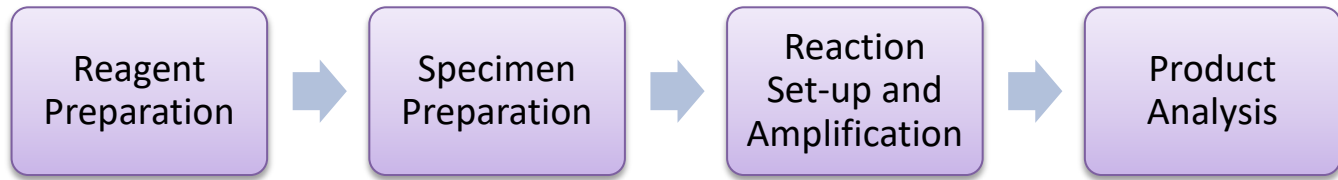
The amplification room is the area in which the PCR machines are housed. It may also contain a containment area in which, for nested PCRs, the second round reaction mixes are inoculated with the primary reaction product. Cloned DNAs should not be brought into this area.

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 5: 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

#### 6.5.6.4. References

Health Protection Agency National Standard Method 'Good Laboratory Practice when Performing Molecular Amplification Assays' Issue no. 3 Issue Date 02.08.06

## 6.6. SOP 6: GeneXpert MTB/RIF (or Xpert MTB /RIF)

### 6.6.1. Purpose

Used by the **Study Laboratories** as a rapid test for:

- Detecting MTBC bacteria on sputum samples at screening,
- Checking for rifampicin (R or RIF) resistance
- Confirmation of presence of MTBC bacteria on positive MGIT cultures at screening, Baseline and at/after Week 16.

#### NOTES:

- If Hain MTBDR*plus* is used (SOP 5) on screening specimens, do not perform GeneXpert.
- Only use GeneXpert for speciation of cultures if no other acceptable method (MPT64 Ag test or Hain LPA) is available.
- Ensure the correct software version is used for the cartridge.

### 6.6.2. Principle

Xpert MTB/RIF is an automated test that uses semi-quantitative, nested real-time PCR for the simultaneous detection of MTBC DNA and RIF resistance associated mutations in the *rpoB* gene in sputum samples (or cultures). The test requires no other instrumentation other than the GeneXpert System and provide results within 2 hours.

Basic step by step instructions required to perform the test are detailed below. For a full description of the system, protocol (e.g. preparing the cartridge) and QC procedures, as well as how to visualize figures and software settings see the Xpert MTB/RIF System Operator Manual – Appendix 2 of this manual.

### 6.6.3. Procedure

#### Equipment and Reagents

- Autoclave bags
- Barcode labels for cartridge identification
- GeneXpert Dx System equipped with GX<sub>2.1</sub> software (catalogue number varies by configuration):
  - o GeneXpert instrument, computer, barcode wand reader, and Operator Manual
- Lockable container with appropriate disinfectant
- Micro tube 1.5 ml
- Rack able to hold 15 ml Falcon tubes
- Sterile Falcon tubes (16.5x120 mm, 15 ml)
- Sterile transfer pipettes (3.5 ml)
- Timer
- Vortex Mixer
- Xpert MTB /RIF kit (CGXMTB /RIF-10) [contains sufficient reagents to process 10 patient or quality-control specimens]

#### Process

##### Step One: Preparing the sputum sediments

1. Using a sterile transfer pipette, transfer 0.5 ml of re-suspended pellet (decontaminated sputum from SOP 2, not untreated sputum sample) to a conical, screw-capped tube.
2. Add 1.5 ml of Xpert MTB/RIF Sample Reagent (SR) to the 0.5 ml of re-suspended sediment using a sterile transfer pipette and shake vigorously 10 to 20 times (a single shake is one back-and-forth movement).
3. Incubate the specimen for 15 minutes at room temperature. Between 5 and 10 minutes of incubation, shake the

specimen vigorously again 10 to 20 times. Samples should be liquefied with no visible clumps of sputum. Particulate matter may exist that is not part of the sample.

#### Step Two: Preparing the cartridge

1. Label each Xpert MTB/RIF cartridge with the laboratory accession number by writing on the sides of the cartridge or attach ID label. Note: do not put the label on the lid or obstruct the existing 2D barcode on the cartridge.
2. Using the sterile transfer pipette provided with the kit, aspirate the liquefied sample into the transfer pipette until the meniscus is above the minimum mark. **Do not process the sample further if there is insufficient volume.**
3. Open the cartridge lid and transfer sample into the open port of the Xpert MTB/RIF cartridge. Dispense sample slowly to minimize risk of aerosol formation.
4. Close the cartridge lid and make sure the lid snaps firmly into place. Remaining liquefied sample may be kept for up to 12 hours at 2-8°C should repeat testing be required.
5. Load the cartridge into the GeneXpert Dx instrument and start the test within 30 minutes of preparing the cartridge.

#### Step Three: Starting the test

1. Before you start the test, ensure the system is equipped with the GX<sub>2.1</sub> software AND the Xpert MTB/RIF assay is imported into the software.
2. Turn on the computer, followed by the GeneXpert Dx instrument (if not already on).
3. On the Windows™ desktop, double-click the GeneXpert Dx shortcut icon.
4. Log on to the GeneXpert Dx System software using your user name and password.
5. In the GeneXpert Dx System window, click **Create Test**. The Scan Cartridge Barcode dialog box appears.
6. Scan the 2D barcode located on the Xpert MTB/RIF cartridge. The Create Test window appears. The software will automatically fill the boxes for the following fields: Select Assay, Reagent Lot ID, Cartridge SN, and Expiration Date based on the barcode information.
7. In the **Sample ID** box, scan or type the sample lab accession number. Cross-check to ensure it is typed or scanned correctly. The sample ID/lab accession number is associated with the test results in the “**View Results**” window and all generated reports.
8. Click **Start Test**. In the dialog box that appears, type your password.
9. Open the instrument module door with the flashing green light and load the cartridge.
10. Close the door. The green light will stop flashing and become steady once the test starts. When the test is finished, the green light will turn off and the system will release the door lock.
11. Once the system releases the door lock at the end of the run, open the module door and remove the cartridge.
12. Used cartridges are considered capable of transmitting infectious agents. Dispose the used cartridges according to your institution’s and country’s safety guidelines.

### **6.6.4. Quality Control**

Each test includes a Sample Processing Control (SPC) and Probe Check Control(PCC):

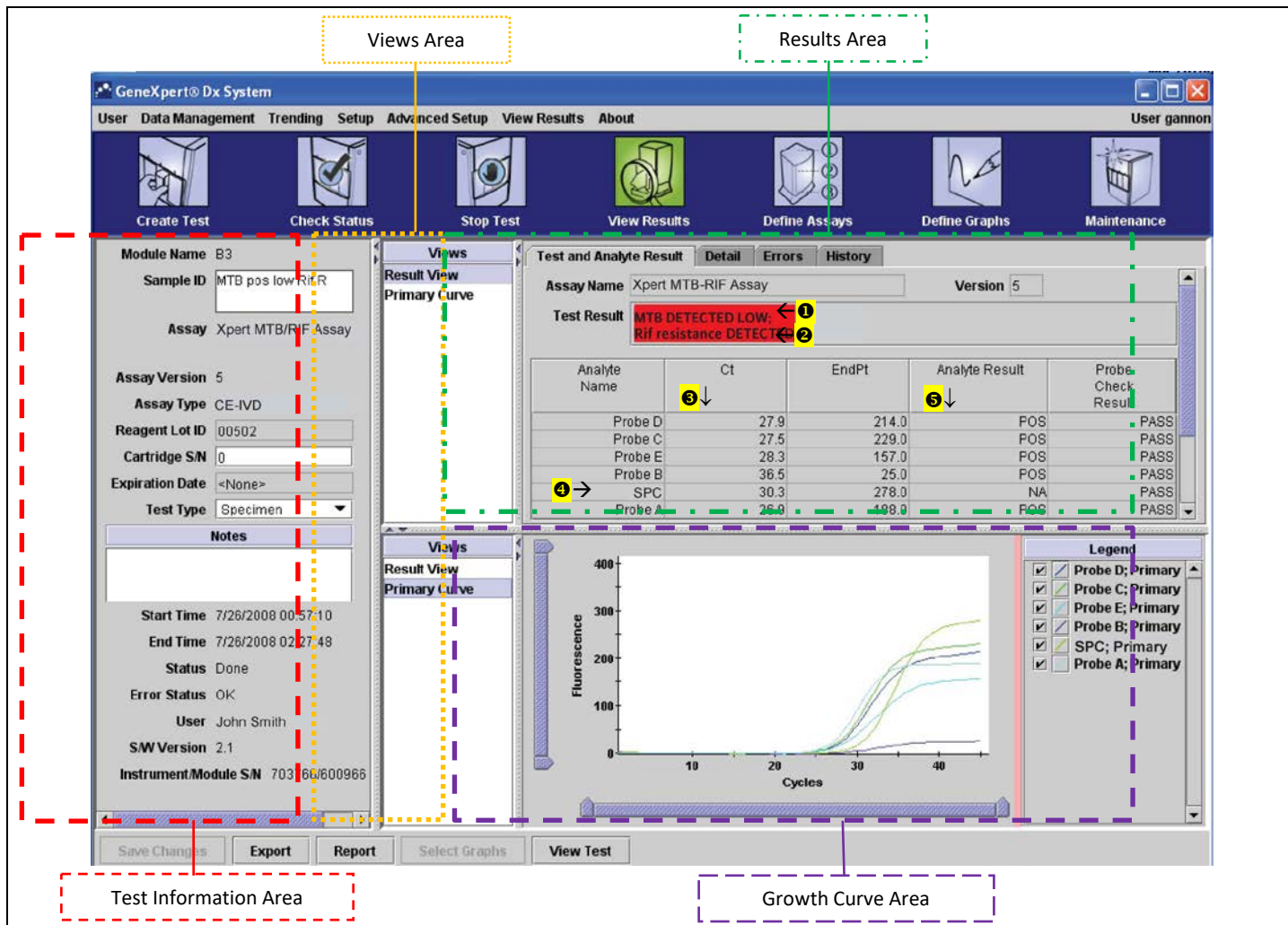
- Sample Processing Control (SPC):  
Ensures the sample was correctly processed. The SPC contains non-infectious spores in the form of a dry spore cake that is included in each cartridge to verify adequate processing of MTB. The SPC verifies that lysis of MTB has occurred if the organisms are present and verifies that specimen processing is adequate. Additionally, this control detects specimen associated inhibition of the real-time PCR assay. The SPC should be positive in a negative sample and can be negative or positive in a positive sample. The SPC passes if it meets the validated acceptance criteria. The test result will be “Invalid” if the SPC is not detected in a negative test.
- Probe Check Control (PCC):  
Before the start of the PCR reaction, the GeneXpert Dx System measures the fluorescence signal from the probes to monitor bead rehydration, reaction-tube filling, probe integrity and dye stability. Probe Check passes if it meets the assigned acceptance criteria.

Incoming batches of Xpert MTB/RIF cartridges should be tested using a sample of cartridges and at least one known positive and one known negative specimen, to ensure expected performance. If this QC fails, contact Cepheid Technical Support.

### 6.6.5. Interpretation of Results

The results are interpreted by the GeneXpert DX System from measured fluorescent signals and embedded calculation algorithms and will be displayed in the “**View Results**” window of the GeneXpert machine. An example of this is shown in Figure 6 below.

Figure 6: Example of GeneXpert View



The results are read from the **Results Area** as follows:

In the “Test Result” field two results (if applicable) will be noted:

- The first result line (1 in Figure 6) will relate to whether MTB is detected or not.
- The second result line (2 in Figure 6) will relate to the Rifampicin resistance result will be recorded, if MTB has been detected.

In the “Ct column” (3 in Figure 6) the starting concentration of the DNA template (sample) is displayed:

- Lower Ct values = a higher starting concentration;
- Higher Ct values = a lower starting concentration.

Quality Control is read:

- Sample Processing Control (SPC) Result (4 in Figure 6) read in the SPC row.

- Probe Check Control (PCC) Column (5 in Figure 6) read via the Analyte Result Column.

#### MTB Detected

MTB target DNA is detected.

- MTBDetected - The MTB result will be displayed as High, Medium, Low or Very Low depending on the Ct value of the MTB target present in the sample.
- Rif Resistance DETECTED, Rif Resistance NOT DETECTED, or Rif Resistance INDETERMINATE will be displayed only in MTBDETECTED results and will be on a separate line from the MTBDETECTED result.
- Rif Resistance DETECTED; a mutation in the *rpoB* gene has been detected that falls within the valid delta Ct setting.
- Rif Resistance INDETERMINATE; the MTB concentration was very low and resistance could not be determined.
- Rif Resistance NOT DETECTED; no mutation in the *rpoB* gene has been detected.
- SPC - NA (not applicable); SPC signal is not required since MTB amplification may compete with this control.
- Probe Check - PASS; all probe check results pass.

#### MTB Not Detected

MTB target DNA is not detected, SPC meets acceptance criteria.

- MTB NOT DETECTED - MTB target DNA is not detected.
- SPC - Pass; SPC has a Ct valid range and endpoint above the endpoint minimum setting.
- Probe Check - PASS; all probe check results pass.

#### RIF Not Detected

RIF target DNA is not detected, SPC meets acceptance criteria.

- RIF NOT DETECTED - RIF target DNA is not detected
- SPC - Pass; SPC has a Ct valid range and endpoint above the endpoint minimum setting.
- Probe Check - PASS; all probe check results pass.

#### INVALID

Presence or absence of MTB cannot be determined, repeat test with extra specimen. SPC does not meet acceptance criteria, the sample was not properly processed, or PCR is inhibited.

- MTBINVALID - Presence or absence of MTB DNA cannot be determined.
- SPC - FAIL; MTB target result is negative and the SPC Ct is not within valid range.
- Probe Check - PASS; all probe check results pass.

#### ERROR

- MTB - NO RESULT
- SPC - NO RESULT
- Probe Check - FAIL\*; one or more of the probe check results fail.

\*If the probe check passed, the error is caused by a system component failure.

#### NO RESULT

- MTB - NO RESULT
- SPC - NO RESULT
- Probe Check - NA (not applicable)

#### **Reasons to Repeat the Assay**

Repeat the test using a new cartridge or initiate alternate procedures if one of the following test results occurs:

- An INVALID result indicates that the SPC failed. The sample was not properly processed or PCR was inhibited.
- An ERROR result indicates that the Probe Check control failed and the assay was aborted possibly due to the reaction tube being filled improperly, a reagent probe integrity problem was detected, or because the maximum pressure limits were exceeded or there was a GeneXpert module failure.



- A NO RESULT indicates that insufficient data were collected. For example, the operator stopped a test that was in progress.
- Rifampicin resistance is indeterminate.

#### **6.6.6. Documentation**

Report test results either using LRF1, if it is a Screening specimen, or LRF3, if the visit required speciation (baseline or at/after Week 16). Alternatively, lab-specific forms or LIMS may be used as long as the required fields (highlighted in LRF 1 and LRF3) are present and they are approved by the Sponsor.

## 6.7. SOP 7: Liquid Culture by Mycobacteria Growth Indicator Tube (MGIT)

### 6.7.1. Purpose

Used by the **Study Laboratories** for culture of sputum samples for:

- Definitive diagnosis of MTB (presence or absence in the sputum sample).
- Assessment of the bacterial load in the original sample by determining the time taken for culture tube to signal positive (time-to-positivity, TTP; or time-to-detection, TTD) in a BACTEC MGIT instrument.

### 6.7.2. 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. And any culture flagged positive, must undergo confirmatory ZN stain microscopy, blood agar culture (BAC) (and speciation if applicable).

### 6.7.3. 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:

- Blood samples are not suitable for use in the MGIT system.
- MGIT tubes can be prepared in a BSC outside of the Containment Level 3 (CL3/BSL3) laboratory. Inoculation of the MGIT tubes with sputum sediment and 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, 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.

#### Equipment and Reagents

- BSC
- Benchguard (or alternative work surface protection)
- Appropriate PPE
- Mycobactericidal disinfectant (specified in local Health and Safety documentation)
- Ethanol 70%
- BACTEC MGIT 960 7 ml MGIT tubes
- MGIT PANTA
- MGIT Growth Supplement
- Graduated Plastic Pasteur Pipettes
- Discard bucket containing appropriate liquid mycobactericidal disinfectant
- Biohazard bags
- 1000 µL pipette and aerosol resistant tips
- Sterile, disposable 10 ml pipettes
- Markers

### 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 below) 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, Nalaxidic acid, Trimethoprim, Azlocillin) with 15 ml of MGIT OADC (Oleic Acid Albumin Dextrose Complex) 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: Inoculation and incubation of the MGIT tubes

1. For each specimen, label a MGIT tube with the patient number (or screening number if pre-enrolment) and the laboratory accession number label. Record the MGIT tube number in the approved laboratory source documentation. If working with a LIMS system, add an accession barcode label to each tube.
2. Add 0.8 ml of the supplements mixed above to each MGIT tube using a sterile pipette. Be careful to avoid contamination. The mixed supplements should be added to the MGIT medium prior to inoculation of specimen in MGIT tube. Do not add the mixed supplements after the inoculation of specimen.
3. Add 0.5 ml of a well-mixed, decontaminated and concentrated sputum specimen (SOP 3) to the appropriately labelled MGIT tube. Immediately recap the MGIT tube tightly and mix well by inversion several times. Wipe the MGIT tube with a paper towel soaked in disinfectant before removing it from the BSC. Use a separate graduated Pasteur pipette or micropipette for each specimen. Dispose of waste pipette tips into the discard bucket containing mycobactericidal disinfectant.
4. Store the remaining sputum sediment at 4°C for 10 days, until confirmed the MGIT tube is not contaminated.
5. The negative control from each batch of processed sputum samples should also be inoculated into a MGIT tube (labelled with 'negative', the date and batch number), and details completed on the local source document.
6. 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).

### **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 Three: Removal and work-up of positive MGIT tubes

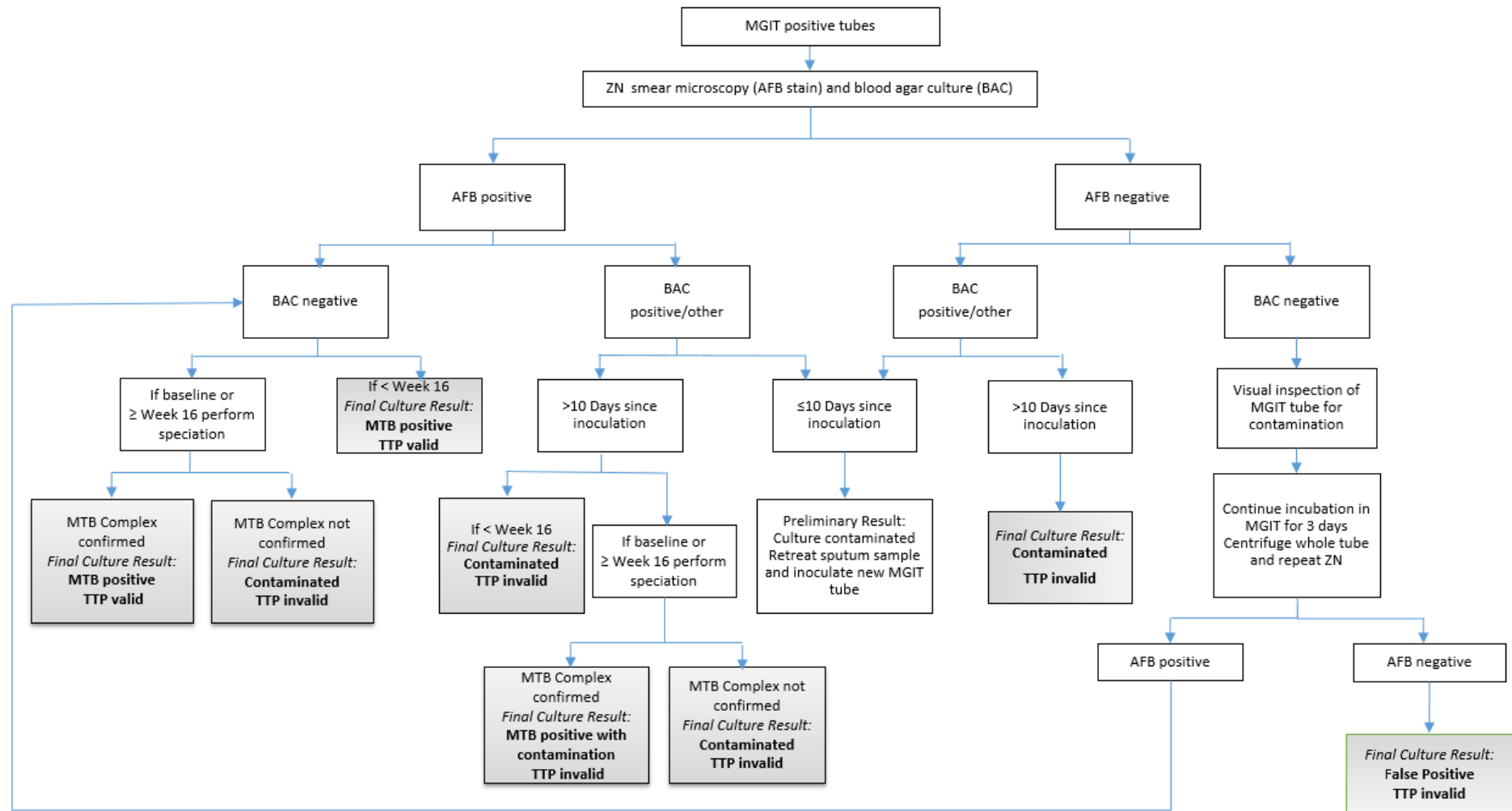
**NOTE:** Only discard positive MGIT tubes after autoclavation

### Equipment and Reagents

- BSC
- Discard bucket containing mycobactericidal disinfectant (specified in local Health and Safety documentation)
- Blood agar (BA) plate
- 10 µl loop
- Plastic tube for centrifugation of sample
- Glass microscope slide
- Sterile Pasteur pipette and aerosol resistant tips.
- LJ 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 (TTP).
3. Record the laboratory accession numbers of all unloaded tubes next to their corresponding results on the print outs. The print outs 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:
  - 4.1. Blood Agar Culture (BAC) on all positive MGIT tubes:
    - a. Label one BA plate with the laboratory accession number and the patient number.
    - b. Vortex the MGIT tube well.
    - c. Inoculate the BA plate with liquid from the MGIT tube using a 10 µL disposable loop, and incubate in a 37°C incubator for 48 hours.
    - d. 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.
    - e. Check for growth or contamination on the BA plate daily.
  - 4.2. Perform ZN stain microscopy (SOP 4) on all positive MGIT tubes even if confirmed to be contaminated.
    - a. Using a Pasteur pipette or micropipette and ART, remove 50 µL of the MGIT culture and place onto a slide.
    - b. Heat fix, stain and examine the slide for the presence of AFB per SOP 4.
    - c. Although it is not necessary to semi-quantify the number of AFBs at this stage, describe the AFB identified in the smear (i.e. typical or atypical morphology and whether cording is seen).
5. Once the results of the ZN and BAC become available, refer to the algorithm shown on Figure 7.

Figure 7: Algorithm for Interpretation of Positive MGIT Cultures



Step Four: Interpretation of results

BAC		AFB (ZN smear)		Result		MGIT TTP
Negative	and	Present (positive)	this is	True Positive	and means	Valid

1. If the sample is from baseline or from Week 16 or beyond, perform speciation – depending upon the selected test – see SOPs 5, 6 or 9.
2. If the MPT64 Ag Test is going to be performed, wait for 48-72 hours (from the time it signalled positive) before removing the MGIT tube. This additional incubation time is recommended to increase the test sensitivity.

BAC		AFB (ZN smear)		Result		MGIT TTP
Positive or Other*	and	Present or Absent	this is	Contaminated	and means	Invalid

\* Situations where BAC conclusion should be marked as Other:

- a. BAC not done (e.g. test not set up, discarded before reading, result missing).
  - b. BAC is negative, but MGIT tube visibly contaminated with fungal or other microbial growth.
  - c. BAC is negative, but evidence of fungal hyphae seen on ZN smear.
1. If the sample is confirmed as **contaminated within the first 10 days of inoculation**, the remaining sputum sediment (from the original decontamination) stored at 4°C is **to be retreated** per SOP 3 and a new MGIT culture to be inoculated.
  2. If the contamination is confirmed after 10 days, the original sample will not be retreated and the overall result will be recorded as contaminated.
  3. If the culture is contaminated (BAC positive or other) but contains AFB (ZN positive) AND corresponds to a sample from baseline or from week 16 or beyond, perform speciation.

BAC		AFB (ZN smear)		Result		MGIT TTP
Negative	and	Absent	this is	Possible False Positive	and means	Confirm it is a true false positive culture by checking if there are no AFBs present.

Confirm it is a true false positive by performing the following:

1. Examine the MGIT tube – record if it is turbid (in particular, if it shows a ‘typical turbidity’, i.e. breadcrumbs at the bottom of the tube) or showing any evidence of microbial growth (MTB or any contaminant).
  - A clear tube may be a true false positive resulting from altered reagent pH.
2. Repeat the ZN smear a second time to double check for AFBs.
3. If AFBs are not detected in the second smear, re-incubate the MGIT tube for at least 3 days (in MGIT machine or incubator) to allow further growth of any MTB present. After 3 days of additional incubation, repeat the ZN smear again.
4. This time, prior to performing the ZN smear, centrifuge the MGIT tube as follows:
  - If the centrifuge cannot accommodate MGIT tubes:
    - Inside the BSC, remove 1 ml of well mixed fluid from MGIT tube with a sterile Pasteur pipette into an Eppendorf tube.
    - Spin the sample using a microfuge.
    - Remove most of the supernatant and re-suspend pellet in remaining fluid (about 250 µL).
    - Label a slide and prepare a smear with the resuspended pellet.
    - OR
  - If the centrifuge can accommodate MGIT tubes:
    - Spin MGIT tubes without decanting at 3,000 xg for 15 min.
    - Tip off all but 2 mL of the supernatant into a discard bucket containing appropriate disinfectant.
    - Resuspend the pellet in the remaining supernatant.
    - Label a slide and prepare a smear with the re-suspended pellet.

#### Step Five: Storage/shipment for further investigations

Two samples must be stored for each isolate, one in 50% glycerol at -70°C to -80°C, and the other on a LJ slope. For the preparation of cultures for storage/shipment, follow SOP 2. A record must be kept of all isolates in storage.

In addition, for select visits, additional LJ subcultures are to be made for shipping to the Central Laboratories – see SOP 2 for details.

#### Step Six: Removal and work-up 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 reported as a negative result. If a sample flags positive after 42 days, this must also be reported as negative.

1. Remove the MGIT negative tubes from the machine according to manufacturer's instructions.
2. Visually inspect all negative tubes. Any tube that appears to have growth must be processed as follows:
  - 2.1. Perform ZN and BAC as described above for positive tubes.
  - 2.2. Contact TB Alliance for instructions on how to report the results, if either AFB or another organism is detected.
3. Print the 'Unloaded Negatives Report', record the accession numbers 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 either in an unloaded negatives folder or with the patient's worksheets.
4. Autoclave negative tubes prior to discarding.

### **6.7.4. Quality Control**

#### New media and supplements

A MTB reference strain (H37Rv/H37Ra) is tested to ensure that the medium supports growth of mycobacteria

#### **Procedure**

1. Prepare a fresh culture of MTB (H37Rv/H37Ra)
2. 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 1 ml of Dilution 1 to 4 mL of sterile saline – Dilution 2 (1:50).
  - Add 1 ml of Dilution 2 to 4 mL of sterile saline – Dilution 3 (1:500).
4. Prepare the MGIT tube following the normal MGIT culture procedure.
5. Label each tube with the 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 fails, contact the manufacturer for troubleshooting.

**Results of this QC are to be reported using NiX-TB Mycobacteriology Quality Manual Attachment Eiii.**

MGIT maintenance must be performed daily and monthly, preferably before unloading or loading of tubes, and **recorded on Quality Manual Attachment J.**

#### MGIT failure/breakdown lasting more than 24 hours:

Follow manufacturer's instructions. Briefly, if power is lost for more than 24 hours remove all tubes and place in a 37°C incubator. Read manually using an ultraviolet (UV) trans-illuminator (365 nm) or a Wood's lamp with a long-wave bulb or black-light (wear eye protection). Once the tubes are removed they must be read off-line daily throughout the 6 week protocol. Tubes must not be returned to the MGIT instrument. Prepare smears and stain any positive tubes for confirmation of AFBs. Before disposing of any negative tubes check for turbidity and perform Z-N microscopy to ensure tubes are negative.

If there is no access to a UV light, take a small sample using aseptic technique from the MGIT tube daily, make a smear and perform Z-N staining. Calculate the TTP from the date the tubes were inoculated to the date the tubes were confirmed positive/negative manually.

#### **6.7.5. Documentation**

The suggested LRF1 (for Screening samples) and LRF2 (Treatment and Follow-up samples) capture the information for completing the eCRF fields (highlighted) related to MGIT culture and follow-up tests. Study Laboratories may use their own forms or LIMS as long as these pieces of information are included and they are approved by the Sponsor.

**NOTE:** TTD/TTP should only be reported for cultures flagged positive, which are not found to be contaminated (BAC negative), and AFB positive (ZN positive). In addition, if the visit requires speciation (SOP 9) wait for the result of this test – in this case, only report the TTD/TTP the culture is found positive for MTBC.



## 6.8. SOP 8: MPT64 Antigen Test

### 6.8.1. Purpose

Used by the **Study Laboratories** to rapidly identify the *M. tuberculosis* complex (MTBC) in acid-fast bacilli (AFB) positive MGIT cultures.

### 6.8.2. Principle

The CE marked BD TBc ID, SD Bioline, or Capilia TB-Neo are all commercial variations of the same test, MPT64 antigen assay (or MPT64Ag test), and all have been shown to be highly sensitive and specific in a number of studies conducted in clinical settings. The MPT64Ag test is a lateral flow, immunochromatographic assay that detects MPT64 antigen, a mycobacterial protein that is specifically secreted from MTBC while growing in culture. When a bacterial suspension is added to the test device, the MPT64 antigen binds to anti-MPT64 antibodies conjugated to colloidal gold particles present on the test strip, forming an antigen-antibody complex. This antigen-antibody complex then migrates across the test strip to the reaction area, where it is captured by a second specific MPT64 antibody fixed to the membrane. If MPT64 antigen is present in the sample, a colour reaction is produced by the labelled colloidal gold particles and is visualized as a pink (or purple) to red line. An internal positive control is included to validate proper test performance. The test will detect the following species of MTBC: *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*.

In this study, the MPT64 Ag test will be used to differentiate MTBC from mycobacteria other than *M. tuberculosis* (MOTT) in AFB positive MGIT cultures **from Baseline (Day 1 or screening to week 4 if Day 1 is contaminated or negative) or at/after Week 16 only**. Also note that **only one baseline sample requires speciation**.

### 6.8.3. Procedure

**To be performed inside a BSC using the appropriate biosafety procedures and PPE.**

#### Equipment and Reagents

- BSC
- Appropriate PPE
- Mycobactericidal disinfectant
- Benchguard (or alternative work surface protection)
- Cotton wool or paper towels
- Biohazard bags
- Screw top bottle for waste
- Extraction buffer (commercially available or in-house prepared) – (KH<sub>2</sub>PO<sub>4</sub>, NaCl, Tween 80)
- MPT64Ag test device
- 200 µL micropipette and sterile ARTs
- 10 µL sterile disposable loops
- Sterile 2 mL cryovials

#### Process

Ideally, test AFB smear-positive MGIT tubes within 2-3 days of instrument positivity.





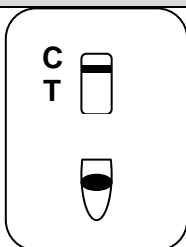
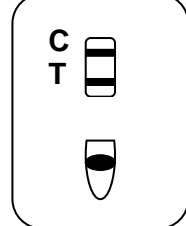
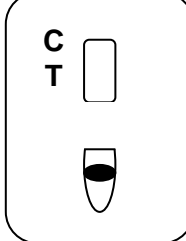
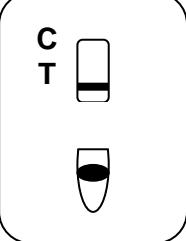
1. If devices are refrigerated, bring to room temperature in the foil pouch prior to testing.
2. Remove the test device from the foil pouch and place on a dry, flat surface.
3. Label the device with the patient or screening number and laboratory accession number.
4. Vortex the tightly capped MGIT tube for 30 seconds to ensure the suspension is well- mixed.
5. Add 100 µl of liquid culture to the sample well.

6. Start timer for 15 minutes. As the test develops, a purple colour will migrate across the result window in the centre of the test device.
7. Interpret the test result 15 minutes after sample application. **Do not read the test after 60 minutes.**

### **Result Interpretation**

The tables below show the proper interpretation of each type of kit. Record the result on the local lab worksheet.

Table 8: Interpretation of the MPT64 Antigen Assays

<b>SD Bioline TB Ag MPT64 (Configuration of cartridge is similar in the Tauns Capilia TB-Neo kit.)</b>	
1. <b>Negative:</b> only the Control Band 'C' appears (purple to red line) in the result window.	
2. <b>Positive:</b> appearance of two colour bands ('T' band and 'C' band) in the result window, no matter which band appears first.	
3. <b>Invalid result:</b> if the control band is not visible in the result window after performing the test, the result is considered invalid.	 
<b>BD TBcID</b>	
1. <b>Negative:</b> A pink/purple to red line forms only on the reading area labelled Control 'C'.	
2. <b>Positive:</b> Pink/purple to red lines form on the reading areas labelled Test 'T' and 'C' of the device.	
3. <b>Invalid result:</b> If no line is observed on the reading area labelled [C], technical errors or product damage has occurred. In this case, the test should be considered invalid and repeated using a new device.	 

### **NOTES:**

- If the MPT64Ag test is negative, but MTB is highly suspected – AFB microscopic morphology and isolate growth pattern in MGIT are consistent with MTB – use a PCR-based test (GeneXpert; or Hain MTBDR*plus*, MTBDR*s*l, MTBC or Mycobacterium CM) to detect bacteria that may have a mutation in the MPT64 gene.
- If the rapid ID test is invalid: investigate causes for the invalid result and check the expiry date of kit, decontaminating a heavily contaminated culture, etc.

#### 6.8.4. Quality Control

##### Frequency:

- Each new lot or shipment of kits.
- Weekly, or with each batch of patient tests, if testing is performed less frequently.

##### Controls:

- Internal reagent control in device.
- Positive control: Culture of MTB reference strain (H37Rv or H37Ra) in MGIT broth.
- Negative control: Culture of a MOTT strain (e.g. a well characterized strain of *M. avium* complex) in MGIT broth or broth from an uninoculated MGIT tube.

##### Acceptable results: Correct results as expected for all controls

- Internal control line is visible.
- MTB must result in a positive test.
- MOTT strain or uninoculated broth must result in a negative test.

##### Corrective actions: If any control result is unacceptable, do not report patient tests.

- Repeat test with new controls; if acceptable, repeat patient tests.
- If repeat results still unacceptable, notify supervisor immediately and investigate potential causes for failure.
- After investigation is complete and QC is acceptable, repeat patient tests and report results.

**Results of this QC are to be recorded on the Nix-TB Quality Manual Attachment Evii.**

#### 6.8.5. Documentation

The suggested LRF3 captures the information for completing the eCRF fields (highlighted) related to speciation. Study Laboratories may use their own forms or LIMS as long as these pieces of information are included and they are approved by the Sponsor.

## 6.9. SOP 9: DNA Extraction

### 6.9.1. Purpose

Used by the **UCL Mycobacteriology Laboratory** to extract DNA from the isolates from the baseline and first positive culture at or after the end of treatment that is indicative of treatment failure or relapse/reinfection [confirmed positive cultures at or after week 26 (6 month treatment arm)/week 36 (9 month treatment arm)].

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

### 6.9.2. 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).

### 6.9.3. Procedure

#### 6.9.3.1. Isolation of genomic DNA from *M. tuberculosis*

##### Equipment/Reagents

- Biological Safety Cabinet
- Waterbath or heating blocks (80-95°C, 60-65°C and 37°C)
- 10 µl loops
- 1.5 ml screw-capped Eppendorf tubes with rubber 'O' ring seal
- 1000 µl pipette and aerosol resistant tips
- 200 µl 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
- Sterile DNAase-free 1.5 ml eppendorf tubes
- 70% ethanol
- Isopropanol
- -20°C freezer
- 4°C refrigerator

### **Preparation of extraction reagents**

#### **Lysozyme solution: 10 mg/ml.**

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

#### **10% SDS**

Add distilled water to 10 g of SDS to make up 100 ml of total solution. . Dissolve by heating at 65 °C for 20 min. Do not autoclave. Store at room temperature for no longer than 1 month

#### **Proteinase K: 10 mg/ml.**

Store in small aliquots at -20 °C. Use a new aliquot each time, do not re-freeze. Any remaining reagent should be discarded. Unless stated otherwise on manufacturing guidelines.

#### **5M NaCl**

29.2 g NaCl/100 ml distilled water. Heat to 65°C and mix until dissolved (this may take hours). Autoclave at 121°C for 20 minutes. Store at room temperature for no longer than 1 year.

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

Dissolve 4.1 g NaCl in 80 ml distilled water. While stirring, add 10 g CTAB. If necessary, heat solution to 65 °C Adjust the volume to 100 ml with distilled water and autoclave at 121°C for 20 minutes. Store at room temperature for no longer than 6 months.

#### **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 or by expiry date indicated.

#### **70% Ethanol**

70 ml 100% ethanol in 30 ml distilled water, store at -20°C.

### **Process**

Vortexing is not recommended at any stage of the extraction as this causes DNA shearing. All steps, until after the heat killing of MTB culture (step 6) and briefly centrifuging the tubes (step 7) must be carried out in an appropriate BSC inside a BSL/CL-3 Laboratory.

1. Fill the waterbath with tap water and set for 80°C. If a waterbath is unavailable use 95°C heating block
2. Label sufficient 1.5mL screw capped tubes containing 'O' rings with patient number and laboratory accession number.
3. Aliquot 400µL volumes of 1x Tris-EDTA (TE) buffer into the tubes using aerosol resistant tips.
4. From LJ slopes with good growth, take all organisms using a 10 µl 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 waterbath/heating block at 80-95°C for 30 minutes or as specified by the local health and safety guidelines.
7. Pulse down the tubes, as above. Tubes can now be removed from the Containment Level 3 Laboratory, unless otherwise stated in local Health and Safety documentation
8. Add 50µL 10mg/mL lysozyme and mix gently with the pipette. Incubate at 37°C in the waterbath, incubator or heating block overnight (if overnight is not possible at least one hour is required).
9. Set the waterbath to 65°C or switch on 60°C heating block. Pre-warm the CTAB/NaCl to 60-65°C.
10. Add 70µL 10% SDS and 5µL 10mg/ml proteinase K. Mix gently with the pipette and incubate at 60-65°C for 10 minutes.
11. Add 100µL 5M NaCl to each tube.
12. Add 100µL CTAB/NaCl (pre-warmed to 60-65°C). Mix gently with the pipette and incubate at 60-65°C for 10 minutes.

13. Switch off and empty the waterbath.
14. Add 750µL chloroform/isoamylalcohol (24:1 v/v). Mix by inversion.
15. Place into correctly labelled 1.5ml universal tubes. Microfuge at 10,000 g for 5 minutes. Ensure the opening of the lids face inwards to develop a clear pellet.
16. Label sterile DNAase free 1.5mL Eppendorf tubes and aliquot 450µL volumes of ice-cold isopropanol to each.
17. Transfer the aqueous supernatants into the Eppendorf tubes containing isopropanol. Take care not to disturb the interface. Mix by gentle inversion.
18. Place at -20°C for 30 minutes. Also place a glass container of 70% ethanol at -20°C.
19. After at least 30 minutes, microfuge at 10 000 g for 15 minutes at room temperature.
20. Remove the supernatants and wash the pellets with 1mL ice-cold 70% ethanol. Invert gently.
21. Microfuge at 10 000 g for 5 minutes at room temperature. Remove and discard as much of the ethanol as possible. Lay or tilt the tubes with open lids to allow the pellets to air-dry (at least 15 min).
22. Rehydrate the pellets in approx. 100µL (depending on pellet size) molecular grade water (or TE buffer with a maximum concentration of 0.1mM EDTA) overnight at 4°C (or 1 hour at 65 °C).

### 6.9.3.2. Estimation of DNA concentration

DNA concentration in each of the samples will be estimated by both of the methods detailed below to ensure the quantity and quality is sufficient for WGS analysis.

#### 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)
- 2 µl Pippette with appropriate tips
- Soft laboratory wipe/tissue
- De-ionised water
- Tris-EDTA (TE)

##### 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 by loading 1 µl distilled or PCR grade water sample to initialize 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 1 µl 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.
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 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 cloth.
11. Pipette 1 µl of sample DNA onto the lower measurement pedestal (if you are unsure about your sample or your pipettor accuracy, a 1.5 – 2 µl 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 should save automatically and at the end of the set of samples click 'Show Report'. This can then be printed and saved. Alternatively, record the DNA concentration and the 260/280 ratio in the table provided.
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.

#### Equipment/Reagents

- Qubit® dsDNA Assay BR Kit. This is the broad range (BR) kit with quantitation range of 2-1000ng. Note: *The high sensitivity (HR) kit (quantitation range of 0.2-100ng) can also be used if required.*
  - o Qubit® dsDNA BR Reagent (Component A)
  - o Qubit® dsDNA BR Buffer (Component B)
  - o Qubit® dsDNA BR Standard #1 (Component C)
  - o Qubit® dsDNA BR 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 (2.0)

**Storage of reagents:** The Qubit® dsDNA BR 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.

#### 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).
2. Prepare Qubit® working solution by diluting the Qubit® dsDNA BR Reagent 1:200 in the Qubit® dsDNA BR 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 10 µl of reagent into 1990µl of buffer.
3. Add 190 µl of Qubit® working solution to each of the standard tubes, then add 10ul of each Qubit® standard to the appropriate tube and mix by vortexing for 2-3 seconds. Be careful not to create bubbles.
4. Add Qubit® working solution to individual sample tubes so that the final volume including the sample is 200 µl. The volume of the sample can be between 1-20 µl, so the volume of working solution can be adjusted accordingly.
5. Add each sample to the appropriate assay tubes and mix by vortexing for 2-3 seconds.
6. Allow all tubes to incubate at room temperature for 2 minutes - the samples are now ready to be read on the fluorometer.
7. On the home screen of the Qubit® Fluorometer, press **DNA** and then select **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 complete, 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 (approx. 3 seconds) remove the tube. The instrument displays the results on the screen. The first value displayed is the concentration in the assay tube.
10. To find out the concentration in the original sample, press **Calculate Stock Conc**. 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. Record this concentration in the table provided in the NiX-TB Quality Manual Attachment M.

11. Press Read Next Sample, and repeat steps 9 and 10 for all remaining samples.

**See SOP 13 for further details on sample shipment and WGS analysis.**



## 6.10. SOP 10: Drug Susceptibility Testing (DST) by Mycobacteria Growth Indicator Tube (MGIT)

### 6.10.1. Purpose

Used by the **UCL Mycobacteriology Laboratory** for Drug Susceptibility Testing (DST) for streptomycin (S), isoniazid (I), rifampicin (R), ethambutol (E), moxifloxacin (M), kanamycin (K) 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. Moxifloxacin will be tested at two concentrations, 2µg/ml and 0.5µg/ml, although only the result from the testing at 0.5µg/ml will be entered into the eCRF.

Susceptibility testing will be performed on pre-treatment isolates from the baseline (Day 1 or Screening to week 4, if Day 1 cultures are negative or contaminated) and the isolates from patients suspected of failure or relapse after treatment (a positive culture at or after week 24 [6 month treatment arm]/week 36 (9 month treatment arm) and any new positive culture thereafter] in order to identify the presence of resistance to any of the study drugs.

**NOTE:** Prior to any drug susceptibility testing (DST), a HAIN MTBC assay (SOP 5) is performed at the UCL Central Mycobacteriology Laboratory to confirm the presence of MTB complex (MTC). If the HAIN test does not confirm MTC, the local laboratory will be contacted, and a new culture will be sent.

### 6.10.2. 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.

### 6.10.3. Procedure

#### Equipment/Reagents

- Biological Safety Cabinet
- Discard bucket containing appropriate liquid disinfectant (specified in local Health and Safety documentation)
- 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 and p200 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

### 6.10.3.1. Preparation of drug stocks for susceptibility testing

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

Table 9: Preparation of DST Drug Stock Solutions

TASK	INSTRUCTIONS
<b>*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</b>	
Prepare BACTEC™ MGIT™ 960 SIRE Drug Kit	<ul style="list-style-type: none"> <li>• Reconstitute each BACTEC™ MGIT™ 960 SIRE Kit Streptomycin lyophilised drug vial with <b>4 ml</b> of sterile distilled/deionised water to make a stock solution of 83µg/ml.</li> <li>• Reconstitute each BACTEC™ MGIT™ 960 SIRE Kit Isoniazid lyophilised drug vial with <b>4 ml</b> of sterile distilled/deionised water to make a stock solution of 8.3µg/ml.</li> <li>• Reconstitute each BACTEC™ MGIT™ 960 SIRE Kit Rifampicin lyophilised drug vial with <b>4 ml</b> of sterile distilled/deionised water to make a stock solution of 83µg/ml.</li> <li>• Reconstitute each BACTEC™ MGIT™ 960 SIRE Kit Ethambutol lyophilised drug vial with <b>4 ml</b> of sterile distilled/deionised water to make a stock solution of 415µg/ml.</li> </ul>
Prepare BACTEC MGIT 960 Pyrazinamide Kit	<ul style="list-style-type: none"> <li>• Reconstitute each BACTEC™ MGIT™ 960 PZA drug vial with <b>2.5 ml</b> of sterile distilled/deionised water to make a stock solution of 83µg/ml.</li> </ul>
Prepare BD Moxifloxacin HCl	<ul style="list-style-type: none"> <li>• Reconstitute each BD Moxifloxacin Hydrochloride drug vial with <b>3 ml</b> of sterile distilled/deionised water to make a stock solution of 166µg/ml. This will be used for the Moxi-2µg/ml set, for the Moxi-0.5 µg/ml this will be diluted 1:4 in sterile distilled/deionised water.</li> </ul>
Prepare BD Kanamycin Sulphate	<ul style="list-style-type: none"> <li>• Reconstitute the BACTEC™ MGIT™ 960 Kanamycin lyophilised drug vial with <b>4 ml</b> of sterile distilled/deionised water to make a stock solution of 207.5µg/ml.</li> </ul>
<b>NOTE:</b> 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.	

### 6.10.3.2. Preparation of MGIT tubes for DST testing

ADD Epi centre info here – for SIRE, K, M.

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 TB eXiST (TB eXtended Susceptibility Testing) module, as is done for the MIC testing (see SOP 11). Using TB eXiST 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. TB eXiST can be used for SIRE, K, M-2 and M-0.5 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 TB eXiST 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 TB eXiST software, refer to SOP11, and associated user guides – ‘Registering TB eXiST 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 10.

Preparation of the drug sets using AST carrier sets:

For preparation of SIRE set:

1. Label five 7 mL MGIT tubes for each test isolate with the appropriate laboratory accession label and the patient study number. 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 100 µl of 83 µg/mL MGIT STR solution to the appropriately labelled MGIT tube.
5. Aseptically pipette 100 µl of 8.3 µg/mL MGIT INH solution to the appropriately MGIT tube.
6. Aseptically pipette 100 µl of 83 µg/mL MGIT RIF solution to the appropriately MGIT tube.
7. Aseptically pipette 100 µl 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.

For preparation of the moxifloxacin 2µg/ml set:

1. Label two 7mL MGIT tubes for each test isolate with the appropriate laboratory accession label and the patient study number. In addition, label tubes with one of each of the following: GC (Growth Control) and MOX-2
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 tubes and 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 166µg/ml MGIT MOX solution to the appropriately labelled MGIT tube.
5. No antibiotics should be added to the MGIT GC tube.

For preparation of the moxifloxacin 0.5µg/ml set:

1. Label two 7mL MGIT tubes for each test isolate with the appropriate laboratory accession label and the patient study number. In addition, label tubes with one of each of the following: GC (Growth Control) and MOX-0.5
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 tubes and supplement from the BD SIRE set can be used. Aseptically add 0.8ml of BACTEC MGIT SIRE supplement to each tube.
4. Dilute the 166µg/ml MGIT MOX solution 1:4 in sterile distilled/deionised water (e.g. 100µl drug stock solution and 300µl of water).
5. Aseptically pipette 100µl of the new drug solution to the appropriately labelled MGIT tube.
6. 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 patient study number. 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 tubes and 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 patient

- study number. In addition, label tubes with one of each of the following: GC (Growth Control) and PZA (pyrazinamide)
- Place tubes in the correct sequence for the 2 tube AST set carrier (see BACTEC MGIT 960 User's manual, AST instructions)
  - 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)
  - Aseptically pipette 100uL of 8000µg/ml MGIT PZA solution to the appropriately labelled MGIT tube.
  - No antibiotics should be added to the MGIT GC tube.

Table 10: 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 -2	166µg/ml	100µl	2.0µg/ml
MOX -0.5	41.5 µg/ml*	100µl	0.5 µg/ml
KAN	207.5µg/ml	100µl	2.5µg/ml
PZA	8000µg/ml	100µl	100µg/ml

\*This concentration is achieved by a 1:4 dilution of the stock solution prepared for MOX-2

#### 6.10.3.3. 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 37C 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 let any remaining clumps settle out for 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 leave for 30 minutes for any remaining clumps to settle out. Dilute 1 ml of supernatant in 4 mL of sterile saline (1:5 dilution). Use this diluted culture for the DST drug tubes.

**>5 Days** – subculture 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. 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 TTP 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.

#### 6.10.3.4. 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 16.5 x 128 mm sterile tube with cap containing 8 – 10 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 16.5 x 128 mm 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 16.5 x 128 mm 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).

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

##### For SIRE, MOX and KAN Growth Control Tubes:

1. Aseptically pipette 0.1 ml of the organism suspension (used to inoculate drug tubes) into a total of 10mL of sterile saline to prepare the 1:100 GC suspension (1% growth control).
2. Mix the GC suspension thoroughly.
3. Inoculate 0.5mL of the 1:100 GC suspension into the MGIT tubes labelled “GC”, using a micropipettor and aerosol resistant tips. Dispose of pipette into discard pot of liquid disinfectant

##### For PZA Growth Control Tubes:

1. Aseptically pipette 0.5 ml of the organism suspension (used to inoculate drug tubes) into a total of 4.5 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 micropipettor and aerosol resistant tips. Dispose of pipette into discard pot of liquid disinfectant
4. Spread 0.1mL of the organism suspension to a BAP.
5. Enclose the blood agar plate in a plastic bag.
6. Incubate at 35 - 37°C.
7. Check the blood agar plate at 48 hours for bacterial contamination. If the blood agar plate shows no growth, then allow AST testing to proceed. If the blood agar plate shows growth, discard the AST set (refer to the BACTEC MGIT 960 User’s Manual, AST Instructions) and repeat testing with pure culture.

#### 6.10.3.6. 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 micropipettor 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 and 0.5) – 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 0.1mL of the organism suspension to a Blood Agar plate.
  6. Enclose the blood agar 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 blood agar plate shows no growth, then allow AST testing to proceed. If the blood agar plate shows growth, discard the AST set (refer to the BACTEC MGIT 960 User’s Manual, AST Instructions) and repeat testing with pure culture.

#### 6.10.3.7. 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 (moxifloxacin or kanamycin) should be documented on the AST print out.

All AST print outs should be labelled with the laboratory accession numbers of the samples and signed off 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- 4-21 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 as a result of the inoculum being prepared incorrectly (adding too many or too few mycobacteria). These samples will need to be repeated.

#### 6.10.3.8. Confirming resistant isolates

All resistant isolates should be verified by preparing a blood agar culture (BAC) to check for growth of contaminating bacteria (SOP 8). All resistant tubes must also be visually inspected to check for typical ‘breadcrumb’ morphology of MTB seen in MGIT tubes. This visual inspection and the blood agar result should be documented on the reverse of LRF 4. 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 or ZN stain (SOP 4). The smear result will also be recorded on the reverse of LRF4. 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 MTC 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 BA. This is because:

- Nix-TB isolates are expected to be resistant to most drugs.
- The culture used to prepare the inoculum/or the subculture for the inoculum has a Hain/Xpert which confirms the presence of MTB before DSTs are set up.
- All resistant results are routinely repeated.
- BA confirms lack of contamination which could be causing false resistance.

Resistant results for SIRE, K, M and Z must be repeated for confirmation. Similarly, if the results for one of these drugs is inconsistent with previous results for the same patient – e.g., baseline 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 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. DSTs may also be required to be repeated to resolve laboratory discrepancies between DST data collected at site laboratories and the UCL Mycobacteriology Laboratory.

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

#### **6.10.4. 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), 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 NiX-TB Quality Manual Attachment Ex.**

## 6.11. SOP 11: Minimum Inhibitory Concentration (MIC)

### 6.11.1. Purpose

This SOP is for use by the UCL Central Mycobacteriology Laboratory to determine the minimum concentration of each of the study drugs – pretomanid (Pa), linezolid (L) and bedaquiline (B) - that inhibits the growth of *Mycobacterium tuberculosis* (MTB) in liquid medium. Minimum Inhibitory Concentration (MIC) testing will be performed, using the BACTEC MGIT 960 instrument, on:

- Pre-treatment isolates from a baseline visit (Day 1, or Screening to Week 4 if Day 1 cultures are negative or contaminated)
- Isolates from patients suspected of treatment failure or relapse or reinfection after treatment (a positive culture at or after week 26 [6 month treatment arm]/week 36 (9 month treatment arm) and any new positive culture thereafter] in order to identify a shift in susceptibility to any of the study drugs.

**NOTE:** Prior to any MIC testing, a Hain MTBC assay (SOP 5) 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.

### 6.11.2. Principle

MIC testing is based on the same principle as the MGIT DST (SOP 11). For MIC, a range of concentrations of the test drugs (pretomanid, bedaquiline and linezolid) 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 as 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.

For standard MGIT-based breakpoint DSTs (see SOP 11), 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 TB eXiST (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 TB eXiST covered in this SOP.

### 6.11.3. 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), linezolid (L) and bedaquiline (B), see Table 1 for manufacturer and storage details. The concentrations tested for these drugs are 2-fold serial dilutions across the following ranges: Pa - 2 to 0.063 µg/ml, L - 8 to 0.25 µg/ml and B - 2 to 0.063 µg/ml (see Table 2).



### 6.11.3.1. Equipment/Reagents

- Biological Safety Cabinet (BSC)
- Discard bucket containing appropriate liquid disinfectant (specified in local Health and Safety documentation)
- Vortex mixer
- Micropipettes (P-1000, P-200 and P-10)
- Filtered pipette tips (100-1000 µl tips, 10-200µl tips and 1-20 µl 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

### 6.11.3.2. Preparation of drug stocks and working solutions

Table 11: Manufacturer and storage instructions for the study drugs

Drug	Code	Solubility	Storage (Powder)	Manufacturer
<b>Pretomanid</b>	Pa	DMSO	4°C	Metrics Inc
<b>Linezolid</b>	L	DMSO	-20°C	Generon
<b>Bedaquiline</b>	B	DMSO	RT	Janssen

Table 12: Drug concentrations used for MGIT MIC testing

Drug	Drug concentration (µg/ml)					
<b>Pa</b>	2	1	0.5	0.25	0.125	0.063
<b>L</b>	8	4	2	1	0.5	0.25
<b>B</b>	2	1	0.5	0.25	0.125	0.063

Drug manufacturer details and storage conditions for each of the study drugs are detailed in Table 11.

For preparation of the **stock solutions** see directions below (and Table 13):

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

**Linezolid (L):** Dissolve 0.025g (25mg) of L in 1.780ml of sterile DMSO (to obtain a 14mg/ml stock solution). Aliquot in 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 in sterile cryotube vials volumes of 50µl 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 13: Drug concentrations used for MGIT Drug Sensitivity 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	<b>DF 1:23.8</b> 42 µl to 958 µl	100 µl	2 µg/ml
Linezolid (L)	25	1.780	14	<b>DF 1:20.8</b> 48 µl to 952 µl	100 µl	8 µg/ml
Bedaquiline (B)	40	10	4	<b>DF 1:23.8</b> 42 µl to 958 µl	100 µl	2 µg/ml

The concentration described in this table corresponds to the highest of the range of Table 12. Starting from this concentration serial 2-fold dilutions in DMSO will be repeated five times 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).

#### 6.11.3.3. 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 LRF5 with MIC values

#### 6.11.3.4. Step 1: Specimen Registration, order TB eXiST tests, print barcodes



For standard MIC testing in the NiX-TB study all 3 drugs will be tested together at the 6 concentrations outlined in Table 12 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 range. For simplicity, the standard 3 drug MIC will be used as the example included in the methods.

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 19 tubes are required for each sample:

- 1 tube - Growth Control
- 6 tubes - Pretomanid concentrations
- 6 tubes - Linezolid concentrations
- 6 tubes - Bedaquiline concentrations

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 screen shots, refer to the Registering TBExiST MGIT tube for MIC-DST Users Guide, at the page numbers shown for each step where appropriate

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 NiX-TB study patient identifier (format 00-0000-000). Assign an accession number – this will be the NiX-TB laboratory accession number and the date in the following format, e.g. P1234567\_20Nov2016.

**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\_20Nov2016\_run1. See Users Guide pages 1-3.

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 12. Select ‘TBeX-Pretomanid’ to add the 6 concentrations for pretomanid to the ‘Ordered Tests’ frame. As above complete Isolate number as ‘1’ and scan each the MGIT tube barcodes in order. Repeat for linezolid and bedaquiline by selecting ‘TBeX-Linezolid’ and ‘TBeX-Bedaquiline’ respectively. There will now be 18 test drug concentrations and a growth control listed in the ‘Ordered tests’ frame, each with a MGIT tube number listed (in sequence # column). Press save.
5. 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 8: Example of printed barcodes



#### 6.11.3.5. 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 4 ml (cat# 245116), BBL Middlebrook OADC Enrichment (cat# 212240) or BACTEC MGIT SIRE supplement can be used.

##### Pretomanid and Bedaquiline

Prior to use, thaw one vial of stock solution for each drug, 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 (2µg/ml final concentration in the MGIT), as per the details in Table 13, for each drug. This 1ml working solution (WS1) is the highest drug concentration and the volume is sufficient for 4 test isolates.
2. Add 500µl of DMSO to 5 screwcap Eppendorf tubes (labelled W2-W6) for the serial dilutions for each drug.
3. For each drug, take 500µl of WS1 and add to the DMSO in the WS2 tube, mix by pipetting. This will create a new, two-fold diluted working solution (1µg/ml final concentration in the MGIT).
4. Take 500µl of WS2 and add to the DMSO in the WS3 tube, mix by pipetting. This will create a new, two-fold diluted working solution (0.5µg/ml final concentration in the MGIT)
5. Repeat for all five serial dilutions for both drugs.
6. Add 100µl of each WS to the corresponding MGIT tubes. The drug and drug concentration are clearly marked on the TBeXiST barcode label.

##### 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 (14mg/ml) and prepare the working solution (8µg/ml final concentration in the MGIT) as per the details in Table 13. This 1ml working solution (WS1) is the highest drug concentration and the volume is sufficient for 4 test isolates.
2. Add 500µl of DMSO to 5 screwcap Eppendorf tubes (labelled W2-W6) for the serial dilutions.
3. Take 500µl of WS1 and add to the DMSO in the WS2 tube, mix by pipetting. This will create a new, two-fold diluted working solution (4µg/ml final concentration in the MGIT)

4. Take 500µl of WS2 and add to the DMSO in the WS3 tube, mix by pipetting. This will create a new, two-fold diluted working solution (2µg/ml final concentration in the MGIT).
5. Repeat for all five serial dilutions.
6. Add 100µl of each WS to the corresponding MGIT tubes.

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

##### **Preparing inoculum from positive MGIT**

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 let any remaining clumps settle out for 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 leave for 30 minutes for any remaining clumps to settle out. 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 19 MGIT tubes per sample.

**>5 Days** – subculture 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 TTP 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 tube should be prepared.

##### **Preparing an inoculum from a LJ slope**

1. Add 4 mL of Middlebrook 7H9 Broth (or BBL MGIT broth) to a 16.5 x 128 mm sterile tube with 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 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 0.5 McFarland standard at this step.
7. 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.
8. Dilute 2 mL of the adjusted suspension in 8mL of sterile saline (1:5 dilutions).

### **Growth Control tube preparation and inoculation**

1. Aseptically pipette 0.1 ml of the organism suspension (prepared as described above from the MGIT or LJ subculture) into a total of 10mL of sterile saline to prepare the 1:100 GC suspension (1% growth control).
2. Mix the GC suspension thoroughly.
3. 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 Patient Number will be clearly marked on TBexiST barcode label.
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 18 remaining drug tubes (6 concentrations for each of the 3 test drugs), 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**

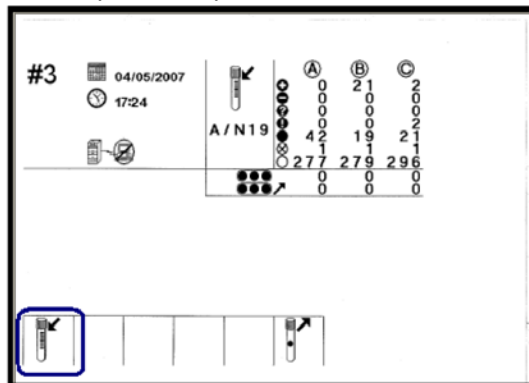
1. To ensure the inoculum that was used to set up the MIC test is not contaminated, spread 0.1mL of the organism suspension to a blood agar plate (BAP).
2. Seal the BAP in a plastic zip-lock bag.
3. Incubate at 35 - 37°C.
4. Check the BAP at 48 hours for bacterial contamination. If it shows no growth, then allow MIC test to proceed. If the BAP shows growth, discard the MIC tubes and repeat testing with pure culture.

#### **6.11.3.7. Step 4 - Tube Loading**

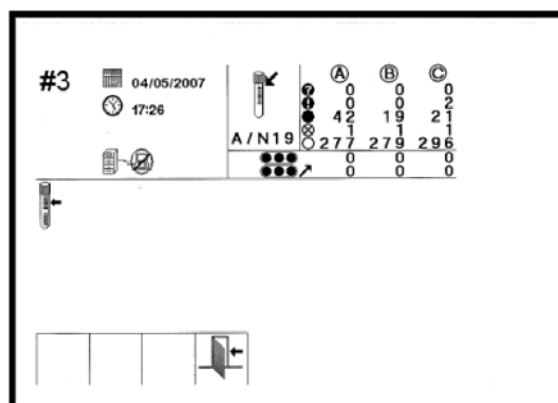
In the BD BACTEC™ MGIT™ software, the option “Accession Barcoding” must be enabled. This will allow the MGIT Instrument to scan both the MGIT tube barcode (sequence #) and the Accession barcode (on the TBexiST generated barcode label)

**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.

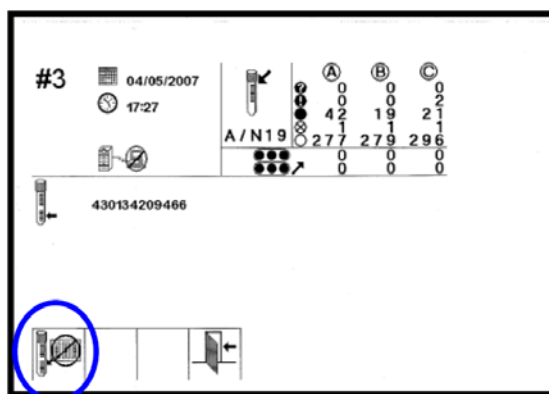
1. Open a drawer and press the “Tube Entry” Soft Key



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 TB eXiST 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.

#### **6.11.3.8. 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 screen shots, 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 'Enter the minimum GU value for the GC' from the table, enter 400 in the space provided, and click 'Run'. This will bring up a listing of ALL MIC samples in the MGIT instrument for which the Growth Control has reached 400GU and therefore the test is complete and ready to be interpreted and finalised.
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 400GU (the first MGIT instrument reading where the GC  $\geq$  400 GU). It also shows the GU at the time of generation of the worklist (if different). It is the column at the timepoint that the GC  $\geq$  400GU that must be used for the interpretation (see Figure 9 for an example TBExiST worklist).  
NOTE: This signed worklist must be kept in the NiX-TB lab file as it is the source data for the GU in each tube at the time of interpretation.

Figure 9: Example of TBExiST worklist

TB eXiST Worklist					24/01/2017 12:49:29
Filter Name: TB eXiST Worklist.Rt					Page 19 Of 72
Enter the Minimum GU Value for the GC = 400					
Sorted By: None					
		GU when GC reached 400	Actual or Last GU (GC on board)	Test Status --- TBExiST/MGIT Tube	Extended GU (GC removed)
Patient Info: ERT11 (H37Rv: 270CT E-F)					
Accession #: H37Rv: 270CT E-F (Mycobacterium tuberculosis)					
Growth Control (days:hours)		410 ( 9:12)	14161 (88:22)	---In Progress/Positive	
Start DT: 27/10/2016 14:02					
Pretomanid H	...8.0 µg/mL	5747 ( 9:12)	15338 (88:22)	--- In Progress/Positive	
Pretomanid H	...16.0 µg/mL	1617 ( 9:12)	12446 (88:22)	--- In Progress/Positive	
Patient Info: ERT2 (H1:136 100CT)					
Accession #: H1:136 100CT (Mycobacterium tuberculosis)					
Growth Control (days:hours)		415 ( 7:6)	22946 (**:19)	---In Progress/Positive	
Start DT: 10/10/2016 17:02					
Bedaquiline H2	...1.0 µg/mL	10401 ( 7:6)	15469 (**:19)	--- In Progress/Positive	
Bedaquiline H2	...2.0 µg/mL	10088 ( 7:6)	19300 (**:19)	--- In Progress/Positive	
Bedaquiline H2	...4.0 µg/mL	7982 ( 7:6)	19403 (**:19)	--- In Progress/Positive	
Bedaquiline H2	...8.0 µg/mL	5561 ( 7:6)	21591 (**:19)	--- In Progress/Positive	
Linezolid H	...1.0 µg/mL	3793 ( 7:6)	26902 (**:19)	--- In Progress/Positive	
Linezolid H	...2.0 µg/mL	1871 ( 7:6)	26984 (**:19)	--- In Progress/Positive	
Linezolid H	...4.0 µg/mL	826 ( 7:6)	27760 (**:19)	--- In Progress/Positive	
Linezolid H	...8.0 µg/mL	185 ( 7:6)	24199 (**:19)	--- In Progress/Positive	

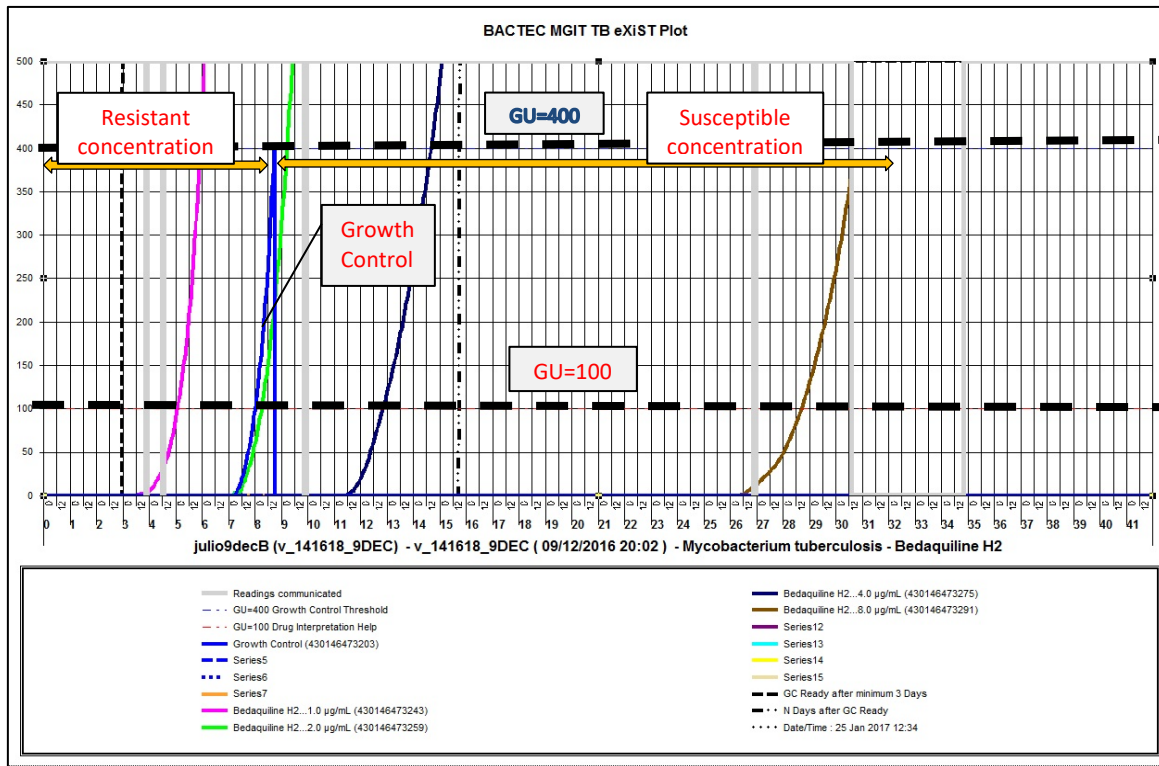
3. Generate TBExiST plots for all of the test drugs – this is a graphical representation of the GU seen in the worklist, with a growth curve shown for each drug concentration. See Figure 10 for an annotated example. To do this, click on the 'Reports' icon, and in the 'Filter Reports' tab, select 'BACTEC MGIT TBExiST plot'. Click on 'Print Preview', select 'Enter Accession Number' from the table and enter the accession number of interest, then select 'Drug' and enter the drug name. 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 NiX-TB eXiST Plots folder using the filename format of Patient No\_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  $\geq$  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. See Users guide pages 11-16.

6. When this is complete for all 18 drug containing tubes, click 'Save'

Figure 10: 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 NiX-TB eXiST Plots folder using the filename format of Patient No\_Lab Accession No\_Date\_Drug.

#### 6.11.3.9. Step 6: Generate Interpretation Report and complete LRF5 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 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 11.
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 into LRF5. If the MIC is out of the range of concentrations tested, it should be reported as >2µg/ml or ≤0.06µg/ml for Pa, >8 µg/ml or ≤0.025µg/ml for L and >2 µg/ml or ≤0.06µg/ml for B. If required, a further round of testing can be performed at a higher/lower set of concentrations as appropriate. This should be noted in the comments section of the LRF if required.
5. 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.

**6.11.3.10. 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. TB-eXiST Completed tubes'.
2. This shows the list of all tubes that have now been assigned a S/R interpretation and finalised. The table shows 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 ZN (SOP4) in the case of suspected contamination or a putative 'resistant' result (see section 1.1.4 Confirming resistant results).
3. Next click on the 'Data View' icon, and select '3. Remove and Finalise'; '3. TB-eXiST 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 Ongoing' 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 'TB-eXiST 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 TB-eXiST complete in the 'Test Status' window, and 'Save'.

Figure 11: Example of TB-eXiST Interpretation Report

TB eXiST Interpretation Report		
Filter Name: TB eXiST Interpretation Report.ftt	24/01/2017 12:36:18	
Enter Accession Number = v_132485_9DEC	Page 1 Of 1	
Sorted By: None		
Antimicrobial	Concentration	Interpretation
<b>Patient Info: julio9decA (v_132485_9DEC)</b>		
<b>FINAL</b>		
<b>Accession #: v_132485_9DEC</b>		
<b>Mycobacterium tuberculosis</b>		
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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24 JAN 2017

#### 6.11.4. Confirming resistant results

1. For any samples where the MIC is greater than the suggested 'resistant' threshold concentrations outlined in Table 14, a BAP 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 ZN results should be recorded in the table provided on the reverse of LRF5. 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.

Table 14: MIC cut-off values for each drug, for a sample to be considered resistant and therefore require further testing

Drug	Pa	L	B
<b>MIC (µg/ml)</b> <b>'Resistant' Threshold</b> <b>Concentration</b>	≥1 µg/ml	≥2 µg/ml	≥1 µg/ml

2. 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 ZN results should be recorded on the reverse of LRF5.
3. For all samples where contamination is detected, the H37Rv QC from the run is out of the acceptable range (see Quality Control section below), or the results are not clear for another reason (clearly state the reason in the comments section of LRF5), the test must be repeated.

**NOTE:** If MTB isolated at/after end of treatment is found to have MIC above cut-off (R), whereas the baseline match for the same patient has MIC below cut-off (S), the repeat, confirmatory test should be done with both isolates in parallel.

#### 6.11.5. 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 and B), 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 NiX-TB Quality Manual Attachment Eiii and Exi. In addition, the H37Rv reference strain should be included on each run to check for MIC performance and control for any variability. 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
<b>MIC (µg/ml)</b>	0.12 and 0.25	0.5 and 1	0.125, 0.25 and 0.5

#### **6.11.6. Documentation**

LRF5 captures the information for completing the eCRF fields (highlighted) related to MIC testing

## 6.12. SOP 12: Whole Genome Sequencing (WGS)

### 6.12.1. Purpose

Used by the **UCL Central Mycobacteriology Laboratory (UCL)**, and Genomic Services and Development Unit, Public Health England (PHE) for molecular typing by Whole Genome Sequencing (WGS) of paired DNA extracts from the isolates at baseline (Day 1, or Screening to Week 4 if Day 1 cultures are negative or contaminated) and the first positive culture at or after the end of treatment [week 26 (6 month treatment arm)/week 39 (9 month treatment arm)]. 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.

### 6.12.2. Principle

DNA will be extracted and quantified as per SOP 9: DNA Extraction at UCL and sent to PHE. 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 processed). At PHE, WGS is performed using the Illumina Nextera XT DNA Library Preparation kit and sequencing performed on Illumina sequencers. Data will be analyzed using the assembly and mapping pipeline outlined in detail below. The WGS data from the paired isolates will be compared and number of single nucleotide polymorphisms (SNPs) different determined. WGS data will also be used to identify mutations in known genes associated with resistance to anti-TB drugs. Isolates may also be sent for WGS to resolve discrepancies in laboratory data.

### 6.12.3. Procedure

#### 6.12.3.1. DNA requirements for WGS analysis

DNA must be extracted and quantified as per SOP 9. Details of the quantification (using Qubit and Nanodrop) must be recorded onto the DNA Quantification Worksheet (Appendix I). To be acceptable to be sent for WGS analysis the extracted DNA from each sample should meet the following criteria:

- 260/280 ratio between 1.8 and 2.0
- Qubit® dsDNA concentration between 6 – 100 ng/μl
- Minimum volume of 60 μl

If this concentration or volume is not achieved, samples with lower concentrations may be able to be processed but this needs to be agreed with PHE beforehand. A copy DNA quantification worksheet (Appendix I) should be emailed to the address stated in Table 16 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 should be diluted in molecular grade water to within the optimal concentration range described above, and dilution details documented on the DNA quantification worksheet (Appendix I). If TE buffer is used for dilution the concentration of EDTA should be below 0.1 mM. If there is sufficient DNA, two aliquots will be prepared – one aliquot will be sent to the PHE, and the other will be stored at UCL as a back-up. If not sufficient DNA, a back-up will not be kept at UCL. DNA should be stored at -20°C at UCL until shipment is required.

If the above DNA requirements criteria are not met for a sample, the DNA extraction must be repeated from a fresh culture stored 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) and new lots prepared as required and a CQIF (Quality Manual Attachment M) completed.

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). 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 online order submission. The online sample sheet must be completed and must include a unique sample ID and the sample names cannot include the following characters: spaces \ / : \* ? " < >). For the NiX-TB study this should be the patient identifier and the lab accession number, e.g. 01-9031-022\_P1136542. This number together with the plate barcode will be used to track samples at all times during processing. Full details of the online submission process are outlined in guidance document 'BW0303 Instructions on sample submission and receiving results' (Appendix 7).

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). Users are required to request a NGS LIMS account through the Infectious Disease Informatics helpdesk and will be required to provide a charge code or purchase order number. When submitting the order details through LIMS the order name for submission on Genesifter should be the same as the submission plate barcode. When the online 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 Nix-TB UCL file.

#### 6.12.3.2. Shipment of DNA from the UCL to PHE.

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

Shipments should be arranged through the courier City Sprint – courier, sender and recipient details are included in the Table 16. City Sprint will provide the required packaging. If necessary, shipments may be delivered by member of the UCL in person.

Table 16: Contact Details for Shipment of DNA from UCL to PHE

<b>Courier Details</b> <b>Arranging delivery of packaging materials and collection of DNA</b>	<b>City Sprint</b> Contact details: General - <a href="mailto:LondonEastHealthcareCT@citysprint.co.uk">LondonEastHealthcareCT@citysprint.co.uk</a> Rebecca Allen - <a href="mailto:RAllen@citysprint.co.uk">RAllen@citysprint.co.uk</a>
<b>Shipper Details</b>	Centre for Clinical Microbiology University College London, 2nd Floor Royal Free Hospital, Rowland Hill St. NW3 2PF, London  Prof. Tim McHugh (Centre Director): <a href="mailto:t.mchugh@ucl.ac.uk">t.mchugh@ucl.ac.uk</a> Tel: +44 (0)207 4726402  Dr. Julio Ortiz-Canseco: <a href="mailto:julio.canseco@ucl.ac.uk">julio.canseco@ucl.ac.uk</a>

	Dr Anna Bateson: <a href="mailto:a.bateson@ucl.ac.uk">a.bateson@ucl.ac.uk</a> Tel: +44 (0)207 794 0500 Ext 31148, 331146
<b>Recipient Details</b>	GSDU/Central Stores National Infection Service Public Health England 61 Colindale Avenue London, NW9 5EQ Contact telephone numbers: 020 83277898  <b>NGS.service@phe.gov.uk</b>

UCL will liaise with PHE to arrange a suitable day for shipment of samples. It is necessary to notify at least one day in advance. Delivery should be between 9:00 and 16:00 Monday - Thursday.

#### 6.12.3.3. Receipt of DNA at PHE

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) submitting the order will receive an email. If there is a discrepancy the customer 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 Nix-TB UCL 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 and the disposal logged onto the disposal log. External customers will have a period of 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. Customer data can be retrieved from the computing infrastructure managed by the PHE Bioinformatics Unit and will be retained for a maximum of 6 months.

#### 6.12.3.4. *M. tuberculosis* genotyping by Whole Genome Sequencing

##### DNA Processing and Sequencing

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 17 below.

Table 17: 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

On completion of a sequencing run GSDU 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.

#### Receipt of Sequence data at UCL.

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 Nix-TB UCL 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 can be downloaded from the FTP server as described in document BW0303 (Appendix 7). It is recommended that the FTP server is accessed using FileZilla software which can be downloaded online. All FASTQ files and the Quality Report for a given run should be downloaded and saved on the UCL 'S:drive' in the 'Nix Sequence data' folder. This is a UCL shared folder with restricted access to staff delegated to be working on the study, and is backed up in accordance with Central UCL IT policy.

#### Data analysis by the designated study bioinformatician/s

Upon receipt of the data, UCL will contact by email the bioinformatician responsible for analysing the sequence data, who will also access the files as required through the FTP site as described above. UCL will send a table to the bioinformatician listing the Patient 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 Nix-TB UCL file. Analysis will be performed as described in Witney et al (2017) and outlined in full below:

##### 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 18)
- 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, Kraken, a sequence classification tool, will be used to speciate the reads.

NOTE: If coverage < 30x and sequence is confirmed MTBC, the bioinformatician will inform UCL and UCL will discuss with PHE 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/regional laboratory. These email correspondences will be printed and kept in Nix-TB UCL file.

##### 2. Sequence analysis pipeline

- Call all genome site positions (this generates a Variant Call Format file (VCF) for all sites – see Table 18). 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 analysis.

- INDELS are identified using SAMtools mpileup as above, but setting the minimum fraction of gapped reads for candidates to 0.05. INDELS are filtered out from phylogenetics analysis (including paired isolate comparison - relapse/reinfection)
- Call all variants from H37Rv (this generates a variant call only VCF – see Table 18). 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 18):
  - 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)
- 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. This will be updated with each new round of WGS data available for the study.
- Assess for the presence of SNPs in the following resistance genes:
  - the 6 genes currently known to be associated with resistance to Pa-824 resistance (*fbiA*, *fbiB*, *fbiC*, *ddn*, *fgd1* and *cofC*). For all resistance genes the following will be reported in the 'Annotated SNP list for PA-824 resistance' (see Table 18):
    - 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)
  - Key resistance determining genes for first and second line drugs – this will be done using a publicly available analysis platform.
- Lineage – the lineage/sub-lineage will be assigned according to the classification described in Coll et al (2014), this will be reported alongside the Pa-824 resistance data output (see Table 18).

NOTE: All analysis will be performed using the current versions (updates to be made annually if applicable) of the software packages listed above. As part of the data output (see Table 18), there is a report which details the version numbers used for each batch of samples analysed.

Table 18: Data files generated during the sequence analysis

File Type*	Details
FASTQ.gz (compressed FASTQ) <i>2 files per sample</i>	Raw sequence data file/s generated by PHE
BAM <i>1 file per sample</i>	Sequence data after alignment with H37Rv (reference strain NC_000962.3)
VCF (all sites) <i>1 file per sample</i>	All site calls
VCF (variant calls only, including additional gene annotation generated using snpEff software) <i>1 file per sample</i>	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 <i>1 file per pair</i>	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-824 resistant genes and lineage) – all samples. Excel file <i>1 file per sample</i>	Study specific proforma, generated from the VCF file to show details of all variants in the Pa-824 resistant genes and the strain lineage
Phylogenetic Tree - initial output as a newick file, and also as png image file. <i>1 file per study</i>	Cumulative phylogenetic tree showing the relatedness of all samples from the study as WGS data is available
Analysis software version <i>1 file per batch of samples analysed</i>	Documents the version of all software used for the sample analysis

**\*All files will be named to include the sample ID which includes the patient identifier and the lab accession number**

**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 18 (excluding the FASTQ which is transferred as described above) will be made available to UCL using a password protected web portal <https://bugs3.sgul.ac.uk/data/bateson/>. These will be downloaded and stored on the UCL S: Drive as described above.

#### **6.12.4 Reporting**

The required paired analysis data (No. of SNPs different between the baseline and follow up samples) will be entered on to LRF 6 (Appendix H) from the appropriate annotated SNP list file (see Table 18 above). For paired isolates where 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 18.

### 6.12.5 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.

# APPENDICES

**APPENDIX A - NIX-TB SPUTUM SPECIMEN TRANSPORT FORM**

This form should accompany each sputum specimen generated from a Nix-TB patient at the clinical site to the laboratory. Use a separate Form for each sample submitted. Once the form is completed, a copy is made and sent to the site, whereas the original should remain in the lab files.

<b>SITE SECTION</b>			
<i>(This section should be completed by the Principal Investigator or delegate)</i>			
Screening or Patient number		_____ - _____ - _____	
Initials	_____ (use a "-" if there is no middle initial)	Date* of birth	____/____/____
Type of Sputum Sample		<input type="checkbox"/> Early Morning <input type="checkbox"/> Spot	
Visit specification	Visit date*	____/____/____	
	Visit in the NiX schedule	<input type="checkbox"/> Screening <input type="checkbox"/> Day 1 <input type="checkbox"/> Treatment, Week ____ <input type="checkbox"/> Unscheduled <input type="checkbox"/> Post-treatment, Month ____ <input type="checkbox"/> Early Withdraw	
Date* of sputum collection		____/____/____	Time <sup>□</sup> of collection
Physician/nurse attending (print name)			
Physician/nurse attending (signature)			

<b>TRANSPORT SECTION</b>	
<i>(This section should be completed by driver, courier or person accompanying sample)</i>	
Date* of sample dispatch	____/____/____
Time <sup>□</sup> of dispatch	____:____
Temperature of transport container (°C)	
Driver/courier/person accompanying sample (print name)	
Driver/courier/person accompanying sample (signature)	

<b>LABORATORY SECTION</b>	
<i>(This section should be completed by the laboratory technician receiving the samples)</i>	
Date* sample received	____/____/____
Time <sup>□</sup> received	____:____
Temperature of transport container on receipt (°C)	
Sample in good condition (yes/no)	
If no please give details (detail problems, is this sample going to be processed? has another sample been requested?)	
Sample processed within 30 minutes (yes/no)	
If no, time sample transferred to fridge (hh:mm and give fridge ID)	
Laboratory technician (print name)	
Laboratory technician (signature)	
Laboratory Accession number	ATTACH LABEL

\*Date format dd/mm/yyyy

□24h clock

# APPENDIX B – LRF1: Screening Samples (page 1)

Screening Number	<div> <div></div> <div></div> <div>-</div> <div></div> <div></div> <div></div> </div>			APPENDIX B: NIX-TB LRF 1 – SCREENING SAMPLES		
Lab Accession Number	<div> <div></div> <div></div> <div></div> <div></div> <div></div> <div></div> </div>			Version No		
ATTACH LABEL				Date		
				Initial		

Patient initials	<div> <div></div> <div></div> <div></div> </div> <div>(use a "-" if there is no middle initial)</div>					
Collection date and time	<div> <div></div> <div></div> <div></div> <div></div> <div></div> <div></div> </div> <div>day month year</div>				<div> <div></div> <div></div> <div>:</div> <div></div> <div></div> </div> <div>Time (24 hour)</div>	
Sputum quality	<input type="checkbox"/> Saliva <input type="checkbox"/> Mucoid <input type="checkbox"/> Mucopurulent <input type="checkbox"/> Purulent <input type="checkbox"/> Bloody <input type="checkbox"/> Mostly Blood					
Estimated specimen volume	<div> <div></div> <div></div> <div>.</div> <div></div> </div> <div>ml</div>				Tech initials _____	
Specimen processing date and time	<div> <div></div> <div></div> <div></div> <div></div> <div></div> <div></div> </div> <div>day month year</div>				<div> <div></div> <div></div> <div>:</div> <div></div> <div></div> </div> <div>Time (24 hour)</div>	
<b>RESULT SECTION – AURAMINE STAIN OR ZIEHL-NEELSEN</b> <input type="checkbox"/> Not Done (add comment)						
Date read	<div> <div></div> <div></div> <div></div> <div></div> <div></div> <div></div> </div> <div>day month year</div>				Tech initials _____	
Smear result	<input type="checkbox"/> No AFB seen <input type="checkbox"/> Scanty <input type="checkbox"/> 1+ <input type="checkbox"/> 2+ <input type="checkbox"/> 3+ <input type="checkbox"/> Missing (add comment)					
Comments (Include the average number of AFBs per field)						
Lab Supervisor * _____ Date & Signature _____ Lab Manager _____ Date & Signature _____						
Date reported	<div> <div></div> <div></div> <div></div> <div></div> <div></div> <div></div> </div> <div>day month year</div>				<div> <div></div> <div></div> <div></div> </div> <div>Signature</div>	

\* Verified that the results are transcribed to the LRF correctly

**APPENDIX B– LRF1: Screening Samples (page 2)**

<b>Screening Number</b>	<div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div>	<b>APPENDIX B: NIX-TB LRF 1 – SCREENING SAMPLES</b>	
<b>Lab Accession Number</b>	ATTACH LABEL		
Version No			
Date			
Initial			

<b>RESULT SECTION – HAIN MTBDR<sub>plus</sub></b> <input type="checkbox"/> Not Done (add comment)			
Date of DNA extraction	<div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div>	Tech initials _____	
	<div style="display: flex; justify-content: space-around; font-size: 0.8em;"> <span>day</span> <span>month</span> <span>year</span> </div>		
Date of DNA hybridisation (Result date)	<div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div>	Tech initials _____	
	<div style="display: flex; justify-content: space-around; font-size: 0.8em;"> <span>day</span> <span>month</span> <span>year</span> </div>		
MTB complex confirmed	yes <input type="checkbox"/> no <input type="checkbox"/>		
Rifampicin banding pattern	<div style="display: flex; justify-content: space-between;"> <div style="width: 30%;"> <input type="checkbox"/> <del>rpoB</del>  <input type="checkbox"/> <del>rpoB WT1</del>  <input type="checkbox"/> <del>rpoB WT2</del>  <input type="checkbox"/> <del>rpoB WT3</del> </div> <div style="width: 30%;"> <input type="checkbox"/> <del>rpoB WT4</del>  <input type="checkbox"/> <del>rpoB WT5</del>  <input type="checkbox"/> <del>rpoB WT6</del>  <input type="checkbox"/> <del>rpoB WT7</del>  <input type="checkbox"/> <del>rpoB WT8</del> </div> <div style="width: 30%;"> <input type="checkbox"/> <del>rpoB MUT1</del>  <input type="checkbox"/> <del>rpoB MUT2A</del>  <input type="checkbox"/> <del>rpoB MUT2B</del>  <input type="checkbox"/> <del>rpoB MUT3</del> </div> </div>		
Rifampicin resistance interpretation	Sensitive <input type="checkbox"/> Resistant <input type="checkbox"/> Missing (add comment) <input type="checkbox"/>		
Isoniazid banding pattern	<div style="display: flex; justify-content: space-between;"> <div style="width: 30%;"> <input type="checkbox"/> <del>katG</del>  <input type="checkbox"/> <del>katG WT</del>  <input type="checkbox"/> <del>katG MUT1</del>  <input type="checkbox"/> <del>katG MUT2</del> </div> <div style="width: 30%;"> <input type="checkbox"/> <del>inhA</del>  <input type="checkbox"/> <del>inhA WT1</del>  <input type="checkbox"/> <del>inhA WT2</del> </div> <div style="width: 30%;"> <input type="checkbox"/> <del>inhA MUT1</del>  <input type="checkbox"/> <del>inhA MUT2</del>  <input type="checkbox"/> <del>inhA MUT3A</del>  <input type="checkbox"/> <del>inhA MUT3B</del> </div> </div>		
Isoniazid resistance interpretation	Sensitive <input type="checkbox"/> Resistant <input type="checkbox"/> Missing (add comment) <input type="checkbox"/>		
Comments			

<b>RESULT SECTION – GENEXPERT MTB/RIF</b> <input type="checkbox"/> Not Done (add comment)				
Date of test	<div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div>	Is the test valid (probe SPC pass)	yes <input type="checkbox"/> no <input type="checkbox"/>	Tech initials _____
	<div style="display: flex; justify-content: space-around; font-size: 0.8em;"> <span>day</span> <span>month</span> <span>year</span> </div>			
MTB complex confirmed	yes <input type="checkbox"/> no <input type="checkbox"/>	If MTB detected: minimum Cycle threshold (the lowest Ct among probes A-E)		<div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div>
IF MTB detected, note semi-quantitative result	High <input type="checkbox"/> low <input type="checkbox"/> medium <input type="checkbox"/> very low <input type="checkbox"/>			
Rifampicin resistance interpretation	Sensitive <input type="checkbox"/> Resistant <input type="checkbox"/> Missing (add comment) <input type="checkbox"/>			
Comments				

Lab Supervisor * _____	Lab Manager _____
Date & Signature	Date & Signature

Date molecular test result reported	<div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div>	_____ <i>Signature</i>
	<div style="display: flex; justify-content: space-around; font-size: 0.8em;"> <span>day</span> <span>month</span> <span>year</span> </div>	

\* Verified that the results are transcribed to the LRF correctly



**APPENDIX B – LRF1: Screening Samples (page 3)**

<b>Screening Number</b>	<div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <span style="font-size: 1.2em; margin: 0 5px;">-</span> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div>	<b>APPENDIX B: NIX-TB LRF 1 – SCREENING SAMPLES</b>	
<b>Lab Accession Number</b>	ATTACH LABEL		
		Version No	
		Date	
		Initial	

RESULT SECTION – MGIT CULTURE				<input type="checkbox"/> Not Done (add comment)	
Date / time protocol started	<div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <span style="font-size: 0.8em; margin: 0 5px;">day</span> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <span style="font-size: 0.8em; margin: 0 5px;">month</span> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <span style="font-size: 0.8em; margin: 0 5px;">year</span>	<div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <span style="font-size: 0.8em; margin: 0 5px;">Time</span>	Tech initials _____		
MGIT tube No.	<div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div>				
MGIT result	<input type="checkbox"/> pos.	<div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <span style="font-size: 0.8em; margin: 0 5px;">day</span> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <span style="font-size: 0.8em; margin: 0 5px;">month</span> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <span style="font-size: 0.8em; margin: 0 5px;">year</span>	Time to detection (TTD/TP):	<div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <span style="font-size: 0.8em; margin: 0 5px;">Days</span>	<div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <span style="font-size: 0.8em; margin: 0 5px;">Hours</span>
	<input type="checkbox"/> neg.	<div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <span style="font-size: 0.8em; margin: 0 5px;">Time</span>	Tech initials _____		
If instrument positive: <b>Blood Agar Plate (BAP) (after 48 h):</b>					
Date Read	<div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <span style="font-size: 0.8em; margin: 0 5px;">day</span> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <span style="font-size: 0.8em; margin: 0 5px;">month</span> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <span style="font-size: 0.8em; margin: 0 5px;">year</span>	Result	<input type="checkbox"/> pos. <input type="checkbox"/> neg. <input type="checkbox"/> other (add comment)		
Comment: Blood Agar Result Other	<input type="checkbox"/> BAP not done				Tech initials _____
	<input type="checkbox"/> BAP is negative, MGIT tube visibly contaminated				
	<input type="checkbox"/> BAP is negative, evidence of fungal hyphae seen on ZN smear				
If instrument positive: <b>Ziehl-Neelsen stain:</b>					
Date read	<div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <span style="font-size: 0.8em; margin: 0 5px;">day</span> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <span style="font-size: 0.8em; margin: 0 5px;">month</span> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <span style="font-size: 0.8em; margin: 0 5px;">year</span>	Result	<input type="checkbox"/> pos. <input type="checkbox"/> neg. <input type="checkbox"/> missing (add comment)	<input type="checkbox"/> cording <input type="checkbox"/> typical <input type="checkbox"/> atypical	
Comments					Tech initials _____
Lab Supervisor * _____ Date & Signature _____ Lab Manager _____ Date & Signature _____					
Date MGIT results reported	<div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <span style="font-size: 0.8em; margin: 0 5px;">day</span> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <span style="font-size: 0.8em; margin: 0 5px;">month</span> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <div style="border: 1px solid black; display: inline-block; width: 20px; height: 20px; margin: 2px;"></div> <span style="font-size: 0.8em; margin: 0 5px;">year</span>	_____ <i>Signature</i>			
RESULT SECTION – ISOLATE STORAGE AND SHIPMENT					
Was the subculture prepared for storage and shipment?			yes <input type="checkbox"/>	no <input type="checkbox"/>	not applicable <input type="checkbox"/>
Was a LJ slope shipped to the Central Lab for characterization?			yes <input type="checkbox"/>	no <input type="checkbox"/>	not applicable <input type="checkbox"/>
Comments					
Lab Supervisor * _____ Date & Signature _____					

\* Verified that the results are transcribed to the LRF correctly

**APPENDIX C – LRF2: Treatment and Follow-up Samples (page 1)**

Subject Number	<input type="text"/> <input type="text"/> - <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> - <input type="text"/> <input type="text"/> <input type="text"/>
Lab Accession Number	<input type="text"/> ATTACH LABEL

APPENDIX C: NIX-TB LRF 2 – TREATMENT AND FOLLOW-UP			
Version No	<input type="text"/>	<input type="text"/>	<input type="text"/>
Date	<input type="text"/>	<input type="text"/>	<input type="text"/>
Initial	<input type="text"/>	<input type="text"/>	<input type="text"/>

Patient initials	<input type="text"/> <input type="text"/> <input type="text"/> (use ' - ' if there is no middle initial)		
Specimen Type	<input type="checkbox"/> Early Morning <input type="checkbox"/> Spot		
Collection date and time	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> day month year	<input type="text"/> <input type="text"/> : <input type="text"/> <input type="text"/> Time (24 hour)	
Visit specification	<input type="checkbox"/> Day 1 <input type="checkbox"/> Treatment, <input type="text"/> <input type="text"/> Week <input type="checkbox"/> Early withdraw <input type="checkbox"/> Post-treatment, <input type="text"/> <input type="text"/> Month <input type="checkbox"/> Unscheduled		
Sputum quality	<input type="checkbox"/> Saliva <input type="checkbox"/> Mucoid <input type="checkbox"/> Mucopurulent <input type="checkbox"/> Purulent <input type="checkbox"/> Bloody <input type="checkbox"/> Mostly Blood		
Estimated specimen volume	<input type="text"/> <input type="text"/> . <input type="text"/> ml	Tech initials _____	
Processed date and time	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> day month year	<input type="text"/> <input type="text"/> : <input type="text"/> <input type="text"/> Time (24 hour)	
<b>RESULT SECTION – MGIT CULTURE</b> <input type="checkbox"/> Not Done (add comment)			
Date / time protocol started	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> day month year	<input type="text"/> <input type="text"/> : <input type="text"/> <input type="text"/> Time	Tech initials _____
MGIT tube No.	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/>		
MGIT result	<input type="checkbox"/> pos. <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> day month year <input type="checkbox"/> neg. <input type="text"/> <input type="text"/> : <input type="text"/> <input type="text"/> Time	Time to detection (TTD/TTP): <input type="text"/> <input type="text"/> . <input type="text"/> <input type="text"/> Days Hours	Tech initials _____
If instrument positive: <b>Blood Agar Plate (BAP) (after 48 h):</b>			
Date Read	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> day month year	Result	<input type="checkbox"/> pos. <input type="checkbox"/> neg. <input type="checkbox"/> other (add comment)
Comment: Blood Agar Result Other	<input type="checkbox"/> BAP not done <input type="checkbox"/> BAP is negative, MGIT tube visibly contaminated <input type="checkbox"/> BAP is negative, evidence of fungal hyphae seen on ZN smear		Tech initials _____

**APPENDIX C – LRF2: Treatment and Follow-up Samples (page 2)**

Subject Number	<input type="text"/> <input type="text"/> - <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> - <input type="text"/> <input type="text"/> <input type="text"/>
Lab Accession Number	ATTACH LABEL

APPENDIX C: NIX-TB LRF 2 – TREATMENT AND FOLLOW-UP			
Version No	<input type="text"/>	<input type="text"/>	<input type="text"/>
Date	<input type="text"/>	<input type="text"/>	<input type="text"/>
Initial	<input type="text"/>	<input type="text"/>	<input type="text"/>

If instrument positive: <b>Ziehl-Neelsen stain:</b>			
Date read	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> day month year	Result	<input type="checkbox"/> pos. <input type="checkbox"/> neg. <input type="checkbox"/> missing (add comment) <input type="checkbox"/> cording <input type="checkbox"/> typical <input type="checkbox"/> atypical
Comments			Tech initials _____
Lab Supervisor * _____ Date & Signature		Lab Manager _____ Date & Signature	
Date MGIT results reported	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> day month year	_____ Signature	
<b>RESULT SECTION - ISOLATE STORAGE AND SHIPMENT</b>			
Was the suture prepared for storage and shipment?		yes <input type="checkbox"/> no <input type="checkbox"/> not applicable <input type="checkbox"/>	
Was a LJ slope shipped to the Central Lab for characterization?		yes <input type="checkbox"/> no <input type="checkbox"/> not applicable <input type="checkbox"/>	
Comments			
Lab Supervisor * _____ Date & Signature			

\*Verified that the results are transcribed to the LRF correctly

**APPENDIX D – LRF3: Speciation**

Screening or Subject Number	<input type="text"/> <input type="text"/> - <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> - <input type="text"/> <input type="text"/> <input type="text"/>
Lab Accession Number	<b>ATTACH LABEL</b>

APPENDIX D: NIX-TB LRF 3 – SPECIATION			
Version No	<input type="text"/>	<input type="text"/>	<input type="text"/>
Date	<input type="text"/>	<input type="text"/>	<input type="text"/>
Initial	<input type="text"/>	<input type="text"/>	<input type="text"/>

Patient initials	<input type="text"/> <input type="text"/> <input type="text"/> (use ' - ' if there is no middle initial)		
Specimen Type	<input type="checkbox"/> Early Morning <input type="checkbox"/> Spot		
Collection date and time	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> day month year	<input type="text"/> <input type="text"/> : <input type="text"/> <input type="text"/> Time (24 hour)	
Visit specification	<input type="checkbox"/> Screening <input type="checkbox"/> Day 1 <input type="checkbox"/> Treatment, <input type="text"/> <input type="text"/> Week <input type="checkbox"/> Post-treatment, <input type="text"/> <input type="text"/> Month <input type="checkbox"/> Unscheduled <input type="checkbox"/> Early withdraw		
<b>RESULT SECTION – SPECIATION</b> <input type="checkbox"/> Not Done (add comment)			
Date of test	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> day month year	Tech initials _____	
Test type	MPT64 Antigen test <input type="checkbox"/> Hain Mycobacterium CM <input type="checkbox"/> Hain MTBC <input type="checkbox"/> Hain MTBDR <sub>plus</sub> <input type="checkbox"/> Hain MTBDR <sub>sl</sub> <input type="checkbox"/> GeneXpert <input type="checkbox"/>		
MTB complex confirmed	yes <input type="checkbox"/> no <input type="checkbox"/> Missing <input type="checkbox"/> (add comment)		
Comments			
Result date	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> day month year	Tech initials _____	
Lab Supervisor *	_____ <i>Date &amp; Signature</i>		
Lab Manager	_____ <i>Date &amp; Signature</i>		
Date Reported	<input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> day month year	_____ <i>Signature</i>	

\* Verified that the results are transcribed to the LRF correctly

**APPENDIX E: Isolate Shipment to UCL**

This form should be completed each time isolates are to be shipped to UCL. A copy of this form should be sent with the LJ slopes.

<b>Laboratory Name:</b>				<b>Name of requestor:</b>
<b>Shipment Date:</b>				
<b>Total number of Slopes:</b>				<b>Signature of requestor:</b>
<b>Patient Number</b>	<b>Laboratory Accession Number</b>	<b>Visit Schedule</b>	<b>Specimen Type</b>	<b>Comment</b>
<b>To be completed upon reconciliation</b>				<b>Additional comments:</b>
<b>Date received:</b>				
<b>UCL Staff Name:</b>				
<b>UCL Staff Signature:</b>				

**APPENDIX F – LRF4: Drug Susceptibility Testing (page 1)**
**APPENDIX F**  
**NIX-TB LRF 4 – Drug Susceptibility Testing**

Version No			
Date			
Initial			

Laboratory Accession Number			
Patient number		□□ - □□□□ - □□□□	
Visit specification		<input type="checkbox"/> Day 1 <input type="checkbox"/> Treatment, <input type="checkbox"/> Week <input type="checkbox"/> Post-treatment, <input type="checkbox"/> Month <input type="checkbox"/> Screening <input type="checkbox"/> Unscheduled	
<b>RESULT SECTION</b>			
Blood Agar Plate Date of evaluation		□□ □□ □□ □□ □□ □□ day month year	
		Result <input type="checkbox"/> pos. <input type="checkbox"/> neg Initial _____	
<b>S I R E</b>	Date of test / started	□□ □□ □□ □□ □□ □□ day month year	Initial _____ Used culture <input type="checkbox"/> MGIT <input type="checkbox"/> LJ
	Streptomycin	<input type="checkbox"/> sensitive <input type="checkbox"/> resistant <input type="checkbox"/> missing (add comment) .....	
	Isoniazid	<input type="checkbox"/> sensitive <input type="checkbox"/> resistant <input type="checkbox"/> missing (add comment) .....	
	Rifampicin	<input type="checkbox"/> sensitive <input type="checkbox"/> resistant <input type="checkbox"/> missing (add comment) .....	
	Ethambutol	<input type="checkbox"/> sensitive <input type="checkbox"/> resistant <input type="checkbox"/> missing (add comment) .....	
Date SIRE removed, or Epicenter Report generated		day □□ month □□□□ year □□□□ <input type="checkbox"/> Epicenter used	
<b>K A N</b>	Date of test / started	□□ □□ □□ □□ □□ □□ day month year	Initial _____ Used culture <input type="checkbox"/> MGIT <input type="checkbox"/> LJ
	Kanamycin	<input type="checkbox"/> sensitive <input type="checkbox"/> resistant <input type="checkbox"/> missing (add comment) .....	
	Date KAN removed, or Epicenter Report generated	day □□ month □□□□ year □□□□ <input type="checkbox"/> Epicenter used	
<b>P Z A</b>	Date of test / started	□□ □□ □□ □□ □□ □□ day month year	Initial _____ Used culture <input type="checkbox"/> MGIT <input type="checkbox"/> LJ
	Pyrazinamide	<input type="checkbox"/> sensitive <input type="checkbox"/> resistant <input type="checkbox"/> missing (add comment) .....	
	Date PZA removed, or Epicenter Report generated	day □□ month □□□□ year □□□□ <input type="checkbox"/> Epicenter used	
<b>M O X</b>	Date of test / started	□□ □□ □□ □□ □□ □□ day month year	Initial _____ Used culture <input type="checkbox"/> MGIT <input type="checkbox"/> LJ
	Moxifloxacin concentration	<input type="checkbox"/> 0.5µg/ml <input type="checkbox"/> 2µg/ml <input type="checkbox"/> Other (please specify) .....	
	Moxifloxacin	<input type="checkbox"/> sensitive <input type="checkbox"/> resistant <input type="checkbox"/> missing (add comment) .....	
	Date Mox removed, or Epicenter Report generated	day □□ month □□□□ year □□□□ <input type="checkbox"/> Epicenter used	
Comments			
Lab Supervisor *		Lab Manager	
Date & Signature		Date & Signature	
Date Reported	□□ □□ □□ □□ □□ □□ day month year	Signature	

\* Verified that the results are transcribed to the LRF correctly

# APPENDIX F – LRF4: Drug Susceptibility Testing (page 2)

## APPENDIX F NIX-TB LRF 4 – Drug Susceptibility Testing

Version No			
Date			
Initial			

Fill out in case of resistant result:

Drug: _____ Start Date: _____ Initials: _____	Blood agar plate sterile	<input type="checkbox"/> yes <input type="checkbox"/> <u>contaminated</u>	_____
	Visible inspection of MGIT tube – Normal TB Morphology seen	<input type="checkbox"/> yes <input type="checkbox"/> no	_____
	Confirmation by ZN-stain (if applicable)	<input type="checkbox"/> AFB+ <input type="checkbox"/> contamination <input type="checkbox"/> No AFB	_____
Drug: _____ Start Date: _____ Initials: _____	Blood agar plate sterile	<input type="checkbox"/> yes <input type="checkbox"/> <u>contaminated</u>	_____
	Visible inspection of MGIT tube – Normal TB Morphology seen	<input type="checkbox"/> yes <input type="checkbox"/> no	_____
	Confirmation by ZN-stain (if applicable)	<input type="checkbox"/> AFB+ <input type="checkbox"/> contamination <input type="checkbox"/> No AFB	_____
Drug: _____ Start Date: _____ Initials: _____	Blood agar plate sterile	<input type="checkbox"/> yes <input type="checkbox"/> <u>contaminated</u>	_____
	Visible inspection of MGIT tube – Normal TB Morphology seen	<input type="checkbox"/> yes <input type="checkbox"/> no	_____
	Confirmation by ZN-stain (if applicable)	<input type="checkbox"/> AFB+ <input type="checkbox"/> contamination <input type="checkbox"/> No AFB	_____
Drug: _____ Start Date: _____ Initials: _____	Blood agar plate sterile	<input type="checkbox"/> yes <input type="checkbox"/> <u>contaminated</u>	_____
	Visible inspection of MGIT tube – Normal TB Morphology seen	<input type="checkbox"/> yes <input type="checkbox"/> no	_____
	Confirmation by ZN-stain (if applicable)	<input type="checkbox"/> AFB+ <input type="checkbox"/> contamination <input type="checkbox"/> No AFB	_____
Drug: _____ Start Date: _____ Initials: _____	Blood agar plate sterile	<input type="checkbox"/> yes <input type="checkbox"/> <u>contaminated</u>	_____
	Visible inspection of MGIT tube – Normal TB Morphology seen	<input type="checkbox"/> yes <input type="checkbox"/> no	_____
	Confirmation by ZN-stain (if applicable)	<input type="checkbox"/> AFB+ <input type="checkbox"/> contamination <input type="checkbox"/> No AFB	_____
Drug: _____ Start Date: _____ Initials: _____	Blood agar plate sterile	<input type="checkbox"/> yes <input type="checkbox"/> <u>contaminated</u>	_____
	Visible inspection of MGIT tube – Normal TB Morphology seen	<input type="checkbox"/> yes <input type="checkbox"/> no	_____
	Confirmation by ZN-stain (if applicable)	<input type="checkbox"/> AFB+ <input type="checkbox"/> contamination <input type="checkbox"/> No AFB	_____
Lab. Manager (Date and Signature): _____			

**APPENDIX G – LRF5: Minimum Inhibitory Concentration (page 1)**

## APPENDIX G

**NIX-TB LRF 5 – Minimal Inhibitory Concentration**

Version No			
Date			
Initial			

Laboratory Accession Number			
Patient number		□□ - □□□□ - □□□	
Visit specification (Tick and complete as appropriate)		<input type="checkbox"/> Day 1 <input type="checkbox"/> Treatment, □□ Week <input type="checkbox"/> Post-treatment, □□ Month <input type="checkbox"/> Screening <input type="checkbox"/> Unscheduled	
<b>RESULT SECTION</b>			
Blood agar plate	□□ □□□□ □□□□	Result: <input type="checkbox"/> pos. <input type="checkbox"/> neg Initials _____	
Date of evaluation	day month year		
<b>BDQ</b>	Test Start Date	□□ □□□□ □□□□	Initials _____
	MIC value	_____ µg/ml or Missing <input type="checkbox"/> Provide reason.....	
	Result Date	□□ □□□□ □□□□	Initials _____
<b>LZD</b>	Test Start Date	□□ □□□□ □□□□	Initials _____
	MIC value	_____ µg/ml or Missing <input type="checkbox"/> Provide reason.....	
	Result Date	□□ □□□□ □□□□	Initials _____
<b>PRE</b>	Test Start Date	□□ □□□□ □□□□	Initials _____
	MIC value	_____ µg/ml or Missing <input type="checkbox"/> Provide reason.....	
	Result Date	□□ □□□□ □□□□	Initials _____
Comments			
Lab Supervisor *		Lab Manager	
Date & Signature		Date & Signature	
Date Reported	□□ □□□□ □□□□	Signature	
	day month year		

\* Verified that the results are transcribed to the LRF correctly



# APPENDIX G – LRF5: Minimum Inhibitory Concentration (page 2)

## APPENDIX G

### NIX-TB LRF 5 – Minimal Inhibitory Concentration

Version No			
Date			
Initial			

Fill out in case of resistant result:

Drug: _____ Conc (µg/ml): _____ Start Date: _____ Initials: _____	Blood agar plate sterile <input type="checkbox"/> yes <input type="checkbox"/> contaminated	_____
	Confirmation by ZN-stain <input type="checkbox"/> AFB+ <input type="checkbox"/> contaminated <input type="checkbox"/> No AFB	_____
Drug: _____ Conc (µg/ml): _____ Start Date: _____ Initials: _____	Blood agar plate sterile <input type="checkbox"/> yes <input type="checkbox"/> contaminated	_____
	Confirmation by ZN-stain <input type="checkbox"/> AFB+ <input type="checkbox"/> contaminated <input type="checkbox"/> No AFB	_____
Drug: _____ Conc (µg/ml): _____ Start Date: _____ Initials: _____	Blood agar plate sterile <input type="checkbox"/> yes <input type="checkbox"/> contaminated	_____
	Confirmation by ZN-stain <input type="checkbox"/> AFB+ <input type="checkbox"/> contaminated <input type="checkbox"/> No AFB	_____
Drug: _____ Conc (µg/ml): _____ Start Date: _____ Initials: _____	Blood agar plate sterile <input type="checkbox"/> yes <input type="checkbox"/> contaminated	_____
	Confirmation by ZN-stain <input type="checkbox"/> AFB+ <input type="checkbox"/> contaminated <input type="checkbox"/> No AFB	_____
Drug: _____ Conc (µg/ml): _____ Start Date: _____ Initials: _____	Blood agar plate sterile <input type="checkbox"/> yes <input type="checkbox"/> contaminated	_____
	Confirmation by ZN-stain <input type="checkbox"/> AFB+ <input type="checkbox"/> contaminated <input type="checkbox"/> No AFB	_____
Drug: _____ Conc (µg/ml): _____ Start Date: _____ Initials: _____	Blood agar plate sterile <input type="checkbox"/> yes <input type="checkbox"/> contaminated	_____
	Confirmation by ZN-stain <input type="checkbox"/> AFB+ <input type="checkbox"/> contaminated <input type="checkbox"/> No AFB	_____
Lab. Manager (Date & Signature): _____		

# APPENDIX H – LRF6: Paired Whole Genome Sequencing

APPENDIX H		Version No			
		Date			
NIX-TB LRF 6 – Paired WGS		Initial			

Patient number		□□ - □□□□ - □□□□			
<b>Isolate 1</b>	Visit specification (tick and complete as appropriate)	<input type="checkbox"/> Screening <input type="checkbox"/> Day 1 <input type="checkbox"/> Treatment, <input type="text"/> <input type="text"/> Week			
	Laboratory Accession Number				
	Collection date	<input type="text"/> <input type="text"/> day <input type="text"/> <input type="text"/> month <input type="text"/> <input type="text"/> year			
<b>Isolate 2</b>	Visit specification (tick and complete as appropriate)	<input type="checkbox"/> Treatment, <input type="text"/> <input type="text"/> Week <input type="checkbox"/> Post-Treatment, <input type="text"/> <input type="text"/> Month <input type="checkbox"/> Unscheduled			
	Laboratory Accession Number				
	Collection date	<input type="text"/> <input type="text"/> day <input type="text"/> <input type="text"/> month <input type="text"/> <input type="text"/> year			
<b>PAIRED WHOLE GENOME SEQUENCING</b>					
Was paired whole genome sequencing performed?		<input type="checkbox"/> Yes <input type="checkbox"/> No (add comment)			
Sequence Run date*		<b>Isolate 1</b> <input type="text"/> <input type="text"/> day <input type="text"/> <input type="text"/> month <input type="text"/> <input type="text"/> year <b>Isolate 2</b> <input type="text"/> <input type="text"/> day <input type="text"/> <input type="text"/> month <input type="text"/> <input type="text"/> year			
Number of Single Nucleotide Polymorphisms (SNP) differences between isolate 1 and isolate 2* <small>Note: For pairs with &lt;20 SNPs different, please print the associated annotated SNP table and attach to this LRF</small>					
Comments					
Result date <small>(Note: this is the date of the Annotated SNP List Report comparing the two isolates)</small>		<input type="text"/> <input type="text"/> day <input type="text"/> <input type="text"/> month <input type="text"/> <input type="text"/> year			
Lab Supervisor *		Lab Manager			
Date & Signature		Date & Signature			

\* Verified that the results are transcribed to the LRF correctly

## DNA QUANTIFICATION WORKSHEET

Lab Manager (Sign and Date): \_\_\_\_\_

APPENDIX I – DNA Quantification Worksheet (page 2)

APPENDIX I

DNA QUANTIFICATION WORKSHEET

If DNA concentration is acceptable and DNA requires dilution prior to sending for WGS, dilution steps should be documented below.

NOTE: Note this table is only required to be completed if DNA is sent for WGS

Lab accession number	Patient ID	QUBIT Final dsDNA <del>conc</del> (µg/ml) in DNA sample	Final <u>DNA</u> <u>concentration</u> (µg/ml) required for WGS	Dilution Factor (add details)	Final volume of sample sent for WGS (µl)

Lab Manager (Sign and Date): \_\_\_\_\_

## **Appendix 1 -- Hain Life Sciences, GenoLyse®, Version 1.0, Instructions for Use, 10/2012**



GenoLyse\_1012\_5161  
0-09-02.pdf

## **Appendix 2 – Xpert *M.tb*/RIF System Operator Manual**



XpertMTB\_Broch\_R9\_  
EU.pdf

## **Appendix 3 – Hain GenoType MTBDR*plus*, Version 2.0, Instructions for Use, 10/2011**



MTBDRplusV2\_1011\_  
304A-01-02.pdf

## **Appendix 4 – Hain GenoType MTBDR*s*/, Version 2.0, Instructions for Use, 06/2015**



MTBDRsIV2\_0615\_317  
A-02-02.pdf

## **Appendix 5 – Hain GenoType MTBC, Version 1.0, Instructions for Use, 06/2015**



GenoType MTBC  
(IFU-301-10).pdf

## **Appendix 6 – Hain GenoType Mycobacterium CM, Version 1.0, Instructions for Use, 08/2016**



MYCCMV2\_0816\_299  
A-01-02.pdf

## **Appendix 7 – BW0303: Instructions on sample submission and receiving results**



Adobe Acrobat  
Document