LABORATORY MANUAL

MYCOBACTERIOLOGY – LABORATORIES

Protocol Title: A Phase 3, Open-Label Partially Randomized Trial to Evaluate the Efficacy, Safety and Tolerability of the Combination of Moxifloxacin plus PA-824 plus Pyrazinamide after 4 and 6 months in Adult Subjects with Drug-Sensitive Smear-Positive Pulmonary Tuberculosis and after 6 months of Treatment in Adult Subjects with Multi-Drug Resistant, Smear Positive Pulmonary Tuberculosis.

Protocol Number: NC-006-(M-Pa-Z)
Protocol Name: STAND (Shortening **T**reatments by **A**dvancing **N**ovel **D**rugs**)**
Version: 1.0; 18January2014

I hereby approve the above document and release it for appropriate amendment to make it Mycobacteriology Laboratory specific and thereafter use at the Mycobacteriology Laboratories:

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TABLE OF CONTENTS

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Master Version Number/Date	Change
1.0/18January2015	Initial version

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1. ABBREVIATIONS

AFB	Acid Fast Bacilli
ATCC	American Type Culture Collection
BA	Blood Agar
BSC	Biological Safety Cabinet
CL3	Containment Level 3
CQIF	Continuous Quality Improvement Form
CRF	Case Report Form
DMSO	Dimethyl Sulfoxide
DST	Drug Susceptibility Testing
E	Ethambutol
FQ	Fluoroquinolones
GC	Growth Control
H	Isoniazid
HYB	Hybridization Buffer
IQC	Internal Quality Control
LM	Laboratory manual
MGIT	Mycobacteria Growth Indicator Tube
MIC	Minimum Inhibitory Concentration
MIN	Minute(s)
M	Moxifloxacin
M.tb	Mycobacterium tuberculosis
MTC	Mycobacterium tuberculosis complex
NALC	N-Acetyl L-Cysteine
NaOH	Sodium Hydroxide
OADC	Oleic Acid Albumin Dextrose Complex
OD	Optical Density
PANTA	Polymyxin B, Amphotericin B, Nalixidic acid, Trimethoprim, Azlocillin
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain reaction
Z	Pyrazinamide
QC	Quality Control
R	Rifampicin
S	Streptomycin
SIRE	Streptomycin, Isoniazid, Rifampicin and Ethambutol
SOP	Standard Operating Procedure
STR	Stringent Wash Solution
T	Temperature
TTP	Time to Positivity
Z-N	Ziehl-Neelsen

2. CONTACT DETAILS

Name	Contact Person	Contact Details		<<Physical and Postal as applicable>>
<<Screening: Z-N smear (AFP +/- grading), Hain MTBDRpl/GeneXpert (R resistance), Hain MTBDRsl (FQ resistance, *M.tb* confirmation).>> MGIT (*M.tb* confirmation and TTP).>>				
<<Local/Regional Laboratory>>	<<>>	Tel:	<<>>	<<>>
		Fax:	<<>>	<<>>
		Cell:	<<>>	<<>>
		E-mail	<<>>	<<>>
<<Local/Regional Courier>>	<<>>	Tel:	<<>>	<<>>
		Fax:	<<>>	<<>>
		Cell:	<<>>	<<>>
		E-mail	<<>>	<<>>
<<Other>>				
<<Other Laboratory>>	<<>>	Tel:	<<>>	<<>>
		Fax:	<<>>	<<>>
		Cell:	<<>>	<<>>
		E-mail	<<>>	<<>>
<<Other courier>>	<<>>	Tel:	<<>>	<<>>
		Fax:	<<>>	<<>>
		Cell:	<<>>	<<>>
		E-mail	<<>>	<<>>
Screening: *pncA* molecular test (Z resistance). Note: Sample prepared and couriered by <<>> laboratory.				
TASK Laboratory	Dr. M. Barnard	Tel:	+27 21 938 9556	Room F519, 5th Floor, Fisan Building Dept of Biomedical Sciences Faculty of Health Sciences Stellenbosch University Francie van Zijl Drive Tygerberg 7505 South Africa
		Fax:	N/A	
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		E-mail	marinusb@sun.ac.za	
Post screening: MICs, resistance, speciation and strain testing				
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3. INTRODUCTION

The STAND/NC-006-(M-Pa-Z) clinical trial is a Phase 3 trial of the PaMZ regimen used to treat pulmonary tuberculosis. The trial is being conducted globally in approximately 15 countries, and at approximately 50 clinical sites. Microbiological assays for the trial will be conducted at a number of local or regional laboratories, and some assays will be conducted at only one of two central laboratories. The diagrams and table that follow before the start of the SOPs give an overall orientation to the individual assays and their location of conduct. The local and regional labs will work with sputum samples from the sites and will conduct the MGIT cultures for *M.tb* that will be the basis of the primary endpoint of the trial. These laboratories will also do the screening evaluation of sputum smears for AFB and will do rapid molecular tests to determine whether the *M.tb* is susceptible to rifampicin and/or fluoroquinolones. The local and regional labs will extract DNA from the screening sample to send to the *pncA* lab at Stellenbosch University, South Africa, where that central laboratory will do a rapid molecular test to determine whether the *M.tb* is susceptible to pyrazinamide. The local and regional labs will also subculture isolates on LJ slopes. An LJ slope and DNA extracted from an LJ slope will be sent to the central laboratory at University College London. At this central laboratory isolates will be evaluated for susceptibility to a standard panel of antibiotics in liquid culture and Minimum Inhibitory Concentrations will be determined to pretomanid (PA-824) and to moxifloxacin. This laboratory will also conduct a genetic analysis of DNA from the isolates of subjects with positive cultures at or after the end of treatment to evaluate if these isolates are identical or not to the baseline isolate.

The laboratory manual is published in two different editions: Both include the overview diagrams and tables that orient the reader to the flow of assays. The full edition includes SOPs for all assays; the central laboratories at Stellenbosch University and at University London will use just the SOPs required for the assays they perform. The other edition for the local and regional labs includes the SOPs and figures only for the work to be done at these laboratories.

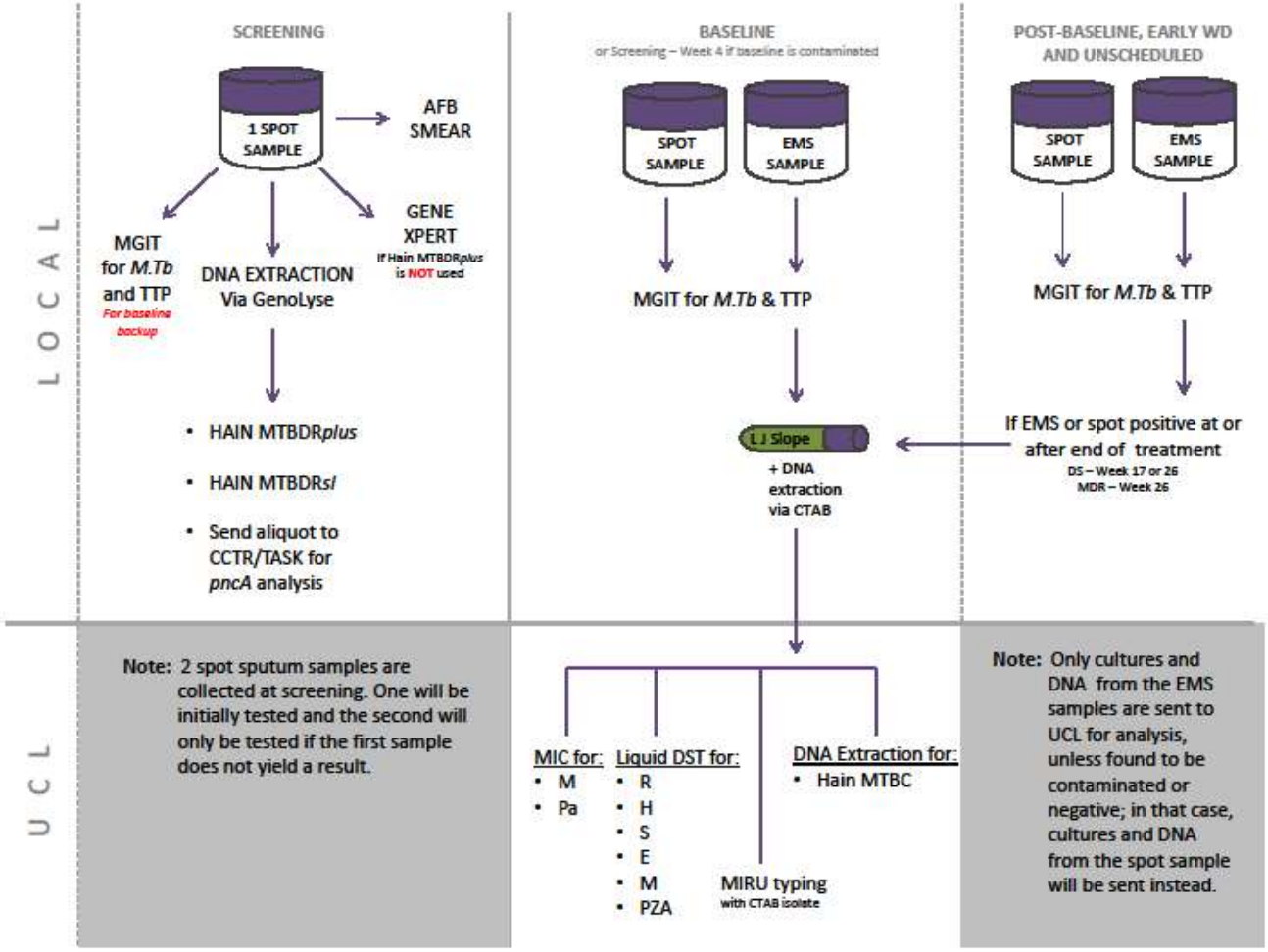
This manual is used in combination with the following documents:

- STAND/NC-006-(M-Pa-Z) Mycobacteriology Laboratory Quality Manual which contains the Quality Control forms to be used throughout the trial;
- STAND/NC-006-(M-Pa-Z) Mycobacteriology Laboratory Data Reporting Manual which will detail the template Mycobacteriology Laboratory Result forms and terminology.

4. MYCOBACTERIOLOGY LABORATORY TESTING

The Mycobacteriology Laboratory Testing that occurs at each laboratory (local plus central (UCL and CCTR/TASK (pyrazinamide resistance testing) is summarized in Figure 2.

Figure 1: Microbiology Testing for STAND Trial per laboratories



The Mycobacteriology Laboratory Testing Schedule is summarized in Figure 2.

Figure 2: Site Visit Flow Chart

Period	**Screening**	**Treatment**													**Follow-Up**						
Visit	Day (-14 (MDR)/ -9 (DS) to -1)	Day 1 (Baseline)	Day 7 (Week 1)	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8 (Month 2)	Week 12 (Month 3)	Week 17 (Month 4) End of treatment: 4 month	Week 22 (Month 5)	Week 26 (Month 6) End of treatment: 6 month	Month 9	Month 12	Month 15	Month 18	Month 24	Early Withdrawal	Unscheduled
Early Morning Sputum		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Spot Sputum	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

The Mycobacteriology Laboratory Testing details are summarized in Table 1: Testing Details

Table 1: Testing Details

TIMING	SAMPLES COLLECTED	ANALYSES PERFORMED
Days (-14(MDR) /-9 (DS) to -1)(Screening):	Two spot sputum: ○ Both collected at the research site under the coaching and observation of the trial staff. ○ The second sample is collected as a back-up sample to the first sample in case it is not possible to obtain a result/s on the first sample. ○ If spot sputum smear shows an indeterminate result or is AFB negative, the test may be repeated on a freshly collected spot sputum/s and that result used.	Performed at <<Local/Regional Laboratory>> Screening Analyses: ○ Direct sputum smear microscopy using Ziehl-Neelsen stain for Acid Fast Bacteria (AFB); ○ *<<Hain* MTB*DRplus or GeneXpert>>* Rapid test for rifampicin resistance; ○ Hain Assay MTBDRsl Rapid test for fluoroquinolones resistance and confirmation *M.tb;* ○ GenoLysed DNA extracted and sent to central *pncA* laboratory for *pncA* molecular test for pyrazinamide resistance. Baseline back up – not for screening purposes: ○ Liquid Culture (MGIT) for presence or absence of *M.tb*; ○ TTP in liquid medium (MGIT).
• All visits from Day 1 (baseline) up to and including Month 24. • If both sputum samples at Month 2 or later are contaminated → Unscheduled visit • Positive culture at or after the end of treatment (Week 17 (4 month treatment arms)/Week 26 (6 month treatment arms)) → Unscheduled visit ≥ 7days from previous sample collection • Unscheduled visits • Early withdrawal visit	Two sputum samples: ○ One early morning collected and brought by subject from home. ○ One spot collected at the site under the coaching and observation of the trial staff.	Performed at <<Local/Regional Laboratory>> Efficacy Analyses: ○ Liquid Culture (MGIT) for presence or absence of *M.tb*; ○ TTP in liquid medium (MGIT).
• Day 1 (baseline) sputum sample (or screening or out to Week 4 if the baseline is contaminated or negative); • Positive Cultures at or after Week 17 (4 month treatment arms)/Week 26 (6 month treatment arms)	N/A. Local laboratory will send LJ slopes and extracted DNA to the central UCL laboratory for above samples already collected.	Performed at University College London Department of Clinical Microbiology The *M.tb* isolates will be processed for: ○ Speciation of the infecting organisms and positive cultures after completion of treatment by HAIN MTBC or GeneXpert to confirm *M.tb;* ○ MIC against moxifloxacin and PA-824 (method to be confirmed); ○ Drug Susceptibility Testing for streptomycin, rifampicin, isoniazid, ethambutol, moxifloxacin, and pyrazinamide (MGIT); ○ Molecular strain typing (MIRU)

Figure 3: Flow chart: Spot sputum sample processing (Screening)

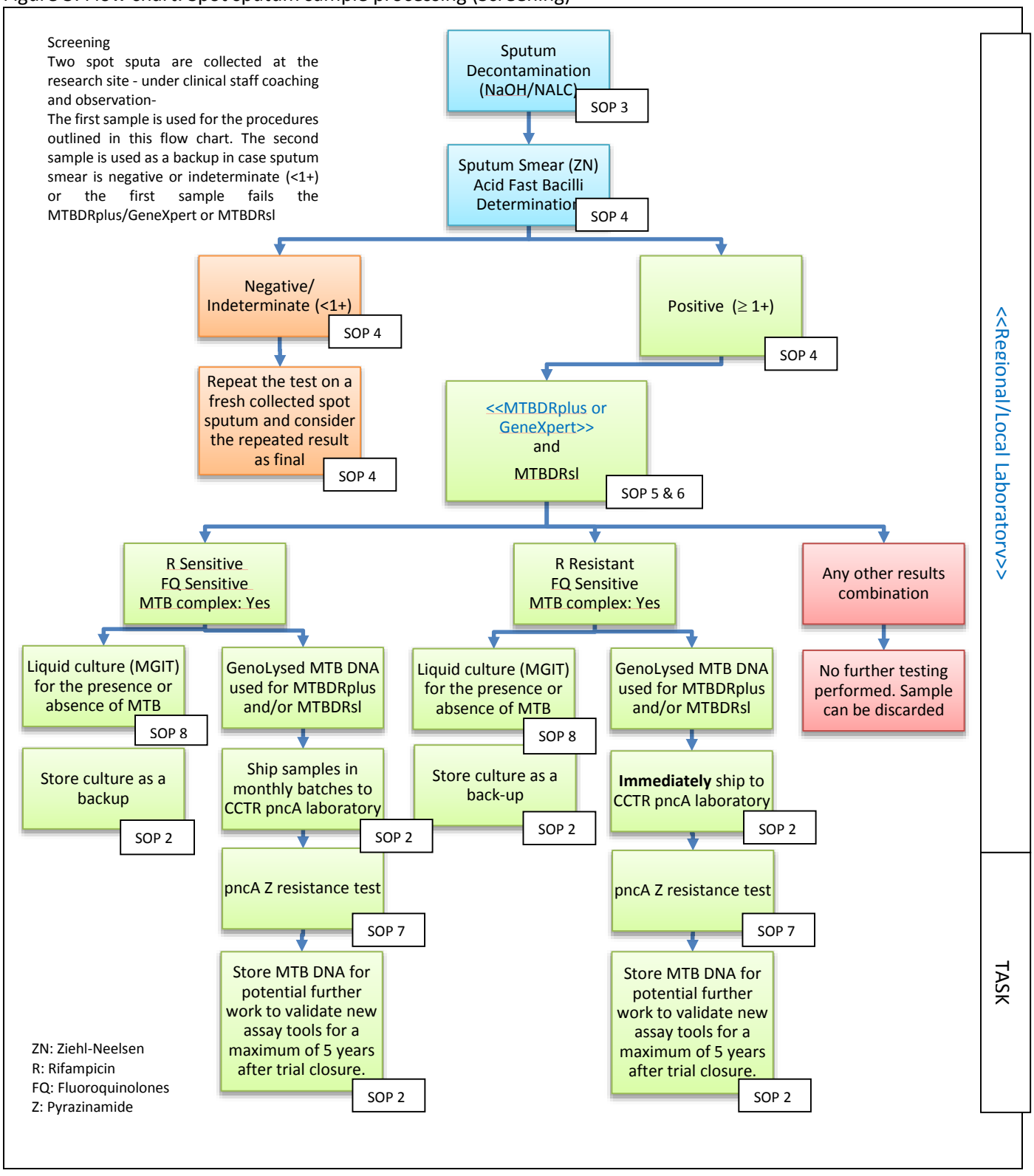


Figure 4: Flow chart sputum sample processing (Day 1 – Month 24)

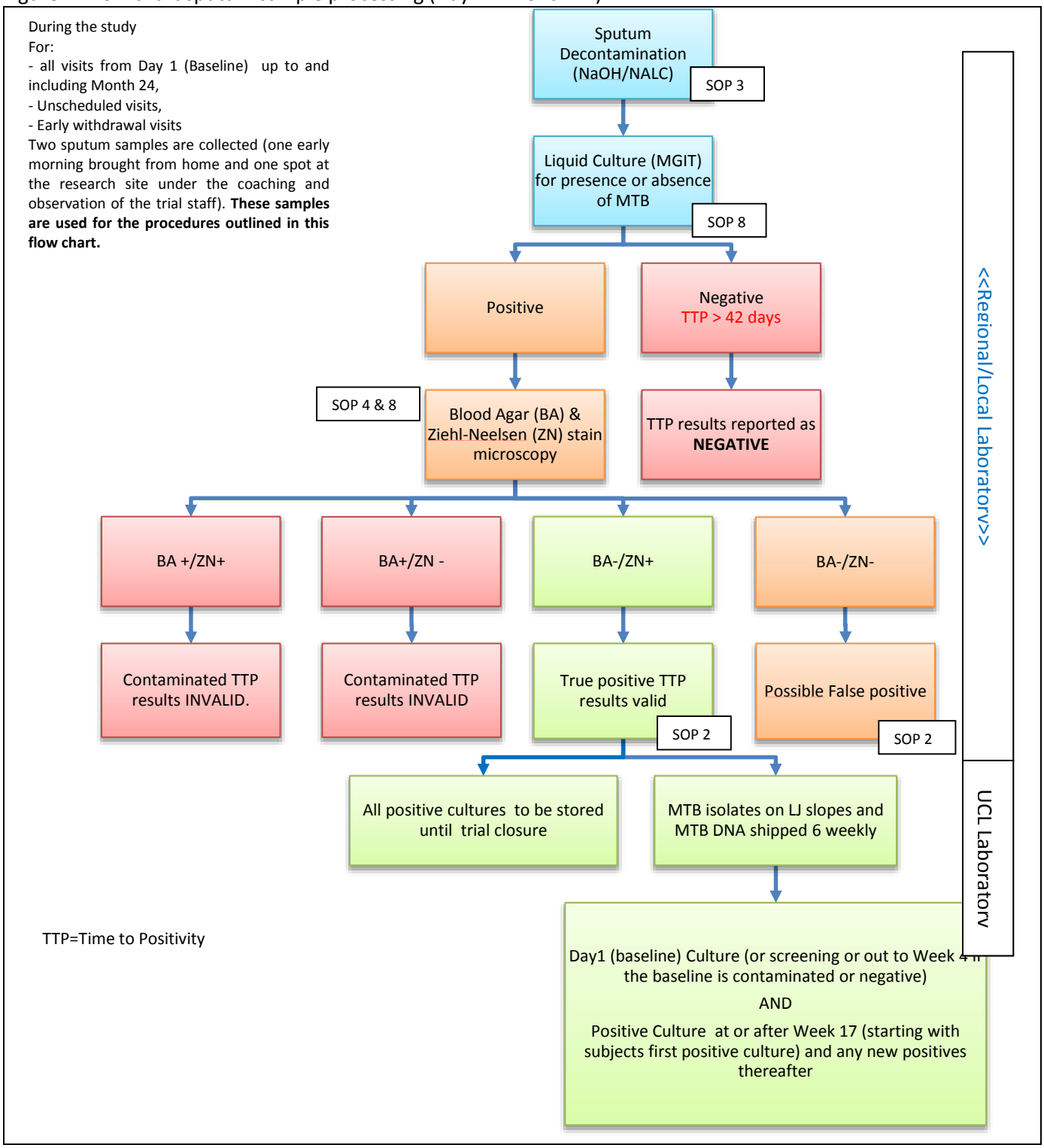
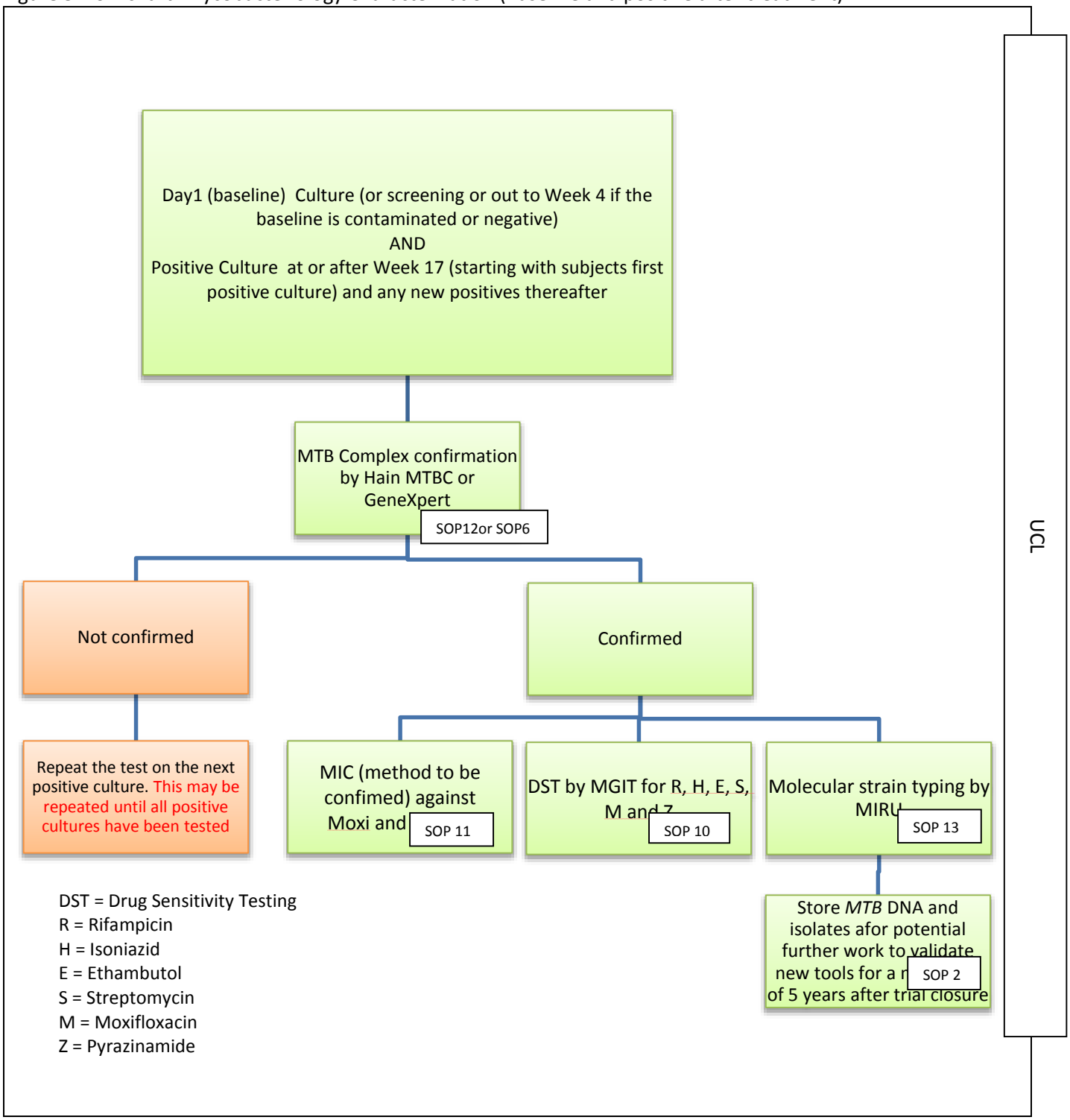


Figure 5 Flow chart: Mycobacteriology Characterization (Baseline and positive after treatment)



5. STANDARD OPERATING PROCEDURES (SOP)

The SOPs which apply to the each mycobacteriology laboratories type are described in Table 2.

Table 2: SOPs applicable to Laboratories

Laboratory	Role	SOP
Local/Regional Laboratory	Screening mycobacteriology (excluding *pncA* resistance testing) and efficacy endpoint testing	SOP 1, 2, 3, 4, 5, 6, 8, 9
pncA Laboratory	Pyrazinamide resistance testing	SOP 2, 7
Central Mycobacteriology Laboratory	Mycobacteriology Characterisation Testing	SOP 2, 4,6, 8, 9, 10, 11, 12, 13

5.1. SOP 1: Sputum Handling

5.1.1. Purpose

Proper collection and transport of sputum specimens if 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 be followed.

5.1.2. Principle

Sputum specimens in this study are collected in the early morning at home ("early morning" samples), and as spot samples at the research site. These are delivered to the local microbiology laboratory where they are received, checked and documented prior to any tests being performed.

5.1.3. Procedure

5.1.3.1. Receipt of specimen at the <<local/regional>> laboratory

The following samples described in Table 3 will be received at the <<local/regional>> laboratory.

Table 3: Sputum Samples Collected

TIMING	SAMPLES COLLECTED
Days (-14(MDR) /-9 (DS) to -1)(Screening)	Two spot sputum collected at the research site under the coaching and observation of the trial staff. • The second sample is collected as a back-up sample to the first sample in case it is not possible to obtain a result/s on the first sample. • If spot sputum smear shows an indeterminate result or is AFB negative, the test may be repeated on a freshly collected spot sputum/s and that result used.
• All visits from Day 1 (baseline) up to and including Month 24. • If both sputum samples at Month 2 or later are contaminated → Unscheduled visit • Positive culture at or after the end of treatment (Week 17 (4 month treatment arms)/Week 26 (6 month treatment arms)) → Unscheduled visit ≥ 7days from previous sample collection • Unscheduled visits • Early withdrawal visit	<u>Two sputum samples:</u> • One early morning collected and brought by subject from home. • One spot collected at the site under the coaching and observation of the trial staff.

5.1.3.2. Logging-in of sputum specimen at the laboratory

- On receipt of samples at the laboratory, assign a unique laboratory accession number to the specimen using the study specific labels. 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, CRF and results.
- Complete the Specimen Transfer Form (Quality Manual Attachment B):
    - 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 24hours 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.
- Check the specimen label details match those on the Specimen Transport Form.
- 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.
- The specimen register should be completed to link the specimen details with the accession number.
- 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

5.1.3.3. Timing of Sample Receipt

- The laboratory must process all specimens as soon as possible **but no later than 48 hours after the specimen is collected.**
- Logistical issues related to the personnel shift or appropriate arrangements for specimens shipping must be pre-arranged to meet this timeframe.
- If laboratories are closed at the weekends or for public holidays short term storage 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,
  - the samples are analysed within 48 hours of sample collection.

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

5.1.3.4. Handling of Specimen Receipt Issues

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

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.

Unlabelled Specimens

Unlabelled specimens must NOT be processed unless the labelling information can be accurately provided by the clinic. If the specimens are not labelled at the time of collection, 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 in the refrigerator as described above. The required labelling information should be provided ideally within 24 hours after collection or at the very latest within 48 hours. The specimen is NOT to be processed until the labelling has been satisfactorily corrected/completed. In case the information cannot be obtained within the appropriate time period, new sample(s) will be requested. Receipt of incompletely labelled specimens must be noted in Continuous Quality Improvement Form (Quality Manual Attachment N).

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 before the 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. However, corrective actions must be taken, and documented on the Continuous Quality Improvement Form (Quality Manual Attachment N) to prevent the problem reoccurring.

Small volume samples

Every effort should be made by the site clinical team to ensure a good quality sputum sample of sufficient volume (>2mL) 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) Time To Positivity (TTP) if required.

4.1.4 Forms

Quality Manual Attachment B – Specimen Transfer Form - Sputum
Quality Manual Attachment N - Continuous Quality Improvement Form

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

5.2.1. Purpose

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

Table 4: Storage of Microbiology Samples

Sample	Prepared By	Stored By	Storage Period	Storage Method
M.tb isolates from: All positive cultures.	Local/Regional Laboratory	Local/Regional Laboratory	Until trial closure	L-J slopes and/or -80°C in 50% glycerol
M.tb DNA and isolates after mycobacteriology characterisation testing	UCL Central Mycobacteriology Laboratory	UCL Central Mycobacteriology Laboratory	A maximum of 5 years after trial closure	Isolates at -80°C in 50% glycerol DNA at -20°C
M.tb DNA after *pncA* pyrazinamide resistance testing	CCTR *pncA* Laboratory	CCTR *pncA* Laboratory	A maximum of 5 years after trial closure	DNA at -80°C

The purpose of this SOP is to describe the methodologies for preparation of these samples.

5.2.2. Procedure

5.2.2.1. DNA Extracts

Introduction

Used by the **local/regional mycobacteriology laboratory** to prepare DNA extracts for shipment to either the UCL central mycobacteriology laboratory or CCTR *pncA* Laboratory.

Note: Refer to courier instructions for shipping details. DNA (either extracted from culture used for MIRU typing or GenoLysed sputum used for *pncA* sequencing) is non-hazardous (not infectious) and should be shipped in below room temperature conditions. Temperature monitoring during transit is not required. Samples can be shipped in an insulated container with wet ice or gel packs that had been kept at -20°C for +36hrs prior to packing.

DNA extracts for MIRU typing

DNA will be extracted, as detailed in SOP 9, from the baseline isolates (it is acceptable to use screening to week 4 isolates if the baseline culture is contaminated or negative) and from the positive isolates of patients suspected of failure or relapse after treatment (positive cultures at or after week 17 (4 month treatment arm)/week 26 (6 month treatment arm)) and any new positives isolates thereafter.

The extracted DNA will be divided into two tubes; one will be stored at the local/regional mycobacteriology laboratory at -20°C or colder and used as a backup and the second will be shipped to the UCL central mycobacteriology laboratory for MIRU typing. A logbook must be kept of all DNA in storage

Before storing the DNA extracts they must be quantified (using Nanodrop or agarose gel) in order to provide the UCL central mycobacteriology laboratory (or the lab performing molecular investigations in the future) with an estimation of the DNA available for each specimen.

The aliquots of extracted DNA should be sent to the UCL central mycobacteriology laboratory in below room

temperature conditions (wet ice or colder) on a 6 weekly basis, except when there are less than 20 vials, in which case send whenever there are 20 or more vials ready or as agreed with the UCL laboratory team. **Any suspected relapse strains should be DNA extracted and sent to UCL immediately**. Quality Manual Attachment P should be completed indicating the estimated concentration and volume of DNA in each vial (a total exceeding 1 µg is required). A copy of this form should then be sent with the vials to UCL.

DNA extracts for *pncA* pyrazinamide resistance testing

The GenoLyse extracted DNA used for the Hain assays (SOP 5) will also be used for the *pncA* pyrazinamide resistance testing. Once the GenoLyse extraction is complete, it is crucial that the top layer of the pure extracted DNA is immediately removed from the pellet containing the debris and aliquotted into a sterile 1.5 mL microcentrifuge tubes with an O-rings.

For DS patients the aliquot will be stored at -20°C and shipped to CCTR *pncA* Laboratory on a monthly basis (see Figure 3). For MDR patients (Rifampicin resistant by MTBDRplus/GeneXpert and Fluoroquinolone sensitive by MTBDRsl) the aliquot will be stored at 4°C and shipped to the CCTR *pncA* Laboratory immediately. All available aliquots from DS patients will also be shipped at this time.

The Quality Manual Attachment S will be completed for each shipment of samples. The top section of the transport form, listing the samples being shipped, will be scanned and emailed to the CCTR *pncA* Laboratory (pnca@task.org.za) in advance of the shipment. A copy of the entire form will be sent with the shipment.

Once *pncA* pyrazinamide resistance testing is complete the CCTR *pncA* Laboratory will store the GenoLyse extracted DNA at -80°C for a maximum of 5 years after trial closure for potential further work to validate new assay tools.

5.2.2.2. Positive Cultures

Introduction

Used by the **local/regional mycobacteriology laboratory** to prepare *M.tb* isolates for shipment to the central mycobacteriology laboratory.

Used by the **local/regional mycobacteriology laboratory and UCL central mycobacteriology laboratory** for storage of all *M.tb* isolates.

LJ slopes for shipment to UCL Microbiology Laboratory

The **local/regional mycobacteriology laboratory** will inoculate an LJ slope (SOP8) from all baseline isolates (it is acceptable to use screening to week 4 isolates if the baseline culture is contaminated or negative) and from the positive isolates of patients suspected of failure or relapse after treatment (positive cultures at or after week 17 (4 month treatment arm)/week 26 (6 month treatment arm)) and any new positives isolates thereafter. These will be incubated at 37°C until good growth is observed (2-4 weeks).

LJ slopes will be shipped to the UCL central mycobacteriology laboratory on a 6 weekly basis. LJ slopes should be sealed with parafilm, wrapped in absorbent issue in bubble wrap, placed securely inside sealable plastic hazard container, inside sealed cardboard hazard box. Packaging should be 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 (Quality Manual Attachment Q) and send a copy with the shipment. The site should keep a copy for their records. The UCL central mycobacteriology laboratory should be informed of a pending shipment (shipment date and airway bill number).

Storage of positive MGIT cultures

The **local/regional mycobacteriology laboratory** will store all isolates for the duration of the trial. The **UCL central mycobacteriology laboratory** will store all isolates after mycobacteriology characterisation testing is complete.

Equipment/Reagents

- Biological Safety Cabinet
- Discard bucket containing appropriate liquid disinfectant (specified in local Health and Safety documentation)
- Cryovial (with rubber o-ring seal), and appropriate storage box
- Sterile pipette and aerosol resistant tips
- PBS/7H9
- Glycerol
- LJ slope
- Pipette and aerosol resistant tips
- Rack for LJ slopes

Two samples should be stored for each isolate. At least one sample should be stored in 50% glycerol at -70°C to -80°C. If only one sample is stored in 50% glycerol, another must be stored on an L-J slope. If two frozen samples are stored, they should be in separate freezers if possible. If not, they should be in separate sections of the freezer.

A logbook must be kept of all isolates in storage.

Storage on LJ slope

To inoculate an LJ slope take 100 - 200µl of the positive MGIT pellet and pipette onto the slope. Securely fasten and label with both the patient number and the lab accession label. 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 subcultured 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- 2 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 and the lab accession label (also handwrite this number in permanent marker in case sticker is removed during freezing). Place in an appropriate storage box and freeze at –70°C to -80°C.

5.2.3. Forms

Quality Manual Attachment P – DNA Extraction and Shipment to UCL
Quality Manual Attachment R – Isolate Shipment to UCL
Quality Manual attachment S - DNA Shipment to CCTR for *pncA* Sequencing

5.3. SOP 3: Sputum Decontamination

5.3.1. Purpose

Sputum processing has two major functions: sputum digestion (liquefaction) of organic debris in the specimen and decontamination of bacteria other than mycobacteria. Although there are several techniques available, none are ideal, i.e., none of them will selectively destroy only contaminating flora and achieve complete liquefaction of the specimen. A reasonable compromise is to destroy as much of the contaminating bacteria as possible while harming as few mycobacteria as possible.

At screening two spot sputum samples are collected. The first spot sample collected will be decontaminated and used for the assays detailed in Figure 2. The second sample is collected as a backup in case the first sample is sputum smear negative or indeterminate (<1+) or the first sample fails the MTBDRplus/GeneXpert or MTBDRsl. If the second sample is not needed it will be discarded. At all other visits an early morning sputum and spot sputum sample are collected. Both samples will be decontaminated and cultured in MGIT.

5.3.2. Principle

The decontamination process is carried out using N-Acetyl-L-Cysteine (NALC) – Sodium Hydroxide (NALC-NaOH), (Equivalent commercially available reagents, e.g. Mycoprep or Alpha Tec NAC-PAC, can also be used as approved by the UCL laboratory team). NALC, a mucolytic agent, is used for rapid digestion, enabling sodium hydroxide (NaOH), the decontaminating agent, to be used at a lower final concentration (in sputum) of 1% – 1.5% than that required in the absence of NALC. 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). **This must be recorded as an additional comment in Quality Manual Attachment F or in the approved laboratory source documentation.**

5.3.3. Procedure

Equipment/Reagents

- 2.9% sodium citrate
- 4% NaOH
- 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)
- Disinfectant with activity against Mycobacteria (specified in local Health and Safety guidelines)
- Pipette and aerosol resistant tips
- 3mL pasteur pipette
- New microscope slides, frosted one side and one end, clean and dry
- NALC powder
- Paper towel soaked in appropriate disinfectant, in case of spills
- Pencil or grease pen for labelling slides
- Permanent marker
- Phosphate buffered saline (pH 6.8)
- Plastic bijoux

- Refrigerated centrifuge with sealed buckets and inserts suitable for 50 mL tubes
- Slide warmer set at 65 to 75°C
- 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 appropriate liquid 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 (details related to the logistics for specimen transportation should pre-arranged so that the samples are processed within the specific timeframe). Samples should be refrigerated if they are not processed within 30 min of receipt in the laboratory (time and fridge identifier should be recorded on Quality Manual Attachment B).
2. The patient data and laboratory accession numbers on Quality Manual Attachment B must then be double-checked. Laboratory accession labels should have been attached to the specimen container and the accompanying laboratory source documentation on receipt of the sample.
    a. In addition attach laboratory accession labels to:
        i. 50 ml centrifuge tube for NaOH/NALC decontamination process,
        ii. plastic bijoux for storage of decontaminated specimen

       Accession labels will also need to be attached to the MGIT tube used to culture the specimen.
    b. 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
    c. 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 decontamination mixture (NaOH/NALC/sodium citrate

NOTE: **UCL Laboratory Team must agree before any change is made to the concentration of the decontamination solution**

1. Add 500 mL 4% NaOH to 10g 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 500mL 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 200mL 4% NaOH to 4g 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 the UCL laboratory team (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 150mL MycoPrep).

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 the 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. Complete Quality Manual Attachment F Form with details of the decontamination solution reagents, the date and time the samples are processed and list the samples that are being processed in the batch. **A batch consists of no more than 7 patient specimens in total.**
4. Include a negative control with each batch of specimens (maximum total of 8 tubes per batch, see 'Quality Control' section below).
5. Work methodically with the tubes on one side and discard buckets close to the specimens, to avoid spillages and/or confusion of samples. Always keep the tubes in the same order as listed on Quality Manual Attachment F.
6. Ensure that tubes, bottles etc. that are removed from the safety cabinet for incubation are free from any droplets/potential contaminants (tubes should be wiped with the paper towel soaked in appropriate disinfectant if there are droplets on tubes).
7. Transfer specimen into a 50 mL centrifuge tube with a screw cap. Make a note of the volume on the approved laboratory source documentation.
8. Immediately add the NaOH-NALC sodium citrate solution in a volume equal to the quantity of specimen. Tighten the cap.
9. Start the timer.
10. Vortex for 15-30 seconds. Invert the tube so all contents are exposed to NaOH-NALC solution
11. Repeat steps 7, 8 and 10 for the subsequent specimens at 30 seconds or 1 min intervals. Record the start time for the first and last sample and the interval time on Quality Manual Attachment F.
12. It is important to mix well during the decontamination period to expose all the sputum to the digestion solution.
13. 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.
14. 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 that each specimen is **only** exposed to decontamination solution for 20 minutes. Record the stop time for the first and last sample on Quality Manual Attachment F to document the exposure time. It is essential buffer is added to each specimen within 20 minutes of adding the decontamination solution since mycobacteria will be killed off if exposed to NaOH beyond the stipulated time.
15. Transfer tubes in a 50ml tube rack to the centrifuge.
16. 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, and temperature should be recorded on Quality Manual Attachment F before use.
17. 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).
18. 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.
19. 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 Healthy 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*.

20. Add a small quantity (1 to 2 mL) of phosphate buffered saline (pH 6.8) to the sediment using a sterile pipette/3mL pasteur pipette and resuspend 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) and for inoculation of MGIT tube.
21. Store any leftover sediment at 4°C, for 10 days until it is confirmed the inoculated media are not contaminated.
22. 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. This repeat decontamination must be noted on the laboratory source documentation (Quality Manual Attachment F and the corresponding MGIT worksheet).

5.3.4. 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 *M. tuberculosis* from one specimen to another. The negative control must be treated the same as the patient samples. The negative control is included in microscopy and MGIT culture. Details of this should be recorded in Quality Manual Attachment F. If there is only 1 specimen in the batch a negative control is not required.

If contamination is present in the control tube (identified either on ZN smear or the blood agar plate that is inoculated from the positive MGIT culture), the results of specimens done in the same batch are checked to determine whether there was an influence from the contamination. If *M. tuberculosis* is present in the negative control tube, the results of specimens done in the same batch are checked to determine whether false positive culture are present, which might indicate carry over from one specimen to another.

The UCL laboratory team must agree to any changes to the concentrations of the decontamination solutions prior to implementation.

5.3.5. Forms

Quality Manual Attachment B – Specimen Transfer Form - Sputum
Quality Manual Attachment F - Processing and Decontaminating Sputum Samples
Quality Manual Attachment C - Containment Level 3 Laboratory Checklist
Quality Manual Attachment D - Equipment Temperature Log Form

5.3.6. 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 Laboratory unless otherwise specified and agreed with the UCL laboratory team due to local availabilities and regulations for Health and Safety. If BSC class I is not available then it must be a class 2A BSC and a negative pressure laboratory. The parameters regarding the CL3 Laboratory must be recorded in the 'Containment Level 3 Laboratory Daily Checklist Form (Quality Manual Attachment C). Also all equipment temperature should be recorded in Quality Manual Attachment D.
- Ensure that reagent containers do not come in contact with the neck of the specimen containers to reduce

the risk of cross-contamination.

- Do not attempt to work with more than 8 specimens (including the negative control) at one time.
- When working with multiple specimens, remove only the caps from the tubes of the same specimen (i.e. same laboratory accession number) at one time, so that caps are not mixed up or specimens cross-contaminated.
- The NaOH decontamination is harmful to mycobacteria so extending the indicated contact time will kill an increasing proportion of tubercle bacilli in the specimen. Thus, it is essential that the time of contact is strictly limited to 20 minutes
- NALC loses activity rapidly in solution, so it MUST be made fresh daily.
- NALC only liquefies the specimen and has no decontamination properties.
- 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.
- It is not necessary to neutralize the processed specimen, especially with the NaOH-NALC method
- With NaOH-NALC digestion, do not agitate the tube vigorously. Extensive aeration causes oxidation of NALC and makes it ineffective.
- Sputum samples and reagents for the digestion/decontamination procedure should be brought to room temperature before processing. Lower temperatures reduce the digestion decontamination process of NaOH-NALC.
- The standard final NaOH concentration is 1%. A range of 1% – 1.5% is tolerated. The concentration should only be changed to address unacceptable contamination rates and must be discussed with the UCL laboratory team prior to implementation.
- Mycobacteria are difficult to pellet by centrifugation as they are hydrophobic. A relative centrifugal force of at least 3,000 g (NOT 3000 rpm) is required to sediment mycobacteria. Lower centrifugation speeds (g-force) will not sediment mycobacteria very well and some bacteria would be lost during decanting the supernatant; this will affect the positivity rate.
- Always use a refrigerated and calibrated centrifuge and record the temperature in the Equipment Temperature Log Form – Quality Manual Attachment D). Temperature build up during centrifugation increases the killing effect on mycobacteria and will adversely affect the positivity rate and time-to-positivity in cultures.

5.4. SOP 4: Ziehl-Neelsen (Z-N) Sputum Smear Microscopy

5.4.1. Purpose

The purpose of staining is to detect acid-fast bacilli by microscopic examination of clinical specimens and cultures. Both living and dead (viable and non-viable) bacilli will stain. A semi-quantitative system is used to report the number of acid-fast bacilli observed in stained smears from clinical specimens.
Z-N sputum smear microscopy of screening samples and cultures allows:
- the identification of Acid Fast Bacilli (AFB), thereby confirming the presence of mycobacteria,
- IUATLD scale recording (screening samples only).

Both of these are required for patient eligibility.

5.4.2. Principle

The property of acid-fastness is due to the presence of mycolic acids in the cell wall of mycobacteria. Primary staining using fuchsin for Z-N staining binds to mycolic acids in the cell-wall. Subsequent decolourization using acid alcohol does not release the primary stain from the cell wall mycolic acids so the mycobacteria retain the purple/blue stain colour. This is known as acid-fastness. A counter stain is added in order to obtain a better contrast and create a background to simplify the focus during examination.

5.4.3. Procedure

Introduction

Z-N smear staining for AFB identification and reporting is performed:
- on screening sputum specimens received only. If the results is negative, indeterminate or <1+, the test may be repeated once on a freshly collected sputum. The result of this would be considered final.
- as part of the MGIT Liquid Culture process (SOP 8) to confirm a positive *M.tb* MGIT result.
- on negative decontamination controls (SOP 3)

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, and at a maximum within 48 hours of sample receipt. Each time a batch of patient smears is carried out, a positive QC smear using *M.tb* (H37Rv) must be stained alongside the samples to ensure the quality that the preparation of the slides is being carried out correctly.

Each new batch of staining reagents must be QC tested before use (see Quality Control section below).

Equipment/Reagents

- Aerosol Resistant Tips
- Biological Safety Cabinet (category CL3 laboratory before heat-fixation of the slide)
- Cover slip
- Disinfectant with activity against mycobacteria (specified in the local health and safety guidelines)
- Distilled water (chlorine free)
- Hot plate (or slide warmer)
- Light microscope, immersion oil, lens paper and lens cleaning solution (70% ethanol)
- Microscope slides, frosted at one end, new and unscratched
- Mounting fluid
- Positive control slide used with each batch (*M. tuberculosis* H37Rv)
- Paper towel soaked in appropriate disinfectant
- Pasteur pipette (pastette)

- Pencil for labelling slide
- Slide drying rack
- Staining rack
- Staining sink
- Wash bottle with distilled water
- Waste containers
- Ziehl-Neelsen stain (carbol fuschin, 3% acid alcohol, malachite green or methylene blue)

Process

Step One: Preparation of Smear

The procedures of 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) Laboratory. Prior to heat-fixation, the slides must remain in the BSC inside the CL3 Laboratory.

Slides used for acid-fast staining should be dry, clean, new and unscratched.

1. Label the frosted end of the 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 30µL of well-mixed resuspended pellet from the decontaminated sputum specimen onto the slide, using a pipette with sterile aerosol resistant tips (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.

Step Two: Ziehl-Neelsen Staining

Once heat-fixed, smears can be stained outside the BSC in the CL3 laboratory and can be examined by microscopy in either the Containment Level 2 (CL2) or CL3 Laboratory once it is dry. Heat-fixing does not kill mycobacteria, so be careful when handling smears.

1. Place the slides on the staining rack and flood with carbol fuchsin.
2. Heat the slide to steaming with a flame, then let stand for 5 minutes.
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 9 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 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!** Once air dry, apply a drop of mounting fluid and a cover slip.

Step Three: Microscopic examination and reading of Ziehl-Neelsen Stained Smear

Examine the Ziehl-Neelsen smears) with the 100x oil objective.

1. Examine the smears 100 fields using a regular pattern, such as the one shown in Figure 6. Start with the positive QC slide. If the QC slide is negative, do not report smear results obtained. Report and resolve the problem.

Figure 6: Scanning scheme for smear examination.

2. Record both the average number of AFBs and assign the corresponding grading as shown in Table 5.

Table 5: Grading system for AFB smears - WHO/IUATLD.

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	+++

Results from the examination of AFB smears are reported according to international standard criteria so they can be compared across laboratories and reported in standard units relevant to patient care. This trial uses the WHO/IUATLD guidelines.

Acceptable results are as follow:

- Positive smear: categorised using the semi-quantitative WHO/IUATLD scaling system above.

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 *Mycobacterium* 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.

3. Discard slides into a covered sharps bin inside the BSC in the all CL3 laboratory.

5.4.4. Quality Control

The following QC is required:
- Each new shipment or lot number of staining reagents (Carbol Fuschin, Malachite Green/Methylene Blue or 3% Acid Alcohol) must be QC tested before use using a positive QC smear using *M.tb* (H37Rv) strain and a negative QC smear containing E. coli. The reagent and QC test details results are to be recorded on the Quality Manual Attachment Ei which will link reagents used with specimens processed. 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 positive and negative controls. If the repeat test fails do not use the reagents and contact the supplier. Any quality control failure and subsequent actions should be recorded on the Continuous Quality Improvement Form (Quality manual attachment N).
- Each time a batch of patient smears is carried out a negative control (decontamination mixture only) and a positive QC smear using *M.tb* (H37Rv) strain to check that each stage of the procedure is working correctly. This must be recorded on Quality Manual Attachments F and H respectively. 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.

For every ten screening slide examined:
1. A review by a second person (i.e. Delegated Laboratory Staff, DLS) is required and the results recorded independently.
2. The results for the IUATLD/WHO scaling system should be the same for both counts. If the results do not match:
   - inform the Laboratory Manager.
   - the counts are to be repeated and confirmed by a third person. The two equivalent accounts from the three are the final result.
   - Re-train the Delegated Laboratory Staff.
   - Document the results and any action taken on Quality Manual Attachment N

5.4.5. Forms

Quality Manual Attachment Ei - Ziehl-Neelsen Stain Reagents
Quality Manual Attachment H – Daily AFB Microscopy
Quality Manual Attachment I - Microscopic Examination of Acid-Fast Smears
Quality Manual Attachment CQIF – Continuous Quality Improvement Form

5.5. SOP 5: Hain Genotype MTBDRplus and Genotype MTBDRsl

Reference: Hain Life Sciences, http://www.hain-lifescience.de/en/instructions-for-use.html.

5.5.1. Purpose

Used by the **local/regional mycobacteriology laboratory** as a rapid test for:
- R resistance
- FQ resistance
- Confirmation of presence of *M.tb.*

Note:
- If Z-N smear is negative or < 1+ (original and repeat), the participant is a screening failure. Do not perform Genotype MTBDR*plus* or Genotype MTBDR*sl.*
- If GeneXpert is used (SOP 6), do not perform Genotype MTBDR*plus.*

5.5.2. Principle

The GenoType MTBDR*plus* and GenoType MTBDR*sl* tests are based on a DNA-STRIP technology that allows molecular identification of *M.tb* complex and resistance to Rifampicin and/or Isoniazid (MTBDR*plus*) and to Fluoroquinolones, Aminoglycosides and Ethambutol (MTBDR*sl*).

Although the assays are limited to detection of known mutations, the high concordance rate with conventional methods and the rapid time to results make the MTBDRplus and MTBDRsl assays useful tests for the diagnosis and management of multi-drug resistant tuberculosis.

5.5.3. Procedure

Equipment /Reagents
- Absorbent paper
- Calibrated thermometer
- Centrifuge
- Graduated cylinder
- Biological safety cabinet (BSC)
- Micropipettors, 10-1000 µL, 200-1000 µL
- Micropipette tips (with filter plug)
- PCR tubes (DNase and RNase free)
- Shaking water bath or TwinCubator
- Sterile water (molecular biology grade)
- Thermostable DNA polymerase with buffer (recommendation: hot start enzyme; extension rate: 2- 4 kb/min at 72°C, half-life: 10 min at 97°C, 60 min at 94°C, amplification efficiency: >10^5 fold)
- Timer
- Tweezers
- Vortex
- Waste receptacles (including splash proof receptacle for liquids containing appropriate liquid disinfectant)
- Water bath or heating block (set to 95°C)
- Thermal Cycler (heating rate: 3°C/sec, cooling rate: 2°C/sec, precision: +/- 0.2°C)

DNA Amplification mix (not provided in kit or mentioned above):
- 10x polymerase incubation buffer
- $MgCl_2$ solution*
- Thermostable DNA Taq polymerase

*Depending on the enzyme/buffer system used, the optimal $MgCl_2$ concentration may vary between 1.5 and 2.5mM. Please note that some incubation buffers already contain $MgCl_2$.

Kit Contents:
- Primer Nucleotide Mix (PNM) contains specific primers, nucleotides, <1% Dimethyl Sulfoxide, dye
- Membrane strips coated with specific probes (STRIPS)
- Denaturation Solution (DEN) ***ready to use*** contains <2% NaOH, dye
- Hybridization Buffer (HYB) ***ready to use*** contains 8 – 10% anionic tenside, dye
- Stringent Wash Solution (STR) ***ready to use*** contains >25% of a quaternary ammonium compound, <1% anionic tenside, dye
- Rinse Solution(RIN) ***ready to use*** contains buffer, <1% NaCl, <1% anionic tenside
- Conjugate Concentrate (CON-C) ***concentrate*** contains streptavidin-conjugated alkaline phosphatise, dye
- Conjugate Buffer (CON-D) contains buffer, 1% blocking reagent, <1% NaCl
- Substrate Concentrate (SUB-C) ***concentrate*** contains dimethyl sulfoxide, substrate solution
- Substrate Buffer (SUB-D) contains buffer, <1% $MgCl_2$, <1% NaCl
- Tray, evaluation sheet
- Manual, template

NB: It should be noted that the kit contents for the MTBDR*plus* and *MTB*DR*sl* differ slightly; therefore, there should be no sharing of reagents between these two kits.

Genolyse extraction:
- 1.5ml screw cap tube
- Lysis Buffer (A-LYS)
- Neutralisation Buffer (A-NB)

Storage and Precautions

1. Primer/Nucleotide Mix (PNM):
   Upon arrival, store at 2 - 8 °C away from any potential sources of contaminating DNA. If storage for longer than 4 weeks is required, store at -20°C and aliquot the PNMs to avoid repeated freezing and thawing.
2. Other kit components:
   Store at 2 – 8 °C.
3. Do not use any of the reagents beyond their expiry date.
4. Sputum samples from patients must always be handled under suitable safety conditions in a Containment Level 3 facility and within a Biological Safety Cabinet.
5. Refer to manufacturer's instruction for precaution in handling reagents (e.g. denaturation Solution (DEN) contains <2% NaOH and Substrate Concentrate (SUB-C) contains dimethyl sulfoxide (DMSO). Both are irritating to eyes and skin).

Process

The procedure for both the GenoType MTBDR*plus* and GenoType MTBDR*sl* molecular assays are divided into three steps:

Step One: DNA isolation.
Step Two: Multiplex amplification with biotinylated primers.
Step Three: Reverse hybridization.

Step One: DNA isolation from decontaminated sputum (screening samples) and from positive cultures (control strain *M.tb* H37Rv)

GenoLyse should be used to manually extract bacterial DNA before amplification and diagnostic tests are

performed. It is essential that all reagents and materials used for DNA isolation and amplification set-up are free from DNAases.

If using bacteria grown on liquid media:

1. Transfer 500µl of the decontaminated sputum into a labelled 1.5ml screw cap tube; when using bacteria grown in liquid media, transfer 1ml.
2. Centrifuge for 15 minutes at 10,000 x g in a standard table top centrifuge with aerosol tight rotor.
3. Discard supernatant and resuspend pellet in 100µl Lysis Buffer (A-LYS) by vortexing
4. Incubate for 5 minutes at 95°C in a water bath. Briefly spin down.
5. Add 100µl Neutralisation Buffer (A-NB) and vortex sample for 5 seconds.

When using bacteria grown on solid media:

1. Collect bacteria with an inoculation loop and suspend in 100µl Lysis Buffer (A-LYS), vortex, and continue to steps 4-5 above.
2. Spin down for 5 minutes at full speed in a standard table top centrifuge with an aerosol tight rotor and directly use 5µl of the supernatant for PCR. The remainder of the sample will be sent to the *pncA* laboratory for PZA resistance testing. It is crucial that the top layer of the pure extracted DNA is immediately removed from the pellet containing the debris and aliquotted into a sterile 1.5 mL microcentrifuge tubes with an O-rings.

Step Two: Amplification

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

1. Prepare the amplification mix (45 µL) in a DNA-free room. **The DNA sample should be added in a separated area.**
    a. Determine the number of samples to be amplified (number of samples to be analysed plus positive *(M. tuberculosis* H37Rv) and negative (sterile, molecular biology grade water) controls).
    b. Prepare a master mix containing all reagents **except for DNA** per Table 4: and mix well, (do not vortex).

Table 4: Hain Genotype Amplification Mix

Reagent	**Volume per tube mix**
Primer Nucleotide Mix (PNM)	35 µL
10 x Polymerase incubation buffer	5 µL
$MgCl_2$ solution *	**X** µL (see below)
1-2 Unit(s) thermostable DNA polymerase (refer to manual)**	Do not consider this volume in total for tube
Molecular biology grade water	**Y** µL (to obtain a volume of 45 µL without DNA)
DNA solution	5 µL (contains 20-100 ng DNA)
Final volume per sample	**50** µL (not considering volume of DNA polymerase)

*Depending on the enzyme/buffer system used, optimal $MgCl_2$ concentration may vary between 1.5 and 2.5 mM. Please note some incubation buffers already contain $MgCl_2$.

**Qiagen Hot Start Taq is recommended. However alternative quality assured Taq polymerases may be used provided the assay has been validated with this enzyme and evidence of optimisation are available for review.

2. Aliquot 45 µL of the amplification mix into each of the amplification PCR tubes.
3. The DNA solution should be added to the PCR tubes in a separate area. A negative control sample contains 5 µl of distilled or PCR grade water instead of DNA solution.

Table 5: Hain Genotype Amplification profile

Step	Number of cycles[a]	Temperature	Time
1	1	95°C	15 min
2	10 (or 20 for culture)	95°C 58°C	30 sec 2 min
	30 (or 20 for culture)	95°C 53°C 70°C	25 sec 40 sec 40 sec
3	1 cycle	70°C	8 min

[a] The protocol for amplification for cultured samples is different (see number of cycles in brackets). When using certain Hot start Taq DNA polymerases, the number of cycles need to be increased (refer to the enzyme's manufacturer).

Depending on the cycler used, the PCR cycling settings might have to be modified (contact local distributor for instructions).

4. Amplification products can be stored between +4 to -20°C
5. For checking the amplification reaction, 5 µl of each samples might be directly applied to a 2% agarose gel without the addition of loading buffer. The amplimers have a length of approximately 63 bp (Amplification control), 115 bp (*M. tuberculosis* complex), 166 bp (*rpoB*), 120 bp (*katG*), 110bp (*inhA*), 123bp (gyrA), 263bp (*rrs*) and 138bp (*embB*) respectively.

Step Three: Hybridization

1. Pre-warm the shaking water bath/TwinCubator to 45°C (+/- 1°C).
2. Pre-warm the Hybridization Buffer (HYB) and Stringent Wash Solution (STR) to 37 – 45°C before use. The reagents must be free from precipitates (NOTE: solution CON-D is opaque). Mix if necessary.
3. Warm the remaining reagents with the exception of Conjugate Concentrate (CON-C) and Substrate Conjugate (SUB-C) to room temperature.
    a. Using a suitable tube, dilute the conjugates with their respective buffer in a proportion of 1:100. Each strip will need 10 µL concentrate added to 1 mL of its respective buffer.
        i. CON-C (orange) with CON-D (NOTE: CON-C must be diluted before each use)
        ii. SUB-C (yellow) with SUB-D (Diluted SUB-C is stable for 4 weeks if stored at room temperature and protected from light)
    b. Mix conjugate and buffers well and bring to room temperature.
4. Dispense 20 µL of Denaturation Solution (DEN, blue) into the corner of each well used.
5. Add to the solution 20 µL of amplified sample. Pipette to mix well and incubate at room temperature for 5 min. Meanwhile, take strips out of the tube using tweezers and mark them with a pencil underneath the coloured marker. Always wear gloves when handling strips.
6. Carefully add to each well 1 mL of pre-warmed Hybridization Buffer (HYB, green). Gently shake the tray until the solution has a homogenous colour. Take care not to spill solution into the neighbouring wells.
7. Place a strip into each well. The strips must be completely covered by the solution and the coated side (identifiable by the coloured marker near the lower end) must face upward. If the strips have turned over as they were immersed in solution, turn them back over using tweezers. Carefully clean tweezers after each use to avoid contamination. This also applies to all the following steps.
8. Place tray in shaking water bath/TwinCubator and incubate for 30 mins at 45°C. Adjust the shaking frequency of the water bath to achieve a constant and thorough mixing of the solution. To allow adequate heat transfer, the tray must be immersed into the water by at least 1/3 of its height.
9. Completely aspirate Hybridisation Buffer. (For example, use a Pasteur pipette connected to a vacuum pump).
10. Add 1 mL of Stringent Wash Solution (STR, red) to each strip and incubate for 15 mins at 45°C in shaking water

bath/TwinCubator.

Work at room temperature from this step forward:

11. Completely remove the STR. Pour out wash solution in a waste container and remove all remaining fluid by turning the tray upside down and gently striking it on an absorbent paper. This also applies to other wash steps.
12. Wash each strip once with 1 mL of Rinse Solution (RIN) for 1 min on shaking platform/ TwinCubator (pour out RIN after incubation).
13. Add 1 mL of diluted Conjugate (from Step 3 in Hybridization Stage above) to each strip and incubate for 30 mins on shaking platform/ TwinCubator.
14. Remove solution and wash each strip twice for 1 minute with 1 ml of Rinse Solution (RIN) and once for 1 minute with approx. 1 ml of distilled water (e.g. use wash bottle) on shaking platform/ TwinCubator (pour out solution each time). Make sure to remove any trace of water after the last wash.
15. Add 1 mL of diluted substrate (from Step 3 in Hybridization Stage above) to each strip and incubate away from the light and without shaking. Depending on the test conditions (e.g. room temperature) the substrate incubation time can vary between 3 and 20 minutes. Extended substrate incubation times can lead to increased background staining and might impair interpretation of the results.
16. Stop reaction by briefly rinsing twice with distilled water.
17. Remove strips from the tray using tweezers, and dry them between two layers of absorbent paper.

5.5.4. Evaluation and Interpretation of Results

An evaluation sheet is provided with each kit and can be photocopied for repeat use.
When using this evaluation sheet, paste the developed strips in the designated fields by aligning the bands CC and AC with the respective lines on the sheet.
Determine the resistance status and note down in the respective column. Each strip has a total of 27 reaction zones.
Note: Not all bands of a strip have to show the same signal strength. Any strips with questionable bands should be scanned and sent to the sponsor for clarification.
Band intensity should be equal to or greater than that of the AC band to be interpreted.

Conjugate Control (CC)
A line must develop in this zone, documenting the efficiency of conjugate binding and substrate reaction. This band should be present in all samples and both controls.

Amplification Control (AC)
When the test is performed correctly, a control amplimer generated during amplification will bind to the Amplification Control zone on the strip. A missing band therefore indicates mistakes during amplification set-up or the carry-over of amplification inhibitors with the isolated DNA. In case of a positive test result, the signal of the Amplification Control zone can be weak. In this case, however, the amplification reaction was performed correctly and the test does not have to be repeated.
This band should be present in all samples and both controls.

***M. tuberculosis* complex (TUB)**
This zone hybridizes, as known, with amplimers generated from all members of the *Mycobacterium tuberculosis* complex. If the TUB zone is negative, the tested bacterium does not belong to the *M. tuberculosis* complex and cannot be evaluated by this test system.
This band should be present in the positive control and any samples containing M. tuberculosis.

Locus Controls MTBDR*plus* (*rpoB, katG,* and *inhA*)
The Locus Control zones detect a gene region specific for the respective locus and must always stain positive. These bands should be present in the positive control and any samples containing M. tuberculosis.

Locus Controls MTBDR*sl* (*gyrA, rrs and embB*)
The Locus Control zones detect a gene region specific for the respective locus and must always stain positive. These bands should be present in the positive control and any samples containing M. tuberculosis.

Wild Type Probes
When all wild type probes of a gene stain positive, there is no detectable mutation within the examined regions. The strain tested is sensitive for the respective antibiotic.
The absence of a signal for at least one of the wild type probes indicates a resistance of the tested strain to the respective antibiotic.

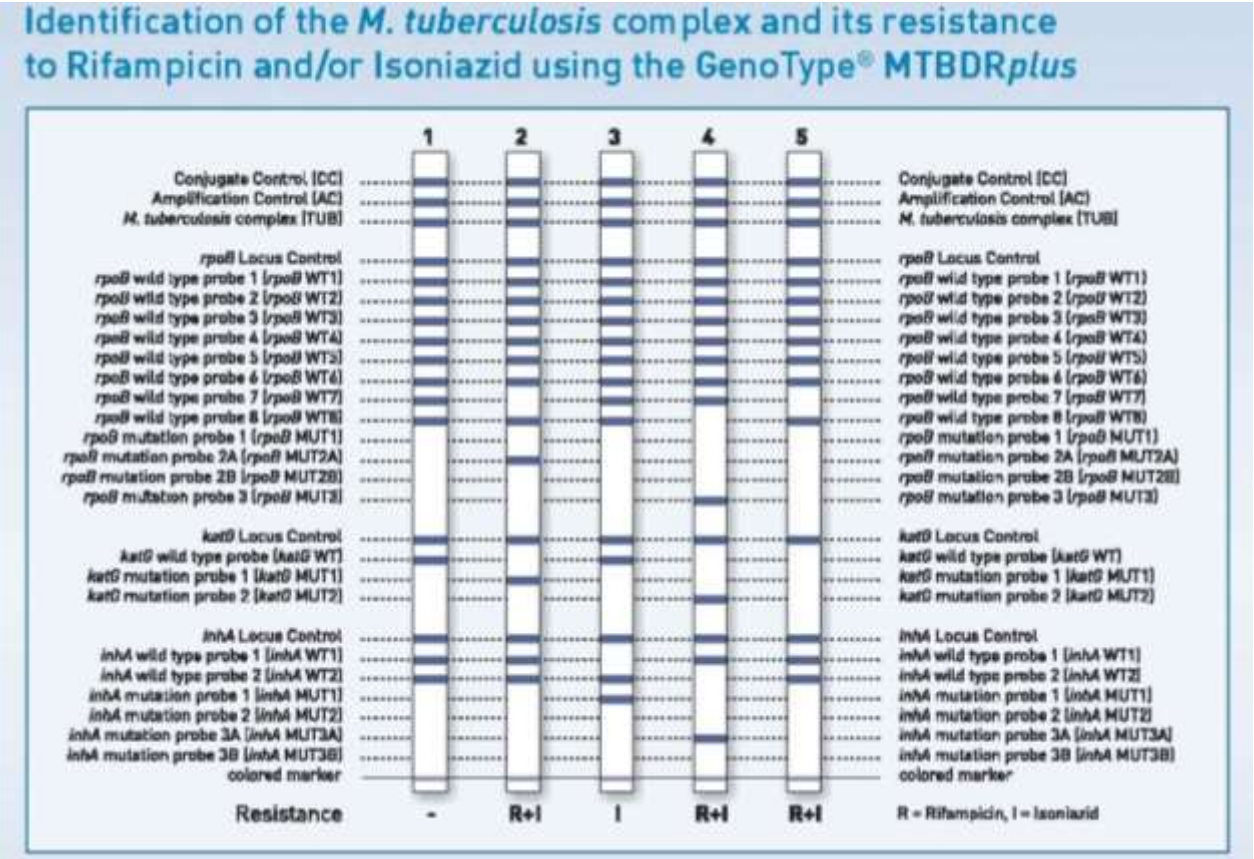
Mutation Probes
The mutation probes detect some of the most common resistance mediating mutations. Compared to other probes, positive signal of the mutation probes *rpoB* MUT2A and MUT2B may show a lower signal strength.
Only those bands whose intensities are about as strong as or stronger than that of the Amplification Control zone are to be considered.
Each pattern that deviates from the wild type pattern indicates resistance of the tested strain.

The banding pattern obtained with the *rpoB* probes allows conclusions to be drawn about rifampicin resistance of the strain tested, the *katG* banding pattern about a high level isoniazid resistance and the *inhA* banding pattern about a low level isoniazid resistance.
The banding pattern obtained with the gyrA probes allows conclusions to be drawn about fluoroquinolone resistance (e.g. moxifloxacin), the banding pattern obtained with rrs probes allows conclusions to be drawn about resistance to aminoglycosides (e.g. Kanamycin) and the banding pattern from the embB probes allows conclusions to be drawn about resistance to ethambutol.

Note the following special cases:
a. There is a possibility that the specimen tested contains a heterogeneous strain. If, at investigation, this strain has developed only a partial resistance, one of the mutation probes as well as the corresponding wild type probe may appear.
b. There is a possibility that the tested specimen contains more than one M. tuberculosis strain (due to mixed culture or contamination). If at least one of these strains harbours a mutation, one of the mutation probes as well as the corresponding wild type probe may appear.

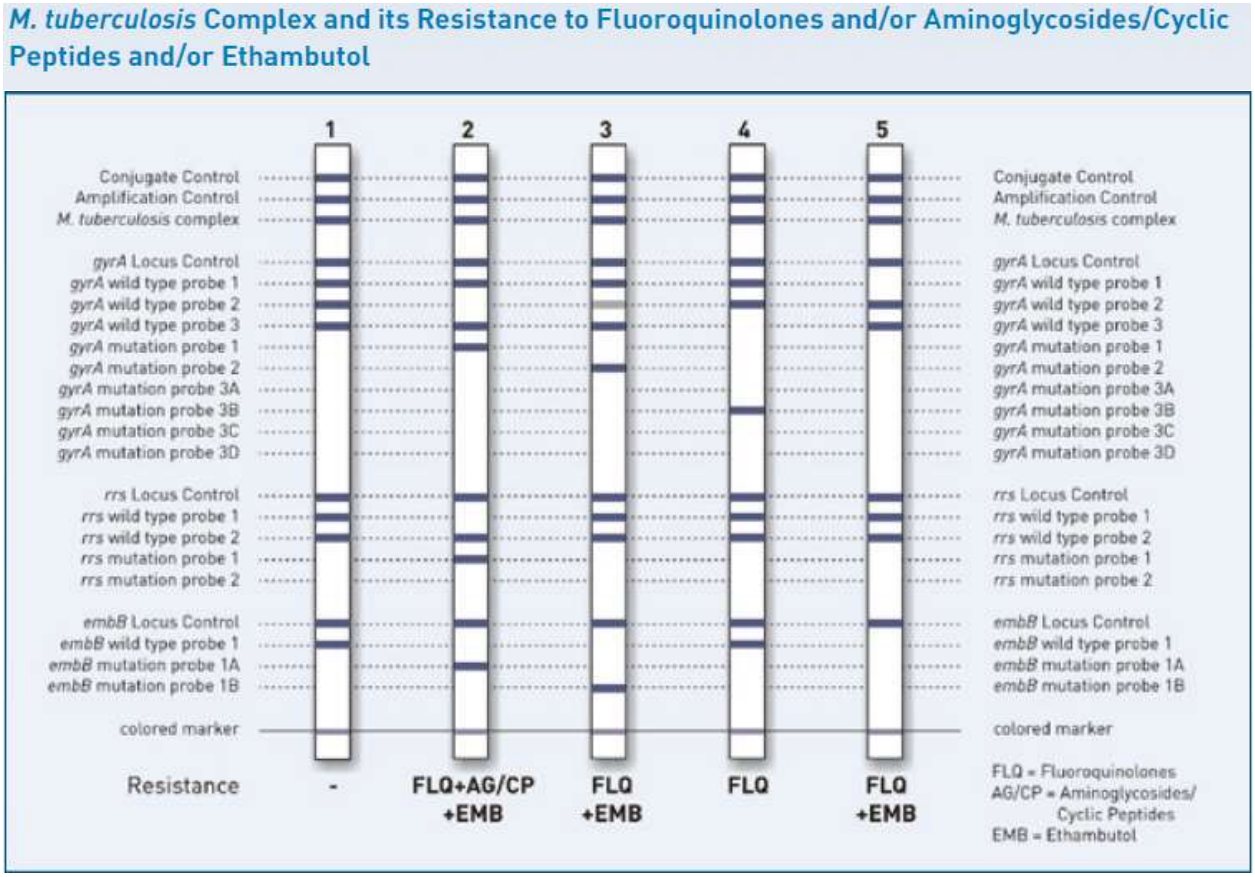
Indeterminate Results:
1. Both wild type and mutation probes present
2. Both wild type and mutation probes absent

Figure 7: Evaluation and interpretation of results: HAIN MTBDR*plus*



Courtesy of Hain Lifescience GmbH

Figure 8**:** Evaluation and interpretation of results: HAIN MTBDR*s*/



Courtesy of Hain Lifescience GmbH

5.5.5. Troubleshooting

Issue	Possible Cause
Overall weak or no signals (including Conjugate Control Zone)	- Room temperature too low or reagents not equilibrated to room temperature. - None or too little amount of CON-C and/or SUB-C mixes used.
Weak or no signals except for Conjugate Control zone	- Isolated DNA was not sufficiently amplified and an inadequate quantity or low quality DNA was used. o Check amplimer on a 2% agarose gel. If no amplimer is visible, repeat DNA isolation and amplification and repeat testing. If necessary, try a different DNA isolation method. - Strip incubation temperature too high.
No Homogenous Staining	- Strips were not completely immersed during incubation steps. - Tray was not shaken properly.

Issue	Possible Cause
High Background Colour	- CON-C and/or SUB-C used too concentrated. - Washing steps were not performed with the necessary care. - Wash solutions too cold.
Unexpected Results	- Wrong incubation temperature - Hybridization buffer and/or Stringent Wash Solution were not properly pre-warmed or mixed. - Isolated DNA and/or amplification agents were contaminated with other isolated and/or amplified DNA. If amplification agents were contaminated, the negative control sample will also show the respective banding pattern - Contamination of neighbouring wells by spillage during addition of Hybridization Buffer. - Depending on the amount of amplified DNA used and certain reaction conditions, a strong and fast colour may develop. In such cases, the substrate incubation should be discontinued as soon as the signals are clearly visible in order to prevent the development of cross-hybridizing bands. - No pure culture used as starting material or more than one mutation was present in the strain. - Silent mutation in probe region (See Limitations paragraph below and refer to the manufacturer's instructions for further details).

5.5.6. Limitations

It is possible that mutations in the probe region that do not cause an amino acid substitution (silent mutations) will still produce the absence of one of the wild type bands. A silent mutation in codon 514 of the *rpoB* gene leading to the absence of the rpoBWT3 band was observed in rare cases (Alonso M et al, 2011).
Refer to the manufacturer's instructions for additional details.

5.5.7. Forms

Quality Manual Attachment Li - GenoType MTBDRplus
- GenoType MTBDRplus (12 Evaluation Sheet – provided with the kit (photocopy the form)

Quality Manual Attachment Lii - GenoType MTBDR*sl*
- GenoType MTBDRsl 12 Evaluation Sheet – provided with the kit (photocopy the form)

5.5.8. Quality Control

In order to validate the correct performance of the test and the proper functioning of reagents, each MTBDR*plus* and MTBDR*sl* strip includes 5 control zones:
- Conjugate Control (CC) zone to check the binding of the conjugate on the strip and a correct chromogenic reaction.
- Amplification Control (AC) zone to check for a successful amplification reaction.
- MTBDR*plus*: three Locus Control zones (*rpoB, katG* and *inhA*) checking the optimal sensitivity of the reaction for each of the tested gene *loci*.

- MTBDR*sl*: three locus control zones (gyrA, rrs and embB) checking the optimal sensitivity of the reaction for each of the tested gene loci.

Each time the test is performed a positive (sensitive *M.tb* H37RV strain) and negative (ddH_2O) control is used

Note:
Positive control (MTBDR*plus*) – AC/CC/TUB positive, all 3 locus control probes positive, all wild type probes positive, all mutation probes negative, overall sensitive to rifampicin and isoniazid.
Positive control (MTBDR*sl*) – AC/CC/TUB positive, all 3 locus control probes positive, all wild type probes positive, all mutation probes negative, overall sensitive to fluoroquinolones, aminoglycosides and ethambutol.
Negative control – AC/CC positive, all other probes negative.
Occasionally there may be non-specific banding patterns in the negative control. This is acceptable as long as the TUB band is absent.

5.5.9. Good Laboratory Practice When Performing Molecular Amplification Assays

5.5.9.1. 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.

5.5.9.2. General Considerations

a. **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 labcoats, gloves and handwashing 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.

5.5.9.3. Specimen Processing

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

a. **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.

b. **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.

c. **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.

d. **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.

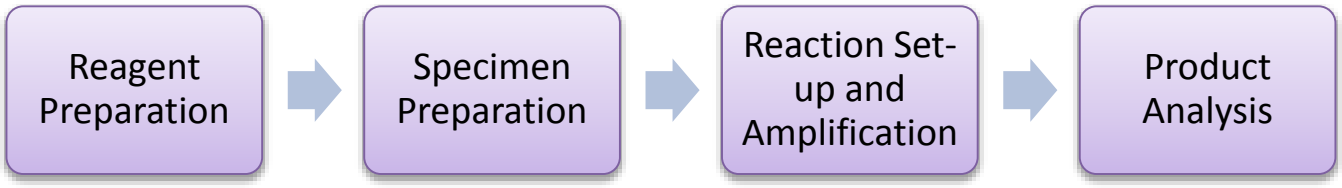
e. **The Amplification Room**

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.

f. **The Product Analysis Room**

This is the room in which post-PCR manipulations are performed eg 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 9: 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 carried out within defined areas of a larger laboratory and amplification and product analysis are in a second laboratory

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

5.6. SOP 6: GeneXpert *M.tb*/RIF (or Xpert *M.tb*/RIF) Test

5.6.1. Purpose

Used by the **local/regional mycobacteriology laboratory** as a rapid test for:
- R resistance
- Confirmation of presence of *M.tb.*

Note: If Genotype MTBDR*plus* is used (SOP 5), do not perform GeneXpert.
Used by the **central mycobacteriology laboratory** as a rapid test for confirmation of presence of *M.tb*
-If Hain MTBC is not used (SOP12), do not perform GeneXpert

5.6.2. Principle

This diagnostic test (also known as the Xpert *M.tb*/RIF) test is based on a semi-quantitative, nested real-time PCR for the detection of *M. tuberculosis* complex DNA in sputum samples and the detection of rifampicin (RIF) resistance associated mutations of the *rpoB* gene in samples from patients at risk for RIF resistance. The *M.tb*/RIF test is intended for use with specimens from untreated patients for whom there is clinical suspicion of tuberculosis, so that the results can be provided within 2 hours.

Basic step by step instructions are detailed below required to perform the test. 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 *M.tb*/RIF System Operator Manual

5.6.3. Equipment/Reagents

- Autoclave bags
- Barcode labels for cartridge identification
- GeneXpert Dx System equipped with $GX_{2.1}$ software (catalogue number varies by configuration):
    - 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 *M.tb*/RIF kit (CGX*M.tb*/RIF-10) [contains sufficient reagents to process 10 patient or quality-control specimens]

5.6.4. Process

<u>Step One: Preparing the Sputum sediments</u>

1. Using a sterile transfer pipette, transfer at least 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 *M.tb*/RIF Sample Reagent (SR) to the 0.5 mL of re-suspended sediment using a sterile transfer pipette and shake vigorously 10 – 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 – 20 times. Samples should be liquefied with no visible clumps of sputum. Particulate matter may exist that is not part of the sample.

<u>Step Two: Preparing the Cartridge</u>

1. Label each Xpert *M.tb*/RIF cartridge with the lab 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 *M.tb*/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 ºC should repeat testing be required.
5. Be sure to 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 $GX_{2.1}$ software AND the Xpert *M.tb*/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 *M.tb*/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.

5.6.5. Quality Control

Each test includes a Sample Processing Control (SPC) and probe check (PCC):

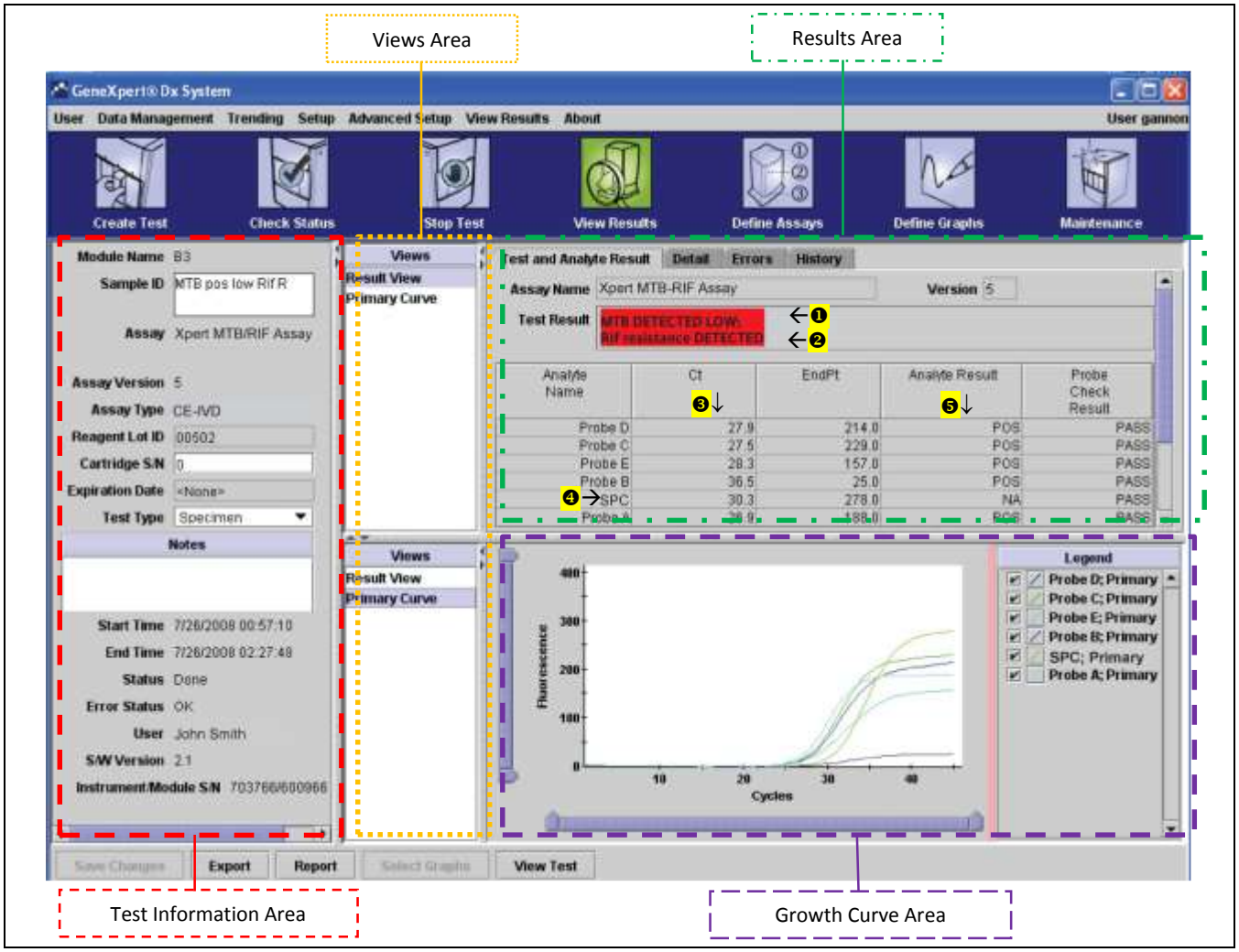
- Sample Processing Control (SPC):
  Ensures the sample was correctly processed. The SPC contains non-infectious spores in the form of a dry spore cake that is included in each cartridge to verify adequate processing of *M.tb*. The SPC verifies that lysis of *M.tb* has occurred if the organisms are present 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.

5.6.6. Interpretation, Recording and Reporting 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 10: GeneXpert View Results Window Example.

Figure 10: GeneXpert View Results Window Example



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 (❶ in Figure 10) will relate to whether *M.tb* is detected or not.
- The second result line (❷ in Figure 10) will relate to the Rifampicin resistance result will be recorded, if *M.tb* has been detected.

In the "Ct column" (❸ in Figure 10) 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 (❹ in Figure 10) read in the SPC row.
- Probe Check Control (PCC) Column (❺ in Figure 10) read via the Analyte Result Column.

M.tb Detected
M.tb target DNA is detected.

- *M.tb* Detected - The *M.tb* result will be displayed as High, Medium, Low or Very Low depending on the Ct value of the *M.tb* target present in the sample.
- Rif Resistance DETECTED, Rif Resistance NOT DETECTED, or Rif Resistance INDETERMINATE will be displayed only in *M.tb* DETECTED results and will be on a separate line from the *M.tb* DETECTED 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 *M.tb* 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 *M.tb* amplification may compete with this control.
- Probe Check–PASS; all probe check results pass.

M.tb Not Detected
M.tb target DNA is not detected, SPC meets acceptance criteria.
- *M.tb* NOT DETECTED—*M.tb* 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 *M.tb* cannot be determined, repeat test with extra specimen. SPC does not meet acceptance criteria, the sample was not properly processed, or PCR is inhibited.
- *M.tb* INVALID–Presence or absence of *M.tb* DNA cannot be determined.
- SPC–FAIL; *M.tb* target result is negative and the SPC Ct is not within valid range.
- Probe Check–PASS; all probe check results pass.

ERROR
- *M.tb*–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
- *M.tb*–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.

5.7. SOP 7: *pncA* molecular test

5.7.1. Purpose

Used by the ***pncA* Mycobacteriology laboratory** to test for pyrazinamide resistance at screening.

5.7.2. Principle

Molecular detection of resistance to PZA resistance is performed using the *pncA* molecular test.

5.7.3. Procedure

Samples are stored and shipped from the local laboratory to the *pncA* laboratory as GenoLysed left over Hain material.

4.7.3.1 Amplification of the *pncA* gene to investigate resistance to PZA

a. **Purpose**

To prepare the amplification master mix for the *pncA* gene in order to amplify the *pncA* gene in *M. tuberculosis*, from GenoLyse extracted bacterial DNA, after which detection of the *pncA* gene will be done to ensure that the amplification was successful prior to sequencing of the *pncA* gene for resistance determination to Pyrazinamide

b. **Procedure**

Due to the sensitivity of the polymerase chain reaction (PCR) it is required that different rooms are used for different procedures as the procedures in itself are incompatible.

c. **Master-Mix Preparation Area**

Equipment & Materials

1. Laminar flow cabinet fitted with a UV light.
2. Dedicated Automatic pipettes (1-10 µl, 2 - 20µl , 20 - 200µl)
3. *pncA* Forward and Reverse primers (Table 1)

Table 6: Gene, primers and target DNA sequences for genotypic Pyrazinamide drug susceptibility testing (DST).

Primer set number	Gene	Primer	Sequence 5’ to 3’	Tm	Fragment Length
1	*pncA*	JpncAs Forward JpncAs_Reverse	ggcgtcatggaccctata gtgaacaacccgacccag	60°C	738 bp

4. RNA and DNA free water.
5. HotStart Taq DNA polymerase (Qiagen).
6. PCR buffers.
7. dNTP stock solutions
8. Intercalating agent (Syto-9).
9. Appropriate filter tips for the pipettes.
10. 1.5 ml Eppendorf screw-cap tubes with O-ring.
11. 1.5 ml tube racks.
12. 0.2 ml PCR tubes.
13. PCR tube racks.
14. Disposable gloves (Small, Medium, Large).
15. Dedicated laboratory coat

Procedure

1. The master mix for a batch of 24 reactions will be prepared in a 1.5ml screw-cap tube with O-ring. The master mix reaction setup is shown in Table 7, and each reagent should be added into the 1.5ml screw-cap tube sequentially.

Table 7: *pncA* Master Mix Recipe

Reagent	**1X**	**24X**
10X Buffer (Qiagen)	2.5 µl	60 µl
dNTPs - 10mM (total), (Qiagen)	2 µl	48 µl
JpncAs Forward Primer (10mM)	1.875 µl	45 µl
JpncAs Reverse Primer (10mM)	1.875 µl	45 µl
Syto-9 (50 µM)	1 µl	24 µl
HotStarTaq DNA polymerase (Qiagen)	0.125 µl	3 µl
Nuclease free water (Qiagen)	13.125 µl	315 µl

2. Once all the components have been added, gently mix them by inversion (5 – 10 times).
3. Aliquot a final volume of 22.5µl into single 0.2 ml PCR tubes.
4. Store the aliquoted master mix at -20°C until required for use for up to one week only.
5. Decontaminate the area after the master mix has been prepared and stored until use.

d. **Template (DNA) Addition Area**

Equipment & Materials

1. 10 µl Automatic pipette (1 - 10 µl range).
2. Appropriate filter tips.
3. Work station fitted with UV light.
4. Disposable gloves (Small, Medium, Large).
5. Dedicated laboratory coat

Procedure

1. Using a new filter tip for each specimen in question, carefully add 2.5 µl crude DNA (prepared, according to TM001) to the corresponding PCR tube (22.5 µl). This will result in a final volume of 25 µl for the amplification procedure.
2. Add 2.5 µl of H37Rv DNA (GenoLyse® or GenoXtract® treated) for the positive control after the test specimens. This step is done to ensure that the extraction procedure was done correctly, as well as that the master mix preparation was correctly formulated.
3. Extraction Negative Control (ENC) – add 2.5 µl of water (in place of DNA) after the positive control. This step is done to detect GenoLyse® reagent contamination.
4. Master mix Negative control (MNC) – add 2.5 µl of water (in place of DNA) after the ENC. This step is done to detect PCR reagent contamination.

e. **Amplification Area**

Equipment & Materials

1. Thermocycler (Veritas).
2. Foil.

Procedure

1. Select the "CCTR *pncA*" thermal cycler setting on the Veriti Thermal Cycler.
2. Double check that the PCR cycle setup is as follow prior to amplification of the template DNA :

Amplification will be initiated by incubation at 95°C for 15 minutes, followed by 50 cycles at 94°C for 30 seconds, Tm = 60 °C (Table 1) for 30 seconds, and 72°C for 1 minute. After the last cycle, the samples will be incubated at 72°C for 10 minutes.

3. Once checked, initiate the PCR program by selecting the final volume of 25 µl and press start.
4. Once completed, remove the PCR tubes and cover them with foil.
5. The successful amplification of the *pncA* gene will be detected in the Detection Area.

f. **Detection Area**

Amplification is confirmed by quantification of Amplification by High Resolution Melt (HRM) analysis by fluorescence detection

Limitations:

1. Cross-contamination may occur if work is not carried out uni-directionally, or if the work area is not properly decontaminated.
2. The incorrect reagent volumes, or lack thereof will result in unsuccessful amplification.
3. Improper DNA extraction will also result in unsuccessful amplification.

4.7.3.2 Confirmation of *pncA* amplification by High Resolution Melt (HRM) Curve Analysis

a. **Purpose**

To confirm the successful PCR amplification of a positive *pncA* amplification with the JpncA primer set

b. **Equipment & Materials**

1. Dedicated RotorGene 6000 to perform a High Resolution Melt (HRM).
2. Alternatively, a Bio-Rad CFX can also be used, for the visualization of PCR products.

c. **Procedure**

After amplification with the *pncA* primer set, as per SOP TM040 "Amplification of the *pncA* gene", has been done, a high resolution Melt (HRM) PCR must be done.

HRM PCR

RotorGene 6000:

a. Keep samples covered in foil at all possible times, until loading them into the RotorGene
b. Load the samples into the RotorGene and conduct a melt at the following settings:

The melt is done at the GREEN channel, at a temperature range of 85 °C – 95 °C. There is a 90 second pre-melt, followed by an HRM of 0.1 °C per step, while each step is held for 2 seconds.

Bio-Rad CFX:

c. Keep samples covered in foil at all possible times, until loading them into the CFX
d. Load the samples into the CFX and conduct a melt at the following settings:

The melt is done at the FAM channel, at a temperature range of 85 °C – 95 °C. There is a 90 second pre-melt, followed by the melt of 0.1 °C per step, while each step is held for 2 seconds.

HRM Curve Analysis

Positive *pncA* melt curve

Below is are typical melt profiles of the *pncA* PCR product. Amplification is confirmed by the detection of a distinct fluorescent melt curve signature consisting of two maxima (±92°C and ±93.5°C, respectively) using high-resolution melt (HRM) analysis (Figure **11**). Figure 16 represents multiplexed HRM curves.

Figure 11: The melt curve of the normalised melt curve depicted in Figure 13

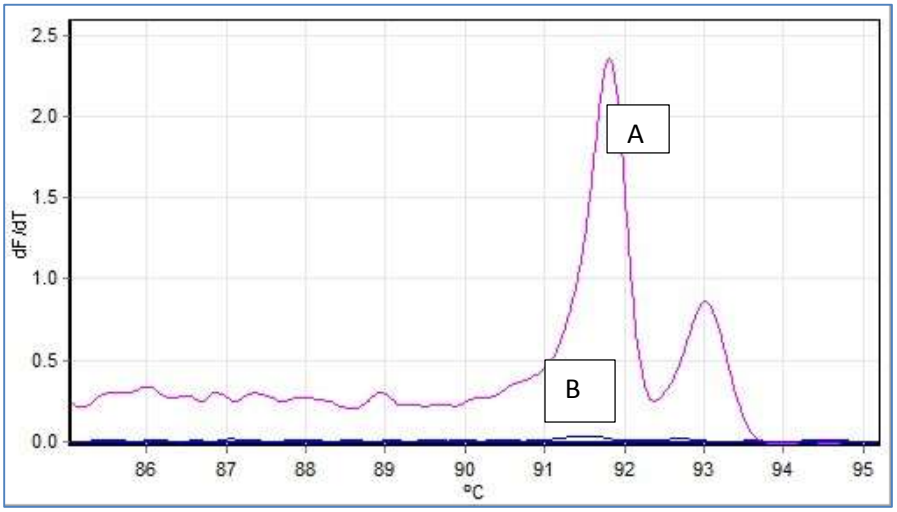
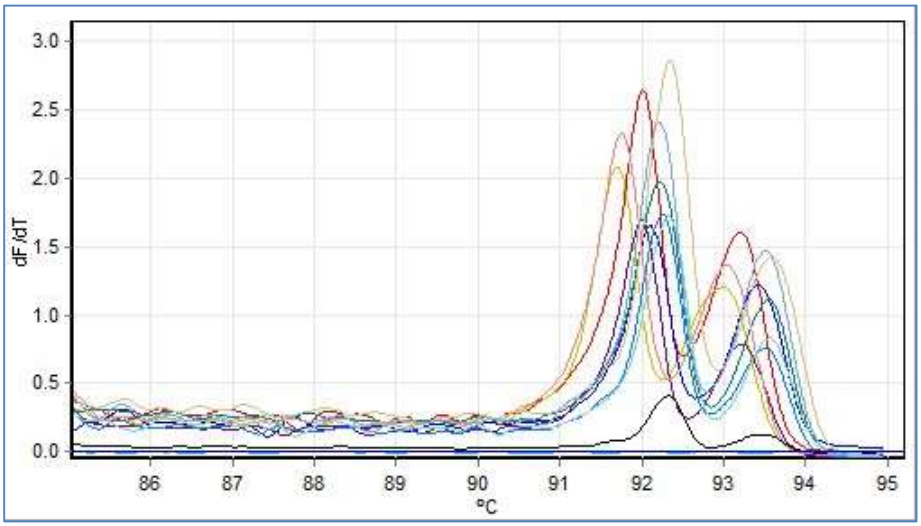


Figure 12: A Fluorescence melt curve of positive *pncA* PCR products.

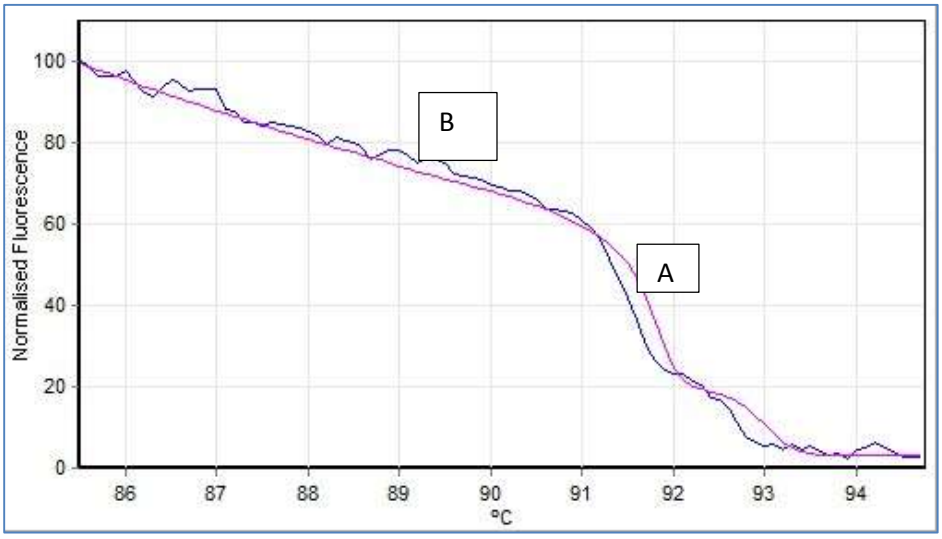


Negative *pncA* melt curve

1. In the case of an HRM revealing the absence of a PCR product, that sample is documented as not containing *M.tb*, and therefore not sequenced.
2. Where the first peak of the *pncA* melt curve (dF/dT) is lower than 0.2 dF/dT, it is usually taken as negative, and not sent for sequencing. Sometimes it is 'border line' and is sent for confirmatory *pncA* sequencing.

3. The Normalised Fluorescence melt curve should also be taken into consideration. If the melt is not smooth, it is also taken as a negative PCR product (Figure **13**).

Figure 13: A normalised melt curve, representing both a positive (A) and a negative (B) *pncA* melt product.



Genotypic Pyrazinamide DST

Once the successful amplification of the *pncA* gene has been established, the amplification products (amplicons) are to be submitted to the Central Analytical Facility at Stellenbosch University for Amplicon purification and DNA sequencing using the ABI3130XL genetic analyser (TM042) in order to determine pyrazinamide susceptibility. Prior to shipment of these amplicons, the Specimen Transport Form, TEST METHOD TRANSPORT FORM NC006-003 (TMTF NC006-003) must initially be completed at the CCTR laboratory,sent to CAF for completion, and sent back to the CCTR for completed and results reporting.

4.7.3.3 Cleanup of *pncA* PCR products post HRM Curve Analysis

a. **Purpose**

Purification is done to remove excess primers and dNTP's as well as small primer dimers from the PCR product

b. **Cleanup For More Than 10 Samples**

If there are more than 10 samples, the purification systems utilized will be that of the Tecan Freedom Evo robotic system.

Equipment And Materials

1. Nucleofast 96-well PCR clean-up plate (Machery-Nagel)
2. NanoDrop
3. Tecan Freedom Evo robotic system
4. Disposable robotic pipette tips
5. 200µl PCR tubes without lids
6. 96 well PCR plate flat, no skirt
7. Strip caps for 200µl PCR tubes
8. PCR tube racks
9. Freshly filtered 18.2 Mega-ohm pure water

Procedure

During the procedure described below ALWAYS maintain the orientation of the racks so that the A1 position in the plate is in the left corner furthest away from you.

1. Order the tubes containing PCR products generated in SOP TM041 "Confirmation of *pncA* amplification by High Resolution Melt (HRM) Curve Analysis", in the appropriate Eppendorf tube rack. If the PCR products are in a 96 well plate, place the plate in the appropriate Eppendorf rack.
2. Completely remove the lids from the tubes by cutting the stem holding the lid to the tube.
3. Select the script entitled PCR_cleanupv4_1_var_tubes_to_tubes from the list generated from the control computer and click "OK".
4. Place the rack with PCR products (source tubes) on the Tecan in the position indicated in the script on the controlling computer.
5. Place the Nucleofast 96-well PCR clean-up plate on the Tecan vacuum station, again maintaining the orientation.
6. Place freshly filtered water in the indicated position on the Tecan.
7. Place a rack with empty 200µl PCR tubes (or empty 96well flat no skirt PCR plate) in the indicated position (destination tubes).
8. Verify that all tubes, racks and containers are in the correct places and are properly seated.
9. Ensure that the correct disposable robotic tips are in the locations as indicated in the script.
10. Click "OK" to start the script.
11. Observe the first cycle of the script to ensure that the procedure is being performed.
12. Monitor the progress of the scrip and ensure that the vacuum engages properly.
13. On completion of the script, remove source tubes with the original PCR products and verify that the PCR products have been removed properly. Store the empty tubes in the rack according to sequencing facility policy.
14. Remove the destination tubes and verify that the tubes contain the appropriate volume of sample (approximately 50µl)
15. If some samples were not transferred properly, immediately inform the laboratory manager of the script malfunction so that the source of the malfunction can be investigated and corrected. Identify the corresponding well positions in the Nucleofast 96-well PCR clean-up plate and transfer the sample manually.
16. Cover the rack and proceed to the NanoDrop to verify the concentration of the PCR products.
17. Follow the laboratory SOP for determining the concentration of the purified PCR product.

c. **Cleanup For 10 Or Less Samples**

If there are 10 samples or less, the purification systems utilized will be that of the Agencourt's solid-phase paramagnetic bead technology for high-throughput purification of PCR amplicons (Figure 1).

Principle

Agencourt AMPure XP utilizes an optimized buffer to selectively bind PCR amplicons 100bp and larger to paramagnetic beads. Excess primers, nucleotides, salts, and enzymes can be removed using a simple washing procedure. The resulting purified PCR product is essentially free of contaminants.

Equipment and Materials:

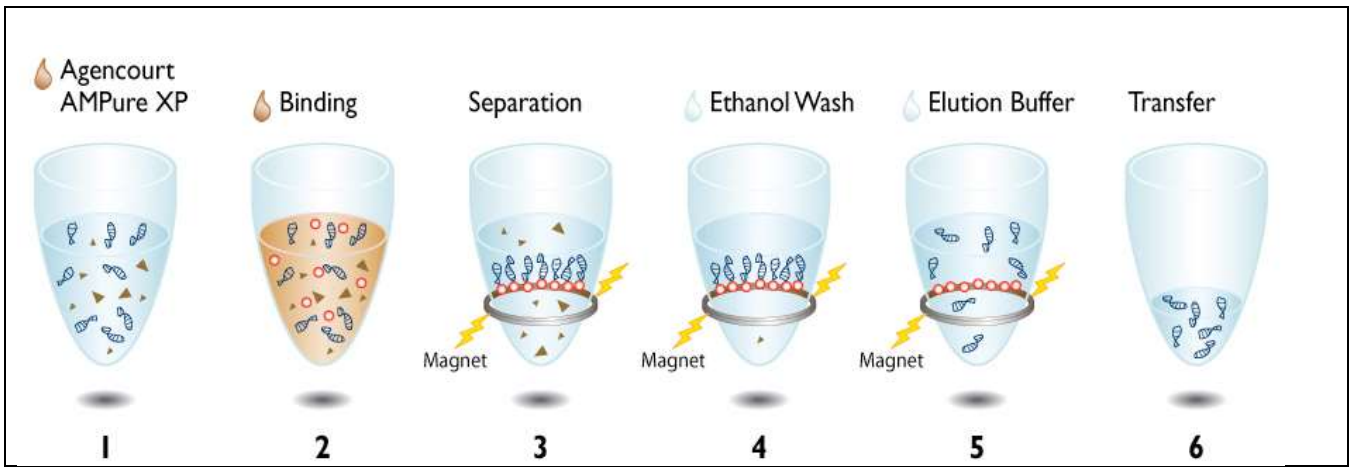
1. Agencourt AMPure XP Magnetic Particle Solution
2. Agencourt Magnetic eppendorf holder
3. 1.5µl tubes
4. Freshly filtered 18.2 Mega-ohm pure water
5. Fresh 70% ethanol

6. Bench top Centrifuge

Procedure

1. Order the tubes containing PCR products generated in SOP TM041 "Confirmation of *pncA* amplification by High Resolution Melt (HRM) Curve Analysis", in the appropriate Eppendorf tube rack.
2. Add 1.8ul AMPure XP per 1.0 µl of PCR product in 1.5µl tubes.
3. Pipette up and down 10 times to mix, without introducing bubbles.
4. Leave at room temperature for 5 minutes in order to bind DNA to the paramagnetic beads.
5. Place the tubes on the magnet and wait approximately 2 minutes for the beads to completely separate from the liquid.
6. Remove the liquid from the tube (containing contaminants and small fragments) without disturbing the beads and discard.
7. Wash beads and PCR product 2x with 70% ethanol to remove contaminants
8. Add 200 µl 70% ethanol to the tubes still on the magnet, but do not disturb the beads.
9. Leave for 30 sec and then remove the ethanol and discard.
10. Repeat a-b once.
11. Use a p10 pipette to remove any traces of ethanol in the bottom of the tube.
12. Allow the excess ethanol around the beads to evaporate by leaving the samples at room temperature for 3 minutes.
13. Elute purified PCR product from beads by adding 50 µl filtered 18.2 Mega-ohm pure water to the beads after removing from the magnet.
14. Pipette up and down 10 times vigorously and vortex to ensure all the product is eluted from the beads.
15. Spin down briefly and place the tubes on the magnet for 3 minutes.
16. Transfer the liquid containing cleaned PCR product into a new tube.

Figure 14: Graphic representation of the Agencourt Cleanup



4.7.3.4 DNA Sequencing of PCR Amplicons using the Sanger Sequencing Method

a. **Purpose**

To determine the specific sequence of a particular fragment or gene.

b. **Equipment And Materials**

1. GeneAmp 9700 thermal cycler

2. Tube holder for the GeneAmp 9700 thermal cycler
3. Bench top Centrifuge
4. Sequencing Primers
5. 5x Sequencing buffer
6. BigDye
7. 8-strip 0.2ml PCR tubes
8. 0.2 ml single PCR tube with cap
9. 8-strip caps for 0.2ml PCR tubes
10. 96 well PCR plate half skirt
11. Rubber sealing mat for 96-well plate
12. Eppendorf tube racks.
13. Freshly filtered 18.2 Mega-ohm pure water

c. **Procedure**

Preparation of cycle sequencing mix:

1. Mix 60µl of BigDye and 140µl of 5 x Sequencing buffer.

Dilution of PCR products after PCR product cleanup SOP TM042 "Cleanup of *pncA* PCR Products post HRM Curve Analysis".

2. Using the PCR product concentration obtained from the NanoDrop, carefully dilute the PCR products to 5ng/µl.

Reaction set-up

3. Reaction set-up is to be done in either single 0.2ml PCR tubes, strips of eight 0.2ml tubes or in a 96-well Axygen plate
    a. Maintain sample order from Post PCR clean-up
    b. When using single tubes, or strips of eight tubes, use the tube holder for the GeneAmp 9700 thermal cyclers
    c. When working in 96 well plates, maintain the plate orientation
    d. Ensure that all plates and tubes are clearly marked.
    e. DO NOT label part of tube that fits in PCR block
    f. DO NOT label the top of PCR tubes.
    g. Use an electronic multi-dispensing pipet to dispense the sequencing primer and sequencing mix
    h. If a large number of reactions are performed a master-mix of primer and sequencing mix can be made. 6µl master-mix must then be dispensed into the Strips of eight or 96-well Axygen plate and 4µl PCR product added.
4. Add 3µl sequencing primer (at 1.1µM) to each well.
    a. Tap the tubes/plate on the bench to collect the primer in the bottom of the plate.
5. Add 3µl sequencing mix (mixture of BigDye and 5x Sequencing buffer)
    a. If multi-dispensing, ensure that the tip does not touch the primer drop
    b. Gently tap the tubes/plate on the bench to collect the mix in the bottom of the plate
6. Add 4µl purified PCR product (final concentration 5ng/µl) using a P10 pipette
    a. Pipette directly into the primer + sequencing mix in the bottom of the plate
    b. Mix by carefully pipetting up and down once WITHOUT introducing bubbles
7. Cap tubes tightly - either singles or those with strip of 8 caps or the 96 well plate with a cover plate with PCR rubber seal
8. Pulse spin the tubes/plates in a plate centrifuge to ensure that all reagents are mixed in the bottom of the tubes/plates.

a. Visually inspect to verify that all reagents are mixed in the bottom of the tubes/plates and that there are no bubbles present at the bottom of the tubes.

9. Place tubes/plate in GeneAmp 9700 thermal cycler
10. Select the following programme on the instrument: “seq55” to initiate the reaction run.
11. Before starting the run, verify the following settings in the programme:
    a. Rapid thermal ramp to 94°C
    b. Denature at 94°C for 5 minutes
    c. Followed by 25 cycles
        i. 10 seconds at 94°C
        ii. Rapid thermal ramp to 55°C for 5 seconds
        iii. Rapid thermal ramp to 60°C for 4 minutes
    d. Rapid thermal ramp to 4°C and hold at 4°C
12. The run can now be initiated.

4.7.3.5 Dye Terminator Removal Post DNA Sequencing

a. **Purpose**

To remove unincorporated dye terminators from cycle sequencing reactions.

b. **Equipment And Materials**

1. Tecan Freedom Evo robotic system
2. GeneAmp 9700 thermal cyclers
3. Eppendorf 5804R Bench top Centrifuge
4. 2 - 20 µl multichannel pipette
5. Princeton separations CENTRI SEP 96 Well Plates
6. Deep well waste collection plate
7. Tube holder for the GeneAmp 9700 thermal cyclers
8. Disposable robotic pipette tips (2-20 µl)
9. 0.2ml PCR tubes without lids
10. Strip caps for 0.2ml PCR tubes
11. 96 well PCR plate half skirt
12. 96 well ABI plate
13. Rubber sealing mat for 96-well plate
14. Eppendorf tube racks.
15. 0.5% SDS
16. Freshly filtered 18.2 Mega-ohm pure water
17. Disposable troughs
18. Septa Covers

c. **Protocol**

Prior to the purification steps described below, remove a CENTRI SEP 96 Well plate from the fridge and let it obtain ambient temperature by placing it on the bench for up to 2 hours.

Purification of sequencing reactions

1. Pulse spin the tubes / plate in which the DNA was cycle sequenced (SOP TM 043 “DNA sequencing of PCR amplicons by the Sanger sequencing method”, at 1500rpm in order to collect all liquid in the bottom of the tubes/plates.

2. Open the tubes by carefully removing the lids from the tubes.
3. Decant the 0.5% SDS into a disposable trough and pipette 4 µl of the 0.5% SDS to each cycle sequencing reaction by using a multichannel pipette.
    a. Hold the tips just above the open tubes and dispense the 4 µl into the open tubes so that the tips do not come into contact with the sequenced amplicons. Should this happen a new set of tips must be loaded onto the multichannel pipette.
4. Close the open tubes with new lids or rubber mat.
5. Return the tubes/plate to the thermal cycler.
6. Select the following program for SDS treatment of the sequenced amplicons: "SDS".
7. Ensure that the thermal cycler is set at 98°C for 5 minutes and 25°C for 10 minutes.
8. Initiate the reaction.
9. Once the reaction has completed, remove the plate from the instrument and keep it for later use in the Tecan Freedom Evo robotic system.

Preparation of the CENTRI SEP 96 Well plate

1. Remove the top and bottom seal of the CENTRI SEP plate and place it on top of a deep well waste collection plate.
2. Centrifuge the "open" CENTRI SEP plate for 2 minutes at 1500rpm in the Eppendorf 5804R bench centrifuge.
3. Replace the collection plate underneath the spun down Sephadex columns with a clean 96 well ABI plate, labelled with the sample sheet number.
4. Use the appropriate Sephadex clean-up script on the Tecan Freedom Evo robotic system to transfer the cycle sequencing reaction from the Axygen plate/tubes to the Sephadex columns.
5. Once done, immediately centrifuge the Sephadex columns again for 2 minutes at 1500rpm in the Eppendorf 5804R bench centrifuge.
6. Dispose of the CENTRI SEP plate and keep the sample ABI collection plate.
7. Place a clean septa over the collection plate and start Electrophoresis (see "TM045 - Electrophoresis of DNA sequencing products").

4.7.3.6 Capillary Electrophoresis of DNA Sequencing Products

a. **Purpose**

To separate sequenced DNA fragments in order to sequence the electropherogram

b. **Equipment And Materials**

These steps are performed in a temperature controlled laboratory with restricted access (Room C116).

1. GeneAmp 9700 thermal cyclers (Room C118)
2. 3130xl or 3730xl Genetic Analyzer (Room C116)

c. **Procedure**

Data Import

1. Import the Sample sheet containing information regarding the samples to be analysed to the genetic analyser. The format of the sample sheet is determined by the analyser used.
2. Verify that the active spectral calibration matches your dye set and capillary array length for all scheduled runs.

(See SOP 3130xl or 3730xl Genetic Analysers for detailed operation.)

Electrophoresis Of Sequencing Samples

1. Use the PCR system 9700 to denature the sequencing reactions by heating at 95°C for 1 minute. Hold at 4°C until ready to load Analyser.
2. Assemble the plate "sandwich" by placing the plate into the plate base. Snap the plate retainer on top of the plate + plate base. Verify that the holes of the plate retainer and the septa strip are aligned.
3. Load the samples as indicated in the Analyser SOP/user's manual and make sure that the correct run module is selected for a specific run. See "Getting started guide" for Applied Biosystems 3130xl Genetic Analysers p132 for sequencing run modules.
4. Close the doors of the Analyser.
5. To start a run, click the "Run" button – Run scheduler. The green LED light on the front panel of the Analyser will flash while the run is in process. When the run is completed, the green light will stop flashing.

4.7.3.7 DNA Sequence Analysis

a. **Purpose**

To identify specific single-nucleotide polymorphisms (SNP-variants), in the *M. tuberculosis pncA* gene, that are associated with pyrazinamide resistance. This section of the SOP gives a step-by-step explanation to new users for importing and analysing data using the Variant Reporter™ v1 software.

b. **Equipment**

PC Software: Variant Reporter™ v1 (Applied Biosystems, 2007)
Sample data path:
G:\<SAF07><"year"_Data><P><PZA_Project>
Tutorial data path:
G:\<SAF07><TB-Project><PZA><Results><2014_07_21

c. **PROCEDURE**

1. Go to Desktop; double-click "Variant Reporter" (VR).
2. VR will open showing a table within the "Dashboard view".
3. Select the line with "PZA-Template" found in the Project Name column.
4. Now see the "Project View" pane that appeared vertically on the left. It contains Setup options, Quality control options as well as Results options.
5. Select the "Import" button found in the "Setup>Import and manage traces" pane that should be open already.
6. Go to the tutorial data folder in the path shown above.
7. Select "2014_07_22", and then click on "Add Selected Traces>>" (File type should be ".ab1 trace files".
8. A list of "Traces to Add" will appear on the right of the "Import Traces" window, whereafter just select "Okay".
9. The trace file names (ab1 file name) should already be assigned with strict nomenclature. Therefore just select "Auto Assign".
10. Keep the specimen name between the 1st and 2nd occurrence of "_" (underscore) and the amplicon name between the 2nd and 3rd occurrence of the "_". Then click "Okay".

Important: The capillary sequencer imports and saves the name in accordance to how the sample names were provided on the samples sheet. Afterwards the ab1 file should have e.g. an accession name as follow: 4_303252_JpncAs_Forward__G11_1.ab1. The sequencer software exports the file with the plate's well position added to the initial name. The "_1" at the end is irrelevant.

11. A trace grid will appear showing the specimens in a vertical orientation, and the amplicon (pncA) horizontally. For each specimen (sample) these must be 2 traces for the amplicon, representing a forward and reverse sequence.

12. Go to the the next Setup option in the "Project View" pane referred to as "Set Analysis Parameters" (Note: The template already has these parameters fixed and saved).
13. Leave "mobility file" unchanged.
14. Set "mixed base threshold" so that a mixed base is not assigned when the secondary peak height is ≤ 10%.
15. Keep analysed data scaling as "True Profile".

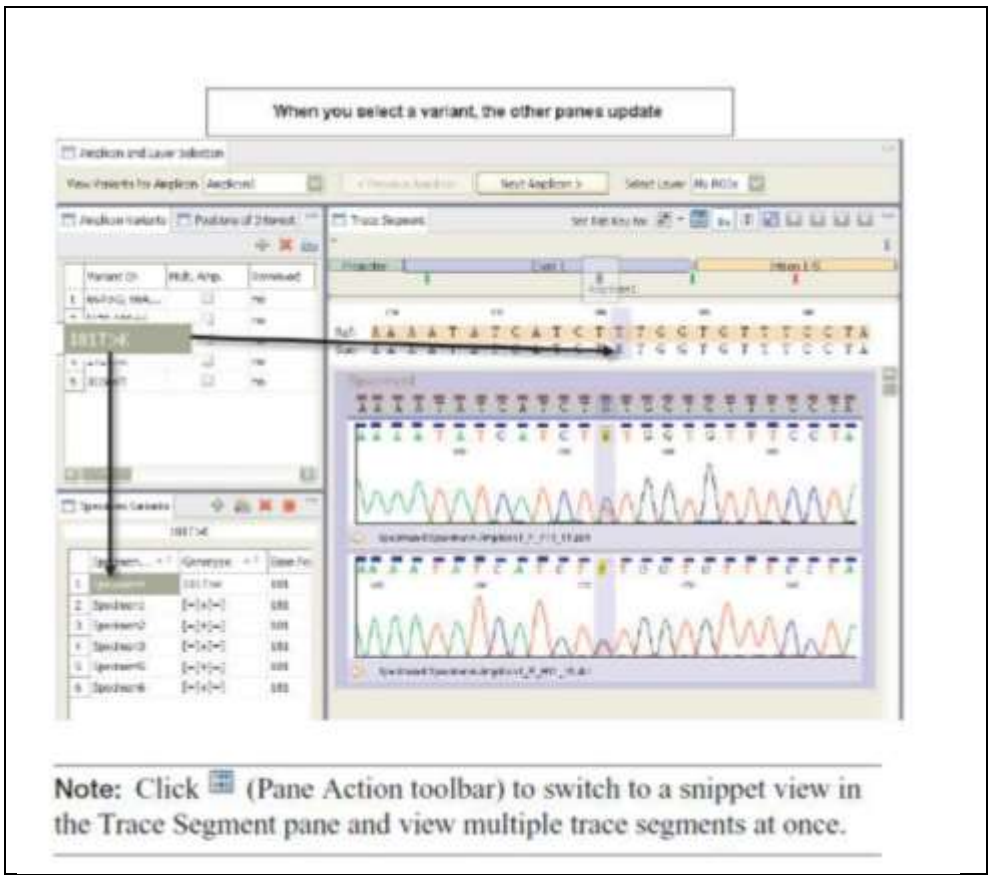
Trimming options

16. Trim using quality values: Mask each end until fewer than 2 bases in a window of 20 bases have QVs < 20.
17. Keep "Trim using base positions unchecked".

Filter options

18. Filter traces until the minimum trace score the less than 25.
19. Filter traces if the % Expected Trimmed Read Length (TRL) is less than 70%.
20. Go to the next option in "Project View" pane known as "Define Reference". Import the reference file i.e. "pncA_reference.txt". This should provide a reference sequence of 961 nt, starting with "cacc" and ending with "cacg". The reference can be found in the path G:\<SAF07><TB-Project><PZA>
21. Now define your amplicon region by submitting the forward and reverse primer sequences. Select "Amplicon Primers/Known Variants". In the drop-down select "Edit primer sequences". Add the primer sequences, click on "Align", then "Okay". The primer sequences are as follow:
    - pncA-F: AGTCGCCCGAACGTATGGTG
    - pncA-R: CAACAGTTCATCCCGGTTCG
22. A yellow line representing the amplicon should be present at this point. When clicking on this line the corresponding sequence at the bottom should be highlighted in yellow.
23. Next, define the pncA gene sequence by highlighting nucleotide numbers 201 to 761, and then clicking on the "ROIs" drop-down. The gene starts with "5'-atgcgggc" and ends with "ctcctga-3'". In the "ROIs" drop-down window select "Create/edit ROI". A "Reference feature manager" window will appear.
24. Set the following:
    a. a. Name: set the name (e.g. PZase, pyramidase or PZA etc.).
    b. b. Type: gene
    c. c. Select the "Translatable" tick box
25. Now select the "Reference" drop-down window and click on "Change codon table".
26. Choose the "bacterial" codon table.
27. Now select the green "Analyze" button in the "Project View" pane. Now the software should successfully align all good-quality trace sequences to the reference sequences.
28. After analysis, the software opens the "Project Results Summary"" page if your project passed the quality threshold settings (Note: If your project does not meet the Quality Threshold settings during analysis, this page allows you to examine where the project failed in detail.
29. In the "Amplicon summary window" click to display the "Variant Legend" and "Amplicon quality legend".
30. Go to the "Variant Review" page in the "Project View" pane to see a full list of all variants, per amplicon, that the software detected during analysis.
31. Select the first variant in the Amplicon Variants table; all specimens containing the selected variant highlight immediately in both the Specimen Variants table and the Trace Segment pane (See Figure 15 below).

Figure 15: Specimen Variants table and the Trace Segment pane



To accept or reject selected specimen genotypes:

32. Select a variant that you want to review in the Variant ID column
Amplicon Variants table
33. Review the specimens (extended trace view or snippet view) associated with each variant (Specimen Variants table).
34. Right-click to accept, or reject, the specimen genotype(s).
    (Note: Click to accept or reject all specimen genotypes at once)
    (Note: When you accept or reject a specimen genotype, both tables (Specimen Variants table, Amplicon Variants table) update simultaneously.
35. Save your project when you have completely reviewed all detected variants.
36. To create a report, click to open the Reports dialog box. Select the checkbox next to the report, or reports, you want to print and/or export.
    (Note: When selecting a Specimen Report, you must also select the specimen from a drop-down list, then specify the file format).
37. Specify the file format as well as the target directory you want the report to export to, then click either “Print Preview”, “Print” or “Export”.
38. Results location:
    G:\<SAF07><”year”_Data><P><PZA_Project><Results><PZA_”year-month-day”>.
39. Once done, the results are to be mailed to the molecular division supervisor of TASK laboratory.

5.8. SOP 8: Liquid culture by Mycobacteria Growth Indicator Tube (MGIT)

5.8.1. Purpose

Used by the **local/regional mycobacteriology laboratory** for culture of mycobacteria for:
- Definitive diagnosis of *M.tb* (presence or absence).
- Assessment of the bacterial load by determining the time taken for culture tubes to signal positive (TTP).

5.8.2. 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 culture 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.

A MGIT tube remaining negative for 42 days and shows no other visible sign of positivity should be removed from the instrument, recorded as negative and autoclaved prior to disposal.

5.8.3. 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).

Step One: Inoculation and Incubation of the MGIT tubes

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) 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 lab.
- Prior to use the user should 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.

Equipment/Reagents

- Biological Safety Cabinet
- 7ml MGIT tubes
- MGIT PANTA
- MGIT Growth Supplement
- Graduated Plastic Pasteur Pipettes
- Discard bucket containing appropriate liquid disinfectant (specified in local Health and Safety documentation)
- 1000µL pipette and aerosol resistant tips

1. Reconstitute MGIT PANTA (Polymyxin B, Amphotericin B, Nalixidic acid, Trimethoprim, Azlocillin) with 15.0 mL of MGIT OADC (Oleic Acid Albumin Dextrose Complex) Growth Supplement. Mix completely until dissolved. 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. This mixture is stable for 5 days if stored at 2° - 8°C.
2. 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.
3. Add 0.8 mL of the supplements mixed above to each MGIT tube using a sterile pipette. Contamination of the

tubes and the supplement should be avoided during this process.

4. Add 0.5 mL of a well-mixed, processed and concentrated sputum specimen 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 pastette or micropipette for each specimen. Dispose of waste pipette tips into the discard bucket containing appropriate disinfection as recommended in the Local Health and Safety Guidelines. Store the remaining sputum sediment at 4°C, for 10 days until confirmed the MGIT tube is not contaminated.
5. 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).

DO NOT PLACE TUBES WITHOUT THE MACHINE ASSIGNING A STATION.

Step Two: Detection of positive MGIT tubes, confirmation and interpretation of results

MGIT tubes must be autoclaved prior to disposal once result(s) have been confirmed and positive samples have been appropriately stored (see step 4).

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/Reagents

- Biological Safety Cabinet
- Discard bucket containing appropriate liquid disinfectant (specified in local Health and Safety documentation)
- Blood agar plate
- 10µl loop
- Plastic universal or tube for centrifugation of sample
- Glass slide
- Sterile pastette and aerosol resistant tips.
- Lowenstein Jenson (LJ) slope
- 37°C incubator
- Centrifuge/microfuge

1. Once complete remove the MGIT tubes from the machine according to manufacturer's instructions.
2. The MGIT will record the date the tube was flagged as positive and the number of days and hours taken to reach positivity (TTP = time to positive, also known as TTD = detection).
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.
4. For Positive Tubes, the following must be performed:
   4.1. Perform Blood Agar culture on all positive MGIT tubes:
      a. Label one blood agar plate with the laboratory accession number and the patient number.
      b. Inoculate the blood agar plate with liquid from the MGIT tube using a 10µL disposable loop. Incubate the blood agar plate along with the MGIT tube in a 37°C incubator for 48 hours.
      c. Check for growth or contamination on the blood agar plate daily.
         i. If contamination is detected within 10 days, re-decontaminate the deposit according to the decontamination process in SOP 3.
         ii. If contamination is detected more than 10 days after inoculation, record the final result as contaminated.
   4.2. Perform Z-N Stain microscopy (SOP 4) on all positive MGIT tubes even if confirmed to be contaminated

a. Sampling
   i. Direct Sampling – Sediment is present at the bottom of the MGIT tube
      - Remove a small amount of the sediment from the positive MGIT tube using a sterile pastette.
      - Label a slide and make film on slide for Z-N staining.

   ii. If Direct Sampling is not possible (sufficient sediment is not present in the tube), follow one of the procedures below to concentrate the sample.

      - If the centrifuge cannot accommodate MGIT tubes:
         - Inside the BSC, remove 1 mL of well mixed fluid from MGIT tube with a sterile pastette 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 make the film on the slide for Z-N staining from the resuspended pellet.

      OR
      - If the centrifuge can accommodate MGIT tubes:
         - Spin MGIT tubes without decanting at 3,000g 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 make film on the slide for Z-N staining from the re-suspended pellet.

b. Heat fix, stain and analyse slide per SOP 4.
c. Examine the Z-N smear for the presence of AFB.
   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).

<u>Step Three: Interpretation of Results</u>
Refer to Figure 16.

Blood Agar Culture		**AFB (Z-N smear)**		**Result**		**MGIT TTP**
Negative	and	Present	this is	True positive	and means	Valid
Positive	and	Present or Absent	this is	Contaminated	and means	Invaild

1. If the sample is confirmed as contaminated within the first 10 days of inoculation, the remaining sputum sediment (from the original decontamination) is to be retreated per SOP 3 and the MGIT TTP will be attempted a second time.
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.

Blood Agar Culture		**AFB (Z-N smear)**		**Result**		**MGIT TTP**
Negative	and	Absent	this is	Possible False Positive	and means	Confirm it is a true false

						positive culture by confirming there are no AFBs present.

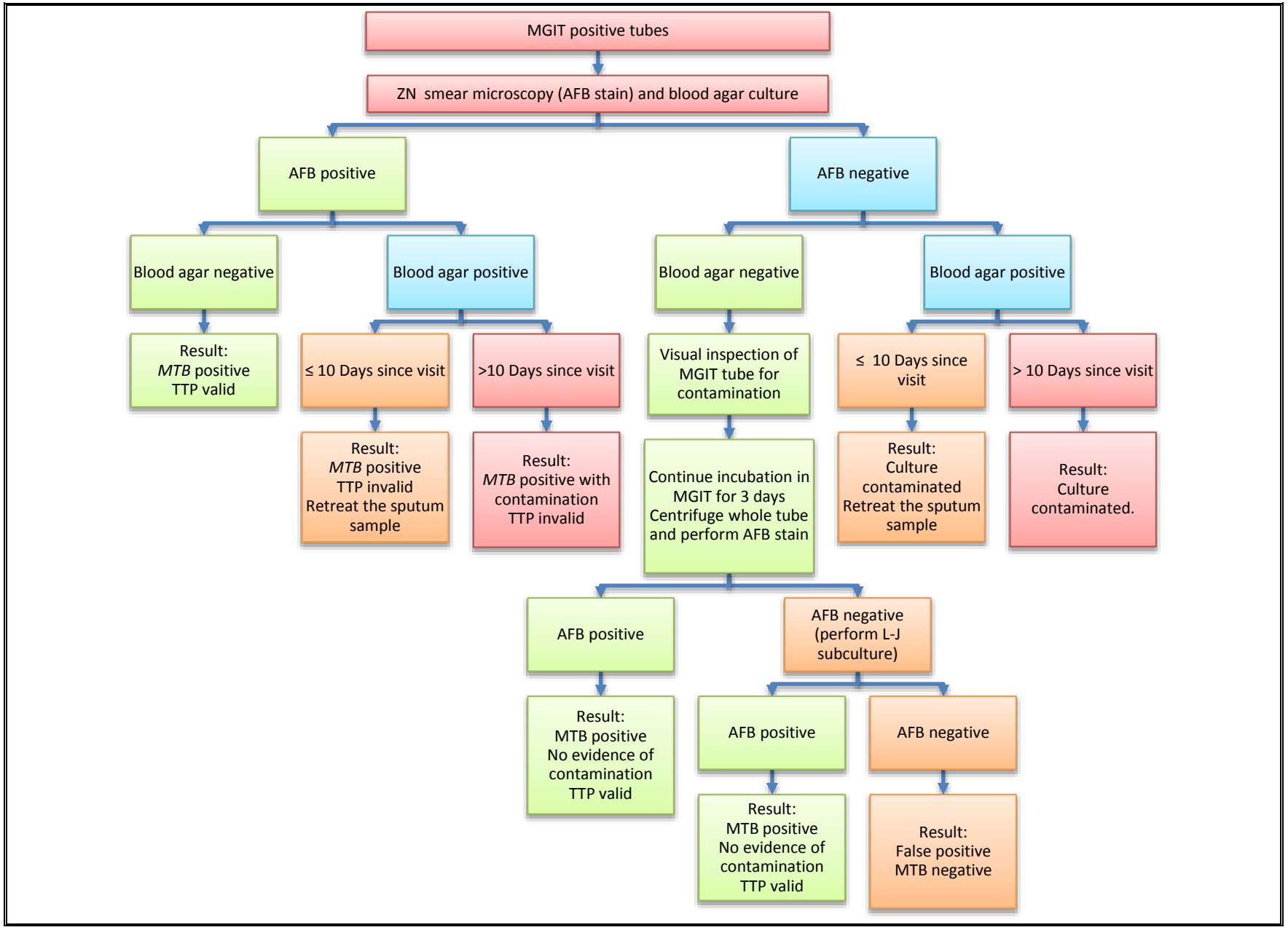
Confirm it is a true false positive by performing the following:

1. Look at the MGIT tube – record if is it 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 (*M. tuberculosis* or any contaminant).
    - A clear tube may be a true false positive resulting from altered reagent pH.
2. Repeat the Z-N smear a second time to double check for AFBs. If the tube was not centrifuged previously because precipitate was present, centrifuge the tube prior to performing the second Z-N to ensure the sample is sufficiently concentrated.
3. If AFBs are not detected in the second smear, re-incubate the MGIT tube for at least 3 days (in MGIT or incubator) to allow further growth of any *M. tuberculosis* present. After 3 days of additional incubation, repeat the Z-N smear again.
    - OPTIONAL: If AFBs are still not detected after this step, inoculate an L-J slope and incubate for 3-4 weeks to check if any growth may occur later.
4. Blood agar culture conclusion should be marked as other when the blood agar culture is negative, but there is concern that the sample is contaminated either by visual inspection of the tube or by the presence of fungal contamination seen on the ZN smear. Also complete the comments section to provide the necessary information as outlined below. It is essential that the comment also clearly states that the **'blood agar culture is negative'**.

    Valid situations where blood agar culture conclusion should be marked as negative:

    a. Blood agar not done (e.g. test not set up, discarded before reading, result missing)
    b. Blood agar plate is negative, MGIT tube visibly contaminated with fungal growth
    c. Blood agar plate is negative, MGIT tube visibly contaminated but no fungal growth seen
    d. Blood agar plate is negative, evidence of fungal hyphae seen on ZN smear
    e. Blood agar plate is negative, contamination seen on LJ slope inoculated from MGIT tube

Figure 16: Algorithm for Interpretation of MGIT results



Step Four: Storage for further investigations

Equipment/Reagents

- Biological Safety Cabinet
- Discard bucket containing appropriate liquid disinfectant (specified in local Health and Safety documentation)
- Cryovial (with rubber o-ring seal), and appropriate storage box
- Sterile pipette and aerosol resistant tips
- PBS/7H9
- Glycerol
- LJ slope
- Rack for LJ slopes

The following processing, storage and shipment of samples is required:

1. All Day 1 *M.tb* isolates and isolates from **all** positive cultures are to be stored at the local/regional mycobacteriology laboratory until trial closure.
2. The central mycobacteriology laboratory will store all isolates after mycobacteriology characterisation testing is complete.
3. Day 1 (baseline) sputum sample (or screening or out to Week 4 if the baseline is contaminated or negative) and Positive Cultures at or after Week 17 are to be stored on L-J slopes and shipped in 6 weekly batched to the central mycobacteriology laboratory (see SOP 2 for shipment information).

Two samples should be stored for each isolate. At least one sample should be stored in 50% glycerol at -70°C to -80°C. If only one sample is stored in 50% glycerol, another must be stored on an L-J slope. If two frozen samples are stored, they should be in separate freezers if possible. If not, they should be in separate sections of the freezer. A logbook must be kept of all isolates in storage

Storage on LJ slope

To inoculate an LJ slope take 100 - 200µl of the positive MGIT pellet and pipette onto the slope. Securely fasten and label with both the patient number and the lab accession label. 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 subcultured 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- 2 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 and the lab accession label (also handwrite this number in permanent marker in case sticker is removed during freezing). Place in an appropriate storage box and freeze at –70°C to -80°C.

Step 5: Detection and removal of negative MGIT tubes

The threshold for tubes to be declared as negative in BACTEC MGIT 960 is 42 days. Any tube that has not flagged positive prior to or at day 42 should be reported as a negative result. If a sample flags positive after 42 days, this must be reported as negative as well. The negative tubes must be autoclaved prior to being discarded.

Remove the MGIT negative tubes from the machine according to manufacturer's instructions and 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.

Contamination rates

The contamination rate must be calculated and monitored as part of internal quality control on ongoing basis to determine which sample has to be repeated in a timely manner (i.e. before it becomes too late to recall the patient).

The rate of contamination is determined by calculating the proportion of contaminated tubes over a given period of time. Contamination rates will be monitored as shown in Quality Manual Attachment G. If the rate is out of this range, Quality Manual Attachment Gi must be completed with details of the contaminated samples and sent to the UCL Laboratory Team.

Table 8: Contamination rates for MGIT culture

	Contamination rates	Comment
Very low	less than 3%	May indicate too harsh a decontamination process, which would also affect growth of mycobacteria and may reduce the positivity rate of and increase time-to-detection of a positive mycobacterial culture.
Acceptable	3% - 8%.	
High	above 8%	May be due to: • Improper or under decontamination of specimen. • Processing of very mucoid specimens that are hard to liquefy. • Long storage and transportation time of the specimen after collection. (In such situations, especially in hot weather, contaminating bacteria tend to overgrow and then are hard to kill by routine decontamination procedure). • Use of non-sterile materials such as pipettes, tubes, etc. (Sometimes if reagents are prepared, stored in bulk and used for long periods of time, they may become contaminated).

The ONLY samples that can be classified as **contaminated** are:

- MGIT positive AND
- Show growth on blood agar within 48 hours.

An exception to this are samples visibly contaminated (e.g. with fungi visible either in the MGIT tube or on the ZN smear) which do not show growth on blood agar.

5.8.4. Quality Control

5.8.4.1. MGIT

M. tuberculosis reference strain (H37Rv) is tested to ensure that the medium supports growth of mycobacteria.

Upon receipt of a new batch of MGIT tubes and supplements, test one tube from each new batch and test the supplements, recording the batch number, expiry date and results on Quality Manual Attachment Eii.

Procedure

1. Prepare a fresh culture of *M. tuberculosis* (H37Rv)
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. Any QC failure and subsequent actions should be recorded on Quality Manual Attachment N.

MGIT maintenance must be performed daily and monthly, preferably before unloading or loading of tubes. Record all readings in the BACTEC MGIT 960 Maintenance Log – Quality Manual Attachment J

MGIT failure/breakdown lasting more than 24 hours:
Refer to and 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.

5.8.4.2. Blood Agar

For each new lot of commercially prepared blood agar plates or batch of blood agar plates prepared in the laboratory, record the details (i.e. the lot number, collection date -for blood if not purchased and expiry date of each ingredient) in the Blood Agar Plate Quality Control Form – Quality Manual Attachment Eiii.

5.8.5. Forms

Quality Manual Attachment C – Equipment temperature log form#
Quality Manual Attachment D – Containment Level 3 checklist
Quality Manual Attachment Eii – MGIT tubes and PANTA/growth supplement
Quality Manual Attachment Eiii - Blood agar plates
Quality Manual Attachment G/Gi – Contamination rates/Contaminated samples
Quality Manual Attachment K – MGIT calibration tube log
Quality Manual Attachment J – BACTEC MGIT 960 daily maintenance log

5.9. SOP 9: DNA Extraction

4.9.1. Purpose

Used by the **local/regional mycobacteriology laboratory** to extract DNA on pre-treatment isolates from the baseline and the isolates from patients suspected of failure or relapse after treatment (positive cultures at or after week 17 (4 month treatment arm)/week 26 (6 month treatment arm) and any new positive culture thereafter). If the baseline MGIT is contaminated and pure culture cannot be obtained, it is acceptable to use the screening to week 4 culture as a back up to perform the DNA extraction.

The method described here is designed to yield microgram quantities of high molecular weight DNA suitable for molecular typing. An aliquot of all extracted DNA should be sent to the **central mycobacteriology laboratory** for further analysis (another aliquot of the same DNA to remain at site).

4.9.2. Principle

Although DNA can be extracted from *M. tuberculosis* 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 pick 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. *M. tuberculosis* 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 and Ca 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).

4.9.3 Procedure

4.9.3.1 Isolation of Genomic DNA from *M. tuberculosis*

<u>Equipment/Reagents</u>

- Biological Safety Cabinet
- Waterbath or heating blocks (80-95°C, 60 - 65°C and 37°C)
- 10µL loops
- 1.5mL screw capped eppendorf tubes with rubber 'O' rings
- 1000µL pipette and aerosol resistant tips
- 200µL pipette and aerosol resistant tips
- Tris-EDTA (TE) buffer
- Microfuge
- 10mg/ml lysozyme
- 10% Sodium dodecyl sulphate (SDS)
- 10mg/ml Proteinase K
- 5M NaCl
- Cetyl trimethylammonium bromide (CTAB)
- Chloroform

- Isoamylalcohol
- Sterile DNAase-free 1.5mL eppendorf tubes
- 70% ethanol
- -20°C freezer
- 4°C refrigerator

Preparation of solution

Lysozyme solution: 10 mg/ml.
Store in small aliquots at -20°C Use one aliquot each time, do not freeze and thaw twice.

10% SDS
10 g SDS/100 ml distilled water. 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 one aliquot each time, do not freeze and thaw twice.

5M NaCl
29.2 g NaCl/100 ml distilled water. Autoclave. 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. Store at room temperature for no longer than 6 months.

Chloroform/isoamylalcohol (24:1)
Mix 1 part of isoamylalcohol with 24 parts of chloroform. Store in cool, dark, ventilated place, use within 6 months or by expiry date indicated.

70% Ethanol
70 ml 100% ethanol in 30 ml dH_2O, 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 addition of chloroform (step 14) must be carried out in an appropriate Biological Safety Cabinet inside a Containment level 3 laboratory.

1. Fill the waterbath with tap water and set for 80°C. If waterbath is not available 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 or 7H11/7H10 plates with good growth, take all organisms using a 10µ 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 20 minutes.
7. Pulse down the tubes, as above.
8. Add 50µL 10mg/mL lysozyme, mix gently with the pipette and incubate at 37°C in the waterbath or incubator 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.
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. Pre-warm the CTAB/NaCL to 60-65°C.
11. Add 100µL 5M NaCl.
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). Mix by inversion.
15. Tubes can now be removed from the Containment Level 3 Laboratory. Microfuge at 10,000 g for 5 minutes.
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 TE (depending on pellet size) overnight at 4°C (or 1 hour at 65 °C).
23. Divide the DNA aliquot into two tubes: one for storage at site and one for shipment to UCL.

4.9.3.2. Estimation of DNA Concentration

DNA concentration will be estimated by one of the methods detailed below.

If DNA concentration cannot be measured at site the DNA can be sent to UCL at the laboratories risk. If there is not enough DNA to use for molecular typing at UCL, the laboratory will have to repeat the DNA extraction for those samples and re-submit to UCL for molecular typing.

4.9.3.2.1 Estimation of DNA Concentration Using Agarose Gel

Equipment/Reagents

- Agarose
- Balance and weighing boats
- Microwave
- Tris borate EDTA (TBE)
- Hybaid gel electrophoresis tank and apparatus
- Ethidium bromide
- 10ul pipette and appropriate tips
- Loading buffer
- Parafilm
- Lambda DNA control standards

- UV transilluminator
- UV protection glasses, a face visor and gloves
- Digital camera

Process

1. Weigh out 1.2g agarose using the weighing balance and weighing boats and place into a 250 mL glass bottle.
2. Add 120mL 0.5 X TBE buffer to create enough for two 1 % gel using the Hybaid Electro-4 gel tank equipment.
3. Loosely place the cap on the glass bottle and place in the microwave.
4. Heat on high power until all the agarose has dissolved, swirling intermittently to prevent clumping. Take care not to overheat, as it will boil over.
5. Leave the gel to cool to 56^{o}C ('hand-hot').
6. Wearing Nitrile gloves and protective goggles add 3µL ethidium bromide to the cooled gel solution, discarding the Gilson pipette tip into the charcoal pot.
7. Prepare the Hybaid Electro-4 gel-casting tray by attaching the black foam ends firmly to each end of the tray on a flat, even surface.
8. Pour the gel into the tray and insert enough combs for the number of specimens. Allow the gel to set.
9. Remove the two foam ends and the combs.
10. Place the gel in the Hybaid Electro-4 electrophoresis tank and cover with 0.5 X TBE buffer.
11. Pipette 1µL of loading buffer for each extract and for 4 Lambda controls onto a piece of Parafilm.
12. Aspirate 4µL of the first extract and mix with a drop of loading buffer. Then load 5µL of the mix into the first well of the gel.
13. Repeat this for each sample and Lambda DNA control standards of approximately 50, 25, 12.5, 6.25 ng/µL.
14. Close the lid on the gel tank and then connect the leads to the tank and to the power pack (red to +, black to -).
15. Set the voltage to 170V and run until the loading buffer front has migrated approximately 1 cm.
16. Switch off the voltage, disconnect, open the lid, take out the gel and place on the UV transilluminator. UV protection glasses, a face visor and gloves must be worn when viewing the gel.
17. Estimate concentrations by making visual comparisons between the band intensities of the extractions and the Lambda DNA standards.
18. Take a picture using the digital camera system or equivalent, print and attach to the worksheet.

4.9.3.2.2. 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 TE buffer 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. Analyze 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. Using 1 µL sample DNA pipette 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.
16. Clean after use by wiping with 70% ethanol followed by distilled water.

4.9.4 Forms

Quality Manual Attachment P - DNA Extraction and shipment to UCL.

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

5.10.1. Purpose

Used by the **central mycobacteriology laboratory** for Drug Susceptibility Testing (DST) for streptomycin (S), isoniazid (I), rifampicin (R), ethambutol (E), moxifloxacin (M), and pyrazinamide (Z).

DST to S, I, R, E and M is performed using the Bactec MGIT 960 SIRE kit, whereas DST to Z is performed using the Bactec MGIT960 PZA kit.

Susceptibility testing will be performed on pre-treatment isolates from the baseline and the isolates from patients suspected of failure or relapse after treatment (a positive culture at or after week 17 (4 month treatment arm)/week 26 (6 month treatment arm) and any new positive culture thereafter) in order to identify the presence of resistance to any of the study drugs. If the baseline MGIT is contaminated and pure culture cannot be obtained, it is acceptable to use the screening to week 4 culture as a back up to perform the susceptibility profile.

Note:
For central mycobacteriology testing, HAIN MTBC assay (SOP12) or GeneXpert (SOP6) is to be used to confirm *M.tb* complex prior to DST by MGIT. If HAIN MTBC does not confirm *M.tb* complex, repeat the test on the next positive culture received from the local mycobacteriology laboratory.

5.10.2. Principle

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.

5.10.3. 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 Pyrazinamide MGIT kit reagents
- Moxifloxacin powder
- BD BACTEC MGIT supplement (for both 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 LJs:

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

5.10.3.1. Preparation of drug stocks for susceptibility testing

Drug stocks and preparation of MGIT tubes can be carried out outside of the CL3 laboratory. Record all details of DST kit lot numbers and expiry dates, and date of drug reconstitution on Quality Manual Attachment Eiv-Evi

TASK	INSTRUCTIONS
***NOTE* – the following may be reconstituted with different volumes. Failure to use the appropriate volume of sterile distilled/deionised water for reconstitution of the drugs will invalidate these tests**	
Prepare BACTEC™ MGIT™ 960 SIRE Kit	• Reconstitute each BACTEC™ MGIT™ 960 SIRE Kit Streptomycin lyophilised drug vial with **4ml** of sterile distilled/deionised water to make a stock solution of 83µg/ml. • Reconstitute each BACTEC™ MGIT™ 960 SIRE Kit Isoniazid lyophilised drug vial with **4ml** of sterile distilled/deionised water to make a stock solution of 8.3µg/ml. • Reconstitute each BACTEC™ MGIT™ 960 SIRE Kit Rifampicin lyophilised drug vial with **4ml** of sterile distilled/deionised water to make a stock solution of 83µg/ml. • Reconstitute each BACTEC™ MGIT™ 960 SIRE Kit Ethambutol lyophilised drug vial with **4ml** of sterile distilled/deionised water to make a stock solution of 415µg/ml.
Prepare BACTEC MGIT 960 Pyrazinamide Kit	• Reconstitute each BACTEC™ MGIT™ 960 PZA drug vial with **2.5ml** of sterile distilled/deionised water to make a stock solution of 8000ug/ml
***NOTE* – On receipt of SIRE and pyrazinamide kit reagents, store the lyophilised drug vials at 2 - 8°C. Once reconstituted, the antibiotic solutions should be aliquotted 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. Once thawed, use immediately. Discard any unused portions.**	
Prepare Moxifloxacin stock for BACTEC MGIT 960	• Moxifloxacin will be provided in powder form by Bayer, and supplied by UCL. The powder can be stored at room temperature. • Prepare a 100x stock solution of 16.6mg/ml in sterile distilled/deionised water and mix/vortex until the powder is **fully** dissolved (clear pale yellow solution). Filter sterilise the solution and aliquot as appropriate. • This solution can be frozen at -20°C or colder for up to 6 months. Once thawed, use immediately and do not refreeze. Discard any unused portions. Note: before using the 100x stock solution must be diluted 1:100 (10µl into 990µl) in sterile ddH_2O to make a working solution of 166µg/ml.

5.10.3.2. Preparation of MGIT tubes for DST testing

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 number. In addition, label tubes with one of each of the following: GC (Growth Control), STR (streptomycin), INH (isonaizid), 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 PZA set:

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

For preparation of the Moxifloxacin set:

1. Label two 7mL MGIT tubes for each test isolate with the appropriate laboratory accession label and the patient number. In addition, label tubes with one of each of the following: GC (Growth Control) and MOX (moxifloxacin)
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. **NOTE- moxifloxacin stock solutions have to be diluted further before use.** Make a working solution of 166µg/ml by performing a 1:100 dilution of the stock solution in sterile distilled/deionised water (e.g. 10ul of moxifloxacin stock in 990µl of ddH2O).
5. Aseptically pipette 100 µL of 166µg/mL moxifloxacin solution to the appropriately labelled MGIT tube. Discard any unused working solution immediately after use.
6. No antibiotics should be added to the MGIT GC tube.

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
MGIT PZA	8000µg/ml	100µl	100µg/ml
MOX	Stock solution (100x) -16.6mg/ml Working solution – 166µg/ml	100µl	2.0µg/ml

5.10.3.3. Using inoculum from positive MGIT – carried out in BSC in CL3 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. Mix well by vortexing to break up the clumps and let the large clumps settle out before taking the supernatant undiluted into the DST drug tubes.

Days 3, 4 and 5 – the growth in the tube should be diluted before use. Mix well by vortexing to break up the clumps and let the large clumps settle out. Dilute the supernatant 1:5 (1mL of broth in 4mL of sterile saline) and use this well mixed 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.

5.10.3.4. Using an inoculum from LJ – carried out in BSC in CL3 laboratory

1. All preparations must be made from the pure cultures of *M. tuberculosis.* 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).

5.10.3.5. Growth Control tube preparation and inoculation – carried out in a BSC in CL3 laboratory

For SIRE and Moxi Growth control Tubes:

1. Aseptically pipette 0.1mL 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 (10% growth control)
2. Mix the GC suspension thoroughly.

3. Inoculate 0.5mL 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

5.10.3.6. Inoculation of tubes containing test drugs – carried out in BSC in CL3 laboratory

1. Aseptically pipette 0.5mL of the organism suspension into each of the six remaining drug tubes (STR, INH, RIF, EMB, PYZ and MOX), 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. 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, PZA)
    - Moxi – 2 tube carrier set – load as undefined drug (GC, Moxi)
5. 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.

5.10.3.7. 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.

The BACTEC MGIT 960 automatically interprets these results and reports a susceptible (S) or resistant (R) result for the SIRE and PZA tests.

For the moxifloxacin, 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, whereas if the growth units are less than 100 the isolate is sensitive. This 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' – this means the run has failed because the growth control tube reached 400 GU outside of the acceptable time frame.
SIRE- 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.

5.10.3.8. Confirming resistant isolates

All resistant isolates should be verified by preparing a blood agar plate and ZN smear from the resistant tube, as described for positive MGIT cultures (see SOP8). This will confirm the culture was pure and the resistant result not caused by growth of contaminating bacteria.

- If the blood agar plate shows no growth **and** the smear shows no concomitant flora, you can accept the resistant result.
- If the blood agar plate shows growth and / or the smear shows concomitant flora, you cannot use the resistant result, repeat the susceptibility testing with a pure *M.tuberculosis complex* culture.

Mono-resistance to ethambutol, rifampicin and pyrazinamide is uncommon and should be repeated to confirm the result is valid. However any resistant result should be repeated for confirmation

- The resistant result must be repeated for confirmation. This must include testing of the complete drug sensitivity panel in the case of SIRE – i.e. if an isolate is identified as isoniazid mono-resistant, all SIRE drugs must be repeated. This serves as an additional quality check to confirm the drug resistance profile for both resistant and sensitive drugs. For PZA and moxifloxacin resistant cases, only the drug for which was identified need to be repeated as these are stand alone tests
- If the results of the confirmatory test match the initial DST (and are ZN positive and BA negative), then the results can be accepted. If the repeat testing shows a sensitive 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 sensitive result is the valid result. Similarly, if any sensitive results in the SIRE panel are resistant on repeat testing, these should be repeated.

  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.

5.10.3.9. 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 *M.tb* strain H37Rv (ATCC 27294), which is sensitive to all of the test drugs. This data is recorded on attachment Eiv/Ev/Evi. If the QC fails, all results for the batch should be reviewed, new reagents purchased and testing of clinical samples repeated.

5.10.3.10. Forms

Quality Manual Attachment C – Equipment temperature log form#
Quality Manual Attachment D – Containment Level 3 checklist
Quality Manual Attachment Ei – Ziehl-Neelsen stain reagents
Quality Manual Attachment Eiii - Blood agar plates
Quality Manual Attachment Eiv – MGIT SIRE drug susceptibility testing kit
Quality Manual Attachment Ev – MGIT PZA drug susceptibility testing kit

Quality Manual Attachment Evi - MGIT moxifloxacin drug susceptibility testing
Quality Manual Attachment K – MGIT calibration tube log
Quality Manual Attachment J – BACTEC MGIT 960 daily maintenance log

5.11. SOP 11 - Drug susceptibility testing (DST) - Minimum Inhibitory Concentration (MIC)

Placeholder statement: At the time of writing the Drug susceptibility testing (DST) - Minimum Inhibitory Concentration (MIC) SOP and associated analytical method is currently under development. Once finalised this manual will be updated, up-versioned and re-issued to incorporate the MIC method.

5.12. SOP 12 – Hain GenoType MTBC

5.12.1. Purpose

Used by the **central mycobacteriology laboratory** as a rapid test for differentiation of M. tuberculosis Complex prior to DST by MGIT.

- If GeneXpert is used (SOP6), do not perform HAIN MTBC

Note:
For central mycobacteriology testing, HAIN MTBC assay or GeneXpert (SOP6) is to be used to confirm *M.tb* complex prior to DST by MGIT. If HAIN MTBC does not confirm *M.tb* complex, repeat the test on the next positive culture received from the local mycobacteriology laboratory

5.12.2. Principle

The GenoType MTBC test is based on a DNA-STRIP technology that allows, based on gyrase B gene polymorphisms, molecular differentiation of the following species/strains belonging to the *Mycobacterium tuberculosis* complex*: M.africanum, M.bovis BCG, M.bovis ssp. bovis, M.bovis ssp. caprae, M.microti,* and *M.tuberculosis/"M.canettii"*.

5.12.3. Procedure

Equipment/Reagents

- Absorbent paper
- Calibrated thermometer
- Centrifuge
- Graduated cylinder
- Biological safety cabinet (BSC)
- Micropipettors, 10-1000 µL, 200-1000 µL
- Micropipette tips (with filter plug)
- PCR tubes (DNase and RNase free)
- Shaking water bath or TwinCubator
- Sterile water (molecular biology grade)
- Thermostable DNA polymerase with buffer (recommendation: hot start enzyme; extension rate: 2- 4 kb/min at 72°C, half-life: 10 min at 97°C, 60 min at 94°C, amplification efficiency: $>10^5$ fold)
- Timer
- Tweezers
- Vortex
- Waste receptacles (including splash proof receptacle for liquids)
- Water bath or heating block (set to 95°C)
- Ultrasonic bath
- Thermal Cycler (heating rate: 3°C/sec, cooling rate: 2°C/sec, precision: +/- 0.2°C)

DNA Amplification mix (not provided in kit or mentioned above):

- 10x polymerase incubation buffer
- $MgCl_2$ solution*
- Thermostable DNA Taq polymerase

*Depending on the enzyme/buffer system used, the optimal $MgCl_2$ concentration may vary between 1.5 and 2.5mM. Please note that some incubation buffers already contain $MgCl_2$.

Kit Contents:

- Primer Nucleotide Mix (PNM) contains specific primers, nucleotides, <1% Dimethyl Sulfoxide, dye
- Membrane strips coated with specific probes (STRIPS)
- Denaturation Solution (DEN) ***ready to use*** contains <2% NaOH, dye
- Hybridization Buffer (HYB) ***ready to use*** contains 8 – 10% anionic tenside, dye
- Stringent Wash Solution (STR) ***ready to use*** contains >25% of a quaternary ammonium compound, <1% anionic tenside, dye
- Rinse Solution(RIN) ***ready to use*** contains buffer, <1% NaCl, <1% anionic tenside
- Conjugate Concentrate (CON-C) ***concentrate*** contains streptavidin-conjugated alkaline phosphatise, dye
- Conjugate Buffer (CON-D) contains buffer, 1% blocking reagent, <1% NaCl
- Substrate Concentrate (SUB-C) ***concentrate*** contains dimethyl sulfoxide, substrate solution
- Substrate Buffer (SUB-D) contains buffer, <1% $MgCl_2$, <1% NaCl
- Tray, evaluation sheet
- Manual, template

Storage and Precautions

6. Primer/Nucleotide Mix (PNM):
   Upon arrival, store at 2 - 8 °C away from any potential sources of contaminating DNA. If storage for longer than 4 weeks is required, store at -20°C and aliquot the PNMs to avoid repeated freezing and thawing.
7. Other kit components:
   Store at 2 – 8 °C.
8. Do not use any of the reagents beyond their expiry date.
9. Sputum samples from patients must always be handled under suitable safety conditions in a Containment Level 3 facility and within a Biological Safety Cabinet.
10. Refer to manufacturer's instruction for precaution in handling reagents (e.g. denaturation Solution (DEN) contains <2% NaOH and Substrate Concentrate (SUB-C) contains dimethyl sulfoxide (DMSO). Both are irritating to eyes and skin).

Process

The whole procedure is divided into three steps:

Step One: DNA isolation.
Step Two: Multiplex amplification with biotinylated primers.
Step Three: Reverse hybridization.

Step One: DNA isolation from positive cultures

1a) 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)

1b) 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.

2) Incubate bacteria for 20 min at 95°C (boiling water bath).
3) Incubate for 15 minutes in an ultrasonic bath
4) 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'). It is essential that all reagents and materials used in the set-up for DNA isolation and amplifications are free from DNAases.

1. Prepare the amplification mix (45 µL) in a DNA-free room. **The DNA sample should be added in a separated area.**
    a. Determine the number of samples to be amplified (number of samples to be analysed plus positive *(M. tuberculosis* H37Rv) and negative (sterile, molecular biology grade water) controls).
    b. Prepare a master mix containing all reagents **except for DNA** per Table **4**: and mix well, (do not vortex).

Table 9: Amplification Mix

Reagent	Volume per tube mix
Primer Nucleotide Mix (PNM)	35 µL
10 x Polymerase incubation buffer	5 µL
$MgCl_2$ solution *	**X** µL (see below)
1-2 Unit(s) thermostable DNA polymerase (refer to manual)**	Do not consider this volume in total for tube
Molecular biology grade water	**Y** µL (to obtain a volume of 45 µL without DNA)
DNA solution	5 µL (contains 20-100 ng DNA)
Final volume per sample	**50** µL (not considering volume of DNA polymerase)

*Depending on the enzyme/buffer system used, optimal $MgCl_2$ concentration may vary between 1.5 and 2.5 mM. Please note some incubation buffers already contain $MgCl_2$.

**Qiagen Hot Start Taq is recommended. However alternative quality assured Taq polymerases may be used provided the assay has been validated with this enzyme and evidence of optimisation are available for review.

2. Aliquot 45 µL of the amplification mix into each of the amplification PCR tubes.
3. The DNA solution should be added to the PCR tubes in a separate area. A negative control sample contains 5 µl of distilled or PCR grade water instead of DNA solution.

Table 10: Amplification profile

Step	Number of cycles[a]	Temperature	Time
1	1	95°C	15 min
2	20	95°C 58°C	30 sec 2 min
	20	95°C 53°C 70°C	25 sec 40 sec 40 sec
3	1 cycle	70°C	8 min

[a] When using certain Hot start Taq DNA polymerases, the number of cycles need to be increased (refer to the enzyme's manufacturer).

Depending on the cycler used, the PCR cycling settings might have to be modified (contact local distributor for instructions).

4. Amplification products can be stored between +4 to -20°C

Step Three: Hybridization

1. Pre-warm the shaking water bath/TwinCubator to 45°C (+/- 1°C).

2. Pre-warm the Hybridization Buffer (HYB) and Stringent Wash Solution (STR) to 37 – 45°C before use. The reagents must be free from precipitates (NOTE: solution CON-D is opaque). Mix if necessary.
3. Warm the remaining reagents with the exception of Conjugate Concentrate (CON-C) and Substrate Conjugate (SUB-C) to room temperature.
    a. Using a suitable tube, dilute the conjugates with their respective buffer in a proportion of 1:100. Each strip will need 10 µL concentrate added to 1 mL of its respective buffer.
        i. CON-C (orange) with CON-D (NOTE: CON-C must be diluted before each use)
        ii. SUB-C (yellow) with SUB-D (Diluted SUB-C is stable for 4 weeks if stored at room temperature and protected from light)
    b. Mix conjugate and buffers well and bring to room temperature.
4. Dispense 20 µL of Denaturation Solution (DEN, blue) into the corner of each well used.
5. Add to the solution 20 µL of amplified sample. Pipette to mix well and incubate at room temperature for 5 min. Meanwhile, take strips out of the tube using tweezers and mark them with a pencil underneath the coloured marker. Always wear gloves when handling strips.
6. Carefully add to each well 1 mL of pre-warmed Hybridization Buffer (HYB, green). Gently shake the tray until the solution has a homogenous colour. Take care not to spill solution into the neighbouring wells.
7. Place a strip into each well. The strips must be completely covered by the solution and the coated side (identifiable by the coloured marker near the lower end) must face upward. If the strips have turned over as they were immersed in solution, turn them back over using tweezers. Carefully clean tweezers after each use to avoid contamination. This also applies to all the following steps.
8. Place tray in shaking water bath/TwinCubator and incubate for 30 mins at 45°C. Adjust the shaking frequency of the water bath to achieve a constant and thorough mixing of the solution. To allow adequate heat transfer, the tray must be immersed into the water by at least 1/3 of its height.
9. Completely aspirate Hybridisation Buffer. (For example, use a Pasteur pipette connected to a vacuum pump).
10. Add 1 mL of Stringent Wash Solution (STR, red) to each strip and incubate for 15 mins at 45°C in shaking water bath/TwinCubator.

Work at room temperature from this step forward:

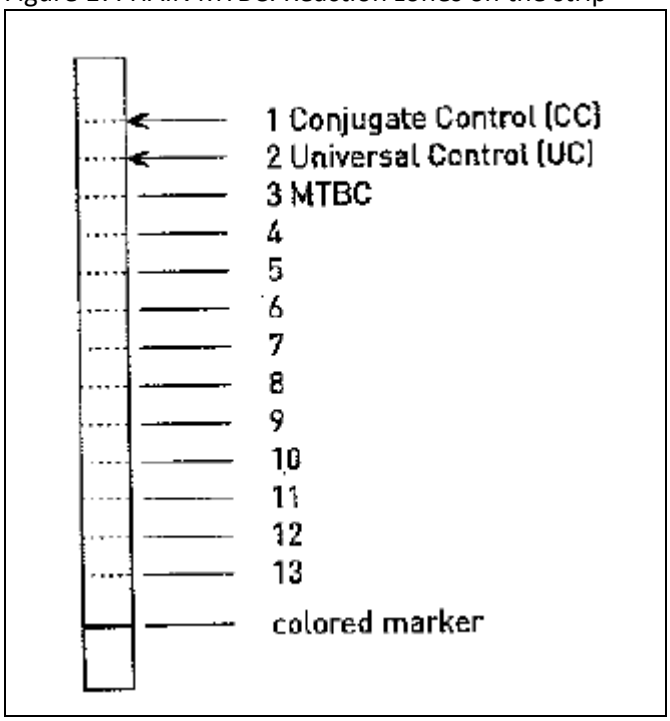
11. Completely remove the STR. Pour out wash solution in a waste container and remove all remaining fluid by turning the tray upside down and gently striking it on an absorbent paper. This also applies to other wash steps.
12. Wash each strip once with 1 mL of Rinse Solution (RIN) for 1 min on shaking platform/ TwinCubator (pour out RIN after incubation).
13. Add 1 mL of diluted Conjugate (from Step 3 in Hybridization Stage above) to each strip and incubate for 30 mins on shaking platform/ TwinCubator.
14. Remove solution and wash each strip twice for 1 minute with 1 ml of Rinse Solution (RIN) and once for 1 minute with approx. 1 ml of distilled water (e.g. use wash bottle) on shaking platform/ TwinCubator (pour out solution each time). Make sure to remove any trace of water after the last wash.
15. Add 1 mL of diluted substrate (from Step 3 in Hybridization Stage above) to each strip and incubate away from the light and without shaking. Depending on the test conditions (e.g. room temperature) the substrate incubation time can vary between 3 and 20 minutes. Extended substrate incubation times can lead to increased background staining and might impair interpretation of the results.
16. Stop reaction by briefly rinsing twice with distilled water.
17. Remove strips from the tray using tweezers, and dry them between two layers of absorbent paper.

5.12.4. Evaluation and Interpretation of Results

An evaluation sheet is provided with each kit and can be photocopied for repeat use.
When using this evaluation sheet, paste the developed strips in the designated fields by aligning the bands CC and UC with the respective lines on the sheet.

Each strip has a total of 13 reaction zones:

Figure 17: HAIN MTBC: Reaction zones on the strip



Not all bands on the strip have to show the same signal strength

If a large amount of amplicon was used, additional bands may occur

Validation:

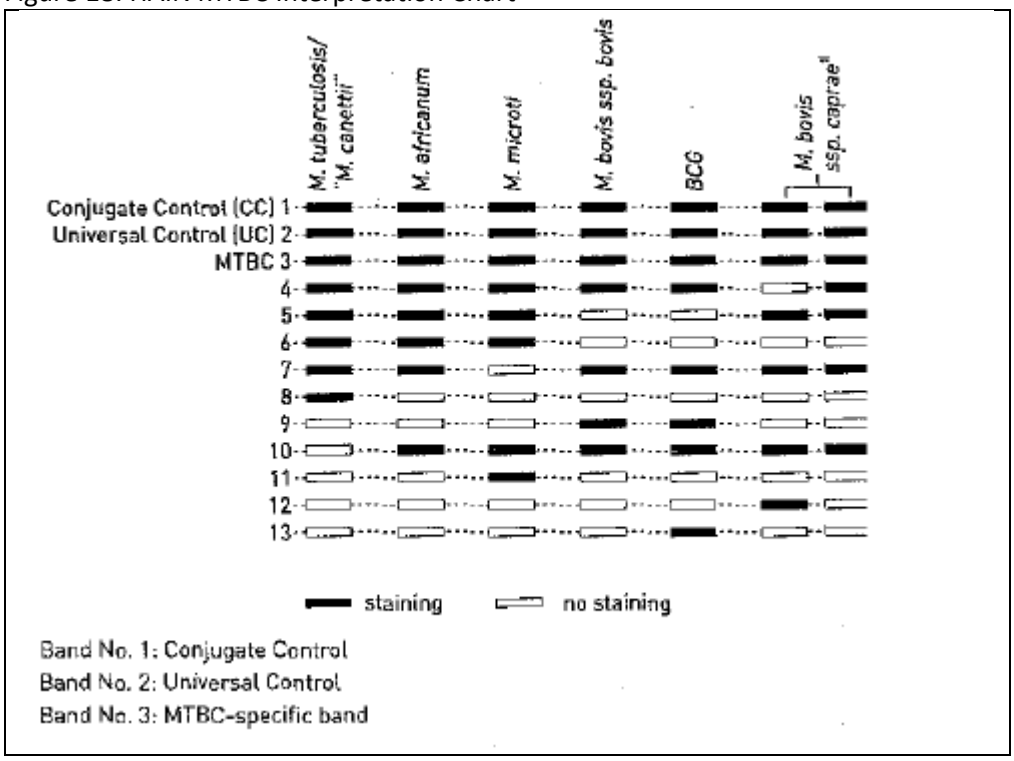
Conjugate control (CC): **A line must develop in this zone,** documenting the efficiency of conjugate binding and substrate reaction. If this is not the case, the test is invalid.

Universal Control (UC): This zone detects **all known mycobacteria and members of the group of gram-positive bacteria with a high G+C content**. If this zone and the Conjugate Control zone stain positive but the remaining banding pattern cannot be assigned to a specific mycobacterium, the respective bacterial species cannot be determined by HAIN.

MTBC: This zone hybridizes with amplicons generated from all members of the *Mycobacterium tuberculosis* complex.

Other bands: Specific probes, for evaluation see interpretation chart.

Figure 18: HAIN MTBC Interpretation Chart



Approximately 5% of the subspecies M.bovis caprae exhibit a binding pattern according to the right column. Should a banding pattern be observed which does not match with the examples above, the test is invalid and should be repeated. If a too large inoculum was used, this may need correction.

5.12.5.Quality Control

Each time the test is performed a positive (sensitive M. *tuberculosis* H37RV strain) and negative (ddH2O) control is used.

5.12.6.Forms

Quality Manual Attachment Liii - GenoType MTBC Quality Control Form

- GenoType MTBC 12 Evaluation Sheet – provided with the kit (photocopy the form)

GOOD LABORATORY PRACTICE WHEN PERFORMING MOLECULAR AMPLIFICATION ASSAYS

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.

5.12.7.Good Laboratory Practice When Performing Molecular Amplification Assays

5.12.7.1. 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.

5.12.7.2. General Considerations

b. **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 labcoats, gloves and handwashing 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.

5.12.7.3. Specimen Processing

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

g. **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.

h. **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.

i. **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.

j. **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.

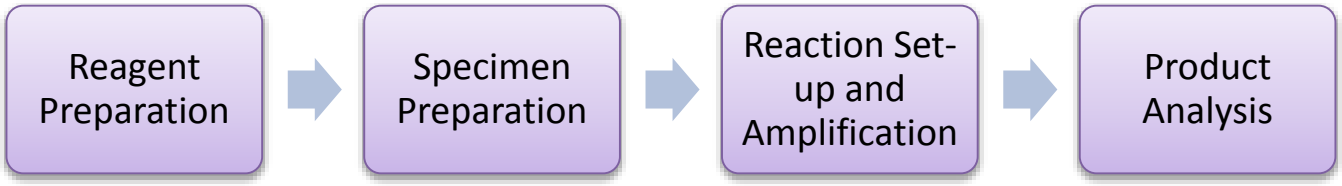
k. **The Amplification Room**

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.

l. **The Product Analysis Room**

This is the room in which post-PCR manipulations are performed eg 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 19: 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 carried out within defined areas of a larger laboratory and amplification and product analysis are in a second laboratory

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

5.13. SOP 13 - MIRU typing of M. tuberculosis

5.13.1. Purpose

Used by the **central mycobacteriology laboratory.** This procedure describes the strain typing of *M. tuberculosis* using Mycobacterial Interspersed Repeating Units (MIRU) and describes the amplification of PCR amplicons, the analysis of these amplicons by capillary electrophoresis and the interpretation of the data produced. The purpose of strain typing *M. tuberculosis* isolates is to identify whether patients relapse during the course of the trial (same strain as at baseline) or experience reinfection/new infection (different strain from baseline).

5.13.2. Principle

MIRU units are 40-100 base pairs (bp) sequences of DNA, often found as tandem repeats. Estimation of their length in bp provides an indication of the number of the repeats in each locus. The *M. tuberculosis* strain H37Rv has 41 such units. PCR and sequence analysis of 31 of these units found 24 to be variable enough be used to type strains of *M. tuberculosis* (See Appendix 1: H37Rv Genotype).

5.13.3. Procedure

Equipment / Materials

- -20°C freezer
- 4°C refrigerator
- Thermocycler
- 3130 Genetic Analyser
- Heating block set at 95°C
- Corbett PCR setup robot
- 1.5mL Eppendorfs
- 0.2µl tubes
- 96 well PCR plates (Applied Biosystems)
- Lids for PCR plates (Applied Biosystems)
- Robot tips
- 50-100µl multichannel pipette and filter tips
- 10µl multichannel pipette and filter tips
- 20µl pipette and filter tips
- 2 µl pipette and filter tips
- GIBCO BRL Electrophoresis Power Supply
- Image Quant 300 UV machine
- 60ml gel tray
- Gel combs
- Tape
- Gel electrophoresis tanks (GIBCO)

Reagents

- Qiagen HotStart taq polymerase kit (contains: 10x buffer, 5x Q solution, 25mM MgCl2, HotStarTaq)
- 5mM dNTPs
- Primer Mixes 1-8 (made from 100mM stocks)
- Hi-Di Formamide
- Bioventures MapMarker ladder
- 100bp DNA ladder (Trackit™)

- 10XTBE buffer (Ultrapure™, GIBCO)
- Molecular grade agarose powder (Bioline)
- Gel loading dye
- Ethidium bromide

PCR Amplification

Mastermixes are made up according to the Table 11:

Table 11: Volumes (µl) per reaction for multiplex PCRs

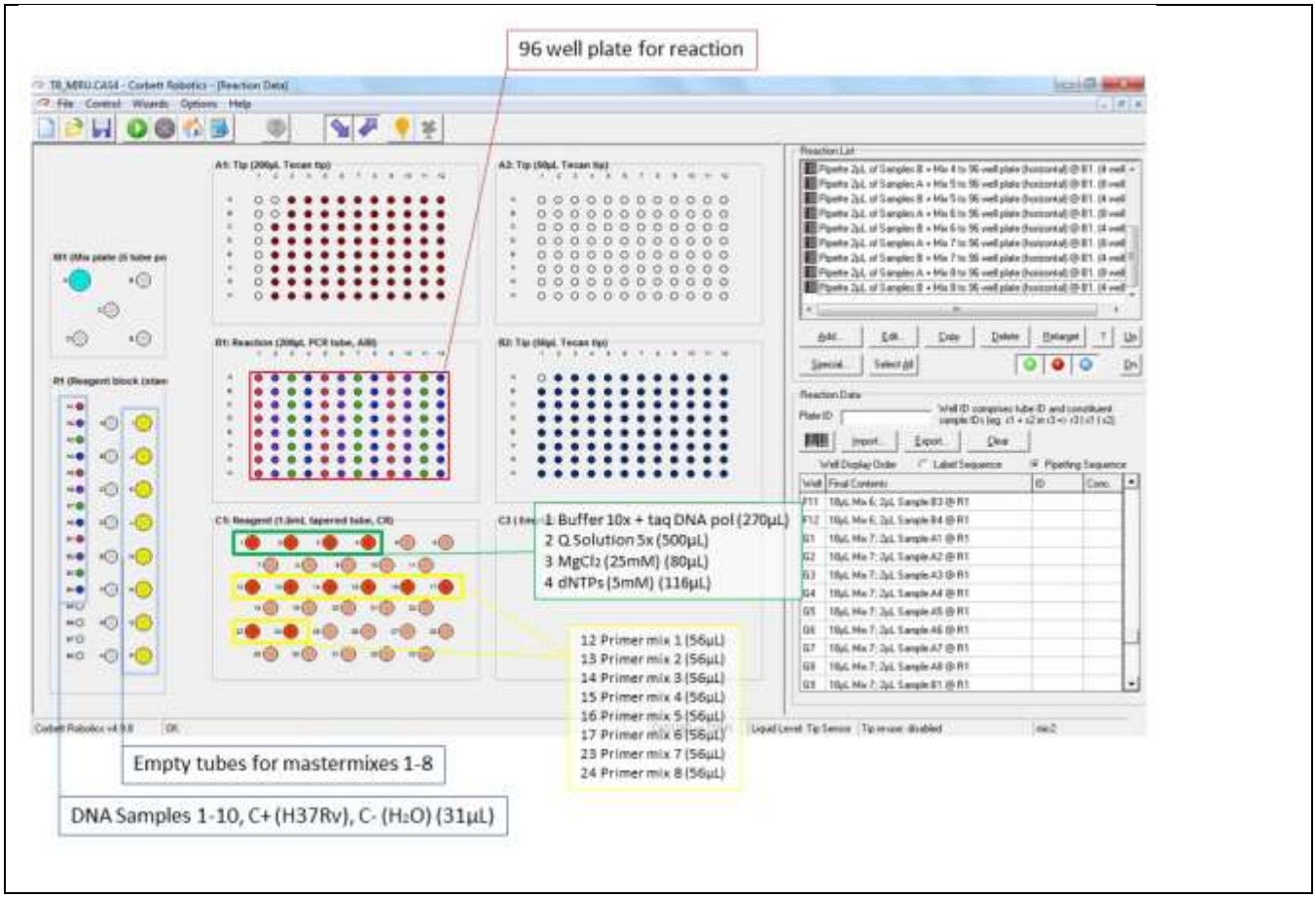
Mix	1	2	3	4	5	6	7	8
Loci	580, 2996, 802	960, 1644, 3192	0424, 0577, 2165	2401, 3690, 4156	2163b, 1955, 4052	154, 2531, 4348	2059, 2687, 3007	2347, 2461, 3171
Alias	MIRU 4, 26, 40	MIRU 10, 16, 31	ETRA	VNTR 47, 52, 53	QUB-11b, -, QUB-26	MIRU 2, 23, 39	MIRU 20, 24, 27	46, 48, 49
Final $MgCl_2$ conc	3mM	2mM	1.5mM	3mM	1.5mM	2.5mM	1.5mM	2mM
H20 (µl)	7.52	8.32	8.72	7.52	8.72	7.92	8.72	8.32
10 x Buffer (µl)	2	2	2	2	2	2	2	2
5 x Q solution (µl)	4	4	4	4	4	4	4	4
25mM MgCl2 (µl)	1.2	0.4	0	1.2	0	0.8	0	0.4
5mM dNTP (µl)	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Primer mix (µl)	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.4
Hotstart Taq (µl)	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
DNA	2	2	2	2	2	2	2	2
Final Vol	20	20	20	20	20	20	20	20

Using the Corbett PCR setup robot

The MIRU method file is opened in the robot software

1. Aliquots of the various reagents (10 x buffer with taq added, 5x Q soln, 25mM $MgCl_2$, 5mM dNTP, primer mixes), an empty 96 well plate, 8 empty eppendorfs for the master mixes, DNA samples and extra tips if required are placed in the correct position on the robot (as shown in the method file and in Figure 20 below)
2. The run button is pressed on the robot software

Figure 20: Robot layout



Alternatively PCR mixes can be set up manually:

1. In the hood in the clean room 8 mastermixes (one for each primer mix) are made up containing correct primer mix, 10x buffer, Q solution, dNTPs and taq, $MgCl_2$ and H_2O, and 18µl aliquotted into each well (according to plate plan below)
2. The plate is then transferred to the hood in MBL and 2ul of the appropriate DNA sample added to each well

For both methods a positive control (H37Rv DNA) and negative control (water) are included for each of the eight multiplexes on each 96 well plate. This leaves space for 10 DNA samples (with 8 multiplexes each). See PCR plate plan (Table 12).

Table 12: PCR plate plan

		Strain 1	Strain 2	Strain 3	Strain 4	Strain 5	Strain 6	Strain 7	Strain 8	Strain 9	Strain 10	+ve control (H37Rv)	-ve control (water)
Primer Set		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	**A**												
2	**B**												
3	**C**												
4	**D**												
5	**E**												
6	**F**												
7	**G**												
8	**H**												

PCR Cycling Settings

The 96 well plate is placed on the thermal cycler in MEL

The following PCR conditions are used (program called MIRU on left hand PCR machine in MEL)

PCR Cycling setting			
Step	**Number of cycles[a]**	**Temperature**	**Time**
1	1	95°C	15m
2	30	94°C 59°C 72°C	1m 1m 1m 30sec
3	1	72°C	10m
		4°C	∞

[a] Number of cycle may vary depending on Taq polymerase used (Contact the manufacturer for details).

The resulting PCR products are stored at 4 °C before being used in capillary electrophoresis, or at -20 °C for longer term storage.

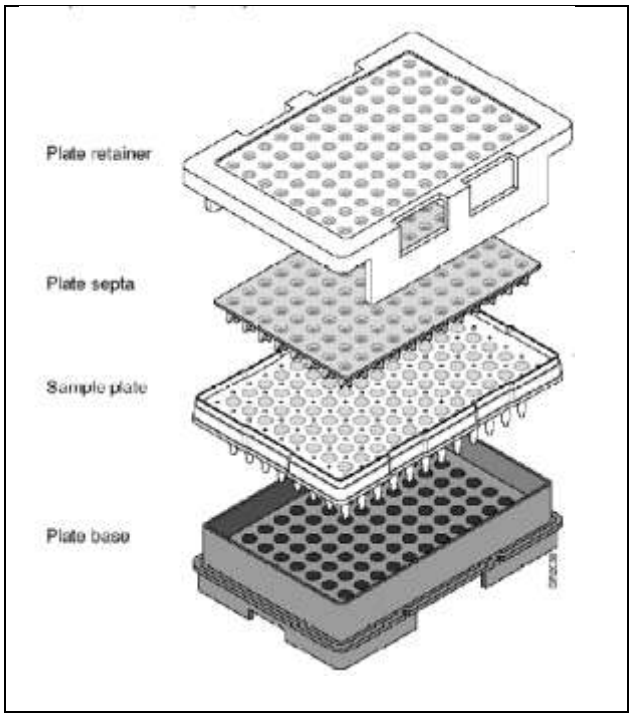
Sizing using Capillary Electrophoresis

Setting up the sequencing plate

1. Set the heating block to 95°C
2. PCR products should be diluted 1:50 (1µl PCR product into 49µl water) into a new 96 well plate
3. In an eppendorf, mix 970µl of formamide with 48.5µl of the MIRU ladder *(note: for 1 well this is 10µl formamide and 0.5µl MIRU ladder).*
4. Dispense 10.5µl of this mix into each well of the 96-well plate using a multi-channel pipette
5. Dispense 1µl of each diluted PCR product into the 96-well sequencing plate
6. Tap down the plate and centrifuge briefly if necessary to ensure that each sample is positioned at the bottom of each well and that there are no air bubbles at the bottom of the wells.
7. Place the plate on the heating block at 95°C for 5 minutes. Once removed from the heating block, place the samples on ice for a few minutes until they have cooled down.
8. Once the plate has cooled place a plate septa on top of the sample plate. Place the plate into the plate base and finish by covering the plate with the plate retainer which should produce a ‘click’ sound when

on securely (Figure **21**).

Figure 21:Plate Set-up



9. Place the plate onto the autosampler in the correct orientation and close the sequencer doors.
10. Open up the Foundation Data Collection software
11. In the left hand column, select 'Plate Manager'
12. To create a new plate, select 'New'
13. Create a plate name in the format: MIRU_STAND yyyy_mm_dd
14. Select the following for one sample:

Sample Type: 'sample', "positive control" and "negative control"
Size Standard: 'MIRU MapMarker1000'
Panel: 'Primer set 1' (2, 3, 4, 5, 6, 7, 8)
Analysis Method: 'TB_MIRU short time'
Results Group: 'TB_MIRU_RESULTS_GRP'
Instrument Protocol: 'MIRU_protocol'
Highlight all the samples to be processed. In the 'edit' tab select 'fill down'.

15. In the left hand menu select 'Run Scheduler'. Search for the newly created file by typing the file name in the search tab. It needs to be exactly the same name.
16. Once the file has been found, select the yellow plate to link the plate on the autosampler to the corresponding plate record. The plate should turn green once a link has been made.
17. Select the green start button (►) to start the run.

The run module used in MIRU_protocol is called MIRU_module and has the following settings:

Oven Temp	60
Poly Fill Vol	5020
Current Stability	5.0
Pre Run Voltage	15.0
Pre Run Time	180
Injection Voltage	1.6
Injection Time	15
Voltage no. of Steps	30
Voltage Step Interval	15
Data Delay Time	200
Run Voltage	15
Run Time	4000

Sizing using Agarose Gel Electrophoresis

For PCR products larger than 1000bp, sizing cannot be accurately performed using capillary electrophoresis. (although the presence of a product should still be visible using this method). If this occurs the sample must be run out on an agarose gel to determine its size.

1. Prepare a stock of 1000ml of 0.5XTBE buffer. To do this add 50ml of 10XTBE to 950ml of distilled water.
2. For a 60ml gel which can run up to 12 samples, weigh out 0.9g of agarose powder and add to 60mls of 0.5XTBE (1.5% gel).
3. Melt the agarose using a microwave oven for approximately 2 minutes, agitating from time to time until the powder has dissolved fully, allow the melted agarose to cool before adding 1.5ul of ethidium bromide in a fume hood.
4. Secure a 60ml gel tray by taping the edges or in gel casting clamp, and place a gel comb into the tray. Pour the melted agarose into the tray and allow to set for 10-15 minutes
5. Once the agarose has solidified remove the tape and place the gel tray into the gel electrophoresis tank. Pour enough 0.5XTBE into the gel tank so the gel is covered.
6. Pipette 2-5µl of the 100bp DNA ladder in the first, middle and last wells of the gel.
7. Mix 2µl of loading dye with 6µl of the DNA sample and pipette into the next well. Do this for all the DNA samples which require sizing on the gel.
8. Include a positive control (H37RV) and negative control (water) for each gel run.
9. Run the gel at 120 V for 60 minutes. The gel may need to be run for longer until the products can be sized against the ladder
10. Carefully remove the gel from the tank and expose to UV light using the Image Quant 300. Take a photo of the gel using the 'Iquant capture 300' program.
11. Determine the size of the PCR product by comparing with the position of the 100bp ladder.
12. Determine the corresponding repeat number, using the allele calling table in the appendices, for each MIRU loci.

Analysis of Data in Genemapper

Analysis of the results is carried out in the Genemapper software. This software analyses the capillary electrophoresis data and assigns alleles based on the 'bin sets' created for MIRU

Adding new samples to a project:

1. In Genemapper, select 'open project'. Select 'MIRU_STAND' and click 'open'.
2. Select the add new samples icon
3. To find the correct datafile for the newly processed samples select:
   E drive/UDC/data collection/data/ga3130/GA3130/completed runs/TB_MIRU/ga3130/GA313
4. Highlight the relevant file and select 'Add to list', then 'Add to project'. The new sample files will have now been added.
5. To analyse the data using the 'bin' sets created, under 'panel', select 'primer set' – these samples will then need to be re-analysed by clicking the green ▶ button

Checking ladders have been assigned correctly:

1. In the size match editor window check to see if the peaks look like they have been labelled correctly by the software
2. If not reassign by right clicking on the relevant peaks and selecting the correct value
3. Click on apply to make sure that any changes are applied
4. Go back to the samples table and reanalyse any samples for which the ladder has been changed by clicking the green ▶ button

Reviewing allele calls:
As the software does not always call the peaks correctly (eg. in presence of pull up peaks or stutter peaks) the allele calls should be reviewed for each sample. To do this:

1. In the samples plot view display the different dyes on separate graphs by selecting "Binning mode" icon in the top left hand corner of the screen.
2. Select the "Genotypes table" icon in the top right hand corner of the screen
3. Turn on the labels by selecting view→labels→horizontal labels
4. Select editing mode as peak selection (Alleles→editing mode→peak selection)
5. To **delete** an incorrect label right click on the peak or associated label and select delete allele(s). A comment box will appear allowing you to give a reason for the change (eg. Pull up peak)
6. To **change** an incorrect label right click on the peak or associated label and select rename allele (can be changed to another allele, ? or a custom label)
7. To **add a new label** to an unlabelled peak, right click on the peak (you may need to zoom in on the peak first). A box will then appear allowing you to label the peak as the appropriate allele
8. Save the project to ensure that any allele changes are saved

Any changes made to the allele calls will appear in the genotypes table for the project. This table should be regularly exported to an excel file to keep as a record of the MIRU results.

13.4 Quality Control

All new batches of reagents (Primers, HotStarTaq kit, dNTPs, Mapmarker ladder) will also be tested with H37Rv DNA, with the presence of the peaks of the expected size for all primers indicating a pass. The H37Rv genotype is in the appendices below. Results are recorded on Quality Manual Attachment M

13.5 Forms

Quality Manual Attachment M – MIRU typing

Appendices

1. H37Rv genotype

Primer set	Position	Alias 1	Alias 2	Dye	Colour	H37Rv genotype	H37Rv size (bp)
1	580	MIRU 04	ETRD	6-FAM	Blue	3'	353
1	2996	MIRU 26		VIC	Green	3	438
1	802	MIRU 40		NED	Yellow	1	408
2	960	MIRU 10		6-FAM	Blue	3	643
2	1644	MIRU 16		VIC	Green	2	671
2	3192	MIRU 31	ETRE	NED	Yellow	3	651
3	424	VNTR 42		6-FAM	Blue	2	639
3	577	VNTR 43	ETRC	VIC	Green	4	382
3	2165	ETRA		NED	Yellow	3	420
4	2401	VNTR 47		6-FAM	Blue	2	363
4	3690	VNTR 52		VIC	Green	5	562
4	4156	VNTR 53	QUB-4156c	NED	Yellow	2	681
5	2163b	QUB-11b		6-FAM	Blue	5	422
5	1955			VIC	Green	2	206
5	4052	QUB-26		NED	Yellow	5	742
6	154	MIRU 02		6-FAM	Blue	2	508
6	2531	MIRU 23		VIC	Green	6	465
6	4348	MIRU 39		NED	Yellow	2	646
7	2059	MIRU 20		6-FAM	Blue	2	591
7	2687	MIRU 24		VIC	Green	1	447
7	3007	MIRU 27	QUB-5	NED	Yellow	3	657
8	2347	VNTR 46		6-FAM	Blue	4	563
8	2461	VNTR 48	ETRB	VIC	Green	3	518
8	3171	VNTR 49		NED	Yellow	3	488

2. Composition of primer mixes for use in PCRs

Primer Mix 1			**Primer Mix 2**		
Loci (relative conc in final mix)	**100ul stock**	**1ml stock**	Loci	**100ul stock**	**1ml stock**
MIRU 4 (4uM)	4	40	MIRU 10 /ETRD (4uM)	4	40
MIRU 26 (4uM)	4	40	MIRU 16 (4uM)	4	40
MIRU 40 (4uM)	4	40	MIRU 31 /ETRE (4uM)	4	40
Each unlabelled primer (20uM)	20	200	Each unlabelled primer (20uM)	20	200
Water	28	280	Water	28	280
Total	100	1000	Total	100	1000
Primer Mix 3			**Primer Mix 4**		
Loci	**100ul stock**	**1ml stock**	Loci	**100ul stock**	**1ml stock**
42/Mtub04 (4uM)	4	40	47/ Mtub30 (4uM)	4	40
43/ETRC (2uM)	2	20	52/Mtub39 (2uM)	2	20
ETRA (4uM)	4	40	53/QUB4156 (20uM)	20	200
Each unlabelled primer (20uM)	20	200	Each unlabelled primer (20uM)	20	200
Water	30	300	Water	14	140
Total	100	1000	Total	100	1000
Primer Mix 5			**Primer Mix 6**		
Loci	**100ul stock**	**1ml stock**	Loci	**100ul stock**	**1ml stock**
QUB-11b (4uM)	4	40	MIRU 2 (4uM)	4	40
Mtub 21 (2uM)	2	20	MIRU 23 (4uM)	4	40
QUB-26 (8uM)	8	80	MIRU 39 (4uM)	4	40
Each unlabelled primer (20uM)	20	200	Each unlabelled primer (20uM)	20	200
Water	26	260	Water	28	280
Total	100	1000	Total	100	1000
Primer Mix 7			**Primer Mix 8**		
Loci	**100ul stock**	**1ml stock**	Loci	**100ul stock**	**1ml stock**
MIRU 20 (4uM)	4	40	46/ Mtub29 (4uM)	4	40
MIRU 24 (4uM)	4	40	48/ETRB (4uM)	4	40
MIRU 27 (4uM)	4	40	Mtub34 (4uM)	4	40
Each unlabelled primer (20uM)	20	200	Each unlabelled primer (20uM)	20	200
Water	28	280	Water	28	280
Total	100	1000	Total	100	1000

3. Allele calling table for the 24 MIRU loci

Locus	MIRU 02	MIRU 04	MIRU 10	MIRU 16	MIRU 20	MIRU 23	MIRU 24	MIRU 26	MIRU 27	MIRU 31	MIRU 39	MIRU 40
Convention	**154**	**580**	**960**	**1644**	**2059**	**2531**	**2687**	**2996**	**3007**	**3192**	**4348**	**802**
Allele												
0	402	175	482	565	437	150	395	285	498	492	540	354
1	455	252	537	618	514	200	447	336	551	545	593	408
2	508	329	590	671	591	253	501	387	604	598	646	462
3	561	406	643	724	668	306	555	438	657	651	699	516
4	614	483	696	777	745	359	609	489	710	704	752	570
5	667	560	749	830	822	412	663	540	763	757	85	624
6	720	637	802	883	899	465	717	591	816	810	858	678
7	773	714	855	936	976	518	771	642	869	863	911	732
8	826	791	908	989	1053	571	825	693	922	916	964	786
9	879	868	961	1042	1130	624	879	744	975	969	1017	840
10	932	945	1014	1095	1207	677	933	795	1028	1022	1070	894
11	985	1022	1067	1148	1284	730	987	846	1081	1075	1123	948
12	1038	1099	1120	1201	1361	783	1041	897	1134	1128	1176	1002
13	1091	1176	1173	1254	1438	836	1095	948	1187	1181	1229	1056
14	1144	1253	1226	1307	1515	889	1149	999	1240	1234	1282	1110
15	1197	1330	1279	1360	1592	942	1203	1050	1293	1287	1335	1164

Locus	VNTR 42	VNTR 43	VNTR 1955	VNTR 2163b	ETRA	VNTR 46	VNTR 47	VNTR 48	VNTR 49	VNTR 3690	QUB-26	VNTR 53
Convention	**424**	**577**	**1955**	**2163**	**2165**	**2347**	**2401**	**2461**	**3171**	**3690**	**4052**	**4156**
Allele												
0	537	171	92	77	195	335	247	347	326	272	187	563
1	588	208	149	146	270	392	305	404	380	330	298	622
2	639	266	206	215	345	449	363	461	434	388	409	681
3	690	324	263	284	420	506	421	518	488	446	520	740
4	741	382	320	353	495	563	479	575	542	504	631	799
5	792	440	377	422	570	620	537	632	596	562	742	858
6	843	498	434	491	645	677	595	689	650	620	853	917
7	894	556	491	560	720	734	653	746	704	678	964	976
8	945	614	548	629	795	791	711	803	758	736	1075	1035
9	996	672	605	698	870	848	769	860	812	794	1186	1094
10	1047	730	662	767	945	905	827	917	866	852	1297	1153
11	1098	788	719	836	1020	962	885	974	920	910	1408	1212
12	1149	846	776	905	1095	1019	943	1031	974	968	1519	1271
13	1200	904	833	974	1170	1076	1001	1088	1028	1026	1630	1330
14	1251	962	890	1043	1245	1133	1059	1145	1082	1084	1741	1389
15	1302	1020	947	1112	1320	1190	1117	1202	1136	1142	1852	1448

(Strains H37Rv, H37Ra, BCG and 1% of clinical strains)

Allele	MIRU 04
0'	122
1'	199
2'	276
3'	353

Appendix 1: Hain Life Sciences, GenoLyse ®, Ver 1.0, Instructions for Use, 10/2011

GenoLyse_1011_51610-08-02.pdf

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

XpertMTB_Broch_R9_EU.pdf