REMOX Clinical TB Trial
Laboratory Manual Section 1

SPUTUM COLLECTION AND TRANSFER

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Approved by: Dr Tim McHugh  Signature and date:

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PURPOSE
Proper collection and transport of sputum specimens is required to ensure quality laboratory results. Adherence to procedural details will result in collecting adequate and quality sputum specimens for analysis in the mycobacteriology laboratory and maintaining correct identity of the specimen.

PRINCIPLE
Sputum specimens collected at a single time point are referred to as 'spot' sputum specimens. These are preferably collected in the early morning, as they have more mycobacteria and are less likely to be contaminated with other microbes. A 'pooled' sputum specimen is all sputum produced by a given patient during 16 hour period, usually, 4pm - 8am that is placed in "honey pot" specimen container. Pooled specimens have a slightly higher yield on culture than early morning spot specimens but also are more likely to be contaminated.

For all routine sputum smear and culture, early morning (first sputum produced by the patient that day) and/or spot specimens will be collected at the timepoints set out in the summary chart of the protocol. One sputum sample will be collected up to and including week 26. Two sputum samples will be collected at months 9, 12, 15 and 18. If a patient is positive post week 17 another sample will be requested to confirm potential relapse or treatment failure. Note; if the month 18 samples are found to be smear positive but culture contaminated additional samples should be requested.

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Pooled specimens will not be accepted for routine sputum smear and culture, and will only be collected from a subset of patients for quantitative colony counts and RNA extraction.

**PROCEDURE**

**Materials**

- Sterile, disposable, single use, screw capped jars for spot samples, or ‘honey pot’ specimen jars (180ml or similar capacity) for 16 hour pooled sputum
- Permanent marker
- Disposable gloves
- Refrigerator
- Certified digital thermometer
- Cool box
- Cold packs
- Six digit labels supplied by REMoxTB – lab accession numbers.

**Forms**

- Quality Manual attachment B – Specimen Transfer Form
- AND/OR
- Sputum Request form – this is a site-specific form used for routine laboratory requests. The patient name should be replaced with either the screening or study number, and the form should be clearly marked ‘REMoxTB Clinical Trial’. It is acceptable for a site to use the REMoxTB specimen transfer form as the request form (the section completed by the clinician covers the same details in many cases) if this is preferable and saves any unnecessary duplication.

**Dispatch of the specimen from the clinic site**

Before transportation from the clinic, a designated staff member of the REMoxTB trial must verify for each transport box that:

1. The total number of sputum containers in the box corresponds to the accompanying Sputum Request forms and/or Specimen Transfer forms (one for each sputum specimen).
2. The identification number (study or screening number) on each sputum container corresponds to that on the Sputum Request form and/or the Specimen Transfer form.
3. The Sputum Request forms and/or Specimen Transfer forms contain the requested information to complete the clinical section in full. The ‘Dispatch section’ of the Specimen Transfer form must be completed and signed by the attending physician and/or driver (if delegated to do so).
4. Ensure that there are an appropriate number of frozen cool packs in the container in order to maintain the temperature between 2°-8°C.
5. The maximum/minimum thermometer must be allowed to reach equilibrium in the container so that an accurate measurement can be taken.
6. Make a copy of the specimen transfer forms to keep filed at the clinic for future reference.

When this verification is completed, a designated staff member of the REMoxTB trial must

1. Put the Specimen Transfer forms and/or the Sputum Request Form in an appropriate envelope, to be transported with the transport container.
2. Reset the max/min thermometer before reading temperature at time of departure, just prior to transport. Record the temperature, and the name and signature of the driver or courier transporting the samples in the ‘Transport section’ of the Specimen Transfer form.
Receipt of sputum specimens in laboratory

1. The laboratory must process all specimens as soon as possible but no later than 48 hours after specimen was collected - record the date and time the samples are received on the accompanying forms.
2. The laboratory resets the max-min thermometer before reading the temperature of the container at the time of receipt. The temperature is recorded in the ‘Laboratory section’ of the Specimen Transfer form.
3. The laboratory matches specimens with Sputum Request/Specimen Transfer forms in the presence of the driver/courier.
4. The laboratory technician receiving the samples records their name and signature on the ‘Laboratory section’ of Specimen Transfer/request form.
5. The laboratory must also carry out a visual check of the specimens to confirm they are in good condition, and record information on the Specimen Transfer form.
6. If the laboratory finds mis-labelling, incomplete labelling, incomplete forms or mismatching of specimen labels and accompanying forms, the following procedures are followed.

Labelled specimens

1. Sputum Request and Specimen Transfer forms and specimen labels must be fully completed and fully match.
2. The laboratory will contact the collecting station or clinic to obtain any needed information before the specimen is processed. The contact with the station or clinic will be documented in writing and initialled.
3. Specimens without the matching forms are not accepted. The laboratory will contact the collecting station or clinic. This contact should be clearly documented on a telephone/email/fax log.
4. The specimen will NOT be processed until a fully completed Specimen Transfer Form is received.

Unlabelled specimens

Unlabelled specimens MUST not be processed.
1. If specimens are not labelled at the time of collection the clinic will be contacted and will be asked to resolve and correct the discrepancies and complete the labelling process.
2. Pending this correction the specimen will be stored in the laboratory in the refrigerator.
3. Receipt of incompletely labelled specimens must be noted in the Continuous Quality Improvement Form (QM attachment Q).
4. The specimen will NOT be processed until the labelling has been reliably corrected/completed, if necessary new sample(s) will have to be requested.

Specimens that arrive outside the designated temperature range

Every effort should be made to maintain the sputum samples within the specified temperature range 2-8°C, following receipt of the sample at the clinic, transport to the lab, and prior to processing at the laboratory. This is essential to minimise the growth of any contaminating bacteria present in the sputum sample.

1. If a sputum sample arrives at the laboratory outside of the agreed range, a repeat sample should be requested as soon as possible.
2. If it is not possible to get another sample (i.e. the patient has left the clinic), the original sample should be processed to avoid losing the time-point.
3. Corrective action should be taken, and documented on QM attachment Q, to rectify the problems with sample transport conditions.
4. Sputum transport temperatures will be reviewed if the site has high contamination rates to determine if this is a contributing factor.

Small volume specimens

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.

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If this is not possible and the sputum sample is less than 2ml (and also a good quality specimen), it should still be processed. Although sputum processing is less accurate when the specimen volume is less than 2ml (because the sputum pellet is re-suspended in 1.5-2ml of PBS after centrifugation), it is still valuable to determine whether acid fast bacilli can be detected.

The volume should be noted as less than 2ml on the laboratory source documents so these samples can be excluded from the quantitative culture analyses (e.g. MGIT TTP) if required.

Note: specimens that comprise of saliva only must not be processed, another sample should be requested

Logging-in of sputum specimens in laboratory

1. Assign a unique laboratory accession number to the specimen using the sticky labels provided.
2. Place a laboratory accession number label on the Sputum Request/Specimen Transfer forms for each specimen.
3. The lab accession number is used to label tubes for all subsequent downstream processing of this specimen - cryotubes, MGIT tubes, agar plates, microscope slides, etc, and for reporting data (CRFs or dockets).
4. The specimen register should be used to link the specimen details with the accession number
5. If the specimens are not processed within 30 minutes of receipt in the laboratory place in the designated sputum refrigerator and record the time and fridge ID on the specimen transfer forms.

The Specimen Transfer forms will be stored at the laboratory,— a copy of the request or transfer form may be sent back to the clinic and/or data office as appropriate.
SPUTUM PROCESSING

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Note: All procedures related to sputum culture need to be carried out in a Containment Level 3 (P3) laboratory

PURPOSE

Processing sputum specimens has two objectives: decontamination of bacteria other than mycobacteria and liquefaction of organic debris in the specimen. 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.

In this study, sputum specimens collected at screening, baseline, weekly during intensive phase treatment, monthly during continuation phase treatment and three monthly during follow-up visits will be processed in this manner, as only qualitative or semi-quantitative tests will be performed at these time intervals.

PRINCIPLE

N-acetyl-L-cysteine (NLC), a mucolytic agent, is used for rapid digestion, enabling the decontaminating agent, NaOH, to be used at a lower final concentration (in sputum) of 1%. NLC loses activity rapidly in solution, so it
MUST be made fresh daily. Sodium citrate is also included in the decontamination solution as it aids in liquefaction by binding heavy metals, thus stabilising NALC. Phosphate buffer is used to neutralise the NaOH and dilute the homogenate to lessen the viscosity and specific gravity prior to centrifugation. Mycobacteria have a low specific gravity and may remain buoyant during centrifugation. A relative centrifugal force of at least 3,000 g (NOT 3000 rpm – the centrifuge must be calibrated) is adequate to sediment mycobacteria. The rate at which mycobacteria sediment is critically dependent on time of centrifugation and relative centrifugal force applied to the specimen. While prolonged time of centrifugation can offset a lower relative centrifugal force, increased centrifugation time increases the temperature of the specimen, which leads to additional killing of mycobacteria (hence the need for a refrigerated centrifuge).

**PROCEDURE**

**Materials**

- Biological safety cabinet (BSC), Class I (NB Class 2 hoods acceptable if conform to British Standard, BS EN 12469:2000 Performance criteria for microbiological safety cabinets)
- Disinfectant with activity against Mycobacteria (specified in local Health and Safety documentation)
- Waste receptacles (including splash proof receptacle for liquids)
- Paper towel soaked in appropriate disinfectant, in case of spills
- 50 mL conical, graduated polypropylene centrifuge tubes with tight screw cap
- NALC powder
- 4% NaOH
- 2.9% Na citrate
- Phosphate buffer (pH 6.8)
- Vortex mixer
- Test tube rack for 50 mL centrifuge tubes
- Refrigerated centrifuge with sealed buckets and inserts suitable for 50 mL tubes
- Timer
- Microscope slides, frosted one side, one end, clean and dry
- Transfer pipette, graduated, about 6 mL capacity, sterile
- Slide warmer set at 65 to 75 °C
- Pencil for labelling slides
- Permanent marker
- Pipette

**Forms**

- Quality Manual attachment C – Equipment Temperature Log Form
- Quality Manual attachment D - Containment Level 3 Laboratory Checklist
- Quality Manual attachment F – Specimen Processing Form
- Quality Manual attachment G/Gi – Weekly Contamination Rates

**Specimen Registration**

1. Follow log-in procedure (LM1. Sputum Collection & Transfer). Sputum samples should be processed as soon as possible and no longer than 48 hours after the sample was produced. Samples should be refrigerated if they are not processed within 30 minutes of receipt in the laboratory (record time and fridge
2. The patient data and laboratory accession numbers on the Specimen Transfer/Sputum Request form must then be double-checked. Laboratory accession labels should have been attached to the specimen container and the accompanying forms 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 bijou for storage of decontaminated specimen
   b) The patient screening number or study number (patient identifier - post enrolment) are also written in permanent marker on all tubes and containers that will subsequently contain the patient specimen
   c) A microscope slide is labelled with the laboratory accession number and the patient screening number or study number using a pencil or grease pen.
   d) The MGIT tube identity number is recorded and will need to be transcribed onto CRF (CRF 5a)/Docket. 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 (eg 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 500ml 4% NaOH to 10g NALC and mix to dissolve. Mix gently (do NOT shake vigorously).
2. Pour into a sterile, break-resistant glass bottle.
3. Add 500ml 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 eg. add 200ml 4% NaOH to 4g NALC, mix well and pour into an appropriately sized sterile bottle. Add 200ml sodium citrate to the NaOH/NALC mix to give 400ml working solution.
5. Transfer some of the working solution into a sterile 50ml tube and use this to add to the specimens. This avoids introducing contamination into the stock bottle.

Process of Decontamination using NALC / NaOH / sodium citrate

1. Before processing specimens, prepare a splash proof waste receptacle with disinfectant at the appropriate concentration and place a paper towel soaked in an appropriate disinfectant on the work surface inside the Biological Safety Cabinet.
2. Ensure refrigerated specimens have been brought to room temperature before processing.
3. Complete Quality Manual attachment F with details of the decontamination solution reagents, and list the samples that are being processed in the batch on Quality Manual attachment F. A batch consists of no more than 7 specimens in total.
4. Include one negative control with each batch of specimens (maximum total of 8 tubes per batch), this should be a blank tube (NaOH-NALC only) placed in the middle of the batch and should be included in microscopy and MGIT culture. It is important this is treated exactly the same as the patient samples. If there is only 1 specimen in the batch a negative control is not required. Details of this should be recorded in quality manual attachment F.
5. Work methodically with the tubes on one side and discard buckets close to the specimens, to avoid spillages/confusion of samples. Always keep 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. If this happens, wipe the tube with the paper towel soaked in disinfectant
prior to removal from the cabinet.

7. Transfer specimen into a 50 ml centrifuge tube with a screw cap. Make a note of the volume on the lab worksheet/lab book.

8. Add NaOH-NALC sodium citrate solution (see notes above) in a volume equal to the quantity of specimen. Tighten the cap.

9. Vortex for about 15-30 seconds. Invert the tube so the whole tube is exposed to NaOH-NALC solution.

10. Start timer.

11. Repeat parts 6-8 for the subsequent specimens at 30 sec or 1 minute intervals. Record the start time for the first and last samples and the time interval on quality manual attachment F.

12. It is important to mix well during the incubation period to expose all the sputum to the digestion solution.

13. Make sure the specimen is completely liquefied. If still mucoid, add a small quantity of NaOH-NALC sodium citrate solution. Mix well.

14. After 20 minutes, add phosphate buffer (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 sec or 1 minute 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 samples on quality manual attachment F to document the exposure time. It is VERY important that the buffer is added to each specimen within 20 minutes of adding the NALC 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 tubes in 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 3000 g (note this is not 3000 rpm) or more for 15 minutes at 4°C.

18. After centrifugation, remove centrifuge buckets and place in the Biological Safety Cabinet before opening up. Do not open the buckets on the open bench in case there has been a spillage or breakage during centrifugation. Carefully decant as much of the supernatant as possible into a suitable splashproof container containing a mycobactericidal disinfectant. 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. Make sure the sediment is not lost during decanting of the supernatant fluid. Add a small quantity (1 to 2 ml) phosphate buffer (pH 6.8) using a sterile 3ml pasteur pipette and resuspend the sediment using a pipette or vortex mixer if required.

19. Use the resuspended pellet to prepare smears for acid-fast bacteria (AFB) microscopy (LM3) and for inoculation of MGIT tube (LM5) and LJ slope (LM5).

20. Store any leftover sediment at 4°C, for at least 7 days until it is confirmed that the inoculated media are not contaminated.

21. If contamination is detected in either the MGIT or LJ cultures within 10 days, the de-contamination procedure should be repeated with this remaining sediment following exactly the same procedure and new cultures inoculated. This repeat decontamination must be noted on the source documentation so as the CRFs/Dockets can be completed correctly.

Note: Store all Month 18 sediments until the MGIT and LJ protocols are complete -- if the sample is found to be smear positive culture contaminated and another sample can not be obtained, the original sediment can be retreated at any time-point within the protocol and new cultures inoculated. The decontaminated sputum sediment can be stored directly at -80°C after 7 days.

NOTES

- All processing must be done in the biological safety cabinet.
- 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 eight specimens in any single batch, including the negative control.

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When working with multiple specimens, remove only the caps from the tubes of the same specimen (same lab 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 that set out in this protocol.

The contamination rate should be monitored as part of internal quality control. This is determined by calculating the proportion of contaminated tubes/slopes out of total number of tubes/slopes completed over a given period of time. An acceptable rate is 3 - 8%. See below for definition of contamination. This should be recorded on quality manual attachment G/Gi.

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.

High pH may also cause transient false fluorescence.

It is not necessary to neutralize the processed specimen, especially with the NaOH-NALC method, although some laboratories routinely neutralize the processed specimen.

With NaOH-NALC digestion, do not agitate the tube vigorously. Extensive aeration causes oxidation of NALC and makes it ineffective.

If the specimen has noticeable quantities of blood mixed with it (not just specks of blood), 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 indicated on the worksheets or lab book.

Mycobacteria, being hydrophobic, are difficult to pellet by centrifugation. Lower centrifugation speed (g-force) would not sediment mycobacteria very well and some bacteria would be lost during decanting the supernatant, which will affect the positivity rate. Higher centrifugation speeds and longer time (maximum 25 minutes) result in a better concentration of mycobacteria, which positively affects smear and culture positivity will affect the positivity.

Temperature build up during centrifugation increases the killing effect on mycobacteria which will affect the positivity rate and time-to-detection adversely. Always use a refrigerated centrifuge.

Reagents for during the digestion/decontamination step should not be chilled but should be at room temperature. Lower temperatures reduce the digestion decontamination process of NaOH-NALC.

**Definition of Contaminated Samples**

Only samples that flag positive in the MGIT and show growth on blood agar plates are to be considered contaminated. An exception to this is those samples visibly contaminated (e.g. with fungi) which do not show growth on blood agar.

For LJ slopes only samples with visible contamination, ZN negative bacterial growth or slopes that have disintegrated should be considered contaminated. Slopes showing colour change only (but no visible growth) should not be considered as contaminated unless confirmed using blood agar and/or microscopy.

**Internal Quality Control Measures**

- Laboratory QC of reagents and media.
- Record of decontamination concentration and times.
- Continuous record of contamination rates.
- Use of negative controls to identify cross-contamination, and contamination of stock solutions
- Record of temperature controlled equipment.
- Record of maintenance and certified servicing of regularly used equipment such as centrifuges, pipettes etc, BSCs.
**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.

**PRINCIPLE**
With the fluorochrome stain Auramine, organisms fluoresce bright yellow, non-specific debris stains pale yellow, and the background is almost black. Fluorochrome stain is more sensitive than Ziehl-Neelsen (ZN) because the smear can be examined under a lower power since the bacilli stand out brightly allowing more fields to be examined in the same amount of time than with Ziehl-Neelsen. For Ziehl-Neelsen stains, the principle of stain, decolouriser and counter stain is the same as for fluorochrome staining. The Ziehl-Neelsen method uses a carbol fuchsin stain, acid alcohol decolouriser, and methylene blue or malachite green counter stain.

**SUMMARY OF PROCEDURE**
- Acid fast bacteria (AFB) smears are performed on all sputum specimens received (for screening and from enrolled patients during the trial according to the schedule in the protocol).
- Smears are processed on the day of decontamination of the specimen and the results of microscopy are entered into the appropriate laboratory worksheet on the day of reading.
- All specimens are decontaminated and concentrated prior to the preparation of the smear (LM2).
Auramine may be used to determine the presence or absence of AFB.

Confirmation and semi-quantification of the presence of AFB is undertaken using a ZN stain. The ZN stain can be performed on the slide which has been auramine stained.

A ZN stain is also used to confirm the presence of mycobacteria in positive cultures using a freshly prepared smear (see LM 5.1 and LM 5.2).

A fixed amount of each specimen (30 µl) is transferred to a frosted slide and heat fixed (65 – 75 °C).

Prior to heat-fixing, the slides must remain in the Biological Safety Cabinet (BSC) inside the Containment Level 3 laboratory. After heat-fixation, smears can be stained outside the BSC in the CL3 laboratory, and once dry can be examined by microscopy in either the CL2 or CL3 laboratory. Slides must be stored in a slide holder (allowing safe storage and organised archiving) in the CL2 or 3 Laboratory. All sputum smear slides should be kept as a reference in an organised manner (easily retrievable if required), slides for confirmation of positive cultures may be disposed of.

PREPARATION OF SMEARS

Materials

- Biological Safety cabinet
- Disinfectant with activity against Mycobacteria (specified in local Health and Safety documentation)
- Waste receptacles (including splash proof receptacle for liquids)
- Paper towel soaked in appropriate disinfectant
- Microscope slides, frosted at one end, new
- Pencil for labelling slide
- Hot plate
- Pipette
- Aerosol Resistant Tips

Forms

- Quality Manual attachments EI/Eii - Reagent QC form

Procedure

1. Label frosted end of slide in pencil with laboratory accession number, Study number or the patient screening number (if pre-enrollment), and date.
2. Working in an appropriate Biological Safety Cabinet, vortex the decontaminated deposit to mix it thoroughly.
3. Place specimen container in a suitable rack whilst working with it. Any lids removed must be placed on a paper towel soaked in appropriate disinfectant whilst the sample is being processed.
4. Using a micropipettor with sterile aerosol resistant tips (or an appropriate loop or pastette) transfer 30µl of well-mixed resuspended pellet from the decontaminated sputum specimen onto the slide, spreading it to cover an area approximately 2cm diameter circle. Allow the slides to air dry before heat fixing.
5. Place the slides on a hotplate set at between 65°C to 75°C for at least 15 minutes to heat fix the samples. Work systematically through the samples with slides on one side and the discard bucket in close proximity (often best at back of cabinet). Dispose of waste pipette tips or loops into the discard bucket. After heat fixing, slides can be removed from the cabinet.

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STAINING OF SMEARS

Materials
- BDH Ziehl-Neelsen stain (carbol fuchsin, 3% acid alcohol, malachite green or methylene blue)
- Auramine stain (auramine, 1% acid alcohol, 0.5% potassium permanganate)
- Staining sink
- Staining rack
- Slide drying rack
- Distilled water
- Wash bottle
- Cover slip
- Mounting fluid
- Positive control slide used with each batch (*M. tuberculosis H37Rv*)
- Negative control slide used with each batch – decontamination mixture only smear

Procedure for auramine staining

1. Place slides on staining rack and flood with auramine stain and let stand for 10 min.
2. Rinse the auramine away with distilled water and tilt slide to drain. Water must be chlorine free.
3. Flood the slide with 1% acid alcohol and leave for 5 min.
4. Wash off the acid alcohol with distilled water
5. Flood slides with 0.5% potassium permanganate for 30 seconds.
6. Rinse the potassium permanganate away with distilled water
7. Air dry.
8. Protect smears from light and examine immediately using the fluorescent microscope.
9. Acid alcohol fast bacilli fluoresce and are recorded as positive and counterstained using Ziehl-Neelsen stain for semi-quantification – see below.

Procedure for ZN staining

1. Place slides on staining rack and flood with carbol fuchsin.
2. Heat the slide to steaming with a flame then let stand for five minutes.
3. Re-flood slide with fresh carbol fuchsin and heat again until steaming, stand for five minutes.
4. Wash away the carbol fuchsin with distilled water.
5. Flood slides with 3% acid-alcohol.
6. Let stand for 9 min (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 minute.
9. Wash away the malachite green with distilled water.
10. Drain slides.
11. Allow slides to air dry in the slide rack DO NOT BLOT!
12. If necessary apply a drop of mounting fluid and a cover slip.

Notes on preparation of smears
- Slides should be dry and clean.
- Temperature and humidity varies by location and time. If slides become moist, put them on the heating block before making the smear. If there are still problems, clean the slides with 70% alcohol and dry on the heating block.
• Slides used for acid-fast staining should be new and unscratched. Acid-fast material from previous smears may be retained in the scratches on old, washed and reused slides.
• Most strains of rapid growing mycobacteria may not be fluorescent, in cases where rapid growers are suspected confirm a negative fluorochrome stain with Ziehl-Neelsen.
• Do not allow potassium permanganate to act over 3 to 4 min or it might quench the fluorescence of acid-fast bacilli.
• Heat fixing (slide warmer or flame) does not always kill mycobacteria, so be careful when handling smears. Always discard slides into sharps bin in the CL3 laboratory.
• Fluorochrome stained smears lose fluorescence with time and exposure to light. Keep smears covered and examine on the same day.

**Internal Quality Control Measures**

• Record the lot number and expiry date of the staining reagents on Quality Manual Attachment El/Eli. Each new batch of staining reagents must be tested using *M. tb* H37Rv as positive control and *E. coli* as a negative control.
REMEX Clinical TB Trial
Laboratory Manual Section 4

Microscopical Examination of ZN & Auramine Stained Smears

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Date: 5th December 2007
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<tr>
<th>Version</th>
<th>Author</th>
<th>Date</th>
<th>Reasons for Revision</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2</td>
<td>SB</td>
<td>05.12.07</td>
<td>First draft to be used in trial</td>
</tr>
<tr>
<td>3.0</td>
<td>EB</td>
<td>13.01.09</td>
<td>To reflect changes to Quality Manual</td>
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<tr>
<td>4.0</td>
<td>EB</td>
<td>23.09.09</td>
<td>To reflect changes instigated after start of trial</td>
</tr>
<tr>
<td>5.0</td>
<td>EB</td>
<td>27.06.11</td>
<td>Changes and clarifications to improve processes in the laboratories</td>
</tr>
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PURPOSE
To semi-quantify the presence acid-fast bacilli in the specimen or culture.

PRINCIPLE
A tentative diagnosis of tuberculosis can be made from observing acid-fast bacilli in sputum smears, but a definitive diagnosis is established only after the isolation and identification of *M. tuberculosis* from the patient by culture. A positive smear is approximately $10^4$ bacilli per mL or greater.

PROCEDURE

Materials
- Light microscope for Ziehl-Neelsen stain
- Fluorescent microscope for auramine stain
- Immersion oil
- Slide holder
- Slide storage box
- Lens paper
- Dark room (for examination under fluorescence)
Forms

- Quality Manual attachment F – Daily batch AFB staining IQC Record form for negative control (decontamination mixture only)
- Quality Manual attachment H – Daily batch AFB staining IQC Record form for positive control
- Quality Manual attachment I – Two way comparison of AFB counts for Z-N slides

Examination of Auramine stains (optional)

1. Examine auramine (fluorescent) stained smears first.
   a) Scan the entire smear with the 25X objective.
   b) Use a scanning pattern of rows either up and down the slide or across and back.
   c) Occasionally use a higher objective to see bacterial morphology closer.

Confirm fluorescent stained smears with Ziehl-Neelsen stain on all positive specimens.

Examination of Ziehl-Neelsen stain

1. Ziehl-Neelsen smears are examined with the 100X oil objective. Examine 100 fields using a regular pattern.
2. Record both the average number of AFBs and corresponding grading as shown in the table below.
3. Save smears in slide boxes (except those prepared for confirmation of positive cultures).
4. Always discard slides in a sharps bin inside the MSC in the CL3 laboratory.

REPORTING AFB SMEAR RESULTS

Principle

The results of examination of acid-fast smears are reported in a standardized way so that one result can be compared to another and in units relevant to patient care. There are several semi-quantitative scaling systems used to report the number of acid-fast bacilli (AFB) observed in stained smears. In the REMoxTB trial reporting MUST use the American CDC scaling system, outlined below. If laboratory is using the WHO/UATLD scaling system use the table below to convert results for recording.

<table>
<thead>
<tr>
<th>No. of AFBs (average over 100 fields)</th>
<th>REMoxTB Reporting</th>
<th>WHO Reporting (for conversion only)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>No AFB seen (NS)</td>
<td>No AFB seen (NS)</td>
</tr>
<tr>
<td>1-9 per 100 fields</td>
<td>+</td>
<td>scanty/or actual number</td>
</tr>
<tr>
<td>1-9 per 10 fields</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>1-9 per field</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>&gt;9 per field</td>
<td>++++</td>
<td>+++</td>
</tr>
</tbody>
</table>

Acceptable results

1. Positive smear: categorised using the semi-quantitative scaling system above
2. Negative smear: no AFB seen.
3. Recording quality control
   Record date, date positive and negative control slides were made, and quality control results on Quality Manual Attachment F and H

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If the quality control results are not acceptable,
   a) Do not report results on smears from that batch.
   b) Resolve the problem.
   c) Stain another QC slide and repeat the specimen slides.

Possible false results

1. Acid-fast artefacts.
2. Contamination of slides by tap water with saprophytic mycobacteria – always use distilled water.
3. Spots of stain deposit (when slide is not properly decolorized) can be mistaken for AFB – review the control slide.
4. Waxes and oils in dirty specimen containers may appear as acid-fast particles or may react with nonacid-fast bacteria and make them appear acid-fast.
5. Heavy metal ions in staining solutions or high chlorine content in water interfere with the fluorescent staining.
6. Only new slides should be used as old one may retain acid fast bacilli although apparently clean.

Notes on examination of smears

- Only open one box of slides at a time.
- Keep the cover on open boxes.
- After examining a positive smear with the oil objective, clean the objective.
- Do not touch immersion oil bottle tip to the smear. Let oil drop onto the slide. Stained bacilli can float from the smear into the bottle.
- If the smear is too thick, debris may cover AFB.
- If the smear is too thin, there may not be enough material to see a low number of AFB.
- Because acid-fast artefacts may be present in the smear, it is essential to view cell morphology carefully. Most artefacts show considerable variation. Mycobacteria show greater uniformity in size, arrangement, and staining. Break down the morphology into:
  - Size (length and width)
  - Colour (shade and intensity)
  - Shape (curved, straight, etc.)
  - Pattern (beaded, banded, etc.)
  - Distribution on smear (e.g., cording)
  - Uniformity of appearance
- When atypical rods are seen, they may represent other pathogenic or non-pathogenic mycobacteria or other partially acid-fast organisms.
- When less than 3 AFB are seen on the entire smear, confirm by ZN stain and by examining other smears from the same or another specimen. These results are doubtful and should not be reported.
- Fluorochrome stained smears lose fluorescence with time and exposure to light. Keep smears covered and examine on the same day.

Internal Quality Control Measures

- All the slide labelling and results records must be complete, accurate and legible. Record the actual number of AFBs per field (or per 10/100 fields) as well as the grading (this will be compared to ensure grading is accurate).
- Each time a batch of patient smears are performed, a negative control (decontamination mixture only) and positive M. tuberculosis (H37Rv) smear are stained to check that each stage of the procedure is working correctly. This must be recorded on Quality Manual attachment F and H respectively.
- To check the accuracy of the AFB counts a two person comparison must be performed on every tenth slide (record on quality manual attachment I).

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• Any QC failure should be recorded on quality manual attachment Q – continuous

**Data reporting**

Smears should be reported within 48 hours of sample receipt. Screening samples and week 17 samples take priority and should be processed, read and reported on the same day whenever possible.
5.1 Liquid culture – Mycobacteria Growth Indicator Tube (MGIT)

PURPOSE
To amplify the number of Mycobacterium tuberculosis bacteria in a sample using a liquid culture (MGIT) and to detect positive samples rapidly. To make a semi-quantitative assessment of the bacterial load by determining the time taken for culture bottles to signal positive.

PRINCIPLE
Bacteria are able to multiply in the nutrient-rich medium, while contaminating organisms are inhibited by the addition of a cocktail of antibiotics. Sufficient bacterial growth for subculture and identification is signalled to the machine by fluorescence detection triggered by an exponential decrease in oxygen in the medium using complex computer algorithms.

Sputum samples are processed (LM2), and inoculated into 7ml MGIT tubes, which are supplemented with OADC and a cocktail of antibiotics (PANTA). The MGIT tubes contain a fluorescent compound embedded in the base of the tube, which is sensitive to the presence of oxygen dissolved in the broth. Initially, the large amount of oxygen quenches the emissions from the compound and little fluorescence is detected. Micro-organisms present in the sputum specimens metabolize oxygen in the culture medium allowing the fluorescence to be detected. Blood samples are not suitable for the MGIT system.

Bactec MGIT 960 – Instrument overview
A single BACTEC MGIT 960 is capable of monitoring a total of 960 7ml MGIT tubes. The tubes are arranged in three drawers, A, B and C, each of which holds up to 320 tubes, and are continuously incubated. Each drawer contains a sample measurement model consisting of:
Stations – wells in the rack into which tubes are inserted.

The detector assembly – this sits below the rack and has 16 detectors, one for each row of stations. The assembly moves from left to right and back, taking test readings for each of the 20 station columns and the calibrator tube.

Drawer status indicators – lamps on the front of each drawer, indicates a POSITIVE, NEGATIVE and station ERROR.

Barcode scanner – at the front of the instrument to scan tube labels for specimen identification. The scanner turns on automatically.

LCD display and keypad – presents information about the system’s status and function key definitions.

Additional details are found in the BACTEC MGIT 960 System’s User’s Manual, Chapter 4, which should be stored within easy access of the MGIT system. The operator of the MGIT MUST be familiar with this manual.

Summary of how the MGIT works

The instrument automatically tests all the tubes continuously. A row of LEDs below the tubes illuminates, activating their fluorescent sensors. Photo detectors take the readings. A test cycle of all drawers is completed every 60 minutes. Positive cultures are immediately flagged by an indicator light on the front of the drawer, an optional audible alarm and are displayed on the LCD screen.

When positive tubes are identified, they should be removed from the instrument for confirmation of results, and for isolation and detection of the organism.

Definition of a Bactec MGIT 960 Growth Unit (GU)

The Growth Unit is an algorithmic measure of sensor fluorescence derived from the raw fluorescence voltage signal produced by optical integration of a MGIT tube in the Bactec 960 instrument. The Bactec 960 takes a reading every hour on the hour. An “Instrument Positive” tube is flagged automatically as a positive via internal algorithms when the GU reaches or exceeds the cut off value of 75 units. This is identified as a True Positive, and is confirmed by further tests such as ZN staining and contamination checks – see ‘Sampling for further analysis’ section.

If the MGIT flags the tube as positive and records a GU of “0” or Higher before 5 hours, this means that the growth has occurred very rapidly and exploded past the 75 unit cut off. Represented graphically, this growth curve would be very steep, compared to the gradual curve generated by the True Positive. If explosive growth has occurred, the software records a “T” in the growth column. Explosive growth usually means that the MGIT tube is contaminated, this will be confirmed by a negative ZN smear, growth on blood agar or both – see ‘Sampling for further analysis’ section

The GU is not an indication of biomass within the vial. However, normally at positivity the biomass is approximately $10^5$ to $10^6$ CFU/ml. There is no direct correlation of biomass and GU at the time of instrument positivity.

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Inoculating a MGIT tube

Materials

- 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)

Forms

- Quality Manual attachment C - Equipment Temperature Log
- Quality Manual attachment D - CL3 Laboratory maintenance checklist
- Quality Manual attachment Eiii – MGIT Reagent QC
- Quality Manual attachment Ev – Blood Agar QC
- Quality Manual attachment G/GI – Weekly Contamination Rate
- Quality Manual attachment J – Daily maintenance and temperature of MGIT
- Quality Manual attachment K – Lot number and expiry MGIT calibration tubes

Perform MGIT daily initiation routine - Internal Quality Control (IQC)

To be performed DAILY every morning, preferably before unloading or loading of tubes.

1. Maintenance button – TOOLS select
2. To check the lights on the drawers, select positive (+ red), negative (- green) and exclamation (! Yellow) respectively – all should light up
3. Open each drawer – select 1st light – all stations should be green.
4. Select same switch to switch off.
5. Select light <: All stations light on red.
6. Close drawer and repeat for all drawers.
7. Press exit – door.
8. Press temperature – FK – each drawer temperature will be recorded. Compare this temperature with temperature in tube thermometers in drawers.
9. Record all readings in the Quality Manual attachment J

Monthly MGIT Maintenance:

10. Filter cleaning – done once a month – remove faceplate at bottom of instrument, grip the bottom and pull firmly forward.
11. Remove the filter and place it in appropriate disinfectant.
12. Dry thoroughly with paper towel and replace.
13. Replace the faceplate, hold it in place – the cut-out should surround the on/off switch and firmly press in towards the instrument. The faceplate will snap into place.

Procedure

**MGIT tubes can be prepared in a BSC outside the CL3 laboratory, but inoculation of MGIT tubes with sputum sediment must be carried out in CL3 BSC.**

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.**

1. While the samples are being decontaminated and digested prepare the antibiotic supplement for the MGIT
Tubes. Reconstitute MGIT PANTA with 15ml of MGIT Growth Supplement. This mixture is stable for 5 days if stored at 2° - 8°C.

2. For each specimen, label a MGIT tube with the Study number (or screening number if pre-enrolment) and the REMoxTB laboratory accession number label. Record the MGIT tube number in the laboratory worksheet/lab book. If working with a LIMS system, add an accession barcode label to each tube.

3. Once dissolved add 0.8 ml of the liquid antibiotic supplement to each bottle using a sterile pipette, taking care not to contaminate them.

4. Following sputum decontamination, re-suspend the sputum deposit in 1-2ml PBS as described in LM2 and add 0.5ml (using graduated pastette or micropipette) to MGIT tube in the BSC. Dispose of waste pipette tips into the discard bucket containing appropriate disinfection.

5. Tightly recap the MGIT tube and mix well.

6. Store the remaining sputum sediment at 4°C, for 10 days or, for month 18 samples, until confirmed the MGIT tube is not contaminated.

**Entering bottles in the machine**

Tube entry – always scan the MGIT barcode first – the machine will assign the stations.

<table>
<thead>
<tr>
<th>FUNCTION</th>
<th>INSTRUCTIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Accession</td>
<td>• Open the desired drawer.</td>
</tr>
<tr>
<td>• Bar-coding disabled (default setting)</td>
<td>• Press the &lt;Tube entry&gt; soft key.</td>
</tr>
<tr>
<td></td>
<td>• Place the tube in the alignment block in front of the scanner with the barcode label facing the scanner. Rotate the tube if necessary.</td>
</tr>
<tr>
<td></td>
<td>• Use a spare barcode label if the tube’s label is damaged.</td>
</tr>
<tr>
<td></td>
<td>• System beeps once if scan is successful.</td>
</tr>
<tr>
<td></td>
<td>• Carefully place the tube in the assigned position.</td>
</tr>
<tr>
<td></td>
<td>• Row and column position are indicated on the main body of the display – assigned station LEDs illuminate GREEN in the drawer.</td>
</tr>
<tr>
<td></td>
<td>• Repeat scanning procedure for each tube.</td>
</tr>
<tr>
<td></td>
<td>• Close the drawer or press &lt;exit&gt; to continue with the next task.</td>
</tr>
</tbody>
</table>

• Accession
• Barcoding enabled
• (used only with LIS systems)

<table>
<thead>
<tr>
<th>FUNCTION</th>
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</tr>
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<tbody>
<tr>
<td>• Accession</td>
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<td>• Use a spare barcode label if the tube’s label is damaged.</td>
</tr>
<tr>
<td></td>
<td>• System beeps once if scan is successful.</td>
</tr>
<tr>
<td></td>
<td>• Scan the accession barcode label (if accession label is missing, torn, etc press the &lt;no accession barcode available&gt; soft key.</td>
</tr>
<tr>
<td></td>
<td>• System beeps once if scan is successful.</td>
</tr>
<tr>
<td></td>
<td>• Carefully place the tube in the assigned position.</td>
</tr>
<tr>
<td></td>
<td>• Row and column position are indicated on the main body of the display – assigned station LEDs illuminate GREEN in the drawer.</td>
</tr>
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<td></td>
<td>• Repeat scanning procedure for each tube.</td>
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<tr>
<td></td>
<td>• Close the drawer or press &lt;exit&gt; to continue with the next task.</td>
</tr>
</tbody>
</table>

**NOTES**
- Do not turn tubes after placing them in the station.
- Do not remove tubes unless they are positive or out-of-protocol negatives (negative at 42 days).
- Do not reassign to a new station.
- The MGIT will record the date each tube was entered.

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27th June 2011
Dealing with positive tubes

<table>
<thead>
<tr>
<th>FUNCTION</th>
<th>INSTRUCTION</th>
</tr>
</thead>
</table>
| Remove positives | 1. Press <silence alarm> to mute the audible alarm  
2. Open the drawer where the RED POSITIVE light is lit.  
3. Press <remove positive tubes> soft key.  
4. Remove the tube from the station with alternating flashing green/flashing red indicator lights. Remove 1 positive tube at a time.  
5. Scan the positive tube’s barcode label by placing the tube in the alignment block in front of the scanner with the barcode label facing the scanner. Rotate the tube if necessary.  
6. The station indicator will extinguish.  
7. Place the tube into a rack or carrier to transport after removal.  
8. Repeat steps 4 to 7 to remove additional positive tubes.  
9. When the task is completed, the instrument will beep 3 times, the drawer indicator light extinguishes, the barcode scanner turns off, and the OK icon appears on the display screen.  
10. Close the drawer or press <exit> to continue with the next task.  
11. Obtain a print out of ‘unloaded positives’. Sign and date print-out. |

- The MGIT will record the date the tube was flagged as positive and the number of days and hours taken to reach positivity (TTD = time to detection).
- Write the accession numbers of all unloaded tubes next to the results on the print outs.
- Print outs should be signed and dated by the laboratory technician unloading the tubes and must be kept in the unloaded positives folder or with the corresponding patients worksheets.
- MGIT tubes must be autoclaved prior to disposal.
- In the unlikely event of a broken tube in the machine – close the drawer and turn off the machine, vacate the room and follow local health and safety guidelines for actions following a spill.

Sampling positive tubes for further analysis – carried out in BSC in CL3 laboratory

Materials
- Discard jar containing appropriate liquid disinfection (specified in local Health and Safety documentation)
- Blood agar plate
- 10µl loop
- Plastic universal or tube for centrifugation of sample
- Glass slide
- Cryovial (with rubber o-ring seal), and appropriate storage box/rack
- Lowenstein Jenson (LJ) slope

Procedure
Blood Agar (this MUST be performed on ALL positive MGIT tubes)
1. Label one blood agar plate – laboratory accession number and study number (or screening number if pre-enrolment)
2. Unscrew MGIT tube lid and use a 10µL disposable loop to inoculate the blood agar plate and spread as normal. Incubate the blood agar plate along with the tube in the incubator at 37°C for 48 hours. If there has been growth on the blood agar plate and if contamination detected within 7 days re-treat the deposit (LM 2). If contamination detected after 7 days then record result as contaminated (except Month 18 samples – see note below).

Ziehl-Neelsen Stain (this should be performed on all positive mgit tubes even if confirmed as contaminated)
There are two possible ways to perform ZN from positive MGIT tubes;

A) Direct Sampling
3. Using sterile pastette remove a small amount of sediment directly from positive MGIT tube
4. Make a film for ZN (on appropriately labelled slide), optional to add albumin to slide before making smear, then heat fix and stain as for staining protocol (LM 3).

B) Concentrating Sample
5. Using sterile pastette remove 1 ml of well mixed fluid from MGIT tube and put into appendorf tube. Using microfuge spin to deposit sample. Remove most of the supernatant and re-suspend pellet (about 250 μL). Label a slide and make film for Ziehl-Neelsen (ZN) staining, then heat fix and stain as for staining protocol (LM 3)

OR
6. If centrifuge can accommodate spin MGIT tube without decanting. Otherwise decant the fluid into a universal in the safety cabinet.
7. Spin the universal at 3000g for 15 minutes.
8. Tip off the supernatant into discard bucket containing appropriate disinfectant leaving 2ml of broth in the universals to resuspend the pellet.
9. Label a slide and make film for Ziehl-Neelsen (ZN) staining, then heat fix and stain as for staining protocol. (LM 3)
10. Examine the ZN film for the presence of acid fast bacteria (AFB) (LM4), although it is not necessary to semi-quantify the number of AFBs. Describe AFB's i.e. typical, atypical and whether cording is seen.

Interpretation
1. If the blood agar is negative and AFBs are detected on the ZN smear this is a true positive and the MGIT time to detection is valid.
2. If the blood agar is positive the sample is contaminated either in the presence of absence of AFB, depending on the result of the ZN smear. The time to positivity will not be valid in either case. If the sample is confirmed as contaminated within the first 10 days, the remaining sputum sediment (leftover from the original decontamination) will be retreated as per LM2 to try to obtain a valid TTP. If the contamination is confirmed after 10 days the original sample will NOT be retreated and it be recorded as contaminated (except Month 18 samples – see note below).
3. If the blood agar is negative, and AFBs are not detected in the ZN smear, it is important to be certain the there are no AFBs present and confirm if this is a real false positive by doing the following—
   a. Look at the MGIT tube – record if is it turbid or showing any evidence of microbial growth (TB or any contaminants). A clear tube may be a true false positive resulting from altered pH problems
   b. Repeat the smear to double check for AFBs (centrifuge the tube if not done previously to ensure the sample is concentrated)
   c. If AFBs are still not detected, re-incubate the MGIT tube for a further 3 days (in MGIT or incubator) to allow further growth of any M. tuberculosis present. Repeat the ZN smear after 3 days.
   d. OPTIONAL: If AFBs are still not detected after this step, inoculate an LJ slope and incubate for 3-4 weeks to look for any growth.

For Baseline and Relapse cultures (any positive at or after week 17, or any NEW positive thereafter):
- Definitive identification will be obtained by using molecular diagnosis (LM 6)
- Sample should be subcultured for drug susceptibility testing (LM5.3)

For screening or baseline and all positive cultures at or after week 17:
- Two positive samples are stored at least one in 50% glycerol at -70°C to -80°C, if only one is stored in 50% glycerol at -70°C to -80°C then another must be stored on an LJ slope as described below. 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.
- To inoculate an LJ slope take 100 - 200μl of the positive MGIT pellet and pipette onto the slope (LM 5.2). Once growth is obtained these positive slopes will be stored 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)
- Spin down the 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 study
number (or screening number if pre-enrolment) and the lab accession label (also handwriting 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. At least 1ml is required for storage.

Note: If baseline AND screening contaminated store week 1 sample.

**For month 18 smear positive culture contaminated samples:**

Keep all sputum sediments for 42 days, if the MGIT is contaminated and another sample can not be obtained the original sediment can be retreated at any time within the 42 day protocol length (not just within 7 days of first decontaminating). Sputum sediments can be stored directly at -80°C after 7 days.

**Dealing with negative tubes**

The protocol for negative tubes should be set at 42 days – any tube that has not flagged positive at/or after 42 days will be reported as MGIT culture negative.

<table>
<thead>
<tr>
<th>FUNCTION</th>
<th>INSTRUCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Remove</td>
<td>12. Press &lt;silence alarm&gt; to mute the audible alarm</td>
</tr>
<tr>
<td>negatives</td>
<td>13. Open the drawer where the GREEN NEGATIVE light is lit.</td>
</tr>
<tr>
<td></td>
<td>14. Press &lt;remove negative tubes&gt; soft key.</td>
</tr>
<tr>
<td></td>
<td>15. Remove the tube from the station with alternating flashing green/flashing red indicator lights. Remove 1 negative tube at a time.</td>
</tr>
<tr>
<td></td>
<td>16. Scan the negative tube’s barcode label by placing the tube in the alignment block in front of the scanner with the barcode label facing the scanner. Rotate the tube if necessary.</td>
</tr>
<tr>
<td></td>
<td>17. The station indicator will extinguish.</td>
</tr>
<tr>
<td></td>
<td>18. Place the tube into a rack or carrier to transport after removal.</td>
</tr>
<tr>
<td></td>
<td>19. Repeat steps 4 to 7 to remove additional negative tubes.</td>
</tr>
<tr>
<td></td>
<td>20. When the task is completed, the instrument will beep 3 times, the drawer indicator light extinguishes, the barcode scanner turns off, and the OK icon appears on the display screen.</td>
</tr>
<tr>
<td></td>
<td>21. Close the drawer or press &lt;exit&gt; to continue with the next task.</td>
</tr>
<tr>
<td></td>
<td>22. Obtain a print out of ‘unloaded negatives’. Sign and date the print-out.</td>
</tr>
</tbody>
</table>

Check the unloaded negative tubes for any sign of growth.

Write the accession numbers of all unloaded negative tubes next to the results on the print outs

Print outs should be signed and dated by the laboratory technician unloading the tubes and must be kept in the unloaded negatives folder or with the corresponding patients worksheets.

The negative MGIT tubes should also be autoclaved prior to disposal

For any negative control tubes record on the appropriate QM attachment F, where possible attach the corresponding MGIT print out.

**In case of MGIT Failure/Breakdown lasting > 24 hours:**

Refer to and follow manufacturer’s guidelines. In summary, 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 (365nm) 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 ZN 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 ZN microscopy (LM 3/4).
Calculate the TTD from the date the tubes were inoculated to the date the tubes were confirmed positive/negative manually.

**Contamination Rates**
The weekly contamination rates for MGIT culture should be 3 – 8%. This will be monitored on a weekly basis as shown in the quality manual attachment G. If the rate is above this range then quality manual attachment Gi should be completed with details of the contaminated samples and sent to UCL. Samples classified as contaminated are ONLY samples that are both MGIT positive AND show growth on blood agar within 48 hours. An exception to this are samples visibly contaminated (e.g. with fungi) which do not show growth on blood agar.

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### 5.2 SOLID CULTURE: LOWENSTEIN JENSON (LJ) SLOPES

**PURPOSE**
The purpose of this procedure is to identify and semiquantify growth on LJ slopes. Slopes will be inoculated from the original decontaminated sputum - these data will be the primary endpoint used to judge the efficacy of treatment in the trial. LJ slopes should also be inoculated from one positive MGIT tube per patient (usually baseline) for archiving, see section 5.1 above.

**Materials**
- LJ slopes
- Graduated plastic pasteur pipettes
- Discard bucket containing appropriate liquid disinfectant (specified in the local Health and Safety documentation)
- Angle-poise lamp

**Forms**
- Quality Manual attachment C – Equipment Temperature Log
- Quality Manual attachment Elv - Reagent QC LJ Slopes
- Quality Manual attachment G/Gi – Weekly contamination rates

**PROCEDURE**
1. Label LJ slope with the laboratory accession number label and the study number (or the screening number if pre-enrolment).
2. Inoculate the LJ slope with 100-200μl of sample (decontaminated sputum or positive MGIT pellet) using a graduated pastette. Dispose of the pastette into a waste bucket containing appropriate disinfectant. Take particular care to minimise aerosol generation when adding sample from positive MGIT tube as this will contain larger numbers of bacteria.
3. Tightly secure lid and ensure there are no droplets around the rim of the tube. Incubate at 37°C for 8 weeks.
4. Examine the slopes weekly, slopes can be read on the bench in the Containment level 3 room, as long as the lids are NOT loosened. Read each slope weekly from the day the slope was incubated. If this is not possible read slopes from 0 to 8 weeks to ensure the slopes are incubated and read up to 56 days.
5. In order to see any fine growth a strong direct light from the angle poise lamp must be shone on to the slope surface. *Mycobacterium tuberculosis* grows as a buff coloured dry colony, which is very distinctive.

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6. Mycobacteria other than \textit{M. tuberculosis} can cause respiratory disease in immunocompetent people and these colonies vary extensively in morphology. \textit{M. malmoense} grows as a fine coating with no colour on the surface of the slope, as do early \textit{M. avium} colonies. \textit{M. fortuitum} appears to be a coliform on the slope with a strong growth that can rapidly cover the slope. It is important that a colony from all fresh growth, not just growth that looks like \textit{M. tuberculosis}, is taken and a ZN stained smear is made (LM3).

Mycobacteria cannot out-compete strong, rapidly growing bacteria and if slopes are contaminated with bacterial growth or the slopes liquefy within 10 days, the original sputum sediment must be retreated and a new LJ slope inoculated. If contamination is detected after 10 days this will be reported as a contaminated sample. Individual slopes that liquefy or are covered by bacterial growth other than Mycobacteria may be safely discarded. If the slopes show colour change but no visible growth, do not class this as contaminated unless confirmed by blood agar or microscopy.

How to record growth on slopes

1. The results of each slope must be recorded in the laboratory worksheet/lab book.
2. There is a standardised method that should be used for reporting mycobacterial growth on LJ-slopes, summarised in the table below. All results must be reported according to the grading outlined in the REMoxTB Reporting column.

<table>
<thead>
<tr>
<th>Colonies</th>
<th>REMoxTB Reporting</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>Negative</td>
</tr>
<tr>
<td>&lt;20 colonies</td>
<td>Record no. of colonies</td>
</tr>
<tr>
<td>20-100 colonies</td>
<td>+</td>
</tr>
<tr>
<td>Innumerable discrete colonies</td>
<td>++</td>
</tr>
<tr>
<td>Confluent</td>
<td>+++</td>
</tr>
<tr>
<td>Contaminated</td>
<td>Contaminated</td>
</tr>
</tbody>
</table>

3. A slope is recorded as negative if there is no growth after 8 weeks.
4. If a slope has a smear prepared this should be recorded on the source document (including the results).
5. If a slope is contaminated and the original sediment is retreated this should be clearly stated on the source document.

For month 18 smear positive culture contaminated samples:

Keep all sputum sediments for 56 days, if the LJ is contaminated and another sample can not be obtained the original sediment can be retreated at any time within the 8 week protocol length (not just within 7 days of first decontaminating). Sputum sediments can be stored directly at -80°C after 7 days.

Contamination Rates

The weekly contamination rates for LJ culture should be 3 – 8%. This will be monitored on a weekly basis as shown in the quality manual attachment G. If the rate is above this range then quality manual attachment Gi should be completed with details of the contaminated samples and sent to UCL.

How to prepare slides from solid culture growth

Materials

- 10% formal saline

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• Glass frosted ended microscope slides
• 10μL loops

PROCEDURE
1. Label the slides in pencil with lab accession number, study number (or screening number if pre-enrolment) and the date
2. Place a 10μL loopful of formol saline on the slide.
3. Pick part of a colony with a separate 10μL loop and emulsify in the formol saline.
4. Heat fix at 65 - 75°C for at least 15 minutes before staining with ZN (LM3).

5.3 DRUG SUSCEPTIBILITY TESTING

PURPOSE OF PROCEDURE
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 and any new positive culture thereafter) in order to identify the presence of resistance to any of the study drugs. If the baseline sample (LJ and/or MGIT) is contaminated and pure culture cannot be obtained, it is acceptable to use the screening or week 1 culture as a back up to perform the susceptibility profile.

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.

BACTEC MGIT 960 SIRE susceptibility test

Materials
• Biological safety cabinet
• Discard jar containing appropriate liquid disinfectant (specified in the Local Health and Safety documentation)
• 7ml MGIT tubes
• BD SIRE MGIT kit reagents
• BD Pyrazinamide MGIT kit reagents
• Moxifloxacin powder (provided by Bayer, supplied via UCL)
• BD BACTEC MGIT supplement (for both SIRE and PZA drug kits)
• McFarland standards
• p1000 and p200 Gilson pipettes (or equivalent) and aerosol resistant tips
• Sterile saline
• Blood agar plates

For DST from LJs:
• Middlebrook 7H9 broth
• Capped sterile tube containing glass beads
• Vortex

Forms
• Quality Manual attachment Evi – Reagent QC MGIT SIRE.
• Quality Manual attachment Evii – Reagent QC MGIT PZA
• Quality Manual attachment Eviii – Reagent QC MGIT Moxifloxacin

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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 QM attachment Evi-viii

<table>
<thead>
<tr>
<th>TASK</th>
<th>INSTRUCTIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NOTE</strong> – 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</td>
<td></td>
</tr>
</tbody>
</table>
| 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 8000µg/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 1.04mg/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 use the 100x stock solution must be diluted 1:100 (10µl into 990 µl) in sterile ddH₂O to make a working solution of 10.4 µg/ml. |

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 study number. In addition, label tubes with one of each of the following: GC (Growth Control), STR (streptomycin), INH (isoniazid), RIF (rifampicin), EMB (ethambutol).
2. Place the tubes in the correct sequence in the 5 tube AST set carrier (see BACTEC MGIT 960 User’s Manual, AST Instructions).
3. Aseptically add 0.8 mL of BACTEC MGIT SIRE Supplement to each SIRE tube. It is important to use the supplement supplied with the kit.
4. Aseptically micropipette, 100 µL of 83 µg/mL MGIT STR solution to the appropriately labelled MGIT tube. Aseptically pipette 100 µL of 8.3 µg/mL MGIT INH solution to the appropriately MGIT tube.
5. Aseptically pipette 100 µL of 83 µg/mL MGIT RIF solution to the appropriately MGIT tube.
6. Aseptically pipette 100 µL of 415 µg/mL MGIT EMB solution to the appropriately labelled MGIT tube.
7. 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

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the study 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 100μL 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 study 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 10.4μg/ml by performing a 1:100 dilution of the stock solution in sterile distilled/deionised water (e.g. 10μl of moxifloxacin stock in 990μl of ddH2O).

5. Aseptically pipette 100 μL of 10.4μg/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.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration of drug after reconstitution*</th>
<th>Volume added to MGIT tubes for test</th>
<th>Final concentration in MGIT tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGIT STR</td>
<td>83μg/ml</td>
<td>100μl</td>
<td>1.0μg/ml</td>
</tr>
<tr>
<td>MGIT INH</td>
<td>8.3μg/ml</td>
<td>100μl</td>
<td>0.1μg/ml</td>
</tr>
<tr>
<td>MGIT RIF</td>
<td>83μg/ml</td>
<td>100μl</td>
<td>1.0μg/ml</td>
</tr>
<tr>
<td>MGIT EMB</td>
<td>415μg/ml</td>
<td>100μl</td>
<td>5.0μg/ml</td>
</tr>
<tr>
<td>MGIT PZA</td>
<td>8000μg/ml</td>
<td>100μl</td>
<td>100μg/ml</td>
</tr>
<tr>
<td>MOX</td>
<td>Stock solution (100x)</td>
<td>100μl</td>
<td>0.125μg/ml</td>
</tr>
<tr>
<td></td>
<td>Working solution – 1.04mg/ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Using inoculum from positive liquid culture – 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 37°C 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.

**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).

**Growth Control tube preparation and inoculation – carried out in 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.

**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)

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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.

**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.

**Confirming resistant isolates –**

All resistant isolates should be verified by preparing a blood agar plate and ZN smear from the resistant tube, as per LMS.1 above. 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 can not 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 results is valid. However any resistant result should be repeated for confirmation.

All resistant isolates should be sent to UCL for independent validation of the results.

**Internal Quality Control Measures**

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 REMoxTB Quality Manual attachment.
If the QC fails, all results for the batch should be reviewed, new reagents purchased and testing of clinical samples repeated. It is optional to include *M. tb* H37Rv in every batch of samples that are being run for DSTs – this may help to determine any false resistant results in the run. If the laboratory is having problems performing DSTs or passing QC then a *M. tb* H37Rv set must be included in every run.
REMOX Clinical TB Trial
Laboratory Manual Section 6

Confirmation of *M.tuberculosis* species

**Version:** 5.0  
**Date:** 27th June 2011

**Approved by:** Dr Tim McHugh  
**Signature:**

**Date:** 5th December 2007  
**Current version:** 5.0

**Revision history:**

<table>
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<th>Reasons for Revision</th>
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<td>SB</td>
<td>05.12.07</td>
<td>First draft to be used in trial</td>
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<td>3.0</td>
<td>EB</td>
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<td>23.09.09</td>
<td>To reflect changes instigated after start of trial</td>
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<td>5.0</td>
<td>EB</td>
<td>27.06.11</td>
<td>Changes and clarifications to improve processes in the laboratories</td>
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**PURPOSE**

The ACCUPROBE MYCOBACTERIUM TUBERCULOSIS COMPLEX CULTURE IDENTIFICATION TEST is a rapid DNA probe test, which utilises the technique of nucleic acid hybridization for the identification of *Mycobacterium tuberculosis* (TB Complex) isolated from culture. The TB Complex consists of the following species: *M. tuberculosis*, *M. bovis*, *M. bovis BCG*, *M. africanum*, *M. microti*, and *M. canetti*.

**PRINCIPLE**

The ACCUPROBE SYSTEM uses a single-stranded DNA probe with a chemiluminescent label that is complementary to the ribosomal RNA of the target organism. After the ribosomal RNA is released from the organism, the labelled DNA probe combines with the target organism’s ribosomal RNA to form a stable DNA:RNA hybrid. The Selection Reagent allows for the differentiation of non-hybridized and hybridized probe. The labelled DNA:RNA hybrids are measured in a GEN-PROBE luminometer. A positive result is a luminometer reading equal to or greater than the cut-off. A value below this cut-off is a negative result. Speciation will be performed on the initial isolate from a patient and any subsequent isolate where there suspected relapse or colonies look morphologically different.
PROCEDURE

Materials
Reagents for the ACCUProbe Mycobacterium Tuberculosis Complex Culture Identification Test are provided in three separate reagent kits (available from Gen-probe):

**ACCUProbe Mycobacterium Tuberculosis Complex Probe Kit** (Cat No. 2860)
- **Probe Reagent**: (4 x 5 tubes)
- **Mycobacterium tuberculosis complex**
- **Lysing Reagent**: (1 x 20 tubes)
- Glass beads and buffer

**ACCUProbe Culture Identification Reagent Kit** (Cat No. 2800)

- **Reagent 1 (Lysis Reagent)**: 1 x 10 mL
  - buffered solution containing 0.04% sodium azide
- **Reagent 2 (Hybridization Buffer)**: 1 x 10 mL
  - buffered solution
- **Reagent 3 (Selection Reagent)**: 1 x 60 mL
  - buffered solution

**GEN-Probe Detection Reagent Kit** (Cat No. 1791)

- **Detection Reagent I**: 1 x 240 mL
  - 0.1% hydrogen peroxide in 0.001 N. nitric acid
- **Detection Reagent II**: 1 x 240 mL
  - 1 N sodium hydroxide

**Storage and handling requirements**

Probes Reagent Tubes must be stored in the foil pouches at 2° to 8°C. The Probes Reagent Tubes are stable in the unopened pouches until the expiration date indicated. Once opened, the pouch should be resealed and the tubes should be used within two months and prior to the expiration date.

Other reagents used in the ACCUProbe Mycobacterium Tuberculosis Complex Culture Identification Test may be stored between 2° to 25°C and are stable until the expiration date indicated.

DO NOT FREEZE THE REAGENTS.

Materials required but not provided in kits

- 1 µL plastic sterile inoculating loops, wire loops, or plastic needles for selecting colonies.
- Control culture strains (M. tb H37Rv and M. avium/M. fortuitum)
- Water bath or heating block (59.5° to 61°C)
- Water bath or heating block (95° ± 5°C)
- Micropipettes (100 µL, 300 µL) and aerosol resistant tips
- Vortex mixer
- MacFarland Standards
- Syscheck
- Polypropylene Tubes (250 tubes)

**Equipment required (available from Gen-Probe)**

- GEN-Probe® LEADER® Luminometer
- GEN-Probe® Sonicator or equivalent
- GEN-Probe® Heating Block (Cat No. 2775)
- GEN-Probe® Sonicator Rack (Cat No. 4027)

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Sample Collection and preparation – carried out in the BSC in the CL3 laboratory

The ACCUPROBE MYCOBACTERIUM TUBERCULOSIS COMPLEX CULTURE IDENTIFICATION TEST is designed to determine the identity of TB Complex isolated from culture.

This test must be performed on ALL baseline cultures (if baseline unavailable then the screening sample may be used) and any positive cultures at or after week 17.

A. Solid Media Method
   Growth from appropriate solid media (Lowenstein-Jensen (L/J) slants) may be tested. Samples may be tested as soon as growth is visible and during the subsequent 60 days of incubation.
   1. Growth can be removed with a disposable plastic loop, a wire loop, or a disposable plastic needle. Swabs should not be used due to the small volume of liquid in which the cells are subsequently resuspended.
   2. Avoid taking any of the solid media with the cells.
   3. The operator may elect to inoculate another culture plate at this time to confirm the purity of the isolate.

B. Broth Culture Method (MGIT Tubes)
   Growth in Middlebrook 7H9 broth (MGIT Tubes) with turbidity equivalent to or greater than a McFarland 1 Nephelometer Standard may be tested with the ACCUPROBE MYCOBACTERIUM TUBERCULOSIS COMPLEX CULTURE IDENTIFICATION TEST. Pipette a 100 µL sample from the well mixed broth suspension into the Lysing Reagent Tube as described below.

Test procedure

This procedure must be carried out in the BSC in the CL3 laboratory until the end of the Sample lysis section, when bacteria have been heat killed at 95°C.

A. EQUIPMENT PREPARATION
   1. For optimal transfer of sonic energy, water must be thoroughly degassed according to the following procedure:
      a. Add enough hot water to fill the sonicator bath to within 1/2 inch of the top of the tank.
      b. Run the sonicator for 15 minutes to thoroughly degas the water.
   2. Adjust one heating block or water bath to 59.5° to 61°C and another heating block or water bath to 95° ± 5°C.
   3. Prepare the GEN-PROBE luminometer for operation. Make sure there is sufficient volume of Detection Reagents I and II to complete the tests.
   4. Perform a ‘wash’ using the detection reagents before starting sample analysis.

B. CONTROLS
   Positive and negative control strains should be tested with every batch of samples analysed. A culture of Mycobacterium tuberculosis (e.g. American Type Culture Collection, ATCC #27294) may be used as the positive control while a culture of Mycobacterium avium (e.g., ATCC #25291) or M. fortuitum may be used as the negative control.

C. SAMPLE PREPARATION
   1. Label a sufficient number of Lysing Reagent Tubes to test the culture isolates and/or controls. Remove and retain the caps.
   2. Pipette 100 µL of Reagent 1 (Lysis Reagent) and 100 µL of Reagent 2 (Hybridization Buffer) into all Lysing Reagent Tubes. IF MGIT CULTURES ARE TO BE TESTED, DO NOT ADD REAGENT 1 TO THE LYISING REAGENT TUBES.
   3. Transfer the sample from the solid media or 100 µL of a well mixed MGIT culture into the labelled Lysing Reagent Tubes as described in the SAMPLE COLLECTION AND PREPARATION Section above (twirl the loop or needle in the Reagent 1 and/or Reagent 2 diluent mixture to remove the cells if testing growth from

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solid media).
4. Recap the Lysing Reagent Tubes and briefly VORTEX.

D. SAMPLE LYSIS
1. Push the Lysing Reagent Tubes through the Sonicator Rack so that the reaction mixture in the bottom of the tube is submerged but the caps are above the water. Place Sonicator Rack on water bath sonicator. DO NOT ALLOW THE TUBES TO TOUCH THE BOTTOM OR SIDES OF THE SONICATOR.
2. Sonicate for 15 minutes.
3. Place the Lysing Reagent Tubes containing the sonicated organisms in a heating block or water bath for 10 minutes at 95° ± 5°C.
4. Carefully remove the Lysing Reagent Tubes from the heating block or water bath.

E. HYBRIDIZATION
1. Open the foil pouch by cutting evenly across the top of the pouch. Remove enough Probe Reagent Tubes to test the culture isolates and/or controls. Reseal the pouch by folding the opened edge over several times and securing with adhesive tape or a clip. Leave the desiccant pillow in the pouch.
2. Label a sufficient number of Probe Reagent Tubes to test the culture isolates and/or controls. Remove and retain the caps.
3. Pipette 100 μL of the lysed specimens from the Lysing Reagent Tubes into the corresponding Probe Reagent Tubes.
4. Recap the Probe Reagent Tubes and incubate for 15 minutes at 59.5° to 61°C in a water bath or heating block.

F. SELECTION
1. Remove the Probe Reagent Tubes from the water bath or heating block. Remove and retain the caps. Pipette 300 μL of Reagent 3 (Selection Reagent) into each tube. Recap the tubes and VORTEX them to mix completely.
2. Incubate the Probe Reagent Tubes for 10 minutes at 59.5° to 61°C in a water bath or heating block.
3. Remove the Probe Reagent Tubes from the water bath or heating block and leave them at room temperature for at least 5 minutes. Remove and discard the caps. Read the results in the luminometer within 1 hour.

G. DETECTION
1. Select the appropriate protocol (M. tb complex) from the menu of the luminometer software.
2. Using a damp tissue or paper towel, wipe each tube to ensure that no residue is present on the outside of the tube and insert the tube into the luminometer according to the instrument directions.
3. When the analysis is complete, remove the tube(s) from the luminometer.

PROCEDURAL NOTES
A. REAGENTS: Reagent 2 (Hybridization Buffer) may precipitate. Warming and mixing the solution at 35° to 60°C will dissolve the precipitate.
B. TEMPERATURE: The Hybridization and Selection reactions are temperature dependent. Therefore, it is imperative that the water bath or heat block is maintained within the specified temperature range.
C. TIME: The Hybridization and Selection reactions are time dependent. Hybridize at least 15 minutes but no more than 20 minutes. Incubate the Probe Reagent Tubes during the SELECTION Step for at least 10 minutes but no more than 11 minutes.
D. WATER BATH: The level of water in the water bath should be maintained to ensure that the Lysing Reagent Tubes are submerged up to, but not above, the level of the sealing ring. It should also be ensured that the entire liquid reaction volume in the Probe Reaction Tubes is submerged.
E. VORTEXING: It is critical to have a homogeneous mixture during the SAMPLE PREPARATION and SELECTION Steps, specifically after the addition of cells to Reagents 1 and 2 and after addition of Reagent 3.
F. TROUBLE-SHOOTING
   1. Elevated negative control values (Mycobacterium avium ATCC #25291) greater than 10,000 RLU (Relative Light Units) in the LEADER luminometer or 300 PLU (Photometric Light Units) in the

Version 2.0
27th June 2011
ACCULDR (formerly PAL) luminometer can be caused by insufficient mixing after adding Reagent 3 (Selection Reagent) or by testing mixed cultures. Because mixed cultures can occur, a portion of the growth may be streaked onto the appropriate agar medium and incubated to check for multiple colony types.

2. Low positive control values (*) less than 30,000 RLU in the LEADER luminometer or 900 PLU in the ACCULDR (formerly PAL) luminometer can be caused by insufficient cell numbers, improper sonication, or by testing mixed or aged cultures. Because mixed cultures can occur, a portion of the growth may be streaked onto the appropriate agar medium and incubated to check for multiple colony types.

G. MAINTENANCE:
- Perform Syscheck once per month (or more often if required) – this must pass before analysing any test samples
- Perform a warm water flush/wash each week to prevent detection reagent tubes becoming blocked
- Perform reagents QC each time a new Lot number of reagents are received in the laboratory using positive and negative controls.
- Record all information on quality manual attachment L – this should be completed each time trial samples are processed to allow batch tracing.

RESULTS

A. INTERPRETATION OF RESULTS
The results of the ACCUPROBE MYCOBACTERIUM TUBERCULOSIS COMPLEX CULTURE IDENTIFICATION TEST are based on the following cut-off values. Samples producing signals greater than or equal to these cut-off values are considered positive. Signals less than these cut-off values are considered negative. Results in repeat ranges should be repeated.

<table>
<thead>
<tr>
<th>ACCULDR (formerly PAL)</th>
<th>LEADER</th>
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<tbody>
<tr>
<td>Cut-off value</td>
<td>900 PLU</td>
</tr>
<tr>
<td>Repeat range</td>
<td>600 - 899 PLU</td>
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</table>

B. QUALITY CONTROL AND ACCEPTABILITY OF RESULTS
Negative control (e.g., *M. avium*, ATCC #25291) and positive control (e.g., *M. tuberculosis*, ATCC #27294) should be included in EVERY run and should satisfy the following values and should be recorded on Quality Manual attachment L.

<table>
<thead>
<tr>
<th>ACCULDR (formerly PAL)</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>&lt;300 PLU</td>
</tr>
<tr>
<td>Positive control</td>
<td>&gt;900 PLU</td>
</tr>
</tbody>
</table>

In addition IQC should include batch testing of reagents and regular maintenance of the luminometer – also documented in Quality Manual attachment L.

LIMITATIONS

This method has been tested using fresh growth from solid media and from broth cultures listed in the SAMPLE COLLECTION AND PREPARATION Section. The efficacy of this test has not been demonstrated on direct clinical samples.

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specimens (e.g., urine, stool, or respiratory specimens).

The ACCUProbe MYCOBACTERIUM TUBERCULOSIS COMPLEX CULTURE IDENTIFICATION TEST does not differentiate between members of the TB Complex, i.e., *M. tuberculosis*, *M. bovis*, *M. bovis BCG*, *M. africanum*, and *M. microti*. The Probe Reagent does not react with any mycobacteria other than tubercle (MOTT) bacilli.

Results from the ACCUProbe MYCOBACTERIUM TUBERCULOSIS COMPLEX CULTURE IDENTIFICATION TEST should be interpreted in conjunction with other laboratory and clinical data available to the clinician.
REMOX Clinical TB Trial
Laboratory Manual Section 7
DNA EXTRACTION

Version: 5.0
Date: 27th June 2011

Approved by: Dr Tim McHugh
Date: 5th December 2007
Signature and date:
Current Version: 5.0

Revision History:

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<td>EB</td>
<td>13.01.09</td>
<td>To reflect changes to Quality Manual</td>
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<td>EB</td>
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<td>To reflect changes instigated after start of trial</td>
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<td>EB</td>
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<td>Changes and clarifications to improve processes in the laboratories</td>
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INTRODUCTION

Purpose
The method described here is designed to yield microgram quantities of high molecular weight DNA suitable for molecular typing. DNA prepared in this way is stored for further analysis. DNA will be extracted from all baseline (or screening) samples and resistant/suspected relapse samples. An aliquot of all extracted DNA should be sent to UCL for further analysis (another aliquot of the same DNA to remain at site).

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).

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1. Isolation of Genomic DNA from *M. tuberculosis*

**Materials**

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

**Procedure**

Vortexing is not recommended at any stage of the extraction as this causes DNA shearing. All steps, until after the addition of chloroform (step 15) 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’s study number and laboratory accession number.
3. Aliquot 400μL volumes of 1x Tris-EDTA (TE) buffer into the tubes using aerosol resistant tips.
4. From LJ slopes with good growth, take 10μL loopfuls of organisms 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.
11. Add 100μL 5M NaCl.
12. Add 100μL CTAB/NaCl (prewarmed 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.

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15. Remove the tubes from the Containment Level 3 Laboratory and microfuge at 10,000 g for 5 minutes.
16. Label sterile RNAase 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.

2a Estimation of DNA Concentration using Agarose Gel

Materials

<table>
<thead>
<tr>
<th>Item</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
</tr>
<tr>
<td>Balance and weighing boats</td>
</tr>
<tr>
<td>Microwave</td>
</tr>
<tr>
<td>Tris borate EDTA (TBE)</td>
</tr>
<tr>
<td>Hybaid gel electrophoresis tank and apparatus</td>
</tr>
<tr>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>10ul pipette and appropriate tips</td>
</tr>
<tr>
<td>Loading buffer</td>
</tr>
<tr>
<td>Parafilm</td>
</tr>
<tr>
<td>Lambda DNA control standards</td>
</tr>
<tr>
<td>UV transilluminator</td>
</tr>
<tr>
<td>UV protection glasses, a face visor and gloves</td>
</tr>
<tr>
<td>Digital camera</td>
</tr>
</tbody>
</table>

Procedure

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 (this will be enough for one DNA quantification gel and one small restriction gel (LM8)).
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°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.
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.

**2b Estimation of DNA Concentration using Nanodrop**

**Materials**

NanoDrop Spectrophotometer
IBM 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)

**Procedure**

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.

**NOTE:** If DNA concentration cannot be measured at site the DNA can be sent to UCL at the laboratory's risk i.e. If there is not enough DNA to use for molecular typing the laboratory will have to repeat the DNA extraction for those samples.

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**Shipment to UCL**

An aliquot of extracted DNA should be sent to UCL in below room temperature conditions (wet ice or colder) at the end of every month, except when there are less than 20 vials, in which case send whenever there are 20 or more vials ready. **Any suspected relapse strains should be DNA extracted and sent to UCL immediately.** Quality attachment P should be completed indicating the estimated concentration and volume of DNA in each vial (ideally more than 1 µg in total). A copy of this form should then be sent with the vials to UCL.

Laboratory Manual 7 : APPENDIX A

PREPARATION OF SOLUTIONS:

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 temp 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 temp 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 temp. 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 dH2O, store at -20°C.
REMOK Clinical TB Trial
Laboratory Manual Section 8

Molecular Genetic Assay for Identification of Resistance to Rifampicin, Isoniazid, Fluoroquinolones, Aminoglycosides and Ethambutol in Mycobacterium tuberculosis complex

Version: 2.0  Date: 27th June 2011

Approved by: Dr Tim McHugh  Signature
Date: 13th January 2009  Current version: 2.0

Revision history:
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<th>Version</th>
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<td>EB</td>
<td>13.01.09</td>
<td>First Draft</td>
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<td>2.0</td>
<td>EB</td>
<td>27.06.11</td>
<td>Changes and clarifications to improve processes in the laboratories</td>
</tr>
</tbody>
</table>

PURPOSE
On the basis of a positive primary culture or pulmonary smear-positive patient material the GenoType® MTBDRplus allows the safe and fast detection of the M. tuberculosis complex and its resistance to rifampicin and/or isoniazid in a single procedure. A further test, the GenoType® MTBDRsl allows the safe and fast detection of the M. tuberculosis complex and its resistance to fluoroquinolones, aminoglycosides and ethambutol.

PRINCIPLE
The GenoType® MTBDRplus test is based on the DNA-STRIP® technology and permits the molecular genetic identification of the M. tuberculosis complex and its resistance to rifampicin and/or isoniazid from pulmonary smear-positive direct patient material (or cultivated samples). The identification of rifampicin resistance is enabled by the detection of the most significant mutations of the rpoB gene (coding for the β-sub-unit of RNA polymerase). For testing of high level isoniazid resistance, the katG gene (coding for catalase peroxidase) is examined and for testing of low level isoniazid resistance, the promoter region of the inhA gene (coding for the NADH enoyl ACP reductase) is examined.

The whole procedure is divided into three steps: DNA isolation from cultured material (LJ slopes/liquid medium) or direct materials (pulmonary, smear-positive, decontaminated), a multiplex amplification with biotinylated primers and a reverse hybridization. The hybridization includes the following steps: chemical denaturation of the amplification
products, hybridization of the single-stranded, biotin labelled amplimers to membrane-bound probes, stringent washing, addition of a streptavidin/alkaline phosphatase (AP) conjugate, and an AP mediated staining reaction. A template ensures the easy and fast interpretation of the banding pattern obtained.

The GenoType® MTBDRs/ test uses the same technology and methodology to detect resistance to fluoroquinolones, aminoglycosides and ethambutol from pulmonary smear-positive clinical specimens. Resistance to fluoroquinolones is enabled by detection of the most significant mutations of the gyrA gene (coding for the A subunit of the DNA gyrase). Aminoglycoside and ethambutol resistance is detected by examining the rrs gene (codes for 16S rRNA) and the embB gene (codes for the arabinosyl transferase) respectively.

SAFETY
Standard Containment level III safety procedures should be adhered to. This procedure must be carried out in a microbiological safety cabinet until heat inactivation is complete.
- Handle sputum as per laboratory procedures for handling human tissues infected with *M. tuberculosis*, a Hazard Group III pathogen.
- The Denaturation Solution (DEN) contains <2% NaOH and is irritating to eyes and skin.
- The Substrate Concentrate (SUB-C) contains dimethyl sulfoxide and is irritating.

Storage and Precautions
Store primer/Nucleotide Mix (PNM) at 2 – 8°C upon arrival, isolated from any potential source of contaminating DNA. If longer storage (more than 4 weeks) is required, store at -20°C. In order to avoid repeated freezing and thawing, aliquot PNM. Store all other kit components at 2-8°C. Do not use the reagents beyond their expiry date. Observe the usual precautions for amplification set-up. It is essential that all reagents and materials used for DNA isolation and amplification set-up are free from DNAses (see Good Laboratory Practice when Performing Molecular Amplification Assays: appendix A attached).

PROCEDURE

Materials
- Microbiological safety cabinet (MSC)
- Absorbent paper
- Micropipetors. 10-1000 μl, 200-1000 μl
- Micropipette tips (with filter plug)
- Waste receptacles (including splash proof receptacle for liquids)
- Calibrated thermometer
- Vortex mixer
- Centrifuge
- Sterile water (molecular biology grade)
- 95°C water bath/ heating block
- Ultrasonic bath

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• Graduated cylinder
• PCR tubes, DNase and RNase free
• Shaking water bath/TwinCubator®
• Thermal Cycler (heating rate: 3°C/sec, cooling rate: 2°C/sec, precision: +/- 0.2°C)
• Thermotable 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: >10^6 fold)
• Timer
• Tweezers

DNA Amplification mix (not provided in kit or mentioned above):
• 10x polymerase incubation buffer
• MgCl₂ solution*
• Thermotable DNA Taq polymerase
*Depending on the enzyme/buffer system used, the optimal MgCl₂ concentration may vary between 1.5 and 2.5mM. Please note that some incubation buffers already contain MgCl₂.

Kit Contents:
• Membrane strips coated with specific probes (STRIPS)
• Primer Nucleotide Mix (PNM) contains specific primers, nucleotides, <1% Dimethyl Sulfoxide, dye
• 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% MgCl₂, <1% NaCl
• Tray, evaluation sheet
• Manual, template

NB: It should be noted that the kit contents for the MTBDRplus and MTBDRsl differ slightly, therefore, there should be no sharing of reagents between these two kits.

Forms
- GenoType® MTBDRplus 12 Evaluation Sheet – provided with the kit (photocopies can be made)
- GenoType® MTBDRsl 12 Evaluation Sheet – provided with the kit (photocopies can be made)
- Quality Manual Attachment M
- Good Laboratory Practice when Performing Molecular Amplification Assays – appendix a

DNA Isolation (Any validated DNA isolation procedure producing amplifiable DNA from mycobacteria can be used)

Smear positive sputum may be used. The working area must be free from amplified DNA. It is crucial to heat samples to 95 – 105°C for at least 15 min in order to totally lyse cells and to inactivate vegetative bacteria.

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1) Use 500μl of decontaminated sputum, pellet bacteria by spinning for 15 min in a
standard centrifuge at approx 10000 x g. Discard supernatant and resuspend
bacteria in 100 μl distilled or PCR grade water by vortexing.
2) Incubate bacteria for 20 min at 95°C (boiling water bath).
3) Incubate for 15 min in an ultrasonic bath.
4) Spin down for 5 min 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.

**Isolation of DNA from positive cultured samples (incl. control strain M. tb H37RV)**

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) to 4) follow method described for sputum DNA isolation above.

**Amplification**
Prepare the amplification mix (45 μl) in a DNA-free room. The DNA sample should be added in a
separated area.

Determine the number of samples to be amplified (number of samples to be analysed plus 2
control samples, positive/negative). Prepare a master mix containing all reagents except for DNA
solution following the table below and mix well (do not vortex). Aliquot 45 μl in each of the
prepared PCR tubes.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume per tube mix</th>
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<tbody>
<tr>
<td>Primer Nucleotide Mix (PNM)</td>
<td>35 μl</td>
</tr>
<tr>
<td>10 x Polymerase incubation buffer</td>
<td>5 μl</td>
</tr>
<tr>
<td>MgCl2 solution *</td>
<td>x μl (see below)</td>
</tr>
<tr>
<td>1 -2 unit(s) thermostable DNA polymerase (refer to manual)**</td>
<td>Do not consider this volume in total for tube</td>
</tr>
<tr>
<td>Molecular biology grade water</td>
<td>y μl (to obtain a volume of 45 μl without DNA)</td>
</tr>
<tr>
<td>DNA solution</td>
<td>5 μl (20-100 ng DNA)</td>
</tr>
<tr>
<td><strong>Final volume per sample</strong></td>
<td>50 μl (not considering volume of DNA polymerase)</td>
</tr>
</tbody>
</table>

*Depending on the enzyme/buffer system used, the optimal MgCl2 concentration may vary
between 1.5 and 2.5 mM. Please note that some incubation buffers already contain MgCl2.
** Quaiagen Hot Start Taq recommended however alternative quality assured Taq polymerase may
be used but the assay must be validated with this enzyme and evidence of optimisation must be
available for review

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.

**Amplification Profile:** (the amplification profile for cultured samples is different – see grey
highlighted box)

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15 min 95°C  1 cycle
30 sec 95°C  2 min 58°C  
25 sec 95°C  40 sec 53°C  40 sec 70°C  
30 cycles or 20 cycles (culture)
8 min 70°C  1 cycle

*When using certain hot start DNA polymerases, this step has to be extended (please refer to manual of enzyme).

Depending on the cycler used, the amplification profile might have to be modified (contact your local distributor). Amplification products can be stored between +4 to –20°C.

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.

### Hybridization

#### Preparation

Pre-warm shaking water bath/TwinCubator® to 45°C; the maximum tolerated deviation from the target temperature is +/-1°C.

Pre-warm solutions HYB and STR to 37 – 45°C before use. The reagents must be free from precipitates (note, however that solution CON-D is opaque). Mix if necessary. Warm the remaining reagents with the exception of CON-C and SUB-C to room temperature. Using a suitable tube, dilute Conjugate Concentrate (CON-C, orange) and Substrate Conjugate (SUB-C, yellow) 1:100 with the respective buffer (CON-C with CON-D, SUB-C with SUB-D) in the amounts needed (for each strip, add 10 µl concentrate to 1 ml respective buffer). Mix well and bring to room temperature. Dilute CON-C before each use. Diluted SUB-C is stable for 4 weeks if stored at room temperature and protected from light.

1) Dispense 20 µl of Denaturation Solution (DEN, blue) in a corner of each of the wells used.
2) Add to the solution 20 µl of amplified sample, pipette up and down to mix well and incubate at room temperature for 5 minutes. 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.
3) 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.
4) 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. Using tweezers, turn over the strips which might have turned when immersed in the solution. Carefully clean tweezers after each use to avoid contamination. This also applies to all the following steps.
5) Place tray in shaking water bath/TwinCubator® and incubate for 30 minutes 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 dipped into the water to at least 1/3 of its height.
6) Completely aspirate Hybridisation Buffer. For example, use a Pasteur pipette connected to a vacuum pump.

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7) Add 1 ml of Stringent Wash Solution (STR₃ red) to each strip and incubate for 15 minutes at 45°C in shaking water bath/TwinCubator®.

Work at room temperature from this step forward:
8) Completely remove stringent wash solution. 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.
9) Wash each strip once with 1 ml of Rinse Solution (RIN) for 1 minute on shaking platform/TwinCubator® after incubation.
10) Add 1 ml of diluted Conjugate (see above) to each strip and incubate for 30 minutes on shaking platform/TwinCubator®.
11) 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.
12) Add 1 ml of diluted substrate (see above) to each strip and incubate protected from the light 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.
13) Stop reaction by briefly rinsing twice with distilled water.
14) Using tweezers, remove strips from the tray and dry them between two layers of absorbent paper.

Evaluation and Interpretation of Results
An evaluation sheet is provided with the 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 amplifier 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 amplifiers 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 can not be evaluated by this test system. This band should be present in the positive control and any samples containing *M. tuberculosis*.

Locus Controls MTBDRplus (*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 MTBDRsI (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 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:
1) 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.
2) 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,
- Room temperature too low or reagents not equilibrated to room temperature.
- No or too little amount of CON-C and/or SUB-C used.

**Weak or no signals except for Conjugate Control zone**
- Quality and/or quantity of isolated DNA do not allow an efficient amplification. Check amplimer on a 2% agarose gel. In case no amplimer is visible, repeat DNA isolation and amplification. If necessary, try a different DNA isolation method.
- Incubation temperature too high

**No Homogenous Staining**
- Strips were not completely immersed during incubation steps
- Tray was not shaken properly

**High Background Colour**
- CON-C and/or SUB-C used too concentrated
- Washing steps were not performed with the necessary care
- Wash solutions to cold.

**Unexpected Result**
- Wrong incubation temperature
- Hybridization buffer and/or Stringent Wash Solution were not properly pre-warmed or mixed
- Contamination of isolated DNA and/or amplification agents with isolated and/or amplified DNA. In case amplification agents are contaminated a negative control sample as also shows the respective banding pattern.
- Contamination of neighbouring wells by spillage during addition of Hybridization Buffer.
- Depending on the amount of amplified DNA used and on the specific reaction conditions, a strong and fast colour development may occur. In such cases, discontinue the substrate incubation as soon as the signals are clearly visible in order to prevent the development of cross-hybridizing bands.
- No pure culture as starting material or more than one mutation in the tested strain.
- Silent mutation in probe region

**Internal Quality Control Measures**
In order to validate the correct performance of the test and the proper functioning of reagents, each MTBDR\(\text{plus}\) and MTBDRs\(\text{s}\) strip includes 5 control zones:
- a Conjugate Control zone to check the binding of the conjugate on the strip and a correct chromogenic reaction
- an Amplification Control zone to check for a successful amplification reaction
- MTBDR\(\text{plus}\): three Locus Control Zones (\(rpoB\), \(katG\) and \(inhA\)) checking the optimal sensitivity of the reaction for each of the tested gene loci
- MTBDR\(\text{s}\): three locus control zones (\(gyrA\), \(rrs\) and \(embB\)) checking the optimal sensitivity of the reaction for each of the tested gene loci.

REMoxTB Quality Manual – Attachment Mi (MTBDR\(\text{plus}\)) and Mii (MTBDR\(\text{s}\))

Each time the test is performed a positive (sensitive M. tb H37RV strain) and negative (dd\(H_2O\)) control is used

**Note:**
Positive control (MTBDR\(\text{plus}\)) – 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\(\text{s}\)) – 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.

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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.
Laboratory Manual 8: Appendix A

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.

2.0 GENERAL CONSIDERATIONS

2.1 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 ie 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.

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

3.1 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.

3.2 THE UNIDIRECTIONAL WORKFLOW
Workflow between these rooms/areas must be unidirectional ie 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.

3.3 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.

3.4 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.
3.5 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.

3.6 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.

DIAGRAM SHOWING WORK FLOW IN A PCR LABORATORY

![Diagram](image)

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.
REMEX Clinical TB Trial
Laboratory Manual Section 9

IS6110 RFLP typing of Mycobacterium tuberculosis

Version: 3.0
Date: 13th January 2009

Approved by: Dr Tim McHugh
Date: 13th June 2007
Signature

Current version: 3.0

Revision history:

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<td>SB</td>
<td>13.06.07</td>
<td>First draft to be used in the trial, originally LM8</td>
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<td>3.0</td>
<td>EB</td>
<td>13.01.09</td>
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PURPOSE
This procedure describes the IS6110 Restriction Fragment Length Polymorphism analysis of Mycobacterium tuberculosis, and describes the molecular strain typing of M. tuberculosis isolates to establish epidemiological links between strains and patients, to monitor contamination and as a research tool.

PRINCIPLE
DNA fingerprinting of Mycobacterium tuberculosis has been shown to be a powerful epidemiological tool. Standardisation of this technique was proposed in 1993 by Van Embden et al to exploit variability in both the number and genomic position of IS6110 to generate strain-specific patterns. The insertion sequence IS6110 is found in almost all isolates of M tuberculosis and is present in up to 26 copies. The variability in the position of these insertion sequences provides the basis of a RFLP typing method. This method is standardised and therefore permits results to be compared between different laboratories and therefore allows investigation of the international transmission of tuberculosis and identification of specific strains with unique properties such as high infectivity, virulence, or drug resistance, as well as monitoring cross contamination.

The DNA from M tuberculosis cultures is extracted and then digested with the restriction enzyme PvuII. Restriction endonucleases recognise short DNA sequences and cleave double stranded DNA at specific sites within or adjacent to the recognition sequences. This cleavage results in the formation of discrete DNA fragments. PvuII cleaves at the following sequence:
The resulting digests are separated by agarose gel electrophoresis and then transferred to a nitrocellulose membrane by Southern hybridisation. Fragments containing the IS6110 insertion sequence are detected using a specific non-radioactively labelled DNA probe. The probe is randomly labelled with Fluorescein and is detected using an anti-fluorescein antibody conjugated with alkaline phosphatase (AP). AP catalyses the breakdown of the substrate dioxetane CDP star, producing a chemiluminescent signal. This signal is then captured by x-ray film, which is developed to visualise the resulting fingerprints. These fingerprints are then inputted into the epidemiology based software package, BioNumerics, which identifies similarities between strains.

**PROCEDURE**

**Materials**

- 10μL loops
- 1.5mL plastic tubes with rubber ‘O’ rings
- Waterbath
- 1000μL pipette and plugged tips
- 200μL pipette and plugged tips
- Fine nose pastettes
- 1.5mL Eppendorfs
- -20°C freezer
- 4°C refrigerator
- Microwave
- Hybaid gel electrophoresis tank and apparatus
- UV light table
- 200μL thin-walled tubes
- Large gel electrophoresis tank and apparatus
- Scalpel
- Bioassay tray
- Measuring cylinder
- Orbital shaker
- Southern blotting apparatus, including:
  - Buffer tank
  - Plastic platform
  - Parafilm
  - Hybond N nitrocellulose membrane
  - 3M paper
  - Quickdraw paper
- UV strip-light
- Heating block
- Oven with shaking platform
- Cling-film
- Plastic envelope
- Hyperfilm, cassettes and developing trays
- Tris EDTA buffer (TE buffer)
- Lysosome
- Proteinase K
- Sodium dodecyl sulphate (SDS)
- CTAB
- NaCl
- Chloroform
- Isoamylalcohol
- Isopropanol
- 70% ethanol
- Agarose
- Tris borate EDTA buffer (TBE)
- Ethidium bromide
- Lambda DNA
- Loading buffer
- pvu II restriction enzyme, buffer and bovine serum albumin (BSA)
- Hind III DNA marker
- HCl
- NaCl
- NaOH
- Tris
- Salt sodium citrate (SSC)
- Hybridisation solution:
  - SDS
  - SSC
  - Dextran sulphate
  - Liquid block
- Alkaline phosphatase conjugate
- Bovine serum albumin (BSA)
- Detection solution

Forms:

- Quality Manual attachments C & I.
1.0 Restriction enzyme digestion of chromosomal DNA

1) Set a waterbath to 37°C.
2) Label 0.2mL Eppendorf tubes with isolate/control numbers.
3) For each restriction digest, 200ng of DNA is required; therefore from the estimated DNA concentrations from (LM8) calculate the volume of each extract required, and note on the RFLP worksheet (see attachments).
4) A final reaction mixture of 25µL is used and made up of Pvu II, buffer, BSA, DNA and water – see the table and examples below.
5) Calculate the volume of sterile water required to complete the reaction mixture and add this, together with the extracted DNA to the appropriate tubes.
6) Remove the Pvu II reagents from the −20°C chest freezer in the gel room and place on ice.
7) In a 1.5mL Eppendorf tube, prepare a mastermix of buffer, Pvu II and BSA on ice as below (x30 is sufficient for 29 extractions). If the volume of each extract required, to obtain 200ng DNA per reaction (see RFLP worksheet), is greater than 21µL, then double the volume of reaction mix should be used.

<table>
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<tr>
<th>Reagent</th>
<th>Volume (µL)</th>
</tr>
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<tbody>
<tr>
<td>10 x Reaction buffer</td>
<td>2.5</td>
</tr>
<tr>
<td>Pvu II (12 units)</td>
<td>1.25</td>
</tr>
<tr>
<td>BSA</td>
<td>0.25</td>
</tr>
<tr>
<td>Total</td>
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Example 1:

- DNA concentration: 50ng/µL
- DNA volume required: 4µL
- Pvu II mixture: 4µL
- Water: 17µL
- Total: 25µL

Example 2:

- DNA concentration: 5ng/µL
- DNA volume required: 40µL
- Pvu II mixture: 8µL
- Water: 2µL
- Total: 50µL

8) Aliquot the mastermix into the labelled tubes in volumes of 4µL
   (or 8µL for those requiring an extra aliquot – see example 2).
9) Mix the tubes by flicking with a finger and pulse down in a microcentrifuge.
10) Incubate at 37 °C for 1 hr.
2.0 Gel Electrophoresis of Restriction Digests

1) Load the gel made previously in LM8, the load restriction digests and electrophorese following the same protocol as in LM8, with the exception that Lambda DNA standards are not required.

2) Place on the UV transilluminator. UV protection glasses, a face visor and gloves must be worn when viewing the gel. Estimate concentrations by making visual comparisons between the band intensities of the restriction digests. The aim of this step is to estimate the volume of each digest that should be added to the final gel to give standardised DNA concentrations and therefore an even image.

3) Note on the RFLP worksheet the volumes required of each extract so that equal concentrations can be loaded onto the large gel.

4) Take a picture using the Kodak digital camera system or equivalent, print and attach to the RFLP worksheet.
### 3.0 Separation of DNA fragments by electrophoresis and Southern blotting

1. Prepare a 0.8% agarose gel as in 6.2 using 2.16g agarose, 270mL TBE and 13.5µL ethidium bromide.
2. Seal the ends of a large casting tray (29 x 25 cm) with autoclave tape and pour the cooled 0.8% gel on a flat, even surface.
3. Insert a 30-toothed comb at the top of the gel and allow to set.
4. Remove the autoclave tape from the ends of the gel and insert it into the Gibco Model H4 electrophoresis tank and cover with fresh 0.5 x TBE buffer. Remove the comb carefully.
5. To each required volume of restriction digest, as calculated above (noted on RFLP worksheet), add 4µL of loading buffer.
6. In the first (left-hand) gel well load 10µL λ *Hind*III marker with 3µL loading buffer.
7. Load the gel with the marker and digests according to the RFLP worksheet.
8. If loading the gel at approximately 17.00 hours, run the gel at 70V overnight.
9. First thing in the morning check the migration of the λ *Hind*III marker, continue electrophoresis until the 2.2 kb fragment has migrated 11 cm. This is checked by placing the entire gel tray on the UV transiluminator UV protection glasses, a face visor and gloves must be worn when viewing the gel.
10. Remove the gel and cut off the bottom right hand corner with a clean scalpel. The gel above the wells can also be removed to allow easier handling.
11. Place the gel in a bioassay tray and take to the blotting room.
12. Immerse the gel in 200mL 0.2M HCl for 10 min with gentle agitation on the orbital shaker (depurination).
13. Rinse briefly (10 sec) in distilled water (x3).
14. Immerse the gel in 1.5M NaCl, 0.5M NaOH (200mL) for 40 min gentle agitation on the orbital shaker (denaturation).
15. Remove the 1.5M NaCl, 0.5M NaOH and immerse the gel in 1M Tris (pH7.4), 1.5M NaCl (200mL) for 40 min gentle agitation on the orbital shaker (neutralisation).
16. Rinse briefly in 10x SSC.
17. Construct the capillary blotting apparatus:
   - Cover the gel support with Whatman 3MM paper (so the paper acts as a wick).
   - Fill reservoir with 10x SSC.
   - Place the inverted gel on the paper (i.e. gel wells face down).
   - Surround the edges of the gel with four parafilm strips.
   - Cut Hybond N+ membrane to fit the gel exactly.
   - Remove the same corner to correspond with the gel and mark the side that will bind the DNA.
   - Place the membrane on the gel with cut corners matching.
   - Remove air bubbles.
   - Place 3 sheets of 3MM paper, pre-wetted with 10 x SSC, over the membrane.
   - Remove any air bubbles.
   - Place 8 sheets of Quickdraw paper over 3MM.
   - Place a bioassay dish lid on top & add a 500g weight.
   - Leave to blot for 2 hours.
18. Dismantle the blotting apparatus, mark the side of the membrane with the DNA on it with pencil and wash briefly with 5x SSC.
19. Air-dry the membrane between sheets of 3MM.
4.0 Hybridisation and detection

1) Set the hybridisation oven to 50°C.
2) Pre-wet the cross-linked membrane with 5x SSC.
3) Place the membrane into a bioassay tray, cover with approximately 80mL hybridisation solution and replace the lid.
4) Prehybridise for 30 min at 50°C shaking gently.
5) Denature the INS1/INS2 probe (stored at −20°C chest freezer in the gel room) at 95°C for 5 min using a heating block or a boiling waterbath then quench on ice. See 6.0 for the production of the probe.
6) Remove 1mL of hybridisation solution, add 15μL of the denatured probe, mix and return to assay dish taking care to distribute the mixture evenly. Check for and remove air bubbles, and seal with Parafilm.
7) Hybridise overnight at 50°C shaking at approximately 80 rpm.
8) In the morning preheat 600 ml 1x SSC, 0.1% SDS (wash solution 1) and 600ml 0.5x SSC, 0.1% SDS (wash solution 2) to 50°C in the oven.
9) Rinse the membrane in 300mL wash solution 1 for 7.5 min at 50°C shaking x2.
10) Rinse the membrane in 300mL wash solution 2 for 7.5 min at 50°C shaking x2.
11) Place the membrane in a fresh bioassay tray.
12) Wash briefly with Buffer A (GI-A). Buffer A must be autoclaved prior to use.
13) Block the membrane with 300mL of 1:10 Liquid Block in Buffer A for 90 min at room temperature with gentle agitation on the orbital shaker.
14) Incubate the membrane with 130mL of Buffer B (1:5000 anti-fluorescein alkaline phosphatase-conjugate in 0.5% BSA in Buffer A) for 60 min at room temperature with gentle agitation on the orbital shaker.
15) Wash the membrane with 300mL Buffer C (Buffer A, 0.3% Tween 20) for 10 min at room temperature with gentle agitation three times.
16) Drain the membrane well and place onto a clean sheet of Clingfilm DNA face up.
17) Pipette on approximately 16 mL of detection solution, enough to cover the whole membrane and leave for 5 min.
18) Drain the membrane thoroughly and place in a plastic envelope ensuring there are no air bubbles. Dry the outer surfaces and edges of the envelope thoroughly with tissue.
19) In dark room, place the membrane in film cassette, DNA side up.
20) Only using orange light, remove a piece of Hyperfilm, cut off a corner and place on top of the membrane with cut corners matching. Seal the cassette.
21) Expose the film for 30 min in the first instance.
22) Place developing solution, fixing solution and tap water (wash) in the correspondingly labelled white trays. These trays must not be mixed up.
23) Using only orange light, open the cassette and place the film in the developing solution. Agitate gently until bands are visible (approximately 1 minute).
24) Wash the film in tap water.
25) Place the film in the fixing solution until the film becomes transparent (approximately 1 minute).
26) Wash the film in tap water.
27) Hang the film up to dry.
28) Review image. If the image is under-exposed, repeat the exposure with a fresh piece of Hyperfilm for a longer period of time. If the image is over-exposed, repeat the exposure for a shorter period of time.
5.0 Photographing/scanning the image and entry into Bionumerics

1) Place the dry image face up on the light table.
2) Turn on digital camera, PC, and light table.
3) Position the digital camera above the image.
4) Open KDSID 2.0 camera programme from shortcut on desktop.
5) Click as follows:
6) File
7) Acquire
8) DC40/DC120 camera
9) Select:
   - Exposure settings
   - Gel type
   - Gel size
   - Compression
   - Delay
   - Open image immediately
   - Delete image in camera
   - Print after opening
   Gel photography
   Coomassie blue
   13 x 17 cm
   On
   Off
   Yes
   Yes
   No
10) Click ‘Take picture’.
11) Select required section of image with dotted square.
12) Save as a TIFF file on the TB RFLP file on the C drive, and also on a floppy disk.
13) Transport to the sequencing lab to transfer image to BioNumerics.
14) The completed worksheet and image are to be filed.
6.0 Preparation of DNA probe by PCR

To avoid contamination with PCR amplifiers a strict 4 room strategy is applied to all PCR protocols. Reagents and equipment must only be moved from room 2 to 4 and never the reverse.

<table>
<thead>
<tr>
<th>Room 1</th>
<th>DNA template preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Room 2</td>
<td>PCR clean room, preparation of the mastermix</td>
</tr>
<tr>
<td>Room 3</td>
<td>PCR grey room, mixing of DNA template with mastermix</td>
</tr>
<tr>
<td>Room 3</td>
<td>PCR 'Dirty' room – handling of all amplifiers</td>
</tr>
</tbody>
</table>

In PCR Room 1:

- Measure out the master mix for 10 PCR reactions (2 +ve/-ve controls, 6 for probe)

<table>
<thead>
<tr>
<th>Component</th>
<th>V (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNTPs</td>
<td>30</td>
</tr>
<tr>
<td>Primers: INS1 (C13)</td>
<td>50</td>
</tr>
<tr>
<td>Primers: INS2 (C14)</td>
<td>50</td>
</tr>
<tr>
<td>KCL buffer</td>
<td>100</td>
</tr>
<tr>
<td>Water</td>
<td>665</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>900</td>
</tr>
</tbody>
</table>

- Aliquots of 90 µL pipetted into 0.5 ml reaction tubes, + 50 µL mineral oil.

**INS probe primer sequences:**

Forward: CGT GAG GGC ATC GAG GTG GC

Reverse: GCG TAG GCG TCG GTG ACA AA

In PCR Room 3:

- Add 10 µl genomic DNA from H37Rv M. tuberculosis reference strain (extracted as stated above in LM8), therefore giving a total reaction volume of 100 µL.

In PCR Room 4:

- Reactions pulsed and loaded onto thermal cycler.
- Programme 42 on block thermocycler
  
  - 94°C for 2 minutes
  - 94°C for 1 minute
  - 65°C for 1 minute
  - 72°C for 2 minutes
  - 72°C for 5 minutes
  
  35 cycles
6.1 Purification of PCR product

1) Prepare a 1.8% agarose gel as in LM8 using 0.54g low melting point agarose, 30 ml 0.5x TBE, 1.5 μL ethidium bromide and large-toothed combs in a mini gel rig.

2) Load 10 μL of PCR product and 2 μL of loading buffer to each of the centre 6 wells and electrophorese at 120 V until the buffer front is 1cm from the bottom edge of the gel.

3) Transfer the gel (on a piece of Parafilm) to the UV light box, and excise the PCR bands with a clean, sharp scalpel. Take care to remove as much excess agarose as possible. UV protection glasses, a face visor and gloves must be worn when viewing the gel.

4) Transfer agar pieces to eppendorf tubes in approximately 300 μg batches.

5) Using Qiagen MinElute Gel Extraction Kit Protocol, add 300 μL buffer QG for each 100 μg of agarose.

6) Incubate at 50°C for 10 minutes (or until the gel has completely dissolved). Vortexing periodically during incubation will assist in this.

7) Add 300 μL isopropanol to the tube and mix by inversion.

8) Apply tube contents to MinElute column and collection tube, and microfuge at 13,000 rpm for 1 minute (the maximum column reservoir is 800 μL, for larger volumes, load column and spin again).

9) Discard flow-through and place column back in same collection tube.

10) Add 500 μL of buffer QG to the column and microfuge for 1 minute. Discard flow-through and place column back in same collection tube.

11) To wash, add 750 μL of buffer PE to the column and microfuge for 1 minute. Discard the flow-through and microfuge for a further 1 minute.

12) Place the column in a clean 1.5 μL collection tube.

13) To elute DNA, add 10 μL of buffer EB to the centre of the column membrane, allow to stand for 1 minute, then microfuge for 1 minute.
6.2 Random labelling of DNA probe

Using the Gene Images random prime labelling protocol - GI labelling of purified PCR product

For each labelling reaction, include 1x control DNA supplied with the kit.

- Thaw components on ice.
- Using a heating block or waterbath, denature DNA at 95°C for 5 minutes. Quench on ice.
- Prepare the reaction mix as follows:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>V (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>29</td>
</tr>
<tr>
<td>Nucleotide mix</td>
<td>10</td>
</tr>
<tr>
<td>Primer</td>
<td>5</td>
</tr>
<tr>
<td>Denatured DNA</td>
<td>5</td>
</tr>
<tr>
<td>Enzyme solution (Klenow) 5 units/µL</td>
<td>1</td>
</tr>
<tr>
<td>Total volume</td>
<td>50</td>
</tr>
</tbody>
</table>

- Mix, pulse down and incubate at 37°C for 2 hours.
- Add 2 µL of 0.5M EDTA to stop the reaction. Store labelled probe in the dark at -20°C for up to 6 months.

6.3 Monitoring incorporation using the rapid labelling assay

- Prepare 1/5, 1/10, 1/25, 1/50, 1/100, 1/250 and 1/500 dilutions of the 5x nucleotide mix in TE buffer.
- Dot out 5 µL of the labelled probe (from 6.10) and 5 µL of the 1/5 dilution of nucleotide mix (negative control) on a strip of Hybond-N+ placed on a non-absorbent backing. Allow the liquid to absorb, (but do not allow to dry) and wash the strip with gentle agitation in excess pre-heated 2x SSC at 60°C for 15 minutes.
- Prepare a reference strip by dotting 5 µL of each nucleotide mix dilution see point 1, above), except the 1/5, on to a separate strip of Hybond-N+. This reference strip can be re-used and can be stored, wrapped in SaranWrap™, for several weeks at ~20°C in the dark.
- Place both the reference and the washed strips on a piece of TE-moistened Whatman 3MM paper and visualise (sample side down) on the UV transiluminator. UV protection glasses, a face visor and gloves must be worn when viewing the gel.
- The labelled probe should be visible as a fluorescent spot with intensity comparable to the reference strip at between the 1/10 and 1/250 dilutions. This indicates that the probe has been successfully incorporated.
- The brighter the intensity of the labelled DNA (compared to the lower nucleotide dilution (1/10), the more efficient the labelling reaction. The negative control should have little or no incorporation.
7.0 Recording of Results (include calculation of results if applicable)

The image is stored in the RFLP folder and once an isolate has been typed and entered into Bionumerics, the gel number is recorded in the Positive Isolate Database. This gel number is also entered in Winpath at the bottom of the Results Entry section.

7.1 Interpretation of Results

Interpretation of results

The software (Bionumerics) calculates the percentage similarity of strains. If two or more strains are termed to be identical or nearly identical (≥95%) then the original images must be examined by eye by a senior HCS.

Repeat criteria and interpretative messages.

Strain typing must be repeated if:

- The extraction fails to yield any DNA
- The restriction fails, leading to a smear on the gel
- The image is unreadable due to high background, low DNA concentrations or poor probe binding.

If a strain has been typed twice and no bands are seen despite sufficient DNA extraction, restriction, probing and detection (i.e. there are no smears and other strains on the gel have produced bands), then that strain is said to possess no copies of IS6110.
# 8.0 Instructions for preparing reagents and buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M EDTA</td>
<td>186.1 g EDTA.2H₂O&lt;br&gt;800 ml dH₂O&lt;br&gt;Adjust to pH 8.0 with NaOH&lt;br&gt;Stir vigorously (does not dissolve until pH 8)</td>
</tr>
<tr>
<td>0.2 N HCl</td>
<td>10 ml concentrated stock&lt;br&gt;490 ml dH₂O</td>
</tr>
<tr>
<td>5 M NaCl</td>
<td>146.1 g / 500 ml dH₂O</td>
</tr>
<tr>
<td>1 M NaOH</td>
<td>20 g / 500 ml dH₂O</td>
</tr>
<tr>
<td>TE buffer</td>
<td>10 ml 1 M Tris pH 8.0&lt;br&gt;2.0 ml 0.5 M EDTA&lt;br&gt;dH₂O to 1000 ml</td>
</tr>
<tr>
<td>1 M Tris</td>
<td>66.1 g Tris HCl&lt;br&gt;9.7 g Tris base&lt;br&gt;500 ml dH₂O&lt;br&gt;PH 7.4</td>
</tr>
<tr>
<td></td>
<td>121.1 g tris base&lt;br&gt;42 ml concentrated HCl stock&lt;br&gt;1000 ml dH₂O</td>
</tr>
<tr>
<td>10% SDS</td>
<td>10 g / 100 ml dH₂O</td>
</tr>
<tr>
<td>20x SSC</td>
<td>175.3 g NaCl&lt;br&gt;88.2 g Na citrate&lt;br&gt;1000 ml dH₂O&lt;br&gt;pH 7.0</td>
</tr>
<tr>
<td>5x TBE</td>
<td>54 g Tris base&lt;br&gt;27.5 g boric acid&lt;br&gt;1000 ml dH₂O&lt;br&gt;20 ml 0.5 M EDTA pH 8.0</td>
</tr>
</tbody>
</table>
### CTAB/NaCl
- 4.1 g NaCl
- 10 g CTAB
- 100 ml dH₂O
- Heat to 65°C to dissolve if necessary

### Lysozyme
- 10 mg / ml dH₂O

### SDS/Proteinase K
- 5 µL proteinase K 10 mg/ml
- 70 µL 10% SDS
- For each sample

### 1.5 M NaCl / 0.5 M NaOH
- 100 ml 1 M NaOH
- 60 ml 5 M NaCl
- 40 ml dH₂O

### 1 M Tris (pH 7.4) / 1.5 M NaCl
- 100 ml 1 M tris
- 60 ml 5 M NaCl
- 40 ml dH₂O

### Hybridisation solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (ml)</th>
<th>Final conc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>138</td>
<td>-</td>
</tr>
<tr>
<td>20x SSC</td>
<td>50</td>
<td>5x</td>
</tr>
<tr>
<td>10% SDS</td>
<td>2</td>
<td>0.1%</td>
</tr>
<tr>
<td>Liquid block</td>
<td>10</td>
<td>5%</td>
</tr>
<tr>
<td>Dextran sulphate</td>
<td>10 g</td>
<td>5%</td>
</tr>
</tbody>
</table>

- Heat to dissolve, dispense 20 ml aliquots, store at ~20°C

### Wash 1
(1x SSC, 0.1% SDS)
- 600mL 1x SSC
- 0.6g SDS

### Wash 2
(0.5x SSC, 0.1% SDS)
- 600mL 0.5x SSC
- 0.6g SDS

### Buffer A
- Quantity: 12.11 g Tris base, 17.53 g NaCl, 1000 mL H₂O
- Final conc: 100 mM Tris base, 300 mM NaCl
- pH 9.5, autoclave before use.

### Buffer B
(0.5% BSA, 1:5000 Anti-fluorescein antibody in Buffer A)
- 130mL Buffer A
- 0.65g BSA
- 25µL anti-fluorescein antibody

### Buffer C
(0.3% Tween 20 in Buffer A)
- 900mL Buffer A
- 2.7mL Tween 20
MIRU typing of *M. tuberculosis*

**Purpose & Scope**
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.

**Principle/Overview of the Procedure**
Mycobacterial interspersed repeating units are 40-100 bp sequences of DNA, often found as tandem repeats. The *M. tuberculosis* strain H37Rv has 41 such units. PCR and sequence analysis of 31 of these units found 24 to be variable enough to be used to type strains of *M. tuberculosis*. The technique has the discriminatory ability approaching that of IS6110 RFLP, but has the advantage of being able to distinguish between strains with few IS6110 copies (<5). Another advantage is that the technique is relatively rapid compared to IS6110 RFLP and can be automated using multiplex PCRs and capillary electrophoresis. The method can still be used in the absence of these technologies, by using conventional PCR and agarose gel electrophoresis to size the products. The product size can be compared with a published table of corresponding copy number or allele. It is notable that if the organism possesses no copy of a particular repeat, then a PCR product will still be generated as the primers used target up and down stream of the repeats. Furthermore, as this technique involves amplification, relatively small amounts of DNA are required.
PROCEDURE

Equipment / Materials

- -20°C freezer
- 4°C refrigerator
- Thermocycler
- 3130 Genetic Analyzer
- 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 following table:

**Table 2: Volumes (μl) per reaction for multiplex PCRs**

<table>
<thead>
<tr>
<th>Mix</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loci</td>
<td>580</td>
<td>960</td>
<td>0424</td>
<td>2401</td>
<td>2163</td>
<td>154</td>
<td>2059</td>
<td>2347</td>
</tr>
<tr>
<td></td>
<td>2996</td>
<td>1644</td>
<td>0577</td>
<td>3690</td>
<td>1955</td>
<td>2531</td>
<td>2687</td>
<td>2461</td>
</tr>
<tr>
<td>Alias</td>
<td>802</td>
<td>3192</td>
<td>2165</td>
<td>4156</td>
<td>4052</td>
<td>4348</td>
<td>3007</td>
<td>3171</td>
</tr>
<tr>
<td></td>
<td>4, 26, 10, 16, 42, 43, 47, 52, 11b, -</td>
<td>MIUR 2,</td>
<td>MIUR 20,</td>
<td>46, 48,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final MgCl₂ conc</td>
<td>3mM</td>
<td>2mM</td>
<td>1.5mM</td>
<td>3mM</td>
<td>1.5mM</td>
<td>2.5mM</td>
<td>1.5mM</td>
<td>2mM</td>
</tr>
<tr>
<td>H20 (μl)</td>
<td>7.5</td>
<td>8.3</td>
<td>8.7</td>
<td>7.5</td>
<td>8.7</td>
<td>7.9</td>
<td>8.7</td>
<td>8.3</td>
</tr>
<tr>
<td>10 x Buffer (μl)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>5 x Q solution (μl)</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>25mM MgCl₂ (μl)</td>
<td>1.2</td>
<td>0.4</td>
<td>0</td>
<td>1.2</td>
<td>0</td>
<td>0.8</td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>5mM dNTP (μl)</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Primer mix (μl)</td>
<td>2.4</td>
<td>2.4</td>
<td>2.4</td>
<td>2.4</td>
<td>2.4</td>
<td>2.4</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>Hotstart Taq (μl)</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>DNA</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Final Vol</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

Using the Corbett PCR setup robot located in the parasitology lab
1. The MIUR method file is opened in the robot software (C:/Program Files/Robotic4/Data/MIUR TB typing/MIUR-12samples)
2. Aliquots of the various reagents (10 x buffer with taq added, 5x Q soin, 25mM MgCl₂, 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)
3. The run button is pressed on the robot software

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, MgCl₂ and H₂O, and 18μl aliquotted into each well (according to plate plan below)
2. The plate is then transferred to the hood in MBL and 2μl 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 below.

**Table 3: PCR plate plan**

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>5</td>
<td>E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>H</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PCR Cycling Settings

Version 4.0
29 Apr 2010
• 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)

<table>
<thead>
<tr>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min</td>
<td>95 °C</td>
</tr>
<tr>
<td>1 min</td>
<td>94 °C</td>
</tr>
<tr>
<td>1 min</td>
<td>59 °C</td>
</tr>
<tr>
<td>1 min 30s</td>
<td>72 °C</td>
</tr>
<tr>
<td>10 min</td>
<td>72 °C</td>
</tr>
<tr>
<td>∞</td>
<td>4 °C</td>
</tr>
</tbody>
</table>

  30 cycles

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 (see diagram below).

![Diagram of a 96 well plate]

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_REMxx dd mm yy
14. Select the following for one sample:
Sample Type: ‘sample’
Size Standard: ‘TB_MIRU_VNTR’
Panel: ‘None’
Analysis Method: ‘TB_MIRU’
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.
16. Once the file has been found, select the yellow plate to link the plate on the autosampler to the
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:

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<th>Parameter</th>
<th>Value</th>
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<td>Poly Fill Vol</td>
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<td>Current Stability</td>
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<td>Injection Voltage</td>
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<td>Injection Time</td>
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<td>Voltage no. of Steps</td>
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<tr>
<td>Voltage Step Interval</td>
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<td>Data Delay Time</td>
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<tr>
<td>Run Voltage</td>
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<tr>
<td>Run Time</td>
<td>4000</td>
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**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 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. In the first well pipette 5ul of the 100bp DNA ladder. DNA ladder can be added to the last well too.
7. Mix 1ul of loading dye with 4ul 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 170 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 “quant 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 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_REMox’ 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.

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 and quality control form is in the appendices below.

Data reporting

All REMox results should be reported on the MIRU/IS6110 worksheet (Appendix C of the UCL REMoxTB Chain of custody SOP), verified and reported to the sites as outlined in the UCL REMoxTB Chain of custody SOP

Appendices

Version 4.0
29 Apr 2010
1. H37Rv genotype

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<th>H37Rv size (bp)</th>
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3. Allele calling table for the 24 MIRU loci
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(Strains H37Rv, H37R, BCG and 1% of clinical strains)

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</table>
4. MIRU Quality Control Form

**Quality Control Test**
Upon receipt of a new shipment or lot number of Qiagen HotStarTaq, dNTPs, Primer mixes 1-8 or MIRU ladder, a PCR amplification of a positive control (M.tb H37Rv DNA) and negative control (UV-treated water) should be analysed following the REMoXTB MIRU SOP (Laboratory Manual LM10) and the results recorded on this worksheet. Record the date the reagent of that lot came into use in the laboratory and the date it finished.

**Quality Control Results**
The positive control must match the H37Rv genotype in LM10 Appendix 1 and the negative control should have no detectable DNA products for the reagents to be used for the analysis of trial samples. QC data should be filed with the Quality Control Forms. If the QC fails, repeat the test with new positive control. 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 Q).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Lot Number</th>
<th>Expiry Date</th>
<th>Date QC Test</th>
<th>QC Passed (Positive and Negative Controls)</th>
<th>Comments</th>
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<tr>
<td></td>
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<td>Yes ☐ No ☐ Yes ☐ No ☐ Yes ☐ No ☐ Yes ☐ No ☐ Yes ☐ No ☐</td>
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<tr>
<th>Staff Signature</th>
<th>Date In Use</th>
<th>Date Finished</th>
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Version 4.0
29 Apr 2010
# REMox Clinical TB Trial

## Quality Control Manual: Tuberculosis Laboratories

**Version:** 4.0  
**Date:** 29th June 2011

---

**Approved by:** Dr Tim McHugh  
**Signature and date:**  
**Date:** 29th June 2011  
**Current Version:** 4.0

---

### Revision History:

<table>
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<th>Version</th>
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<th>Reasons for Revision</th>
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<td>1.0</td>
<td>SB</td>
<td>13.06.07</td>
<td>First draft to be used in trial</td>
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<tr>
<td>1.2</td>
<td>AE</td>
<td>21.01.08</td>
<td>Changes to ‘Specimen Transfer Form’ attachment B</td>
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<tr>
<td>2.0</td>
<td>EB</td>
<td>13.01.09</td>
<td>Re-write of Quality Manual, additional QC forms to reflect the requirements of UCL Laboratory Team</td>
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<td>3.0</td>
<td>EB/AE</td>
<td>11.11.09</td>
<td>Amendments to QM Forms reflecting changes required after start of trial</td>
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<tr>
<td>4.0</td>
<td>HC/RH</td>
<td>29.06.11</td>
<td>Amendments to QC Forms reflecting changes in the Lab manuals</td>
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The Quality Control of several parameters occurring during the specimen processing stages of the clinical trial are essential to ensure the consistency and validity of the results obtained.

The points at which factors need to be monitored and recorded will be dealt with by individual procedure. Some of these factors reoccur at several stages throughout the different procedures, such as the need to monitor the temperature of equipment. These will be highlighted in each section, but will have a common Quality Form whenever possible.

The precise location of the work to be undertaken and the staff responsible must be recorded – attachment A.

When any Quality Control fails, a Continuous Quality Improvement Form must be completed to document the failure and the corrective action, it also includes a grading system with severe QC failures being reportable to UCL - attachment Q.

A day 50 Form has been included to monitor for divergence of results between tests, this form should be scanned or faxed to UCL on a weekly basis – Attachment N.
1. **Sputum Collection and Transfer**

1.1 Record the temperature of the package containing the specimens on receipt at the laboratory using an digital minimum/maximum thermometer – attachment B.

2. **Sputum Processing**

2.1 Record the pressure of the Containment Level 3 Laboratory, the airflow of the Microbiological Safety Cabinet and the manufacture of disinfectants – attachment D.

2.2 Record the Lot numbers, expiry date and date of manufacture of the NALC and NaOH – attachment F.

2.3 Record the working temperature of the refrigerated centrifuge – attachment F.

2.4 Record the temperature of all refrigerators and freezers containing reagents – attachment C.

2.5 Record the order samples processed, decontamination times and contamination rates – attachment F.

2.6 Record the Weekly Contamination Rates – attachment G.

2.7 Record details of the contaminated cultures if weekly contamination rates outside 3 – 8% - attachment Gi.

3. **Tuberculosis smear (AFB) Preparation and Staining &
4. Microscopic Examination of Acid-Fast Smears**

For each new batch of staining reagents (Auramine and ZN) perform and record a positive (*M. tuberculosis* H37Rv) and negative (*E. coli*) smear – attachment Ei and Eii.

For each batch of specimens, perform and record a positive (*M. tuberculosis* H37Rv) and a negative (negative decontamination) smear – attachment H and F respectively.

For every 10 Ziehl-Neelsen slides compare AFB counts between technicians – Attachment I.

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29th June 2011
5. **Culture Methods**

5.1 Record the daily maintenance and temperature of the MGIT, printout the Quality Control Report from the MGIT. Attachment J

5.2 For each new batch of MGIT bottles and antibiotic supplements record the manufacture and quality control. Attachment Eiii

5.3 Record the manufacture and Quality Control of LJ slopes - attachment Eiv.

5.4 Record the manufacture and Quality Control of the blood agar – attachment Ev.

5.5 Record the temperature of any incubators used – attachment C.

5.6 Record the temperature of any refrigerators and freezers containing reagents –attachment C.

5.7 Record the MGIT SIRE Drug Susceptibility Testing Internal quality Control (IQC: *M. tuberculosis* H37Rv) – attachment Evi

5.8 Record the MGIT PZA Drug Susceptibility Testing Internal quality Control (IQC: *M. tuberculosis* H37Rv) – attachment Evii

5.9 Record the MGIT Moxifloxacin Drug Susceptibility Testing Internal quality Control (IQC: *M. tuberculosis* H37Rv) – attachment Eviii

5.10 Record the Lot number and expiry date of the MGIT calibration tubes – Attachment K

5.11 Record drug sensitivity profile of reference strain (H37Rv) if is included in each batch of patient samples

---

6. **Confirmation of Mycobacterium tuberculosis Complex**

6.1 Record the internal quality control for the Accuprobe Test - attachment L.

6.2 Record the internal quality control for the Hain MTBDRplus Test – attachment Mi

6.3 Record the internal quality control for the Hain MTBDRsl Test – attachment Mii

---

7. **DNA Extraction**

7.1 Record the estimated DNA concentration for each sample to be shipped to UCL – attachment P

---

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29th June 2011
OTHER

- Location and Staff Record - start of trial and when changes occur (A)
- Reagent QC - when new lots of reagents arrive in lab (Ei to Evii)
- MGit Calibration log - start of trial and when calibration tubes replaced (K)
- Accuprope - Baseline and relapse samples (L)
- Hain - screening samples (Mi & Mii)
- DNA Extraction and Shipment - when batches of DNA extracted and shipped to UCL (P)
- Continuous Quality Improvement Form - whenever there is a QC Failure (Q)
- Drug susceptibility IQC form (R)

DAILY

- Specimen Transfer Form (B)
- Temperature Charts (C)
- Daily Laboratory Maintenance (D)
- Specimen processing (F)
- Daily AFB Microscopy (H)
- Microscopic examination of acid fast smears - every 10 slides (I)
- MGit Maintenance (J)

WEEKLY

- Weekly Contamination Rates (G/Gi)
- Day 50 Form (N)

Version 4.0
29\textsuperscript{th} June 2011
## Attachment A: Location & Staff Record

### Location of work

<table>
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<tr>
<th>Full Postal Address</th>
<th>Room identification (Building &amp; room number)</th>
<th>Procedures to be performed (LM number and sections if applicable)</th>
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</tbody>
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**Version 4.0**  
**29th June 2011**
**Attachment B: Specimen Transfer Form- SPUTUM**

This form should accompany each sputum specimen generated from a REMox patient at the clinical site to the laboratory.

**Clinical Details**

This section should be completed in the clinic

| Patient number | _ _ _ _ _ _ _ _ _ _ | OR | Screening number | _ _ _ _ _ _ _
|----------------|---------------------|----|-----------------|----------------|

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<th>Date of birth (dd/mmm/yyyy)</th>
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| Type of Sputum Sample | □ Early Morning | □ Other |

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<th>Week ___</th>
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- Unscheduled treatment phase
- Unscheduled post-treatment phase

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<th>Physician/nurse attending (signature)</th>
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*screening = SC, baseline = 00

**Transport Details**

This section should be completed by the driver, courier or person accompanying specimen

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<table>
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<th>Driver/courier (print name)</th>
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<tr>
<th>Driver/courier (signature)</th>
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**Laboratory Receipt**

This section should be completed by the laboratory technician receiving the specimens.

---

Version 4.0

29th June 2011
Laboratory Name

Date sample received (dd/mmm/yyyy)

Time sample received (hh:mm)

Temperature of transport container on receipt (°C)

Sample in good condition (y/n)

If no please give details (detail problems, is this sample going to be processed? has another sample been requested?)

Sample processed within 30 minutes (yes/no)

If no, time sample transferred to fridge (hh:mm, and give fridge ID

Laboratory technician (print name)

Laboratory technician (signature)

Laboratory Accession number

Attachment C: Equipment Temperature Log Form

EQUIPMENT TYPE and No:

MONTH:     YEAR:

LOCATION IN DEPT:  RANGE:  TEMPERATURE

Temperatures should be recorded twice a day at different times each day, when possible.

NOTE: When the temperature is out of range inform the laboratory manager. Refer to site specific or REMx SOP for Temperature Monitoring and Evaluation for corrective actions (appendix 1). Record below ‘minor’ action taken. Record major action on a Continuous Quality Improvement Form (attachment Q).

During periods when the lab is not staffed the max and min temperatures over that time period must be recorded in the action box of the last unrecorded date. Draw a line through all other unfilled boxes.

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<th>Time</th>
<th>Signature</th>
<th>Temp PM</th>
<th>Time</th>
<th>Signature</th>
<th>Action (for out of range temperatures)</th>
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Version 4.0
29th June 2011
Attachment D: Containment Level 3 Laboratory Daily Checklist

*This work area must be clean, uncluttered and well maintained*

- The individual Laboratory Technician MUST date and sign this record upon completion of each housekeeping duty (daily). Records of readings must be noted where applicable.
- The Laboratory Manager MUST date and sign at the end of each week to ensure the duties have been performed.
- All surfaces and the cabinet MUST be disinfected at the end of EACH working day.
- All other areas must be disinfected at least once a week.

<table>
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<th>Thu</th>
<th>Fri</th>
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<td>Pressure with hoods off (&lt;-5Pa)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Pressure with hoods on (-10 to -60 Pa)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSC Reading</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSC Anemometer Reading (record average of five readings taken at different positions)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Equipment temperatures acceptable and recorded</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>MGIT maintenance performed (QC printed/checklist completed)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Fresh disinfectants made (including bottled surfanios for MSC)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benches and MSC cleaned</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Floor mopped (at least weekly)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Centrifuge cleaned (at least weekly)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Laboratory Coats changed (weekly)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Laboratory Manager – name and date</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

If the room pressure or MSC reading is out of range, do not start any work in the laboratory until an appropriately qualified person has fixed the problem and the readings are within range. Record any actions on a continuous quality improvement form (attachment Q).

Version 3.0
16<sup>th</sup> November 2009
**Attachment Ei: Auramine Stain Reagents**

**Quality Control Test**
Upon receipt of a new shipment or lot number of Auramine Stain, Potassium Permanganate or 1% Acid Alcohol a positive (H37Rv) and negative (E. coli) smear should be made following the laboratory manual guidelines and the results recorded on this worksheet. Record the date the reagent of that lot came into use in the laboratory and the date it finished (this will be used to link reagents used with specimens processed).  

**Quality Control Results**
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 Q).

<table>
<thead>
<tr>
<th>Reagent*</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lot Number</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expiry Date</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Date QC Test</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive Smear Result</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative Smear Result</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QC Passed</td>
<td>Yes ☐ No ☐</td>
<td>Yes ☐ No ☐</td>
<td>Yes ☐ No ☐</td>
<td>Yes ☐ No ☐</td>
<td>Yes ☐ No ☐</td>
<td>Yes ☐ No ☐</td>
<td>Yes ☐ No ☐</td>
</tr>
<tr>
<td>Comments</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staff Signature</td>
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<td></td>
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<tr>
<td>Date</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Date In Use</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Date Finished</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Version 3.0  
16th November 2009
* Either Auramine Stain, Potassium Permanganate or 1% Acid Alcohol

**Attachment E11: Ziehl-Neelsen Stain Reagents**

**Quality Control Test**
Upon receipt of a new shipment or lot number of Carbol Fuschin, Malachite Green and/or 3% Acid Alcohol a positive (H37Rv) and negative (E. coli) smear should be made following the laboratory manual guidelines and the results recorded on this worksheet. Record the date the reagent of that lot came into use in the laboratory and the date it finished (this will be used to link reagents used with specimens processed).

**Quality Control Results**
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 Q).

<table>
<thead>
<tr>
<th>Reagent*</th>
<th>Lot Number</th>
<th>Expiry Date</th>
<th>Date QC Test</th>
<th>Positive Smear Result</th>
<th>Negative Smear Result</th>
<th>QC Passed</th>
<th>Comments</th>
<th>Staff Signature</th>
<th>Date</th>
<th>Date In Use</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Yes ☐</td>
<td>No ☐</td>
<td>Yes ☐</td>
<td>No ☐</td>
<td>Yes ☐</td>
</tr>
</tbody>
</table>

Version 3.0
16th November 2009
* Either Carbol Fuschin, Malachite Green/Methylene Blue or 3% Acid Alcohol

**Attachment Eiii: MGIT TUBES and PANTA/Growth Supplement**

**Quality Control Test**

Upon receipt of a new shipment or lot number of BBL MGIT 7 ml tubes, Growth Supplement and/or PANTA suspensions of *M. tuberculosis* control organism (ATCC 27294 – H37Rv) should be adjusted to 0.5 McFarland, diluted 1:500 in saline and inoculated into MGIT tubes (as described in the operating manual). Record the date the reagent of that lot came into use in the laboratory and the date it finished (this will be used to link reagents used with specimens processed).

**Quality Control Results**

The BBL MGIT tubes should be detected as positive within 6 – 10 days. If the QC tubes do not give the expected results do not use the remaining tubes of the lot until you have contacted Becton Dickinson Technical services. Any Quality Control Failure and subsequent actions should be recorded on the Continuous Quality Improvement Form (Quality manual attachment Q).

<table>
<thead>
<tr>
<th>Item*</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lot Number</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expiry date</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Date of QC Test</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Date Tube Positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to detection (TTD)</td>
<td>Yes ☐ No ☐</td>
<td>Yes ☐ No ☐</td>
<td>Yes ☐ No ☐</td>
<td>Yes ☐ No ☐</td>
<td></td>
</tr>
<tr>
<td>QC Passed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Comments</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staff Initials</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staff signature</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Date</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Attachment Eiv: Lowenstein Jenson Slopes**

**Quality Control Test**
Upon receipt of a new shipment/lot number of LJ slopes, suspensions of *M. tuberculosis* control organism (ATCC 27294 – H37Rv) should be prepared and inoculated onto an LJ slope (two drops at 0.5 McFarland). Another un-inoculated slope should be incubated at the same time.

**Quality Control Results**
The slope inoculated with H37Rv should show growth within 2 to 3 weeks and no longer than 4 weeks. The un-inoculated slope should show no growth within 1 week. If the QC fails, do not use this batch and contact the supplier. Any Quality Control Failure and subsequent actions should be recorded on the Continuous Quality Improvement Form (Quality manual attachment Q). Record the date the reagent of that lot came into use in the laboratory and the date it finished (this will be used to link reagents used with specimens processed).

| Lot Number |  |
| Expiry date |  |
| Date of QC Test |  |
| Date H37Rv Slope Positive |  |
| Number Week Growth Seen |  |
| Un-inoculated Slope Negative | Yes ☐ No ☐ Yes ☐ No ☐ Yes ☐ No ☐ Yes ☐ No ☐ Yes ☐ No ☐ |
| QC Passed | Yes ☐ No ☐ Yes ☐ No ☐ Yes ☐ No ☐ Yes ☐ No ☐ Yes ☐ No ☐ |
| Comments |  |
| Staff Initials |  |
| Staff Signature |  |

Version 3.0
16\textsuperscript{th} November 2009
### Attachment EV: Blood Agar Plates

Each time a new Lot of commercially prepared plates or batch of blood agar plates are made up in the laboratory the Lot numbers, collection dates (for blood if not commercially bought) and expiry dates of each ingredient should be recorded. Record the date the reagent of that lot came into use in the laboratory and the date it finished (this will be used to link reagents used with specimens processed).

**Quality Control Test**

Inoculate one plate with *Staphylococcus aureus* and leave one plate un-inoculated. After two days check for growth; the plate inoculated with *S. aureus* should show cream coloured colonies, un-inoculated plates should show no growth. If the QC fails, do not use this batch and contact the supplier or prepare a new batch. Any Quality Control Failure and subsequent actions should be recorded on the Continuous Quality Improvement Form (Quality manual attachment Q).

<table>
<thead>
<tr>
<th>Lot Number and Expiry Date (commercially bought plates)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch Number</td>
<td></td>
</tr>
<tr>
<td>Date Batch Prepared</td>
<td></td>
</tr>
<tr>
<td>Blood Agar</td>
<td>Lot Number</td>
</tr>
<tr>
<td></td>
<td>Expiry Date</td>
</tr>
<tr>
<td>Blood</td>
<td>Lot Number/ Collection Date</td>
</tr>
<tr>
<td></td>
<td>Expiry Date</td>
</tr>
<tr>
<td>QC Test – Colonies Visible</td>
<td>S. aureus plate</td>
</tr>
<tr>
<td></td>
<td>Yes ☐ No ☐</td>
</tr>
<tr>
<td></td>
<td>Yes ☐ No ☐</td>
</tr>
<tr>
<td></td>
<td>Yes ☐ No ☐</td>
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<tr>
<td></td>
<td>Yes ☐ No ☐</td>
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<tr>
<td></td>
<td>Yes ☐ No ☐</td>
</tr>
<tr>
<td></td>
<td>Yes ☐ No ☐</td>
</tr>
<tr>
<td>QC Passed</td>
<td>Yes ☐ No ☐</td>
</tr>
<tr>
<td></td>
<td>Yes ☐ No ☐</td>
</tr>
<tr>
<td></td>
<td>Yes ☐ No ☐</td>
</tr>
<tr>
<td></td>
<td>Yes ☐ No ☐</td>
</tr>
<tr>
<td>Expiry Date for Batch</td>
<td>Yes ☐ No ☐</td>
</tr>
<tr>
<td></td>
<td>Yes ☐ No ☐</td>
</tr>
<tr>
<td></td>
<td>Yes ☐ No ☐</td>
</tr>
</tbody>
</table>

| Comments |  |

Version 3.0
16th November 2009
**Attachment Evi: MGIT SIRE Drug Susceptibility Testing Kit**

**Quality Control Test**

Upon receipt of a new shipment or lot number of MGIT SIRE Kits, positive control testing must be carried out following BD MGIT protocols. Suspensions of *M. tuberculosis* control organism (ATCC 27294 – H37Rv) should be tested as per the normal drug susceptibility testing protocol.

**Quality Control Results**

The Kits pass if the *M. tuberculosis* H37Rv is fully susceptible to all drugs within 4 – 13 days. If the QC tubes do not give the expected results do not use the SIRE kits of the lot until you have contacted Becton Dickinson Technical services. Any Quality Control Failure and subsequent actions should be recorded on the Continuous Quality Improvement Form (Quality manual attachment Q). Record the date the kit of that lot came into use in the laboratory and the date it finished (this will be used to link reagents used with specimens processed).

<table>
<thead>
<tr>
<th>Lot Number</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Expiry date (kit)</td>
<td></td>
</tr>
<tr>
<td>Date of QC Test</td>
<td></td>
</tr>
<tr>
<td>H37Rv GC TIP</td>
<td></td>
</tr>
<tr>
<td><strong>H37Rv S/R/C</strong></td>
<td>S</td>
</tr>
<tr>
<td>QC Passed</td>
<td>Yes</td>
</tr>
<tr>
<td>Comments</td>
<td></td>
</tr>
<tr>
<td>Vials Aliquoted</td>
<td>Yes</td>
</tr>
<tr>
<td>Expiry Date of aliquots (6 months if earlier than kit expiry)</td>
<td></td>
</tr>
<tr>
<td><strong>Staff Initials</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Staff signature</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Date</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Date In Use</strong></td>
<td></td>
</tr>
</tbody>
</table>

Version 3.0
16<sup>th</sup> November 2009
Attachment Evii: MGIT PZA Drug Susceptibility Testing Kit

Quality Control Test

Upon receipt of a new shipment or lot number of MGIT PZA Kits, positive control testing must be carried out following BD MGIT protocols. Suspensions of *M. tuberculosis* control organism (ATCC 27294 – H37Rv) should be tested as per the normal drug susceptibility testing protocol.

Quality Control Results

The Kits pass if the *M. tuberculosis* H37Rv is fully susceptible to PZA within 4 – 20 days. If the QC tubes do not give the expected results do not use the PZA kit of the lot until you have contacted Becton Dickinson Technical services. Any Quality Control Failure and subsequent actions should be recorded on the Continuous Quality Improvement Form (Quality manual attachment Q). Record the date the batch of aliquots came into use in the laboratory and the date they finished (this will be used to link reagents used with specimens processed).

<table>
<thead>
<tr>
<th>Lot Number</th>
<th>Expire date (kit)</th>
<th>Date of QC Test</th>
<th>H37Rv GC TIP</th>
<th>H37Rv PZA S/R/C</th>
<th>QC Passed</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td>Yes [ ] No [ ]</td>
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<td>Yes [ ] No [ ]</td>
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<td>Yes [ ] No [ ]</td>
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<td>Yes [ ] No [ ]</td>
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<td></td>
<td></td>
<td>Yes [ ] No [ ]</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vials Aliquoted</th>
<th>Expiry Date of aliquots (6 months if earlier than kit expiry)</th>
<th>Staff Initials</th>
<th>Staff signature</th>
<th>Date</th>
<th>Date in Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes [ ] No [ ]</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Yes [ ] No [ ]</td>
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<td>Yes [ ] No [ ]</td>
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<tr>
<td>Yes [ ] No [ ]</td>
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</tbody>
</table>

Version 3.0
16th November 2009
**Attachment 5B: Quality Control: Moxifloxacin Drug Susceptibility Testing**

**Quality Control Test**

Upon receipt of a new shipment or lot number of Moxifloxacin, positive control testing must be carried out following BD MGIT protocols. Suspensions of *M. tuberculosis* control organism (ATCC 27294 – H37Rv) should be tested as per the normal drug susceptibility testing protocol.

**Quality Control Results**

The test passes if the *M. tuberculosis* H37Rv is fully susceptible to moxifloxacin within 4 – 13 days. If the QC tubes do not give the expected results do not use the moxifloxacin of the lot until you have contacted the Sponsor (UCL). Any Quality Control Failure and subsequent actions should be recorded on the Continuous Quality Improvement Form (Quality manual attachment Q). Record the date the batch of aliquots of that lot came into use in the laboratory and the date it finished (this will be used to link reagents used with specimens processed).

<table>
<thead>
<tr>
<th>Lot Number</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Expiry date (Moxi)</td>
<td></td>
</tr>
<tr>
<td><strong>Date of QC Test</strong></td>
<td></td>
</tr>
<tr>
<td>Amount moxifloxacin weighed (mg)</td>
<td></td>
</tr>
<tr>
<td>Volume ddH₂O added (ml), detail any further dilutions</td>
<td></td>
</tr>
<tr>
<td>H37Rv GC TIP</td>
<td></td>
</tr>
<tr>
<td>H37Rv MOX S/R/C</td>
<td></td>
</tr>
<tr>
<td>QC Passed</td>
<td>Yes ☐ No ☐ Yes ☐ No ☐ Yes ☐ No ☐ Yes ☐ No ☐ Yes ☐ No ☐</td>
</tr>
<tr>
<td>Comments</td>
<td></td>
</tr>
<tr>
<td>Vials Aliquotted</td>
<td>Yes ☐ No ☐ Yes ☐ No ☐ Yes ☐ No ☐ Yes ☐ No ☐ Yes ☐ No ☐</td>
</tr>
<tr>
<td>Expiry Date of aliquots (6 months if earlier than moxi expiry)</td>
<td></td>
</tr>
<tr>
<td><strong>Staff Initials</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Staff signature</strong></td>
<td></td>
</tr>
<tr>
<td>Date</td>
<td></td>
</tr>
<tr>
<td>Date In Use</td>
<td></td>
</tr>
</tbody>
</table>

Version 3.0
16<sup>th</sup> November 2009
Attachment F: Processing and Decontaminating Sputum Samples

This quality control form should be completed each day that sputum samples are processed. This form will be used to record the timings of NaOH/NALC decontamination, the order samples are processed including the blank control (placed in the middle of the run). This is a ‘decontamination mixture only’ control, and should be processed in exactly the same way as the test samples. This negative control will ensure there is no carry over of bacteria between samples (e.g. from contamination of stock solutions), that could result in false positives. MGIT tubes and LF slopes should also be inoculated in the same order (including blank control for MGIT). Record results of negative control for this batch in the table below. If the smears for the negative control fail, new stocks must be prepared and a repeat QC test performed, if it fails again fill out the continuous quality improvement form (send a copy to UCL). *Label all the negative control tubes/slides with date, run/batch number and ‘negative control’*

<table>
<thead>
<tr>
<th>Date (dd/mm/yyyy):</th>
<th>Run/Batch Number:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date NaOH/NALC prepared:</td>
<td>Working Concentration NaOH/NALC:</td>
</tr>
<tr>
<td>NaOH Lot No:</td>
<td>NaOH Expiry Date:</td>
</tr>
<tr>
<td>NALC Lot No:</td>
<td>NALC Expiry Date:</td>
</tr>
<tr>
<td>Sodium Citrate Lot No:</td>
<td>Sodium Citrate Expiry Date:</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Staff Name</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staff Signature</td>
<td>Centrifuge °C:</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Order samples processed (max 8 samples only)</th>
<th>Laboratory Accession Number (for negative control write ‘negative’)</th>
<th>Study (Patient) Number (for negative control write ‘negative’)</th>
<th>Time Decontamination Started (or time on stop clock):</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>First Sample:</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>Last Sample:</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>Interval Time:</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td>Time Decontamination Stopped (or time on stop clock):</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td>First Sample:</td>
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<td>6</td>
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<td>Last Sample:</td>
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<td>7</td>
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<td>8</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Result +/-</th>
<th>Staff Name</th>
<th>Date</th>
<th>Acceptable (y/n)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZN Stain</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Auramine</td>
<td></td>
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<tr>
<td>MGIT Tube no:</td>
<td></td>
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</tr>
</tbody>
</table>

If MGIT positive perform blood agar to distinguish between cross contamination of *M. tb* or other contamination:

<table>
<thead>
<tr>
<th>Blood Agar</th>
<th>Comments:</th>
</tr>
</thead>
</table>

**Lab Manager (signature)**

Version 3.0

16th November 2009
Negative Control Results:
Attachment G: Weekly Contamination Rates

This QC sheet is used to monitor the contamination rates. The weekly contamination rate should be 3 – 8%. If small numbers of samples calculate the contamination rate over a longer specified period.

**QC Failure:** If the contamination rate is below 3% it is possible the samples are being over decontaminated, possibly due to the concentration of NaOH being too high for this population or the time samples are decontaminated is too long. If the samples are consistently contaminated at a rate higher than 8% this could be due to the NaOH solution not being strong enough or the specimens not being decontaminated for long enough.

<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total Number Completed Tubes (positive and negative) A</td>
<td>Number (Positive) Tubes Contaminated B</td>
<td>Contamination rate % C C = (B/A) x 100</td>
</tr>
<tr>
<td>MGIT</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>LJ</td>
<td></td>
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</tbody>
</table>

Contamination Rates between 3 – 8% Yes ☐ No ☐

**IF NO,** List ONLY the contaminated samples in the table on attachment Gi. The QC Attachment F should be used to calculate and check to see if the decontamination times are accurate and the concentration of NaOH/NALC is correct. If this is shown to be accurate, before changing the concentration of the NaOH/NALC a quality control improvement form should be completed, requesting such a change and sent with this form to the UCL Laboratory Team for review and action.

Attachment Gi Completed Yes ☐ No ☐

Comments:

<table>
<thead>
<tr>
<th>Staff Name</th>
<th>Staff Signature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
</tbody>
</table>

Version 3.0
16th November 2009
<table>
<thead>
<tr>
<th>Study (Patient) Number</th>
<th>Laboratory Accession Number</th>
<th>Sputum processed (Sample)</th>
<th>Staff Name (decontamination process)</th>
<th>NaOH/NALC Conc.</th>
<th>Time (min)</th>
<th>Process in Decon</th>
<th>Order Number</th>
<th>y/n contaminated</th>
<th>Prep. date</th>
</tr>
</thead>
</table>

*Attachment Gi; Contaminated Samples Only complete this form if the weekly contamination rate is outside 3 – 8% for either MGIT or LJ or both (form G).*
Attachment H: Daily AFB Microscopy

**Quality Control Test**
Every time smears are stained a positive control slide (M.tb H37Rv) MUST be included and the results reported in the table below.
If MORE THAN ONE sputum is being processed at a time, a negative control sample must also be prepared and stained, the results should be recorded on QC attachment F.

**Quality Control Results**
The positive and negative control slides must pass for the microscopy results to be reported. If the positive QC fails new smears must be prepared and re-stained, check that the QC attachment Ei and/or Eii have been completed and passed for these batches of reagents. Any Quality Control Failure and subsequent actions should be recorded on the Continuous Quality Improvement Form (Quality manual attachment Q).

<table>
<thead>
<tr>
<th>Date</th>
<th>Batch Number</th>
<th>Positive Control Result</th>
<th>Staff Name and Signature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Auramine</td>
<td>Ziehl-Neelsen</td>
</tr>
</tbody>
</table>

Version 3.0  
16th November 2009
**Attachment I: Microscopic Examination of Acid-Fast Smears**

For every tenth slide examined there will be a two-person comparison recorded in the table below.

<table>
<thead>
<tr>
<th>Initials Lab Staff 1</th>
<th>Date</th>
<th>Slide Laboratory Accession No.</th>
<th>Slide Study (Patient No)/Screening No.</th>
<th>Av. No. AFBs (over 100 fields)</th>
<th>REMoxTB Reporting*</th>
<th>Initials Lab Staff 2</th>
<th>Date</th>
<th>Slide Laboratory Accession No.</th>
<th>Slide Study (Patient No)/Screening No.</th>
<th>Av. No. AFBs (over 100 fields)</th>
<th>REMoxTB Reporting*</th>
<th>Comparison acceptable y/n</th>
</tr>
</thead>
<tbody>
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</tbody>
</table>

* not seen/ 1+ / 2+ / 3+ / 4+

The results for the REMoxTb reporting column should be the same for both counts. If results do not compare inform the Laboratory Manager. The counts should be repeated and confirmed by a third person and staff retrained. Please describe action taken in Continuous Quality Improvement Form, attachment Q.

Version 3.0  
16th November 2009
### Attachment J: MGIT 960 Daily Maintenance Log

(Month/Year) _____ / _____

This Log should be filled out daily (a line should be drawn through weekends and public holidays if not done). In addition, the Quality Control Report from the MGIT should be printed and attached. If any of the sections fail inform the Laboratory Manager. Refer to the MGIT Standard Operating Procedure and/or Operating Manual for troubleshooting. If this fails contact the local Becton Dickinson representative. Any Quality Control Failure and subsequent actions should be recorded on the Continuous Quality Improvement Form (Quality manual attachment Q).

<table>
<thead>
<tr>
<th>Date</th>
<th>Check printer paper</th>
<th>Check Temperature Probes 37 °C + 1 / - 2 °C</th>
<th>Check Drawer Indicators A</th>
<th>B</th>
<th>C</th>
<th>Check Station Indicators A</th>
<th>B</th>
<th>C</th>
<th>Initials</th>
<th>Monthly – clean/replace air filters</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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</table>

Version 3.0
16th November 2009
Attachment K: MGIT Calibration Tube Log

This log should be used to track the expiry date and replacement dates for the MGIT calibration tubes. Calibration tubes should be replaced before they are due to expire. Contact your local Becton Dickinson representative for ordering information.
### Attachment L: Accuprobe Worksheet and IQC form

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Lot number</th>
<th>Expiry date</th>
<th>Positive control M. tuberculosis Acceptable result = &gt;30,000</th>
<th>Negative control M. avium complex Acceptable result = &lt;10,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture identification reagents</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Accuprobe M. tuberculosis reagents</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Detection reagent 1</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Detection reagent 2</td>
<td></td>
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</tr>
</tbody>
</table>

**Maintenance log**

<table>
<thead>
<tr>
<th>Maintenance Log</th>
<th>Date</th>
<th>Comment/Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wash cycle – each use</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Reagent volume check – each use</td>
<td>OK</td>
<td></td>
</tr>
<tr>
<td>Warm water flush – weekly</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Optics check Tritium std* – monthly</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Optics check SYSCHECK** – monthly</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Clean optical chamber (if applicable) – monthly</td>
<td>✓</td>
<td></td>
</tr>
</tbody>
</table>

*Enter ratio of observed/expected value, acceptable range Tritium std = 0.95–1.05 attach result OR
**Enter ratio of observed/expected value, acceptable range Syscheck std = 0.85-1.15 attach result

<table>
<thead>
<tr>
<th>No</th>
<th>Study/Patient Number</th>
<th>Lab. Accession Number (for control detail strain)</th>
<th>LJ or MGIT</th>
<th>RLU</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em><strong>POSITIVE CONTROL</strong></em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em><strong>NEGATIVE CONTROL</strong></em></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>3</td>
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</tbody>
</table>

Comments

Check the accuprobe user manual for troubleshooting options or contact your local accuprobe representatives for any QC failure relating to the equipment or reagents. Record all actions on the continuous quality improvement form Q.

Version 3.0
16\textsuperscript{th} November 2009
### Attachment Mi: GenoType® MTBDRplus

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Performed By (Name)</th>
<th>Date of Test</th>
<th>Product detail</th>
<th>Product Lot Number</th>
<th>Product Expiry Date</th>
<th>Equipment</th>
<th>Recorded Temp °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Extraction</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Heating Block/Water bath</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplification</td>
<td></td>
<td></td>
<td>Primer Nucleotide Mix (PNM)</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>DNA Taq Polymerase</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
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<tr>
<td>Hybridisation</td>
<td></td>
<td></td>
<td>GenoType® MTBDRplus Kit</td>
<td>Waterbath/Twincubator</td>
<td>Substrate Incubation</td>
<td></td>
<td>Time (min)</td>
</tr>
</tbody>
</table>

**Evaluation of Results** performed by ___________________________ Date ___________________________ Evaluation Sheet No: ___________________________

<table>
<thead>
<tr>
<th>Strip No</th>
<th>Patient Screening Number</th>
<th>Laboratory Accession Number</th>
<th>Control Bars Visible (y/n)</th>
<th>Result accepted yes/no</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CC</td>
<td>AC</td>
<td>LC rpoB</td>
</tr>
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<tr>
<td><strong>POSITIVE CONTROL (H37Rv)</strong></td>
<td></td>
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</tr>
<tr>
<td><strong>NEGATIVE CONTROL (ddH₂O)</strong></td>
<td></td>
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</tr>
</tbody>
</table>


If there is a QC failure refer to the REMox Laboratory Manual or MTBDRPlus User guide for troubleshooting options, record all actions on the continuous quality improvement form Q. If this does not resolve the issue send this form and the attachment Q to UCL stating the QC failure.

Version 3.0
16th November 2009
### Attachment Mii: GenoType® MTBDRsI

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Performed By (Name)</th>
<th>Date of Test</th>
<th>Product detail</th>
<th>Product Lot Number</th>
<th>Product Expiry Date</th>
<th>Equipment</th>
<th>Recorded Temp °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Extraction</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Heating Block/Water bath</td>
<td></td>
</tr>
<tr>
<td>Amplification</td>
<td>Primer Nucleotide Mix (PNM)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Waterbath/Twincubator</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>DNA Taq Polymerase</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Substrate Incubation</td>
<td></td>
</tr>
<tr>
<td>Hybridisation</td>
<td>GenoType® MTBDRsI Kit</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Time (min)</td>
</tr>
</tbody>
</table>

**Evaluation of Results** performed by __________________________ Date __________________________ Evaluation Sheet No: __________

<table>
<thead>
<tr>
<th>Strip No</th>
<th>Patient Screening Number</th>
<th>Laboratory Accession Number</th>
<th>Control Bars Visible (y/n)</th>
<th>Result accepted yes/no</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CC</td>
<td>AC</td>
<td>LC gyrA</td>
</tr>
<tr>
<td><strong>POSITIVE CONTROL (H37Rv)</strong></td>
<td></td>
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<tr>
<td><strong>NEGATIVE CONTROL (ddH₂O)</strong></td>
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</tr>
</tbody>
</table>

CC – conjugate control, AC – amplification control, LC – locus control, S – sensitive, R – resistant, y – yea, n – no

Version 3.0
16<sup>th</sup> November 2009
Attachment N: Day 50 Form
This form should be completed each week to include all available baseline data for every study participant that has reached day 50 (week 7) on this week. This is to monitor any discrepancies between smear positivity and culture positivity (MGIT and LJ), to check the MGIT sensitivity has been performed and check data if available. Also included should be results for Accuprobe and Hain (from screening sample if applicable). Each week once this form has been completed it should be scanned/faxed to the Laboratory Team at UCL for review. Use one form per patient, if data is missing at week 7 complete what is available in the first column. When additional data is available continue to record in the extra columns. If results do not concur attach a QC continuous improvement form Q stating the QC failure and any actions taken and fax with this form to UCL (e.g. sample is MGIT -ve LJ +ve).

<table>
<thead>
<tr>
<th>Patient/Study Number:</th>
<th>Lab Accession No. (baseline):</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date (Day 50):</td>
<td>Date (baseline sample):</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date</td>
</tr>
<tr>
<td>Z-N Result</td>
</tr>
<tr>
<td>MGIT TTP (days)</td>
</tr>
<tr>
<td>LJ Growth Visible (week)</td>
</tr>
<tr>
<td>Accuprobe (✓)</td>
</tr>
<tr>
<td>M.t.b complex</td>
</tr>
<tr>
<td>MOTT</td>
</tr>
<tr>
<td>MGIT Sensitivity Set-up (y/n)</td>
</tr>
<tr>
<td>MGIT Sensitivity Results (S/R/C)</td>
</tr>
<tr>
<td>Streptomycin</td>
</tr>
<tr>
<td>Isoniazid</td>
</tr>
<tr>
<td>Rifampicin</td>
</tr>
<tr>
<td>Ethambutol</td>
</tr>
<tr>
<td>Pyrazinamide</td>
</tr>
<tr>
<td>Moxifloxacin</td>
</tr>
<tr>
<td>Two samples in storage* (y/n)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Screening</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hain (if applicable)</td>
</tr>
<tr>
<td>M. tb complex (✓)</td>
</tr>
<tr>
<td>Isoniazid (S/R)</td>
</tr>
<tr>
<td>Rifampicin (S/R)</td>
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<tr>
<td>Fluoroquinolone (S/R)</td>
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</tbody>
</table>

*at least one sample should be frozen in glycerol at -70 to -80°C for long term storage

Version 3.0
16th November 2009
**Attachment P: DNA extraction and shipment to UCL**

This form should be completed each time DNA isolates are to be shipped to UCL. Estimated DNA concentration should be performed after DNA extraction and the values used to complete the table below. Total DNA exceeding 1 ug total per sample is required. A copy of this form should be sent with the DNA samples. The samples should be sent on a monthly basis but should not be sent if less than 20 samples per shipment. The samples should be shipped at below room temperature (wet ice or colder) and UCL advised when a shipment is to be sent.

<table>
<thead>
<tr>
<th>Patient/Study Number</th>
<th>Laboratory Accession Number</th>
<th>A Estimated DNA Concentration (ng/µL)</th>
<th>B Estimated Volume (µL)</th>
<th>C Estimated Total DNA (µg) (C = (A \times B)/1000)</th>
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</tbody>
</table>

**Staff Name:**

**Staff Signature:**

---

Version 3.0

16th November 2009
Attachment Q: Continuous Quality Improvement Form

This form should be completed every time there is a QC failure.

Continuous Quality Improvement Form  N°:

Categorise QC Failure:

☐ Severe
Likely to result in exclusion of data, unable to resolve in-house, likely to delay processing of samples, requires amendment to laboratory manual and/or REMox protocol, repeat QC failure.

Contact UCL Laboratory Team immediately before taking any action, complete section 1 and send (fax/scan) this document and QC form showing failure

☐ Moderate
Does not affect data or delay processing samples, can repeat analysis if necessary, able to resolve in-house by laboratory management and/or Quality Assurance officer, same QC failure may have occurred on more than one occasion, does not require amendment to laboratory manual and/or protocol.

Resolve problem in-house, complete this form but contact and send to UCL Laboratory team to make aware

☐ Mild
Does not affect data or processing samples, easily resolved in-house by laboratory staff, one-off QC failure. Resolve in-house, complete and file this form, no need to contact UCL

<table>
<thead>
<tr>
<th>1. Description of QC Failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC Form:</td>
</tr>
</tbody>
</table>

Print Name & Sign:  Date:

| 2. Detail In-house Action to Fix Immediate Problem |

Print Name & Sign:  Date:

| 3. Root Cause(s) of the Problem |

Print Name & Sign:  Date:

| 4. Action Required to Eliminate the Root Causes (and Implementation Timeframe) |

Print Name & Sign:  Date:

| 5. Action Taken to Verify Effectiveness of Action (and changes to documentation if necessary) |

Version 5.0
24th June 2011
Attachment R: Drug Susceptibility quality control

This form should be completed if the *M. tuberculosis* reference strain H37Rv is included with the patient samples when performing drug susceptibility testing. This is in addition to the reagent testing which is documented on attachments Evi-Eviii.

H37Rv cultures should be prepared as per instructions in LM5.3 and the strain should be fully sensitive to all antibiotics tested. If H37Rv shows resistance to any of the test drugs the results of the patient samples included in the run should not be accepted and should be repeated.

If not all drugs are tested, please write not applicable (N/A)

<table>
<thead>
<tr>
<th>Date</th>
<th>Batch</th>
<th>Result (S/R/C) or X (test invalid)</th>
<th>Comments</th>
<th>Initials</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S I R E P M</td>
<td></td>
<td></td>
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</tbody>
</table>