TB Alliance Protocol NC-005-(J-M-Pa-Z)

Microbiology Manual

Issued by: Kathy D. Eisenach	**Current Version: 1.0** **Version Date: 19 December 2014**
Authors: Kathleen Eisenach Sam Ogwang Marinus Barnard Amour Venter Almari Conradie	**Protocol: 31 January 2014**
Signatures: Kathy Eisenach Kathy D. Eisenach (Microbiology Consultant) _______________________________ Dr Stephen Murray (Study Physician)	_______________________________ Almari Conradie (Global Clinical Project Manager) _______________________________ Dr Marinus Barnard (TASK – CCTR Laboratory Director)
Manual Revision Date: N/A **<<Amendments>>: <<Date>>** **<<Amendments>>: <<Date>>**	

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A. TRIAL PROCEDURES

SCREENING COACHED SPOT SPUTUM

Time Point:
Day -9 to Day -3

Purpose 1:
Direct or concentrated sputum microscopy using fluorescent staining to test for acid-fast bacilli (AFB). This serves as confirmation of presence of AFB and ensures adequate bacterial load (at least 1+ on the IUATLD/WHO grading scale).

Purpose 2:
Hain Line Probe Assay to confirm *M. tuberculosis (M.tb)* and test for resistance to isoniazid, rifampicin (Hain MTBDR*plus*) and fluoroquinolones (Hain MTBDR*sl*); thus distinguishing between Drug Susceptible (DS)-TB and MDR-TB. Hain MTBDR*sl* will be performed only on specimens from patients designated “MDR-TB”, which are *M.tb* positive and resistant to isonaizid and rifampicin.

COACHED SPOT SPUTUM

Time Points:
Day -2, Day -1, Day 1, 3, 7, 14, 21, 28, 35, 42, 49 & 56

Purpose 1:
Quantitative sputum culture on solid media to determine colony forming units (CFU) of *M.tb* and to calculate the change of sputum CFUs ($\log_{10}$CFU) over time.

Purpose 2:
MGIT culture to detect *M.tb* and determine time to culture positivity (TTP) in the MGIT system and to calculate the change in sputum TTP ($\log_{10}$ TTP) over time.

Purpose 3:
Comparison of CFU and MGIT TTP results with those obtained from overnight pooled sputum samples.

OVERNIGHT POOLED SPUTUM

Time Points:
Day -2, Day -1, Day 1, 3, 7, 14, 21, 28, 35, 42, 49 & 56

Purpose 1:
Day -2 and Day -1 only: Direct or concentrate sputum microscopy using fluorescent staining to test for acid-fast bacilli (AFB). This serves as confirmation of presence of AFB and ensures adequate bacterial load (at least 1+ on the IUATLD/WHO grading scale).

Purpose 2:
All visits: Quantitative Sputum Culture and MGIT Culture to calculate efficacy variables. These are the change of sputum colony forming units (CFU) of *M tuberculosis* ($\log_{10}$CFU) over time on solid media and the change of time to culture positivity ($\log_{10}$TTP) in the MGIT system over time in liquid media.

Purpose 3:
Drug Susceptibility Testing (DST) to ensure safety of patients, obtain more information about the level of susceptibility of the experimental (study) drugs, and compare the phenotypic and genotypic PZA results. These tests will be performed on a positive culture from either D-2 or D-1 sputum specimen. Additionally, positive cultures from patients fulfilling the following criteria will be tested: 1) the last positive sputum from withdrawn subjects who have not converted to culture negative status, 2) subjects who are still culture positive at 8 weeks, and 3) the first positive sample after conversion to culture negative status for subjects who have relapsed.

a. MGIT Drug Susceptibility Testing: Positive cultures at the above mentioned time points will be used to test for susceptibility to streptomycin, isoniazid, rifampicin, ethambutol and pyrazinamide (SIREZ).

b. Minimum Inhibitory Concentration (MIC): *M.tb* isolates from sputum cultures at the above mentioned time points will be tested for minimum inhibitory concentration (MIC) of bedaquiline, PA-824 and moxifloxacin. *M.tb* isolates will be shipped every 3-6 months to CCTR/Stellenbosch, where MIC testing will be performed.

c. PZA testing by *pncA* sequencing: DNA samples from sputum sediments at Day -2 or Day -1 will undergo sequencing of the *pncA* gene. DNA samples will be the same as those prepared for the Hain Line Probe Assays. DNA samples will be stored at -80°C until shipped to the CCTR/Stellenbosch lab. Samples will be sent every 3-6 months.

B. MICROBIOLOGY TRIAL PLAN

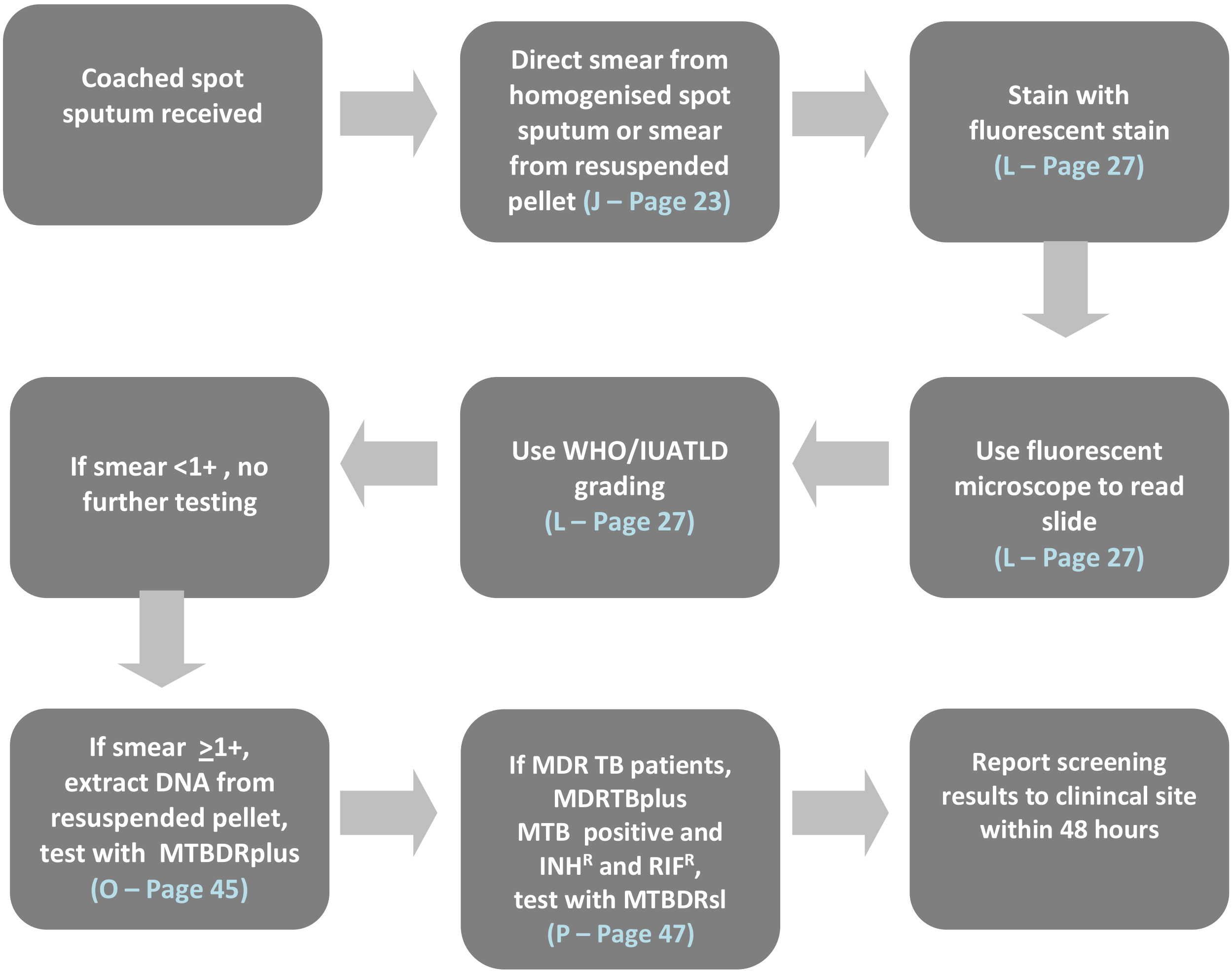
NC-005-(J-M-Pa-Z)			**Screening**			**Treatment**										**Relapse or culture+ D56**
Day Number (when specimen collection started) – micro testing designation	**Sample Type**		**-9 to -3**	**-2**	**-1**	**1**	**3**	**7**	**14**	**21**	**28**	**35**	**42**	**49**	**56**	
Visit Number (day/week subject attended study visit)			**-9 to -3**	**-2**	**-1**	**1**	**4**	**8**	**15**	**22**	**29**	**36**	**43**	**50**	**57**	
Laboratory Assessment/Week Number	**Coached Spot**	**Pooled Overnight**	**-1**			**1**			**2**	**3**	**4**	**5**	**6**	**7**	**8**	
Sputum Collection	**X**		**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	
Sputum AFB Smear[A]	**X**		**X**													
MTBDR*plus*[B]	**X**		**X**													
MTBDR*sl* [B, C]	**X**		**X**													
MGIT Culture and TTP[D]	**X**			**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	
Quantitative Culture	**X**			**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	
Sputum Collection		**X**		**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	
Sputum AFB Smear[A]		**X**		**X**	**X**											
MGIT Culture with TTP[D]		**X**		**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	
Culture Id with MPT64 Ag test[E]		**X**		**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	
Quantitative Culture		**X**		**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	
MGIT DST for SIREZ[F]		**X**		**X**												**X**[G]
Ship MTB Isolates for MIC of J, Pa, M[H]		**X**		**X**												**X**[K]
Ship DNA for *pncA* sequencing[I]		**X**		**X**												

A	AFB microscopy performed with direct or concentrated sputum, fluorescent stain, and WHO grading scheme.
B	If indeterminate result is obtained with either Line Probe Assay, repeat with newly extracted DNA. If repeat is still indeterminate, request a 2nd screening specimen.
C	Only performed for MDR patients when *M.tb* detected and resistant to rifampicin.
D	Report time-to-positivity (TTP) MGIT culture for all positives yielding pure cultures of *M. t*b.
E	Culture confirmation of *M. tb* is done using a lateral flow test detecting MPT64 antigen on all Pooled Overnight sputum MGIT cultures that are AFB positive by ZN, regardless of the BAP result. If D-2 MGIT culture is not done, negative or no AFB observed in positive MGIT culture, use D-1 MGIT culture if possible. For Days 1-56, if MGIT culture is not done, negative or no AFB observed in positive MGIT culture and 7H11S/7H11S+C has AFB positive growth, perform Id test with the AFB positive colonies from the 7H11/7H11S+C. Refer to the MGIT flow charts, which provide algorithms for working up MGIT cultures.
F	Performed on 1 Baseline isolate. If a pure isolate or culture of MTBC is not available, perform DST with the next positive culture yielding pure growth, e.g. use D-1 sample if D-2 sample is not usable.
G	Performed on the last positive culture from withdrawn subjects who have not converted to culture negative status OR on the 8-week specimen from patients who are still culture positive at 8 weeks OR the 1st positive specimen after conversion to culture negative for subjects who have relapsed. The status of such patients must be indicated by the study staff.
H	Performed on 1 Baseline isolate. If a pure isolate or culture of MTBC is not available, perform DST with the next positive culture yielding pure growth, e.g. use D-1 sample if D-2 sample is not usable.
I	Performed on the last positive culture from withdrawn subjects who have not converted to culture negative status OR on the 8-week specimen from patients who are still culture positive at 8 weeks OR the 1st positive specimen after conversion to culture negative for subjects who have relapsed. The status of such patients must be indicated by the study staff.
J	Performed on one Baseline isolate, either D-2 or D-1.

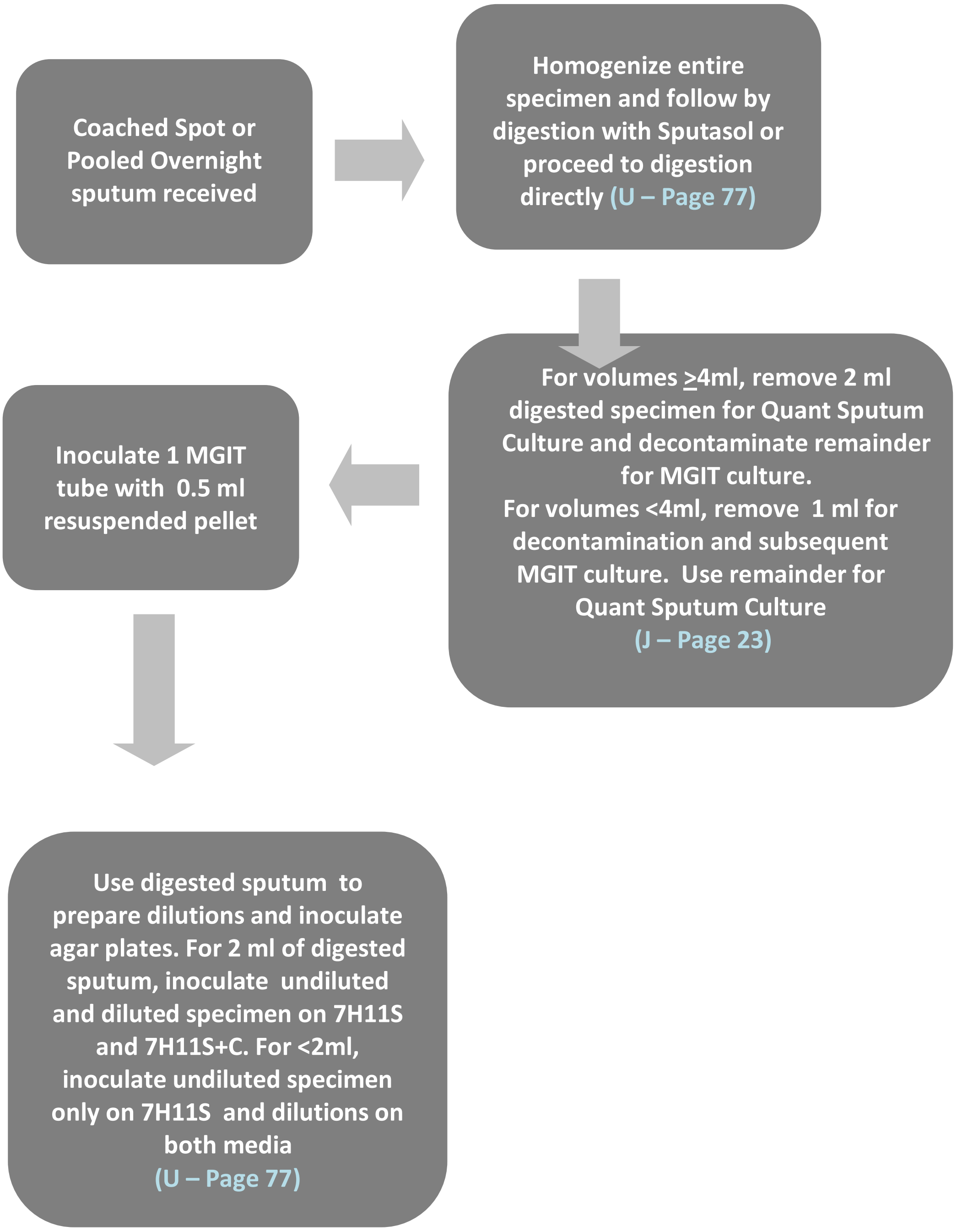
C. MYCOBACTERIOLOGY VISIT LIST

Day -9 to -3 (Screening)
Day -2
Day -1
Day 1 (Treatment, all subsequent days)
Day 3
Day 7
Day 14
Day 21
Day 28
Day 35
Day 42
Day 49
Day 56
Unscheduled

D. MICROBIOLOGY FLOW CHART: SCREENING



E. MICROBIOLOGY FLOW CHART: D-2, D-1 AND TREATMENT COACHED SPOT AND POOLED OVERNIGHT SPUTA



Coached Spot or Pooled Overnight sputum received

Homogenize entire specimen and follow by digestion with Sputasol or proceed to digestion directly (U – Page 77)

For volumes ≥4ml, remove 2 ml digested specimen for Quant Sputum Culture and decontaminate remainder for MGIT culture.
For volumes <4ml, remove 1 ml for decontamination and subsequent MGIT culture. Use remainder for Quant Sputum Culture (J – Page 23)

Inoculate 1 MGIT tube with 0.5 ml resuspended pellet

Use digested sputum to prepare dilutions and inoculate agar plates. For 2 ml of digested sputum, inoculate undiluted and diluted specimen on 7H11S and 7H11S+C. For <2ml, inoculate undiluted specimen only on 7H11S and dilutions on both media (U – Page 77)

F. REPORTING OF STUDY DATA

Results from laboratory tests are recorded on the laboratory's worksheets/workbooks by the technologists performing the tests. The data sheets, worksheets and workbooks are considered the "source documents" of the laboratory test results. Upon completion of the tests, the data are either transferred to a study-specific laboratory report form, the designated "Mycobacteriology Results Report Form" (MRRF), or directly entered into the laboratory database. An example MRRF appears in Appendix 1. The form consists of Completion Guidelines and six pages for completion. The Mycobacteriology Report Form should be completed according to the Mycobacteriology Results Report Form Completion Guidelines included with the MRRF.

New lab test results should be submitted using the MRRF to QLab on a daily basis, or as they become available (if results are expected less frequently). The MRRF pages are sent to QLab either by FAX or scanned transmissions via email:

- E-mail: QLABLocalLab@quintiles.com
- Fax: +27 12 671 2210

Should changes be required to laboratory data previously submitted to QLab, a corrected form should be completed, clearly indicating the specific page/data it replaces previously submitted to QLab. The person responsible for documenting and submitting the change(s) should sign and date the corrected form before submitting it to QLab.

The report forms, both those that are completed and those without results, are kept in the laboratory in a file labeled with the patient ID number.

A technologist transcribes the data from the source documents either onto the Mycobacteriology Results Report Form or directly into the laboratory database, where after the results are reviewed by a second technologist to ensure there are no transcription errors and the results have been interpreted correctly.

Electronic data transfer: data captured into the laboratory database (using local laboratory SOPs) should be transferred to QLab on a weekly basis in Excel or .csv format. QLab will upload the data into the clinical study database. Transfer specification are described in the "Laboratory Data Transfer Specifications" document agreed between the Laboratory and QLab.

Once results have been obtained and/or the respective sheets of the Mycobacteriology Results Report Form have been completed, the Laboratory Director or his/her designee will review all the results to determine if they are logical in the context of the patient for the visit number. Any questionable results should be brought to the attention of Kathy Eisenach or Sam Ogwang.

G. LABORATORY SUPPLIES AND MATERIALS

The following bulk supplies will be sent by each local laboratory to the clinical sites before the start of the study. Alternative materials may be used as available to the laboratories and sites:

1. 50 ml volume-marked specimen tubes/containers for collection of coached screening spot sputum
2. Small Pathoseal (95) specimen bags for packing and transport of spot sputum
3. 125 ml volume-marked specimen containers for collection of pooled overnight sputum
4. Large Pathoseal (Liquitite) specimen bags for packing and transport of overnight sputum to the lab
5. Specimen labels
6. Cooler bags with ice packs and Ziploc bags for patient collection and transport
7. Specimen transport form
8. Specimen transport log form
9. Transport/cooler boxes
10. Ice bricks, Correx (corrugated) sheets and absorbent material

Coached Spot Sputum Collection (example):

Pooled Overnight Sputum Collection (example):

Specimen Transport Box and Ice Bricks (example):

Packing Specimens (example):

Absorbent Material (example):

H. SPECIMEN COLLECTION, STORAGE AND TRANSPORT

PURPOSE

To ensure high quality laboratory results are obtained from the specimens collected. Adherence to procedural details regarding collection and transport of specimens will result in collecting adequate and quality specimens for analysis in the laboratory and maintaining correct identity of each specimen.

PRINCIPLE

Sputum specimens collected at a single time point are referred to as “coached spot” sputum specimens. These will be collected at the site by the study site personnel who will coach the patient to collect a good quality sample. Since specimens collected upon rising from bed in the morning yield higher numbers of *M. tb*, it is preferable for the patient to come to the clinic first thing in the morning before eating breakfast and before expectorating any sputum.

Sputum specimens collected overnigt and pooled during collection are referred to as “pooled overnight” sputum specimens. These specimens consist of all the sputum expectorated by a patient during a 16 hour overnight collection, e.g. from 16:00 to 08:00. Pooled specimens have a higher yield on culture than early morning spot specimens, but are more likely to be contaminated. To reduce the possibility of overgrowth on the quantitative cultures, it is preferable for the patient to keep the pooled specimen in the refrigerator or in a cooler box with ice packs during 16-hour collection period and transport to the clinic.

To ensure quality specimens, target volumes for collection are specified by the specimen type and for the collection time points in the procedure below.

PROCEDURE

Materials (Alternative materials may be used as available to the laboratories and sites.)

Sterile 125 ml volume-marked wide-mouth specimen containers for pooled overnight sputum collections
Sterile 50 ml volume-marked wide-mouth specimen containers or tube for coached spot sputum collections
Specimen labels
Specimen transport forms, study-specific
Specimen transport log, study-specific
Permanent marker
Disposable gloves
Refrigerator
Certified thermometer
Cooler bags with ice packs and Ziploc bags for patient collection and transport
Packaging suitable for transport of infectious materials, including plastic pouches, absorbent material, cool packs/ice bricks, inner containers, transport boxes, and cooler boxes

Procedure

Collection and Storage:

1. Sputum will be collected with the assistance of the clinic specimen collection staff. These are coached spot specimens. A 50 ml tube or wide-mouth container is used for this collection.
2. Patients will collect the overnight specimens. They will be trained by the collection staff on how to collect these specimens at home and be provided with a Participant Booklet, which contains instructions on collecting the overnight specimen. A 125 ml wide-mouth container is used for this collection.
3. Label the specimen container with the study number, patient screening number/patient ID number, specimen type, visit “Day”, and collection date. For coached specimens, include collection time. For overnight specimens, record collection start time and (once returned) the stop time. The labelling must be done on the container and not on the lid.
4. Screw caps must fit tightly to avoid leakage.
5. After the specimen has been collected in the clinic or received from the patient who collected it at home, estimate the volume and determine if the amount of specimen is acceptable.
    a. Coached spot sputum specimens – screening:
        i. Target – 5-7 ml
        ii. Minimum – 3 ml*
    b. Coached spot sputum specimens – treatment:
        i. Target – 3-5 ml
        ii. Minimum – 2 ml
    c. Pooled overnight sputum specimens – Day -2 and Day -1:
        i. Target – 20-30 ml
        ii. Minimum – 10 ml*
    d. Pooled overnight sputum specimens – treatment:
        i. Target – 10-20 ml
        ii. Minimum – 2 ml

    *__Note: If the minimum volumes cannot be obtained at screening, most likely the patient will not be able to produce sputum in the volumes required during the course of the study. Contact the study coordinator to indicate this problem.__
6. Inadequate or low volumes of spot specimens can be addressed in the following way. If the patient is unable to expectorate sufficient volume at one time, the patient can be kept in the clinic and additional attempts made to collect more sputum, up to 1 hour from the first collection. Pooling these collections in one tube is acceptable as long as the collections are supervised, the tube is kept in a refrigerator/on ice packs in between collections, and collections are accomplished within 1 hour.
7. Inadequate or low volumes of overnight pooled specimens cannot be addressed. If volumes are low or insufficient, the collection staff should re-train the patient how to produce sputum.
8. If the patient omits to collect an overnight pooled sputum specimen, the patients should be instructed to start collecting the sample that same day and to bring the specimen to the site the next day. All study procedures for that visit should then also be postponed to the next day.
9. Once the specimen is determined to be acceptable, the site should complete the Specimen Transport or Laboratory Accession Form.
10. If specimens are not transported immediately, refrigerate at 2°-8^{o}C until ready for transport to the laboratory. Refrigerators in which sputum specimens are stored must be monitored with daily temperature readings using a certified thermometer. The acceptable range is 2°-8^{o}C. Record the refrigerator temperture daily on the appropriate temperature record form. When out of range, follow the clinic’s procedure for resolving the problem and documenting the action.

11. If a refrigerator is not available, specimens must be stored in cooler boxes with ice packs. Sputum specimens must never be frozen.

Transport of Specimens:

1. Transport of specimens to the laboratory should be pre-arranged so that the laboratory personnel are prepared to receive and process specimens. A delivery time should be pre-arranged with the sites before the study is started.
2. If applicable, complete the Specimen Transport Log Form for all specimens being transported in one shipment.
3. Enter the details of only one specimen per line on the log.
4. Follow the IATA or United Nations Recommendations on the Transport of Dangerous Goods when packing specimens for transport. These rules apply, no matter how short the distance of transport (road or air).
5. Do the following for road transport:
    a. The screw caps of specimen containers must be properly closed to ensure that specimens do not leak. Leaking containers will be discarded without processing.
    b. Seal each specimen inside a Pathoseal 95kPa ADR (road transport) pouch; only one specimen container per pouch. Place the appropriate Specimen Transport or Laboratory Accession Form in the side pocket of the pouch.
    c. If using transport boxes which require the use of a rigid inner container (container with screw top), place absorbent material in the bottom of the inner container.
    d. Place the sealed pouches, with specimen containers, in an upright position into the inner container.
    e. Place the inner container in an upright position into the transport box and place enough cool packs around the specimens to maintain a temperature of 2°-8°C.
    f. If making use of SABS approved transport boxes, which do not require the use of a rigid inner container, do the following:
        ii. Unclasp the handle of the box.
        iii. Open the lid.
        iv. Place two ice bricks along each of the two shorter sides of the box.
        v. Place Correx sheets in front of the ice bricks to prevent the specimens from touching the ice bricks.
        vi. Place a sheet of absorbent material in the bottom of the box.
        vii. Place the specimen bags in an upright position on the absorbent material.
        viii. If applicable, seal the Specimen Transport Log in a Ziploc bag and place on top of the specimens.
        ix. Close the lid of the box tightly and clasp the handle into position.
    g. DO NOT cover specimens with absorbent material. In case of leakage, it is important to be able to see through the plastic specimen bag and identify the specimen inside without opening the sealed transport pouch.
    h. Transport the specimens to the laboratory without further delay.
6. Designated couriers/drivers will transport specimens from the sites to the laboratory.
7. Specimens must be transported immediately after packing is completed.
8. For air transport, follow steps 1-7 above and follow all the regulations and procedures specified by the courier company. Air transport regulations are strict and beyond the scope of this manual.
9. When the laboratory is located at the clinical site or within the same city, follow steps 1-3 and 6-7. And do the following for local transport:

a. The screw caps of specimen containers must be properly closed to ensure that specimens do not leak. Leaking containers will be discarded without processing.
b. Seal each specimen inside a Pathoseal 95kPa ADR pouch; only one specimen container per pouch. Place the appropriate specimen transport form in the side pocket of the pouch.
c. Place each pouch upright in the transport box/cooler so tubes/containers are upright.
d. Place a sufficient number of ice packs in transport box to maintain the temperature in a range of 2°-8^{o}C. The cooler does not need to be monitored with a thermometer.
e. If applicable, seal the specimen transport log in a Ziploc bag and place inside the transport box.
f. Secure the lid/top of the transport box.

I. SPECIMEN RECEIPT AND HANDLING

PURPOSE

To maintain the integrity of the specimen once received in the laboratory. Proper receipt and handling of the specimen includes verifying that the specimen label and Specimen Transport Form/Laboratory Accession Form coincide, keeping the specimen at the proper temperature (2°-8°C) until processed, and processing the specimen as soon as possible upon receipt.

PRINCIPLE

The laboratory will follow their procedure for logging in specimens and assigning accession numbers to the specimens. Special instructions regarding the receipt and handling of study specimens are described in this procedure.

Receipt of Specimens in the Laboratory:

1. Specimens are received from the courier/driver at a designated specimen reception area.
2. If applicable, sign the driver's log and give the driver an empty transport box to take back to the site. The driver should not wait for specimens to be unpacked. When signing for specimens, discrepancies are not noted in the log book. This can only be done once the transport box has been opened and specimen transport forms and specimen labels have been compared.
3. Open the transport box inside a biosafety cabinet (BSC). Proceed quickly through steps 4-11, to keep the specimens cool.
4. Remove specimens from the box (or inner container if used) and remove the Specimen Transport or Laboratory Accession Forms from the side pocket of the specimen bag and if applicable, the Specimen Transport Log from the Ziploc bag.
5. DO NOT open the transport bags. Compare the details on the label of the specimen container with that written on the specimen transport form.
6. If there is a mismatch between information written on the label and on the Specimen Transport or Laboratory Accession Form, report to the supervisor and follow the relevant procedure (electronic/paper) to document, report and follow up with the investigator site on discrepancies found.
7. Enter the date and time of receipt on the specimen transport forms and sign.
8. If applicable, the Specimen Transport Log will be completed at the laboratory after the specimens have been unpacked.
9. If applicable, compare the list on the Specimen Transport Log with the specimens unpacked from the box and tick off each specimen received. If there are missing specimens, report to the supervisor. Follow the relevant procedure (electronic/paper) to document, report and follow up with the investigator site. Provide a copy of the Specimen Transport Log to the investigator site if required.
10. Allocate laboratory numbers to the specimens that have been confirmed as received, by placing a laboratory number label or writing the laboratory number in the column allocated for the purpose. Do not allocate laboratory numbers to missing specimens.
11. If applicable: if a specimen is unlabelled, do not place a tick mark on the Specimen Transport Log. Unlabelled specimens cannot be identified and are not processed. Allocate a laboratory number to the Specimen Transport or Laboratory Accession Form received with the unlabelled specimen. Follow the relevant procedure (electronic/paper) to document, report and follow up with the investigator site.
12. If being transported to a separate BSL3/BLS2 laboratory, pack the specimens into a specimen box, add the completed Specimen Transport Log (if applicable), seal the box and take this to the laboratory for processing. There should be no delay in getting the specimens to the processing laboratory. If being

processed in the same laboratory, place the specimen box with transport log in the refrigerator designated for specimens.

13. Decontaminate the inside of the used specimen transport box with disinfectant, followed by cleaning with 70% ethanol on the in- and outside of the box.
14. Replace the absorbent material and make sure that there are 4 ice bricks and 2 Correx sheets in the box.
15. Take the box to the receiving/reception area for future transport.

Handling of Specimens:

1. Cut-off processing times will be decided by the laboratories. For example, sputum specimens for quantitative culture received by 12:00 will be processed the same day. Screening specimens must be received by 14:00 in order to be processed on the same day.
2. Specimens received after the cut-off time will be refrigerated and processed the following day, unless pre-arranged otherwise.
3. If there is a large load of specimens on a day, the specimens with earliest collection date/time must be processed first and specimens with a later collection date/time may be left in the refrigerator at 2°-8°C for processing the following day.
4. If specimens are kept in a refrigerator in the laboratory for next day processing, a note must be made on the specimen transport form to indicate where the specimens were stored and why processing was delayed.
5. If a specimen container has leaked:
    a. DO NOT open the plastic pouch.
    b. Check the details on the label through the pouch and either make a note on the Specimen Transport or Laboratory Accession Form and in the ’Comments’ field of the Microbiology Results Report Form (Appendix 1) or laboratory database (if electronic data transfer).
    c. Discard the specimen.
    d. Immediately notify the study coordinator in case another specimen should be collected. This is only applicable to coached spot sputum samples where there is a possibility of collecting another sample on the same day.
6. If there is a mismatch between the information on the label and the Specimen Transport or Laboratory Sccession Form:
    a. Contact the sender (collection staff at site) to obtain any information needed before the specimen is processed. If the identification cannot be ensured, the specimen will not be processed.
    b. Immediately notify the study coordinator in case another specimen should be collected. This is only applicable to coached spot sputum samples where there is a possibility of collecting another sample on the same day.
7. Specimens without Specimen Transport or Laboratory Accession Forms are unacceptable and will not be processed until the laboratory has contacted the sender. The specimen will be kept in the refrigerator until a Specimen Transport or Laboratory Accession form has been received.
8. If a specimen container is unlabelled:
    a. Do not process the specimen.
    b. Discard the specimen.
    c. Contact the sender (collection staff at site).
    d. Make a note on the Specimen Transport or Laboratory Accession form and in the ’Comments’ field of the Mycobacteriology Results Report Form (Appendix 1) or laboratory database (if electronic data transfer).

9. For all problems encountered regarding specimen leakage, Speciment Transport or Laboratory Accession Forms, and specimen labeling, document occurrence and how problem was resolved or handled on the laboratory's form for reporting problems. For example, complete a "Problem Reporting" form and send to the Quality Assurance Officer.

Quality Control

Frequency:

Daily, for all specimens received

Procedure:

1. Check that specimen transport boxes have been packed correctly and enough ice bricks used to maintain a temperature of 2°-8°C.
2. Check that plastic transport bags have been sealed.
3. Make sure that information on labels and Specimen Transport or Laboratory Accession Forms is complete and correct.
4. If applicable, check the Specimen Transfer Log to ensure that all specimens have been received.
5. Check the state of specimen transport boxes.

Documentation:

1. Sign and date the Specimen Transport or Laboratory Accession Forms; discrepancies are noted in the 'Comments' section of the Microbiology Results Report Form (Appendix 1) or laboratory database (if electronic data transfer).
2. Tick or check off the specimens on the specimen transport log.
3. Appropriately document and follow up on issues and report problems to the laboratory manager.

J. PREPARING DIRECT SPUTUM SMEARS FOR MICROSCOPY

PURPOSE

To prepare smears directly from sputum for microscopic examination.

PRINCIPLE

Direct smears are made with sputum specimens that are digested with Sputasol (DTT) and prepared for fluorescence microscopy. For the NC-005 study, the smear can be made with either the DTT-digested specimen, referred to as the direct smear, or the DTT-digested and NaOH-decontaminated specimen, referred to as the concentrated smear. If the latter is preferable because of the workflow (have to decontaminate and concentrate the specimen for the Hain Line Probe Assays or MGIT culture), refer to Preparing Concentrated Sputum Smears for Microscopy SOP (K).

PROCEDURE

Materials

Biological safety cabinet (BSC)
Disinfectant
Receptacles for waste
Benchguard
Paper towels
Gloves
Biohazard bags
10% Sputasol (see Quantitative Sputum Culture for preparation)
Serological pipets, 1 ml
    Or micropipettor and tips
Platform shaker
Vortex
Microscope slides, clean, new, frosted one end
Pencil for labelling slides
Sterile disposable pipet
Slide transport box (if transporting slides to another lab for staining or moving from BSC to lab oven)

Procedure

Steps 1-8 in this procedure must be performed inside the biosafety cabinet (BSC) using BSL3 procedures and practices and appropriate PPE.

1. Remove the sputum specimen from the refrigerator and allow warming to room temperature. If not already in a sterile, 50 ml conical tube, label such tube with the subject ID number, visit interval, date, and lab accession number. Carefully decant the sputum from the wide-mouth container into the conical tube.
   Pre-homogenization using mechanical stirring (optional step). If not used, proceed to step 3:
2. Homogenise the entire sputum specimen by adding a sterile 30 mm cylindrical magnet with pivot ring and stirring mechanically for 30 minutes.
3. Estimate the total volume of the pooled sputum specimen according to the gradations on the tube

(preferably comparing to a 50 ml tube with gradations marked for every ml) or using a serological pipet, and record on the Quantitative Culture Worksheets, along with the subject ID number, visit interval, specimen accession number, and date of plating.

4. Add an equal volume of 10% Sputasol to the specimen. Record the amount of Sputasol added to each tube on the Quantitative Culture Worksheet.
   Example: If the total sputum volume is 10 ml, add 10 ml of 10% DTT for a total volume of 20 ml and a final concentration of 5% DTT. If the volume is 1 ml, add 1 ml of 10% DTT.
5. Vortex the specimen for 20 seconds and then incubate at room temperature for 20 minutes to digest. **Note: If sputum specimens was not mechanically homogenized prior to adding Sputasol, place tube on platform rocker to shake mechanically at 60-80 rpm for 15-20 min**.
6. Label slide at a minimum with specimen number and date of smear preparation. Follow local laboratory procedure for additional labelling as required (e.g. add Visit #, Subject #, Subject initials, etc.).
7. With a diposable pipet place 1-2 drops of digested sputum onto the slide and spread over an area of 1 X 2 cm, approximate a rectangle. Do not let the specimen touch the edges of the slide.
8. Air-dry the smear.
9. Place slides on a slide warmer or hot plate at 65°-75°C for **at least** 2 hours (longer time is preferable to ensure killing of *M. tb*) to heat-fix the samples. It is acceptable to heat-fix the slides in an oven at 80 °C for same length of time. In this situation, the slides are removed from the BSC and transported in a slide box.
10. Slides are stained according to the AFB Microsocopy SOP (L).

K. PREPARING CONCENTRATED SPUTUM SMEARS FOR MICROSCOPY

PURPOSE

To prepare smears from processed and concentrated sputum for microscopic examination.

PRINCIPLE

For the NC-005 study, the smear can be made with either the DDT-digested specimen, referred to as the direct smear, or the DDT-digested and NaOH-decontaminated specimen, referred to as the concentrated smear. Results from direct and concentrated specimens are comparable when the AFB density in the sputum is high, which is typical of screening and baseline specimens. The procedure for decontaminating the sputum specimen is described in Sputum Processing For Decontamination (M).

PROCEDURE

Materials

Biological safety cabinet (BSC)
Disinfectant
Receptacles for waste
Benchguard
Paper towels
Gloves
Biohazard bags
Concentrated sputum specimen (pellet resuspended in PBS)
Microscope slides, clean, new, frosted one end
Pencil for labelling slides
Sterile disposable pipet
Slide transport box (if transporting slides to another lab for staining or moving from BSC to lab oven)

Procedure

Steps 1-6 in this procedure must be performed inside the biosafety cabinet (BSC) using BSL3 procedures and practices and appropriate PPE.

1. Label slide at a minimum with specimen number and date of smear preparation. Follow local laboratory procedure for additional labelling as required (e.g. add Visit #, Subject #, Subject initials, etc.).
2. Vortex specimen to ensure pellet is completely suspended in PBS.
3. With a diposable pipet place 1-2 drops of digested sputum onto the slide and spread over an area of 1 X 2 cm, approximate a rectangle, or a fixed area that is etched on the slide. Do not let the specimen touch the edges of the slide.
4. Air-dry the smear.
5. Place slides on a slide warmer or hot plate at 65°-75^{o}C for **at least** 2 hours (longer time is preferable to ensure killing of *M. tb*) to heat-fix the samples. It is acceptable to heat-fix the slides in an oven at 80 ^{o}C for same length of time. In this situation, the slides are removed from the BSC and transported in a slide box.

6. Slides are stained according to the AFB Microsocopy SOP (L).

L. ACID-FAST MICROSCOPY

PURPOSE

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

PRINCIPLE

Acid fast microscopy can be performed with the Ziehl-Neelsen or fluorescence method. Laboratories are encouraged to use fluorescence microscopy with direct and concentrated sputum smears due to its increased sensivity. For the NC-005 study, fluorescent microscopy will be used. Fluorescence staining includes a stain, decolouriser and counter stain as for Ziehl-Neelsen staining. With Auramine-O stain organisms fluoresce bright yellow, non-specific debris stains pale yellow, and the background is almost black. With Auramine-Rhodamine stain organisms fluoresce yellow-red in an almost black background.

Ziehl-Neelsen staining is used for confirming the presence of AFB in positive MGIT cultures and on Middlebrook agar, if the colonies do not resemble *M. tb*. The Ziehl-Neelsen (ZN) method uses a carbol fuchsin stain, acid alcohol decolouriser and methylene blue counter stain. Acid-fast organisms stain red and the background of debris stains blue.

PROCEDURE

Materials

Direct or concentrate smears (slides prepared in J and K)
Staining sink
Staining rack
Distilled water in wash bottle
Slide drying rack
Slide holder
Slide storage box
AFB positive and negative slides

For Auramine-O Stain or Auramine-rhodamine stain:
Auramine-O or Auramine-rhodamine staining kit
  Or auramine O/auramine-rhodamine, 0.5-1% acid alcohol, 0.5% ptoassium permanganate prepared in-house (see lab's procedure for preparation)
Fluorescence microscope or LED microscope
Dark room

For Ziehl-Neelsen stain:
Ziehl-Neelsen staining kit
  Or carbol fuschin, 3% acid alcohol, methylene blue (see laboratory's procedure for preparation)
Light microscope
Immersion oil
Lens paper & lens cleaning solution

Materials to prepare slides (see Materials in L)

Procedure for Fluorescent Staining

1. Include an AFB positive and AFB negative slide in each staining batch.
2. Place slides on a staining rack and flood with Auramine-O/Auramine-rhodamine stain and let sit for 15 minutes.
3. Rinse the Auramine-O/ Auramine-rhodamine away with distilled water and drain the slides.
4. Decolourise with 0.5% acid alcohol for 2 minutes.
5. Rinse again with distilled water and drain.
6. Flood slides with 0.5% potassium permanganate for 2 minutes; no longer than 4 minutes.
7. Rinse potassium permanganate away with distilled water.
8. Air-dry the slides. **Do not blot dry.**
9. Protect smears from light by placing in a storage box and examine as soon as possible since the fluorescence deminishes quickly.

Examination of Fluorescent Smears

1. Scan the entire smear with the 20x or 25x objective, with 10x eyespiece this is a 200x or 250x magnification.
2. Use a scanning pattern of rows either up and down the slide or across and back. Using 200x or 250x magnification, one 2 mm length is equivalent to 30 fields, which is sufficient to report a negative result.
3. The 40x or 45x objective, 400x or 450x magnification, is used for closer examination of the bacterial morphology. Confirming morphology at higher magnification avoids false positive results due to fluorescing debris. Often this magnification is preferred for grading smears; 1 x 2 mm length is equivalent to 40 fields.
4. Examine the control slides first. If expected results are not observed, do not proceed to examine the slides from the cultures.
5. The mycobacteria can appear as rod, coccoid, and filamentous shapes. In sputum smears, individual rods of *M. tb* may be aggregated side-by-side or end-to-end to form "cords".
6. Count bacilli in the number of fields appropriate for degree of positivity, e.g. the higher the smear positivity, the fewer fields need to be counted. Average the count for the number of fields and record on the laboratory's microscopy worksheet according to the IUATLD/WHO grading scale below.

	Fluorescence Microscopy	
Result	**20x - 25x** (200–250x magnification: 1 length = 30 fields = 300 HPF)	**40x** (400x magnification: 1 length = 40 fields = 200 HPF)
Negative	Zero AFB / 1 length or per 30 fields	Zero AFB / 1 length or per 40 fields
Scanty*	1–29 AFB / 1 length or per 30 fields	1–19 AFB / 1 length or per 40 fields
1+	30–299 AFB / 1 length or per 30 fields	20–199 AFB / 1 length or per 40 fields
2+	10–100 AFB /field on average	5–50 AFB /field on average
3+	>100 AFB /field on average	>50 AFB /field on average

Ref: http://www.stoptb.org/wg/gli/documents.asp?xpand=2

*Have another microscopist re-examine the smear and if still scanty, prepare another smear from the same specimen and examine before reporting results.

7. All slides must be kept until results have been checked, verified and reported.

Preparation of Ziehl-Neelsen Smears

All steps in this procedure must be performed inside the biosafety cabinet (BSC) using BSL3 procedures and practices and appropriate PPE.

1. Label the slide on the frosted end with specimen ID number, source (MGIT or 7H11), and date smear prepared.
2. For ZN staining positive MGIT cultures, mix MGIT tube by vortexing, remove cap, and sample the broth using a sterile disposable pipet. If inoculating a BAP at the same time, do this before preparing the smear. Place 1-2 drops on the slide and spread to cover a 1.5 X 1 cm area.
3. If examining colonies on 7H11S or 7H11S+C media, dispense about 100 ul of sterile distilled water on a glass slide with a transfer pipet. Using a sterile loop or applicator stick, transfer 2-3 colonies to the water and gently mix to make a smooth suspension.
4. Allow the smear to air dry completely.
5. Place the slides on a hot plate or slide warmer at 65°-75°C for a least 2 hours (longer time is preferable) to heat-fix. As described above, a laboratory oven is a suitable alternative.

Procedure for Ziehl-Neelsen Staining

1. Include an AFB positive and AFB negative slide in each staining batch.
2. Place slides on a staining rack and flood with carbol fuchsin.
3. Heat slides from underneath with the flame of a Bunsen burner until the stain starts steaming.
4. Let stand for 5 minutes.
5. Flood slides again with carbol fuchsin and heat again until steaming. Do not allow stain to dry.
6. Let stand for 5 minutes.
7. Wash away the carbol fuchsin with distilled water and drain the slides.
8. Flood slides with 3% acid-alcohol.
9. Let stand for 9 minutes.
10. If the liquid becomes heavily stained, add more acid-alcohol until the stain is washed out.
11. Wash away the acid-alcohol with distilled water and drain the slides.
12. Flood the slides with methylene blue.
13. Let stand for 1 minute.
14. Wash away the methylene blue with distilled water.
15. Drain slides and allow to air dry. **Do not blot dry**.

Examination of Ziehl-Neelsen Smears

1. Using a bright field microsope, examine smears using the 100x oil objective, together with a 10x ocular lens the magnification is 1000x.
2. Examine and grade the control slides first. If expected results are not observed, do not proceed to examine the slides from the cultures.
3. For MGIT cultures, follow the same scanning pattern as for the fluorescent stain. A minimum of 50 fields must be examined to rule out the presence of AFB in the MGIT broth.
4. For 7H11S or 7H11S+C cultures, peruse the smear to examine the morphology.
5. The mycobacteria can appear as rod, coccoid, and filamentous shapes. In culture, *M. tb* form clumps and serpentine cords.

6. Record results (AFB positive or AFB negative) on laboratory's MGIT culture worksheet. Note if cording is seen. For 7H11S and 7H11S+C, record AFB positive or AFB negative on the quantitative sputum culture worksheet.

Reporting of Fluorescent Results for Study

Report type of smear, type of stain, and results on the Mycobacteriology Results Report Form or in the laboratory database.
Select only one option for each parameter:
- Direct or Concentrated
- Fluorescence or Ziehl-Neelsen
- Positive or Negative
- If positive, Scanty, 1+, 2+, or 3+

Quality Control

Internal Quality Control

A negative control smear allows detection of AFB if introduced in the staining procedure or if present on the microscope lens (immersion oil on lens). The use of a negative control smear also ensures that non-AFB organisms do not stain acid-fast.

A positive control smear assesses if the staining reagents are working properly. If using the same stock suspension of *M. tb* to prepare the positive smears, the consistency of the smear grade can also be assessed.

Frequency: With every staining batch

Controls:

Positive control – *M. tuberculosis*
Negative control – *E. coli*

Preparation of Control Slides:

1. Commercially prepared slides may be used.
2. If control slides are prepared in-house, prepare a large batch of smears at one time.
3. Positive and negative control suspensions are placed on separate slides.
4. Using slides frosted on one end:
    a. Spread 30 µL of control evenly in a circle.
    b. Heat-fix as described above for preparation of ZN smears.
    c. Label slides as "positive control" or "negative control" and with the date they were prepared.
    d. Store unstained control slides in a sealed slide box.

Acceptance Criteria:

Negative control – no acid-fast bacilli seen
Positive control – acid fast-bacilli seen, smear grade depends on what was prepared

Documentation:

1. Record results for positive and negative controls on the laboratory's Microscopy worksheet.
2. If the results of the QC slides are not acceptable, do not use the results from the specimen/culture smears in that batch.
3. Resolve the problem and repeat staining with another smear from the same specimens/cultures, including new QC slides.
4. Document the problem and how the problem was resolved on the laboratory's corrective action form.

Internal Quality Asssessment

One of the following must be used to assess proficiency of the microcopists. Record this activity on the laboratory's IQA form.

1. At least 10% of all smears read are re-examined by a 2nd technologist. If there is a discrepancy between the results, the 2 technologists must read the slide together and obtain an accurate result. If they still do not agree, an experienced person must verify the result.
2. Monthly, 10 slides are chosen that represent a range of results (negative, 1+, 2+, and 3+). These are read in a blinded manner. Results are compared to the reported results and among the microscopists. Discrepancies can be examined and steps taken to improve peformance.

Reagent Quality Control

For each new batch of in-house prepared staining reagents record the date prepared, expiration date, and name of person preparing these on the laboratory's QC form. When using commercial processing kits, record the lot number and expiration date of each reagent on the laboratory's QC form. Test each new batch or lot number using a positive and negative smear. Acceptable results must be obtained for the staining reagents to be used.

References

1. Betty A. Forbes, Daniel F. Sahm, Alice S. Weissfeld. *Bailey & Scott's Diagnostic Microbiology*, 12th Edition, 2007, Mosby Inc. St Louis, MO.
2. Garcia L.S., Isenberg, H.D. *Clinical Microbiology Procedures Handbook,* 3rd Edition, 2007, Vol. II & III. American Society for Microbiology, Washington, D.C.

M. SPUTUM PROCESSING FOR DECONTAMINATION

PURPOSE

For the NC-005 study, the screening, coached spot sputum specimens are decontaminated and concentrated before DNA extraction for the rapid susceptibility tests (Hain MTBDR*plus* and Hain MTBDR*sl*) is carried out. All other coached spot sputum specimens and the pooled overnight sputum specimens are decontaminated and concentrated for MGIT culture. The specimens are digested with Sputasol (DTT) beforehand as described in two procedures – Preparation of Concentrated Smears for Microscopy (K) and Quantitative Sputum Culture (U).

For the NC-005 study, the MGIT TTP is a primary endpoint. When decontaminating coached spot sputum specimens and the pooled overnight sputum specimens for MGIT culture, the volume to be decontaminated is determined on the basis of the initial sputum volume. See guidelines in Microbiology Flowchart: D-2, D-1 and Treatment Coached Spot and Pooled Overnight Sputa (E). If the sputum specimen is of low volume (e.g., 1-1.9 ml), there is only about 2- 3.8 ml of DTT- digested sputum for MGIT and Quantitative Sputum Culture. Thus, 1 ml is removed for processing and preparing inoculum for the MGIT culture before plating the DTT-digested sputum directly onto Middlebrook agar or making the first 10-fold dilution for the Quantitative Sputum Culture (U). When the sputum volume is 2 ml or greater, 2 ml of digested sputum is removed for Quantitative Sputum Culture (U) and remainder decontaminated according to this procedure.

PRINCIPLE

The active ingredient in Sputasol is Dithiothreitol (DTT), a strong reducing agent, which is used for digestion. Sodium hydroxide (NaOH) is a decontaminating agent, and should be used at a lower final concentration of 1 - 1.5%. The laboratory should use the lowest concentration of NaOH which results in acceptable MGIT culture contamination rates. **The concentration must not be changed during the study without consulting the sponsor.** Sodium citrate chelates heavy metal ions present in the specimen and stabilizes activity of NALC. The use of sodium citrate is optional as it does not have the same effect on DTT. The phosphate buffer neutralises the NaOH and dilutes 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 3,000 *g* for 15-20 minutes is necessary to sediment mycobacteria.

PROCEDURE

Materials

Biological safety cabinet (BSC), Class II
Mycobactericidal disinfectant
Splash-proof waste container
Waste discard bucket
Benchguard (or alternative work surface protection)
Paper towels
Gloves
Biohazard bags
50 ml centrifuge tubes
Test tube rack for 50 ml centrifuge tubes
In-house prepared decontamination reagents and phosphate buffer

Or commercial kits, e.g., BD MycoPrep or AlphaTec NAC-PAC
Sterile serological pipets with pipet aid
Sterile disposable pipets
Vortex mixer
Platform shaker
Refrigerated centrifuge with biosafety bucket with inserts for 50 ml tubes
Timer (2)

Procedure

All processing steps must be performed inside the biosafety cabinet (BSC) using BSL3 procedures and practices and appropriate PPE.

Preparation of BSC and Special Microbiology Practices:

1. Place a clean sheet of Benchguard (or alternative work surface protection) in the BSC.
2. Prepare a splash-proof waste container with disinfectant at the appropriate concentration and place inside BSC.
3. Place a discard bucket with a biohazard bag containing the appropriate disinfectant inside BSC for disposal of contaminated materials.
4. Process only the number of specimens that will fit in the centrifuge. Example, for an 8-place rotor or buckets holding up to 8 tubes, process 8 specimens at a time.
5. When processing multiple batches on the same day, upon completion of each processing batch clean the BSC with disinfectant, turn on UV lights for 20 minutes, allow air to circulate in BSC for the same time, and place a clean sheet of Benchguard before beginning the next processing batch.
6. Work methodically with specimen tubes on one side of BSC and discard bucket and waste receptacle on other side. Remove only 1 cap from specimen tube at a time.
7. When working with multiple specimens, remove only the caps (specimen tube, MGIT tube, PBS tube) of tubes from the same specimen. This will avoid cross-contamination and a mix-up of specimens.
8. During transfer of liquids from reagent bottles to tubes, take care not to touch the sides of the tubes.
9. Minimize production of aerosols.
10. Minimize disruption of the airflow in the BSC, e.g. use proper arm/hand movements, use minimum number of items in BSC, and avoid placing tall or large equipment in BSC.

Specimen Decontamination – using prepared reagents:

Follow the laboratory's procedures for preparing the NaOH solution (omitting the Na citrate) and phosphate buffer (pH 6.8.)

1. Allow refrigerated reagents and specimens to come to room temperature before testing.
2. Label slides (if microscopy to be performed) and tubes with the laboratory accession number, screening ID or subject ID number, "Visit Day" number, and date of processsing.
3. Record all specimens processed in a batch on the laboratory processing worksheet.
4. Include the following controls in one batch of specimens per week, preferably one batch per day:
   a. One negative control sample = tube with 4 ml of 7H9 broth
   b. One positive control sample = tube with 4 ml of 7H9 broth and 100 µl of 1:100 dilution of H37Rv at 0.5 McFarland standard density (frozen stock)

These samples should be included near the end of the batch and handled as normal specimens being processed for smear microscopy and MGIT culture.

5. If a specimen is not collected in a 50 ml tube, transfer it to a 50 ml centrifuge tube.
6. Determine the amount of sputum by comparing to gradations on the container/tube or milliliter markings placed on a 50 ml tube. Record the volume on the worksheet.
7. Using a sterile pipet add a volume of 2% NaOH equal to that of the specimen volume (1:1) to the tube to achieve a final concentration of 1% or add an equal volume of 3% to achieve a final concentration of 1.5%.
8. Vortex until the suspension is well mixed.
9. Start the timer for 15 minutes when the NaOH solution is added to the 1st specimen.
10. Place tube in rack on shaker platform and set to rotate at 60 rpm. If a shaker is not available, vortex tube gently 2-3 times during decontamination period to mimic the shaking action.
11. Repeat steps 7-10 for subsequent specimens at 30 seconds or 1 minute intervals.
12. When time has elapsed, remove tubes from the shaker. Add phosphate buffer (PBS) to the 40-45 ml mark on the centrifuge tube. Mix by inversion or vortex.
13. Continue to add PBS to all specimens at 30 seconds or 1 minute intervals, so that each specimen is exposed to NaOH for a maximum of 20 minutes. **It is important that the PBS is added within 20 minutes of adding the NaOH since the mycobacteria can be killed with longer exposure.**
14. Transfer tubes to the centrifuge and place in buckets. Ensure the tubes are equally balanced and the biosaftety lids are properly placed on each of the buckets.
15. Centrifuge at 3,000 *g* for 15-20 minutes at 4°-12°C, if centrifuge is refrigerated.
16. When time has lapsed, remove the centrifuge buckets and carry them to the BSC prior to removing the biosafety lids.
17. Carefully decant the supernatant into the splash-proof container, taking care not to disturb the sediment on the bottom of the tube.
18. Add PBS to the pellet using a 2 ml serological pipet to a final volume of 2 ml. Re-suspend the sediment using the pipet to gently aspirate and expell the buffer. May need to re-cap tube and vortex to ensure the pellet is dislodged from bottom and well suspended in PBS.
19. Using a sterile pipet place 0.5 ml into the MGIT tube and 1-2 drops on slide, if making a concentrated smear. The same pipet from step 18 can be used if no vortexing was needed.
20. The leftover sediment (resuspended pellet) may be stored at 4°C for 7 days until it is confirmed that the MGIT culture is not contaminated. This is rarely used as decontaminating the MGIT culture is preferable, if AFB are detected and a pure culture is needed.

Specimen Decontamination – using a commercial kit

1. Follow manufacturer’s instructions.
2. Do not add PBS to the specimen to increase the volume as this dilutes out the mycobacteria.
3. Do not add the NALC.

When adding PBS to the pellet, do not add 2 ml but bring the volume up to 2 ml.

Quality Control

Internal Quality Control

A negative control tube is processed to ensure that the reagents are not contaminated and there is no carry-over of *M. tb* from 1 specimen to another. If contamination is present in the control tube, the results of specimens processed in the same batch are checked to determine whether the contaminants came from a patient specimen (i.e. has the same contaminant as the control). If there is *M. tb* present in the control tube, the results of specimens processed in the same batch are checked to determine if there was potential carryover of *M. tb* from a patient specimen to the control tube and whether there are any false positive cultures, which would suggest carryover from one specimen to another.

A positive control is added near or at the end of the processing batch. The positive control is used to ensure that the NaOH solution has the same effect on the H37Rv suspension and the culture media (MGIT) remain consistent in supporting growth of mycobacteria. This can be assessed by monitoring the MGIT TTP of the positive control.

Frequency:

Negative control – once a week
Positive control – once a week

Controls can be run more frequently if this is the laboratory's routine practice, e.g. a negative control is included in one batch daily.

Preparation of Controls:

Negative control – 7H9 broth is used instead of PBS or water in order for the quality (sterility) of these to be tested with this control.
Positive control – a standard suspension of *M. tuberculosis* H37Rv, which is prepared as follows:

1. Adjust a suspension of H37Rv to turbidity equal to that of 0.5 McFarland standard.
2. Dilute the 0.5 McFarland suspension as follows to obtain a 1:500 dilution:
    a. Add 1 ml of the suspension to 4 ml of sterile 7H9 broth – Dilution 1 (1:5)
    b. Add 1 ml of Dilution 1 to 4 ml of sterile 7H9 broth – Dilution 2 (1:50)
    c. Add 1 ml of Dilution 1 to 4 ml of sterile 7H9 broth – Dilution 3 (1:500)
3. Use the 1:500 dilution as a positive control. It is preferable to have aliquots of this frozen so that they are readily available for use. Note, this suspension can be used as the internal control for the quantitative culture procedure.

Acceptance Criteria:

1. There must be no evidence (on smear or in culture) of contaminants, non-tuberculous mycobacteria (NTMs), or *M. tb* in the negative control.
2. The positive control must show consistent results. The smear must show AFB and the smear grade should be the same. The MGIT culture should always be positive for *M. tb* and the TTP should be in the same range, e.g. 6-10 days. The laboratory should establish baseline for the smear grade and MGIT TTP.

Documentation:

1. Record results for positive and negative controls on the laboratory's QC form.
2. If the expected results are not obtained follow the laboratory's procedure for addressing the problem and taking corrective action.

Reagent Quality Control

For each new batch of in-house prepared processing reagents record the date prepared, expiration date, and name of person preparing these on the laboratory’s QC form. Phosphate buffer must be checked with pH paper or pH meter to confirm that the pH is 8.6. Record this reading on the QC form.

When using commercial processing kits, record the lot number and expiration date of each reagent on the laboratory’s QC form.

N. DNA Extraction for Hain Line Probe Assays and pncA Sequencing (Genolyse Method)

PURPOSE

To extract bacterial DNA using either the manual Hain GenoLyse® DNA extraction kit or the automated Hain GenoXtract® instrument, to screen patients for susceptibility/resistance to the first and second line drugs using the Hain MTBDR*plus* and MTBDR*sl* line probe assays, as well as to screen for *pncA* mutations inferring resistance to pyrazinamide (PZA) in *M. tuberculosis*. Bacterial DNA can be extracted either directly from sodium hydroxide (NaOH)-NALC treated decontaminated smear-positive clinical specimens or from AFB positive cultures.

PRINCIPLE

Various bacterial DNA extraction methods exist and the skills and equipment required for these vary. The Hain DNA extraction methods described here yield high quality DNA and are recommended for the Hain MTBDR*plus* and MTBDR*sl* assays.

The extraction of the DNA from the acid-fast bacilli in the specimen is done by either using the commercially available manual GenoLyse® DNA extraction kit, which makes use of chemical cell lysis with the GenoLyse® lysis buffer during a heat-killing step after which neutralizing buffer is added to stop the reaction, or by making use of the automated Hain GenoXtract® instrument which uses magnetic bead technology.

Both DNA extraction methods requires an initial centrifugation step where the NaOH-NALC treated bacilli/cultured isolate is concentrated at the bottom of the 1.5ml microcentrifuge tube to form the bacteria-containing "pellet". The supernatant, which may consist of either the phosphate buffered saline neutralising buffer from the NaOH-NALC decontamination procedure, Tween® 80 saline, MGIT growth liquid, or sterile distilled water (if colonies were picked from solid media), is discarded. For the manual DNA extraction method, the pellet is resuspended in the GenoLyse® lysis buffer and for the automated DNA extraction system, the pellet is resuspended in sterile distilled water. The heat generated by the heating-block or water-bath, in addition to the GenoLyse® lysis buffer, aids in the killing of the bacilli and assists in the lyses of the cells, thereby rendering the solution non-infectious.

Once the lysis step has elapsed, the GenoLyse® neutralizing buffer, which is transparent, is added to the solution to stop the chemical degradation of the DNA. A final centrifugation step is used to separate the cell debris from the DNA. The heavier debris will form the pellet and the lighter DNA (free from impurities) will be suspended in the supernatant. It is crucial that the top layer of the pure extracted DNA is immediately removed from the pellet containing the debris and aliquoted into sterile 1.5 ml microcentrifuge tubes with O-rings.

The magnetic beads used in the GenoXtract® system captures the bacteria with subsequent lysis thereof using the various reagents in the extraction cartridges, and the bacterial DNA is deposited into a clean tube.

The extracted DNA is divided into tubes. The 1st aliquot should be used for the MTBDR*plus* and MTBDR*sl* line probe assays (screening samples Days -9 to -3) and the 2nd aliquot should be stored at -20°C, until it is sent to the CCTR lab for *pncA* sequencing.

MATERIALS

GenoLyse® DNA Extraction Kit (Hain LifeScience) consisting of:

- GenoLyse® Lysis Buffer (A-LYS)
- GenoLyse® Neutralization Buffer (A-NB)
- The order number for 12 tests is 51612, and for 96 tests it is 51610.
- This can be ordered from the local Hain LifeScience distributor. Details on your local distributor can be obtained from http://www.hainlifescience.de/en/company/contact/distributors/list.html.

GenoXtract® Reagent Cartridges (GenoXtract instrument only)
1.5ml screw-cap microcentrifuge tubes with O-ring (DNAse-free)
Rack for microcentrifuge tubes
50 ml conical tube filled with 10 ml of 3.5 – 5% sodium hypochlorite
Rack for 50 ml conical tube
Sterile disposable loops
3ml Pasteur pipettes
Micropipettor tips (aerosol-resistant)
Micropipettors (20-100 µl, 200-1000 µl)
Pump tips (GenoXtract instrument only)
Waste container for solid waste
70% ethanol
1% sodium hypochlorite
Tween-saline
Sterile distilled water
NALC digested and NaOH decontaminated sputum specimens
(resuspended pellets in PBS)
Positive MGIT cultures or 7H11S plates with AFB-positive colonies
PPE: gown, gloves, respirator, and shoe covers

Shipment of appropriately labelled DNA specimens are to be done in appropriate specimen transport bags below ambient temperature on sealed ice-packs. The 1.5 ml tubes are to be placed into absorbent material, after which it is to be placed into a zip-style biohazard bag for transport.

Equipment

Biological Safety Cabinet
Microfuge with aerosol tight rotor
Heating Block / Water Bath (precision +/- 1°C)
GenoXtract® Instrument
Timer
Vortex
Thermometer
Floaties (available from Hain Lifescience)
4°C refrigerator
-20°C freezer
Absorbent material
Zip-style biohazard specimen transport bags
Sealed ice packs

Ziplock bag

FORMS

FORM TMTF NC005-001 - DNA Transport Form (Appendix 2)
FORM TMTF NC005-002 - DNA Sample Transport Log (Appendix 3)
FORM TMTF NC005-003 – Sample Transfer Log for pncA Sequencing (Appendix 4) – used by CCTR Laboratory only

PROCEDURE

Handling of sputum specimens and viable M. tb cultures must be done inside the biosfety cabinet using BSL3 procedures and practices and appropriate PPE. The samples remain in the BSC until they are rendered non-infectious.

Equipment Start-up

The air in the BSC should be allowed to purge for 20 minutes prior to working inside it. Whilst the BSC is purging, the heating block/water bath should be switched on, and should have reached the desired temperature of 95°C before the DNA extraction procedure is carried out. If using a water bath, it should be filled with tap water before it is switched on. The temperature must be verified by placing a suitable thermometer (up to 110°C) into the hole provided in the heating block. If a water bath is used, “floaties” must be used to secure the thermometer. The microcentrifuge should be placed inside the BSC.

MANUAL DNA EXTRACTION USING THE GENOLYSE® METHOD

Labelling of tubes

1. The labelling of the microfuge tubes can be done whilst the heating block/water bath is attaining the desired temperature of 95°C.
2. Three tubes will be labeled for each sample to be extracted. The side of the tube will be labeled with the laboratory accession number, and the cap of the tube labelled according to sequence numbers. That is, 3 tubes will have the same laboratory accession number on the side and the number "1" on the cap, if it is the 1st number of the batch of NaOH-NALC treated decontaminated sputum specimens/cultured isolates to be processed.
3. For subsequent specimens, the 3 tubes will be labeled with the laboratory accession number and the caps with the next number, e.g. "2", “3”, etc.
4. The 1st tube will be used for the DNA extraction process. 500 µl of NaOH-NALC decontaminated sputum or 1000 µl of a MGIT broth or growth suspension from culture plates will be placed in the 1st tube.
5. When DNA is extracted, 50 µl the DNA sample will be placed into the 2nd and 3rd tubes.
6. The DNA in the 2nd tube will be used on-site to perform the Hain MTBDR*plus* and/or MTBDR*sl* line probe assays.
7. The 3rd tube, also containing 50 µl of the DNA sample, will be shipped to the Centre for Clinical Tuberculosis Research (CCTR) laboratory after a successful Hain MTBDR*plus* and/or MTBDR*sl* line probe assay result has been obtained on-site.

Extraction of bacterial DNA from NALC-NaOH treated sputum specimens

1. Clean all surfaces, pipettes and tip boxes included, with 1% sodium hypochlorite followed with 70% ethanol.
2. Using a 3ml Pasteur pipette, aliquot approximately 500µl of homogenized decontaminated specimen (raw material) into a 1.5ml screw-cap microcentrifuge tube. If there is insufficient residual material, Tween 80 saline may be added to increase the volume to the desired amount required. If desired, the lid of the Eppendorf tube can be marked to indicate which side of the tube faced towards the inside of the microfuge to aid with collection of pellet. The resulting pellet will deposit diagonally at the opposite side of this mark.
3. Centrifuge the specimens at 10,000 *g* or 13,200 rpm for 15 minutes so that a pellet is concentrated on the side of the tube. Carefully take the tubes out of the microcentrifuge after it has spun down, so as to not loosen the pellet from the walls of the tube.
4. Search for the pellet by eye. Once found, turn the tube so that the pellet is positioned relative to the top. This allows for easy access to the supernatant.
5. Remove the supernatant slowly and carefully, as the pellet might be sucked up if removed too fast. If the pellet has been sucked up, transfer the contents back into the same 1.5ml microfuge tube and centrifuge again. If the lid was marked initially, place the tube into the microfuge with the marking facing towards the middle of the microfuge. Once the tube has been re-centrifuged, use the mark to find the approximate position of the pellet and carefully remove the supernatant by slowly sucking up the supernatant from the top and moving down as the volume decreases.
6. Discard supernatant into a 50 ml conical tube which is filled with 10 ml 3.5 - 5% sodium hypochlorite.
7. Re-suspend the pellet in 100 µl lysis buffer (A-LYS). Ensure that the pellet is thoroughly re-suspended and that there are no clumps prior to incubating the sample.
8. Incubate the specimens in the heating block or water bath at 95^{o}C for a minimum of 5 minutes.
9. Add 100 µl of neutralization buffer (A-NB) and briefly vortex.
10. Centrifuge the specimens at top speed for 5 minutes.
11. Carefully take the tube out after it has spun down so as not to loosen the pellet from the walls.
12. Search for the pellet by eye. Once found, turn the tube so that the pellet is positioned relative to the top. This allows for easy access to the supernatant.
13. Immediately pipette 100 µl of the supernatant (which now contains the DNA) slowly and carefully into the 2nd tube, the DNA of which is to be used for the Hain MTBDR*plus* and/or MTBDR*sl* line probe assays.
14. Divide the aliquoted DNA in half, by pipetting 50 µl of the aliquoted DNA in the 2nd tube slowly and carefully into the 3rd tube, which is to be sent to the CCTR laboratory for *pncA* sequencing. Both the 2nd and 3rd tube should now have equal volumes of the same DNA.
15. Switch off the heating block/water bath. If a water bath was used, empty the contents thereof.
16. Once done, clean all surfaces, pipettes, tip boxes, storage boxes, etc., with 1% sodium hypochlorite and then with 70% ethanol.
17. The 2 sets of aliquoted DNA must be stored at either 4°C or -20°C until required for subsequent analyses and/or shipment to the CCTR laboratory.
18. The 2nd tube containing the now aliquoted DNA, is to be used on-site for 1st- and 2nd line drug susceptibility testing screening using the MTBDR*plus* and/or MTBDR*sl* line probe assays, respectively.
19. Once a successful MTBDR*plus* and/or MTBDR*sl* result has been obtained, the 3rd tube, also containing the same aliquoted DNA, can be shipped to the CCTR laboratory for *pncA* sequencing.
20. If the MTBDR*plus* and/or MTBDR*sl* results were unsuccessful the DNA duplicate cannot be sent for *pncA* sequencing, and the DNA extraction must be done anew. Either from any residual resuspended sediment or from another sputum specimen (see Hain LPAs procedures for instructions on handling indeterminate results).

21. The genolysed DNA is stable at room temperature for up to two weeks, but should preferably be stored at 4°C after the extraction procedure. If it is stored for longer than a week, it must be placed in a 20°C freezer.
22. Complete Form TMTF NC005-001 once ready to ship to CCTR. Once the specimen has been assigned to the DNA Sample Transport Log (TMTF NC005-002), make a copy of Form TMTF NC005-001 ande-mail it to pnca@task.org.za. The original DNA Transport Forms should be placed in a sealed Ziploc bag and included in the shipment sent by courier.
23. Shipment of appropriately labelled DNA specimens are to be done in appropriate specimen transport bags below ambient temperature on sealed ice-packs. The 1.5 ml tubes are to be placed into absorbent material, after which it is to be placed into a zip-style biohazard bag for transport.
24. Complete the DNA Sample Transport Log (TMTF NC005-002) for all specimens being transported in one shipment. Enter the details of only one specimen per line on the log.
25. Seal the DNA Sample Transport Log in a Ziploc bag and place on top of the specimens.
26. It is the onus of the shipping laboratory to ensure the IATA and/or UN shipping regulations are adhered to.
27. All DNA samples should be shipped at ambient temperature within a 20°-26°C range to the following address:

    Room **F561**
    Faculty of Medicine and Health Sciences
    Division of Molecular Biology and Human Genetics
    Department of Physiology
    Fisan Building, 5th Floor
    Francie van Zijl Drive
    Tygerberg, 7505
    Cape Town, South Africa

28. The sponsor will assist with arranging of courier shipment of samples where required. If applicable, Material Transfer Agreements should be in place prior to shipment of samples.

Extraction of bacterial DNA from MGIT cultures or colony suspensions

1. The same method is to be followed as the one used for sputum samples, except that approximately 1000 µl of a well-mixed MGIT culture is placed into the screw-cap microcentrifuge tube (1st of 3 tubes).
2. Colonies are to be scrapped form the solid media and re-suspended into 500 µl Tween-saline, after which the same procedure should be used.

AUTOMATED DNA EXTRACTION USING THE GENOXTRACT® INSTRUMENT

Labelling of tubes

Tubes are labeled as described above for the manual extraction method.

Extraction Steps

Extraction of bacterial DNA from NALC-NaOH treated sputum specimens

1. Vortex the resuspended pellet/sediment.
2. Transfer 700 µl into a 1.5 ml labelled screw-cap microcentrifuge tube.
3. Heat kill the prepared samples in a heating block set at 80°C for 20 minutes.

4. Whilst the heat kill step is taking place, prepare the instrument and reagent cartridges.

Extraction of bacterial DNA from liquid and solid media cultures

1. Aliquot approximately 1000 µl of the MGIT culture into a labelled screw-cap microcentrifuge tube.
2. Spin for 5 minutes at 10,000 *g* in the microcentrifuge.
3. Remove the supernatant from the pellet and re-suspend in 1000 µl of sterile distilled water.
4. Vortex the tube.
5. Remove 300 µl from the vortexed tube and discard the removed liquid into the 3.5 – 5% sodium hypochlorite solution, resulting in a final volume of 700 µl.
6. When using growth from solid media, scrape a few colonies using a sterile disposable loop and re-suspend in 700 µl of sterile distilled water in a 1.5 ml screw-cap microcentrifuge tube.
7. Heat kill the prepared samples in a heating block set at 80°C for 20 minutes.
8. Whilst the heat kill step is taking place, prepare the instrument and reagent cartridges.

Instrument Preparation

The following preparatory steps have to be taken before a run is commenced:

1. Take the instrument-specific pump tip and press it down onto the 1000 µl fitler tip until the pump and the tip are well connected and sealed.
2. Mount the Pump-Tip assembly into the designated slot in the array arm.
3. Open the tip holder clip and insert the pump-tip.
4. Ensure that the lower rim of the filter tip is on top of the lower metal edge.
5. Close the tip holder clip.
6. Ensure that all tips are placed correctly and that there is a pump-tip for each corresponding sample as well as a reagent cartridge.
7. Remove the foil from the reagent cartridge(s).
8. Place the open cartridges on the GenoXtract rack and make sure that the front is secured by the plastic lock on the cartridge.
9. Ensure that there is a pump-tip and an eluate tube in each row with a sample and a cartridge.

Loading of the heat-inactivated bacilli

1. After the 20 minutes have elapsed, remove the tubes from the heating block and vortex briefly.
2. Allow the aerosols to settle and open the lid of the tube.
3. Place the open heat-killed samples into the 2nd row of the instrument.
4. Place the corresponding labelled open empty tube in the front row (1st row).

Initiating the Run

1. The GenoXtract is loaded according to the touch screen instructions displayed on the instrument.
2. Switch on the GenoXtract with the “On-Off” switch at the back of the instrument and the “ON” button at the front.
3. Press [CONTINUE] on the initializing menu, after which the main menu is shown on screen.
4. Press [START NEW BATCH] to start a new run.
5. The [SELECT PROTOCOL] menu is opened.
6. Select a protocol from the list of all available protocols. To scroll up and down, use the arrow keys on the right.

7. Load the required number of cartridges for processing the selected protocol.
8. Confirm all steps by pressing [OK].
9. Close the door and press [START] on the "Confirm" menu.
10. When the protocol is completed, the touch screen confirms that the run was successful.
11. Close the screw-cap tubes, which now contains the eluate (DNA), with the corresponding lid.
12. Remove the closed tubes from the instrument and place them in the 1.5ml tube rack for further analysis.
13. Once the DNA has been safely stored, remove all waste from the instrument, and discard as per BSL3 guidelines.
14. Press [TURN OFF] in the "Main" menu and confirm with [YES] before you switch off the On-Off switch at the back of the instrument.

Maintenance of the GenoXract® instrument

1. Disconnect the machine from the power supply and close the door before cleaning the outside of the instrument.
2. Avoid getting the touch screen wet.
3. In order to clean the inside of the instrument; allow the heating block to cool down first.
4. After each run, remove the GenoXtract rack and magnet plate and clean the heating block and inside of the instrument with 70% alcohol.
5. Perform a UV decontamination process at the end of each day by pressing [START NEW BATCH] in the "Main" menu and select [UV DECONTAMINATION].
6. Clean the outside of the instrument with mild detergent on a monthly basis or as required.

QUALITY CONTROL

- Ensure that the heating block/water bath has reached 80°C prior to use, and record this.
- Ensure that the equipment such as the pipettes and microfuge are calibrated.
- If using a heating block, ensure that the wells of the heating block allow for conical 1.5 ml microfuge tubes.
- If the tubes do not fit the wells correctly, heat loss will occur which will result in the ineffective lysis and killing of the bacteria contained within the specimen, which in turn will result in improper DNA extraction resulting in a low yield of bacterial DNA. This applies to the GenoXtract® instrument as well.
- Include an H37Rv positive extraction control at the end of each batch of specimens. See description of the positive internal control in the Hain Line Probe Assay procedures (P & Q).
- Include a GenoLyse® reagent control which will serve as the "blank" control after the H37Rv positive control for each batch as well.

DNA EXTRACT RETAINMENT PERIOD (CCTR Laboratory)

All extracted *M. tb* DNA samples will be stored at CCTR laboratory for a maximum of 5 years after trial closure at -80C^{o}, as these will be used for potential future validations of new assays.

LIMITATIONS

1. Cross-contamination may occur when concentration is disturbed.

2. The incorrect centrifugal speed and/or incorrect incubation temperature will result in unsuccessful DNA extraction due to improper cell lysis. Use of this DNA extraction method is limited to qualified personnel that are well trained in the procedure and familiar with molecular biological methods.
3. The DNA yield may vary if the volume of the patient material is not 700 µl when using the GenoXtract® system.
4. The pellet may loosen from the wall of the Eppendorf tube, which may result in the pipetting of undesirable material, which in turn affects the DNA quality and quantity, which may influence the amplification thereof.
5. The pellet may loosen from the wall of the Eppendorf tube, which may result in the pipetting of undesirable material, which in turn affects the DNA quality and quantity, which may influence the amplification thereof.

REFERENCES

Refer to the Manufacturer's instructions; Hain GenoLyse ® Version 1. Instructions for Use (IFU) on http://www.hain-lifescience.de/en/downloads/microbiology.html, IFU code: IFU-51610-09.

O. GENOTYPE® MTBDRplus ASSAY

PURPOSE

Molecular identification of *M tuberculosis* complex and detection of susceptibility/resistance to rifampicin and isoniazid in sputum specimens. For study NC005 study, this procedure is performed with a specimen collected in the screening stage (Days -9 to -3). If an indeterminate result is obtained, the test must be repeated with newly extracted DNA from the same specimen or another specimen. Thus, a second screening specimen will have to be requested. A conclusive result must be obtained in order for the patient to be enrolled in the study. Results must be reported to the study staff within 48 hours of receipt of the sputum specimen.

PRINCIPLE

The identification of rifampicin resistance is enabled by the detection of the most significant mutations of the *rpo*B gene. For isoniazid resistance testing, the *kat*G and *inhA* genes are examined. The procedure consists of 3 steps: DNA extraction from resuspended sputum sediments, a multiplex amplification with biotinylated primers, and a reverse hybridization. See the DNA Extraction for Hain LPAs and *pncA* Sequencing procedure (P) for these instructions.

SPECIAL NOTE

Refer to package insert supplied with the kit for full details of procedure.

PROCEDURE

1. Refer to the Manufacturer’s instructions; Hain MTBDR*plus* Version 2.
2. Instructions for Use on http://www.hain-lifescience.de/en/downloads/microbiology.html, code IFU-304A-01.

Reporting of Study Results

Results are recorded on the Mycobacteriology Results Report Form or directly into the laboratory database. Refer to Section F for more details.

Select only one result option for *M. tuberculosis* complex:
Detected, Not detected, or Indeterminate

Select only one option for the Isoniazid result:
Susceptible, Resistant, or Indeterminate

Select only one option for the Rifampin result:
Susceptible, Resistant, or Indeterminate

Quality Control

Internal quality control tests for the conjugate reagent (CC) and PCR amplification (AC) are included on the Hain MTBDR*plus* test strip. Expected results must be obtained for the test to be valid. In addition, an internal control consisting of a suspension of pan-susceptible *M. tuberculosis* H37Rv and/or an MDR clinical *M. tuberculosis* isolate must be run with each new lot number and weekly with a batch of patient specimens.

These must consistently give the expected results for *M. tuberculosis* and isoniazid and rifampicin to ensure the quality of patient test results.

P. GENOTYPE® MTBDRsl ASSAY

PURPOSE

Molecular identification of *M. tuberculosis* complex and detection of resistance to fluoroquinolones (e.g. ofloxacin and moxifloxacin), aminoglycosides/cyclic peptides (e.g. kanamycin, amikacin, capreomycin, and viomycin), and ethambutol in sputum specimens. For the NC-005 study, the MTBDR*sl* test will be used only with specimens from suspected MTB TB patients resistant to rifampicin obtained from either GeneXpert or MTBDR*plus* results. Screening specimens (Day -9 to -3) are first tested with the MTBDR*plus* assay and then tested with the MTBDR*sl* assay if results show resistance to isoniazid and rifampin. The same DNA sample used for the MTBDR*plus* will be used for the MTBDR*sl*. Only the result for the fluoroquinolones is reported. If an indeterminate result is obtained, the test must be repeated with newly extracted DNA from the same specimen or another specimen. Thus, a second screening specimen will have to be requested. A conclusive result must be obtained in order for the patient to be enrolled in the study. Results must be reported to the study staff within 48 hours of receipt of the sputum specimen.

PRINCIPLE

The identification of resistance to fluoroquinolones is enabled by the detection of the most significant mutations of the *gyrA* gene (coding for the A sub-unit of the DNA gyrase). For detection of resistance to aminoglycosides/cyclic peptides, the 16S rRNA gene (*rrs*) and for detection of resistance to ethambutol the *embB* gene (which, together with the genes *embA* and *embC*, codes for the arabinosyl transferase) are examined. The procedure consists of three steps: DNA extraction from resuspended sputum sediments, a multiplex amplification with biotinylated primers, and a reverse hybridization. See the DNA Extraction for Hain LPAs and *pncA* Sequencing procedure (P) for these instructions.

SPECIAL NOTE

Refer to package insert supplied with the kit for full details of procedure.

PROCEDURE

a. Refer to the Manufacturer's instructions; Hain MTBDR*sl* Version 1.
b. Instructions for Use on http://www.hain-lifescience.de/en/downloads/microbiology.html, code IFU-317-03.

Reporting of Study Results

Results are recorded on the Mycobacteriology Results Report Form or directly into the laboratory database. Refer to Section F for more details.

Select only one result option for *M. tuberculosis* complex:
Detected, Not detected, or Indeterminate

Select only one option for the fluoroquinolone result:
Susceptible, Resistant, or Indeterminate

Quality Control

Internal quality control tests for the conjugate reagent (CC) and PCR amplification (AC) are included on the Hain MTBDR*sl* test strip. Expected results must be obtained for the test to be valid. In addition, an internal control consisting of a suspension of pan-susceptible *M. tuberculosis* H37Rv and/or an MDR clinical *M. tuberculosis* isolate must be run with each new lot number and weekly with a batch of patient specimens. These must consistently give the expected results for *M. tuberculosis* and isoniazid and rifampicin to ensure the quality of patient test results.

Q. MGIT CULTURE

PURPOSE

To amplify the number of *Mycobacterium tuberculosis* bacteria in a specimen using a liquid culture media (MGIT) and to detect positive specimens rapidly. To make a semi-quantitative assessment of the bacterial load in the sputum by determining the time for the MGIT instrument to signal positive. This is referred to as the time-to-positive MGIT culture (MGIT TTP).

For the NC-005 study, the MGIT instrument result along with the ZN, BAP (blood agar plate) and culture confirmation (MPT64 antigen test) results are used to determine the presence of viable *M. tb* in the sputum. This is done for all positive MGIT cultures of overnight collected sputum specimens. Positive MGIT cultures of spot sputum specimens will be tested only with presumptive ZN and BAP tests; however, MPT64 identification will be performed if the corresponding overnight MGIT culture is negative or not done and the MGIT culture is ZN positive.

For the NC-005 study, the MGIT TTP is a primary endpoint thus when the sputum specimen is of low volume (e.g., 1 ml) a 1 ml aliquot of DTT-digested sputum is removed for processing and preparing inoculum for the MGIT culture (Sputum Processing for Decontamination – M) before proceeding to the Quantitative Sputum Culture (U). This ensures there is sufficient sputum for the MGIT culture.

PRINCIPLE

A fluorescent compound is embedded in silicone on the bottom of 16 x 100 mm round bottom tubes. The fluorescent compound is sensitive to the presence of oxygen dissolved in the broth. Initially, the large amount of dissolved oxygen quenches emissions from the compound and little fluorescence can be detected. Later, actively respiring micro-organisms consume the oxygen and allow the fluorescence to be detected.

Tubes entered into the BACTEC MGIT 960 system are continuously incubated at 37°C and monitored every 60 minutes for increasing fluorescence. Analysis of the fluorescence is used to determine if the tube is instrument positive, i.e. the test sample contains viable organisms. An instrument-positive tube contains approximately 10^5 to 10^6 colony-forming units per millilitre (CFU/ml). Culture tubes which remain negative for a minimum of 42 days and show no visible signs of positivity are removed from the instrument as negatives.

Sputum specimens are processed (i.e. digested and decontaminated) and inoculated into 7 ml MGIT tubes, which are supplemented with OADC and PANTA (cocktail of antibiotics). Contaminating organisms are inhibited by the PANTA.

BACTEC MGIT 960 – Instrument Overview:

A single BACTEC MGIT 960 is capable of monitoring a total of 960 MGIT tubes. The tubes are arranged in three drawers; A, B and C, each of which holds up to 320 tubes that 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 indicate 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. The manual should be stored with the MGIT system. The operator 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 (and an optional audible alarm) and are displayed on the LCD screen. When positive tubes are identified, the operator removes them from the instrument for confirmation of results and for isolation and detection of the organism.

Definition of a BACTEC MGIT 960 Growth Unit:
The Growth Unit (GU) 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. If the GU reaches or exceeds the cut-off value of 75 units, the instrument flags this as a positive. This is identified as a True Positive. Subsequently, further tests are required to confirm positivity (i.e. ZN staining and contamination checks). If the MGIT flags the tube as positive (at least 5 readings must occur before a positive reading is recorded) and records a GU of "0" or higher before 5 hours, then this is a Threshold (T) Positive, meaning that the growth has exploded past the 75 cut-off and has occurred very rapidly. If represented graphically, this growth curve would be very steep, whereas the regular positive curve would normally be a gradual curve up to and beyond the 75 cut-off. If explosive growth has occurred, the software records a "T" in the growth column. Explosive growth usually means that the MGIT tube is contaminated and the contents of these tubes are likely to show contaminating organisms by ZN smear or growth on blood agar or both. The GU is not an indication of biomass within the tube. However, normally at positivity the biomass is approximately 105 to 106 CFU/ml. An "Instrument Positive" tube is flagged as a positive via internal algorithms when it reaches a GU of at least 75. There is no direct correlation of biomass and GU at the time of instrument positivity.

PROCEDURE

Materials

Biological safety cabinet (BSC), Class II
Mycobacterial disinfectant
Splash-proof waste container
Benchguard
Paper towels
Gloves

Biohazard bags
Digested and decontaminated sediments (re-suspended pellets from L)
Sterile 7 ml MGIT tubes
MGIT PANTA
MGIT Growth Supplement
Micropipette
Sterile filtered pipette tips
Sterile disposable pipets
Sterile serological pipets (1 ml, 20 ml)
Pipet aid

Procedure

Inoculating Tubes:

1. Prepare the antibiotic supplement for the MGIT tubes by reconstituting MGIT PANTA with 15 ml of MGIT Growth Supplement. This mixture is stable for 5 days if stored at 2°-8°C.
2. Label the appropriate number of MGIT tubes with the patient ID number, specimen accession number, and date MGIT culture inoculated on the side of the tube.
3. Once dissolved, add 0.8 ml of the liquid antibiotic supplement to each tube using a sterile pipette, taking care not to contaminate the tubes and supplement.
    a. Vortex the resuspended pellet and add 0.5 ml to the MGIT tube using a sterile disposable pipet.
    b. Tightly recap the MGIT tube and mix well.
    c. Wipe the MGIT tube with a paper towel soaked in disinfectant before removing it from the BSC.

Tube Entry: – always scan the MGIT barcode first; the machine will assign the stations.

Function	Instructions
1. Accession	1. Open the desired drawer. 2. Press the <**tube entry**> soft key. 3. Place the tube in the alignment block in front of the scanner with the barcode label facing the scanner. Rotate the tube if necessary. The instrument will beep once to indicate that the tube was scanned. 4. If the tube's label is damaged, use a spare barcode label. 5. The assigned station is shown in the main body of the display (along with the scanned sequence number). In addition, the station LED of the assigned station for that tube will illuminate GREEN. 6. Carefully and completely place the tube in the appropriate position. 7. Repeat steps 1-6 for each tube. 8. When finished, close the drawer or press <**exit**> to continue with the next task.

Notes:

1. Do not turn tubes after placing them in the station.
2. Do not remove tubes unless they are positive or out-of-protocol negatives (negative at 42 days).
3. Do not re-assign to a new station.

Dealing with Positive Tubes:

The instrument positive signal is a + sign and illuminates red. This indicates that 1 or more positive tubes are in the drawer. The signal remains on until all positive tubes are removed through the following 'Remove Positive Tubes Operation'.

All positive tubes should be worked up as soon as possible.

Function	**Instructions**
1. Remove positives	1. Press <**silence alarm**> to mute the audible alarm (if alarm is activated). 2. Open the drawer where the RED positive light is lit. 3. Press the <**remove positive tubes**> soft key. 4. All positive tubes will be indicated by flashing GREEN and RED lights. 5. Remove one tube at a time. 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 GREEN and RED lights 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, placing them in sequential order in the rack. 9. When all positive tubes have been removed, 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.
2. Print Unloaded Positive Tubes Report	1. Press <**print report**> soft key and select <**unloaded positive tubes**> soft key. 2. When report finishes printing, compare unloaded tubes with report. Resolve any discrepancies. If tubes match report, press the <**ok**> soft key. **When you press the <ok> soft key, the information contained in the report is removed from the database.**

Notes:

1. The MGIT instrument will record the date the tube was flagged positive and the number of days and hours taken to reach positivity (TTP).
2. "Unloaded Positive Tube" reports must be kept with the corresponding MGIT culture worksheet.
3. Tubes should be examined visually for growth. *M. tb* growth appears granular/particulate while contaminating bacteria produce turbid growth. If such growth is seen record on the MGIT worksheet.

4. All instrument positive tubes must be stained for AFB (ZN stain) and sub-cultured to blood agar plate (or alternative suitable medium) upon removal from the instrument.
5. In the event of a broken tube in the instrument, close the drawer and turn off the instrument. Vacate the room and follow the laboratory's SOP for a handling a spill.

Working-up positive MGIT tubes:

Manipulations with positive MGIT cultures must be performed inside the biosafety cabinet (BSC) using BSL3 procedures and practices and appropriate PPE.

1. On the laboratory's MGIT culture worksheet record the specimen accession numbers and other pertinent information for the MGIT tubes that are to be worked up.
2. Vortex MGIT tube to thoroughly mix the culture broth. Remove about 200 µl of broth (~4 drops in disposable pipet) and inoculate a blood agar plate with 2 drops and place 2 drops on a slide (AFB Microscopy - J). If multipe tubes are being subcultured, blood agar plates can be divided into 4 quadrants so 4 MGIT tubes can be sub-cultured onto 1 plate.
3. Incubate BAP at 35°C for 72 hours. If a 35°C incubator is not available, use one set at 37°C.
4. Stain smears with ZN and examine for the presence of AFB. Note description of AFB on the MGIT worksheet, i.e. typical, or atypical morphology and if cording is seen.
5. Re-enter the tube into the instrument within 5 hours of removal to ensure original culture results are retained in the instrument database.
6. Once ZN and BAP results are obtained, follow the algorithms detailed on the MGIT Flow Charts 0 to 3 (Appendices 5-8) for working up the cultures. For the overnight collected specimens, final results are based on these outcomes and reporting terminology is specified on the flow charts. Note that there is no provision for reporting non-tuberculous mycobacteria (NTM), thus catergorize them as contaminants, i.e. report "contaminated".
7. For the spot collected specimens, final results are based on the presumptive ZN and BAP test results according to the following table.

MGIT Instrument	**Zehl Neelsen smear**	**Blood agar plate**	**Identification: MTB complex**	**Interpretation and Study Reporting**
Positive	Positive	Negative	Not done	Positive - AFB Growth
Positive	Positive	Positive	Not done	Positive - AFB growth and contaminated
Positive	Negative	Positive	Not done	Contaminated

8. If the MPT64 antigen ID test is going to be performed, wait for 48-72 hours before removing the tube to perform test. This additional incubation time is recommended to increase the sentitivity of the MPT64 antigen ID test. Follow instructions in the MPT64 Antigen Identification Test SOP (R).
9. For the overnight collected specimens, if AFB are present and there is no evidence of contamination, sub-culture the MGIT tube to a LJ slope following the Preparation of *M. tb* Isolates on LJ procedure (V). This slope is for short-term storage of the isolate and may be the source of the isolate submitted to the CCTR laboratory for MIC testing (Addendum 1).
10. If MGIT DST is to be performed (Day -2 specimen), follow the procedure in the MGIT DST SOP (S).
11. All MGIT tubes with positive growth should be kept until the end of the study.

12. If AFB are present on the ZN smear, the BAP has growth (contaminated), and MGIT DST is required (Day -2 or later time point), there are three options for obtaining pure growth for DST. These are listed in order of preference.
    a. Pure growth from another positive MGIT culture collected at a similar time interval (Days -1 or 1 for Day -2).
    b. MGIT culture can be decontaminated and inoculated into a new MGIT tube.
    c. The original sediment, if kept in a refrigerator, can be decontaminated again and another MGIT culture set up. Follow the laboratory's usual procedure for re-treating with NaOH.
13. Note source of growth for DST must be indicated on the laboratory DST worksheet.
14. If the instrument signals at < 7 days, AFBs are not seen on ZN smear, and the BAP has growth, reincubate the tube to allow enough time for the *M. tb*, if present, to grow and be detected by the instrument. Detecting *M. tb* in MGIT cultures is an important result in the analysis of study endpoints, thus the tubes are incubated an additional 14 days to ensure enough time for *M. tb* to grow. For the same reason, the MPT64 antigen ID test is performed on AFB positive and BAP positive (contaminated) cultures, i.e. it is important to document the presence of *M. tb* even in the presence of contaminants.

Dealing with Negative Tubes:

Tubes that are not indicated as positive remain in the instrument until the end of the 42 day incubation period. When no growth is detected after 42 days, the negative tube indicator, marked with a – sign, illuminates green. The indicator remains lit until all negative tubes are removed through the following 'Remove Negative Tubes' operation.

Note the instrument continues to read the tubes if not removed after 42 days. Thus the incubation time on the "Unloaded Negative Report" recorded as TIP (time in protocol) will be higher than 42 days, which the instrument records as "Length of Protocol". For negative culutures unloaded on time, TIP is 42 days. Report as No Growth (NG) detected after 42 days. Do not report TIP of more than 42 days. Also, if the tube is not removed at the completion of the protocol, the instrument may detect growth. This becomes problematic if the growth is *M. tb* as this occurred out-of-protocol. If *M. tb* is detected beyond 42 days, contact Kathy Eisenach or Sam Ogwang for guidance on how to report this result.

FUNCTION	**INSTRUCTIONS**
1. Remove negatives – batch (removal of *all* final negative cultures from the instrument without having to scan the individual tube barcodes)	1. Open drawer where the GREEN light is on. 2. Press <**remove negative tubes**> soft key. 3. Press <**remove negative-batch**> soft key. The barcode scanner turns off, and tubes cannot be scanned. 4. Stations with negative tubes light up with flashing GREEN lights. 5. Remove all tubes in the flashing green stations ***prior*** to closing the drawer. If negative tubes remain in the drawer after it is closed, the instrument will register these tubes as newly entered tubes. 6. Start at front of drawer and remove in sequential order placing in same order in rack. 7. When all negatives are removed, press the <**ok**> soft key. 8. When all negatives have been removed, the instrument beeps 3 times, the GREEN drawer indicator light extinguishes, the barcode scanner turns off, and the <**ok**> icon appears on the display screen. 9. Close the drawer gently or press <**exit**> to continue with the next task. Repeat the steps for other drawers indicating negative tubes.
2. Remove negatives – single tube (each negative tube removed must be scanned by the barcode scanner)	1. Follow steps 1-2, as above. 2. Stations with negative tubes light up with flashing GREEN lights and the barcode scanner turns on. 3. As each negative tube is removed, the barcode label should be scanned prior to removing the next tube. The instrument will beep, indicating that the correct tube was scanned. The LED at the empty station extinguishes. 4. Place the tube in a rack in the sequential order of removal. 5. Continue to remove desired tubes and scan their barcode labels. 6. Follow steps 7-9 above.
3. Print Unloaded Negative Tubes Report	1. Press <**print report**> soft key and select <**unloaded negative tubes**> soft key. 2. When report finishes printing, compare unloaded tubes with report. Resolve any discrepancies. If tubes match report, press the

	<**ok**> soft key. When you press the <**ok**> soft key, the information contained in the report is removed from the database.

Notes:

1. Visualy check all instrument negative tubes.
2. If the tube appears visually positive (i.e. non-homogenous turbidity, small grains or clumps),

a. Prepare a smear and do Ziehl-Neelsen staining to determine whether AFB are present.
b. If AFB are present, sub-culture to BAP and follow the algorithms. If culture has *M. tb*, consult Kathy Eisenach or Sam Ogwang for guidance on reporting.

3. If the tube shows no signs of positivity, it may be autoclaved and discarded.
4. “Unloaded Negative Tube” reports must be kept with the corresponding MGIT culture worksheets.

Reporting of Study Results

MGIT culture results are recorded on the Mycobacteriology Results Report Form or directly into the laboratory database. Refer to Section F for more details.

Select only one option for the final result.

For overnight collected sputum specimens or when spot sputum must be used to confirm identification:
- Negative for *M. tuberculosis* complex
- Positive for *M. tuberculosis* complex
- Positive for *M. tuberculosis* and contaminated
- Contaminated

For spot collected sputum specimens:
- No growth
- Positive for AFB growth
- Positive for AFB growth and contaminated
- Contaminated

Report the time-to-positive (TTP) MGIT culture in days and hours only for “positive for *M. tuberculosis* complex” and “Positive for AFB growth”.

Quality Control

New Media and Supplements:
Each new lot number of MGIT tubes, growth supplement, and PANTA must be tested for its ability to support the growth of *M. tb*. In addition the TTP should be evaluated by testing strains with different growth rates. Although it is not critical for TB clinical trials that the MGIT medium supports the growth of other mycobacteria, it is important to test the instrument for consistency in the time it takes to detect positive cultures. This can be achieved either by inoculating a range of *M. tb* concentrations or three mycobacterial strains that differ in their growth rates.

Frequency:
Upon receipt of a new batch number of MGIT tubes, growth supplement, and PANTA

Controls:

M tuberculosis H37Rv ATCC27294
M. kansasii ATCC 12478*
M. fortuitum ATCC 6841*
*Clinical isolates or strains from proficiency test panels may be used instead of ATCC reference strains if these are not available.

Procedure:

1. Test 1 tube from each new batch number and test the supplement along with the tubes.
2. Prepare a fresh LJ culture of each of the three strains indicated above.
3. Adjust the suspension to turbidity equal to that of 0.5 McFarland standard using standard techniques.
4. Dilute the 0.5 McFarland suspension as follows to obtain a 1:500 dilution:
    a. Add 1 ml of the suspension to 4 ml of sterile saline – Dilution 1 (1:5).
    b. Add 1 ml of Dilution 1 to 4 ml of sterile saline – Dilution 2 (1:50).
    c. Add 1 ml of Dilution 2 to 4 ml of sterile saline – Dilution 3 (1:500)
    d. Add 1 ml of Dilution 3 to 4 ml of sterile saline – Dilution 4 (1:5000)
5. Prepare the MGIT tubes following the normal MGIT culture procedure.
6. Label three tubes with the date, lot number, expiry date and strain designation.
7. Inoculate the *M. tb* MGIT tube with 0.5 ml of Dilution 3.
8. Inoculate the *M. kansasii* MGIT tube with 0.5 ml of Dilution 4.
9. Inoculate the *M. f*ortuitum MGIT tube with 0.5 ml of Dilution 5.
10. Place the three tubes in the instrument following the ‘Tube Entry’ steps.
11. Unload tubes when positive following the ‘Remove Positive Tubes’ operation.
12. Obtain results from the “Unloaded Positive Report” form.

Acceptance Criteria:

1. The medium must support growth of the *M. tb* H37Rv ATCC 27294 reference strain and the other two mycobacterial strains.
2. The TTP of the MGIT cultures of these strains must be in the following ranges:
    M. tuberculosis – 6 to 10 days
    M. kansasii – 7 to 11 days
    M. fortuitum – 1 to 3 days

Documentation:

1. Record results on the laboratory’s QC form.
2. If the expected results are not obtained follow the laboratory’s procedure for addressing the problem and taking corrective action.

Internal Quality Controls

As described in the Quality Control section of the Sputum Processing for Decontamination (M), positive and negative internal controls are processed in a run with patient specimens. The re-suspended sediments of these controls are inoculated into MGIT tubes. Thus, these serve as internal quality controls for the MGIT culture procedure. See the pertinent details in K.

Monitoring of Contamination Rate

The contamination rate of MGIT culture is a Quality Indicator. An acceptable contamination rate for MGIT culture is 8-10% according to the manufacturer. However, some laboratories have consistently higher rates (12%-15%), which are acceptable if they have attempted to reduce contamination by addressing all related processes and procedures and the rate does not go above their established rate. Note that there are no published contamination rates for culture of overnight collected sputa or specimens collected after treatment has been initiated.

For the NC005 study, regular monitoring of MGIT culture contamination will be done to ensure the rate of contamination is not higher than the laboratory's established rate and there is not a significant loss of culture results due to contamination. A MGIT culture contamination spreadsheet must be filled in and submitted bi-weekly to Dr. Stephen Murray, Dr. Christo van Niekerk and Sam Ogwang. The MGIT Culture Contamination Excel spreadsheet is included in the Appendices (Appendix 9).

References

1. Siddiqi SH and S. Rusch-Gerdes. 2006. MGIT Procedure Manual, FIND Diagnostics.
2. BACTEC MGIT 960 User's Manual. Document Number MA-0117. 2004. Becton, Dickinson and Co., Sparks, MD.

R. MPT64 ANTIGEN TEST FOR CULTURE CONFIRMATION

PURPOSE

To rapidly (< 1 hour) and accurately detect *Mycobacterium tuberculosis* in AFB-positive MGIT and 7H11S/7H11S+C cultures. To differentiate *M. tb* from non-tuberculous mycobacteria (NTM). Only CE marked test kits will be used, e.g. BD TBc ID, SD Bioline, and KAT. Culture confirmation will be performed at every visit day from all AFB-positive MGIT cultures of overnight collected sputum specimens. 7H11S or 7H11S+C will be used only when colonies are suspicious of not being *M. tb* or when the corresponding MGIT culture is not AFB positive. If the agar plates do not have AFB positive growth, the MGIT culture of the corresponding spot collected sputum should be used to identify *M. tb* complex, providing this MGIT culture is instrument positive and ZN positive.

PRINCIPLE

These commercial tests are lateral flow, immunochromatographic assays which detect the MPT64 antigen, a mycobacterial protein that is specifically secreted from *M. tb* while growing in culture. When a bacterial suspension is added to the test device, the MPT64 antigen binds to anti-MPT64 antibodies conjugated to colloidal gold particles present on the test strip, forming an antigen-antibody complex. This antigen-antibody complex then migrates across the test strip to the reaction area, where it is captured by a second specific MPT64 antibody fixed to the membrane. If MPT64 antigen is present in the sample, a color reaction is produced by the labeled colloidal gold particles and is visualized as a pink (or purple) to red line. An internal positive control is included to validate proper test performance. The test will detect the following species of *M. tb* complex: *M. tuberculosis, M. bovis, M. africanum*, and *M. microti*. These tests have been shown to be highly sensitive and specific in a number of studies conducted in clinical settings.

PROCEDURE

Materials

TBc ID Test , SD Bioline, or KAT
Extraction buffer (commercially available or in-house prepared)
    TBc ID extraction buffer (KH2PO4 , NaCl , Tween 80)
Sterile saline
Clean cylinder
Flask or bottle with screw cap
Analytical balance
Weigh boats
Distilled water
Vortex mixer
Timer
200 µL micropipette
200 µL sterile aerosol resistant tips
Sterile 2 mL cryovials
10 µL sterile disposable loops
Waste receptacle with biohazard bag and tuberculocidal disinfectant
Permanent marker

Preparation of TBc ID Extraction Buffer:

Buffer formula:
KH_2PO_4 - 0.01 M
NaCl - 0.145 M
Tween 80 - 0.01%

To prepare 1 L of buffer:

1. Using weigh boat and analytical balance, weigh out 1.36 g of KH_2PO_4.
2. Weigh out 8.5 g of NaCl.
3. Dissolve powders in 500 mL distilled water in a volumetric flask.
4. Using a micropipette, add 100 µL of Tween 80; pipette up-and-down repeatedly to dislodge all Tween material.
5. Mix thoroughly and bring to a final volume of 1 L with distilled water, using a cylinder.
6. Aliquot buffer into smaller volumes, e.g. 250 ml, before autoclaving. Label containers with the buffer name, date prepared, expiry date, batch lot number, and initials of preparer.
7. Sterilize for 15 minutes at 121°C, 15 psi.
8. Store buffer at 2 to 8°C for up to 6 months.
9. Before each use, check buffer visually for signs of contamination or degradation, and bring to room temperature.

Sample preparation and inoculation of MPT64 antigen test device must be performed in a BSC using full PPE.

Sample Preparation:

1. From AFB-positive MGIT tubes:
   a. Test AFB smear-positive MGIT tubes within 2 to 5 days of instrument signaling positive.
   b. Vortex the tightly capped MGIT tube for 30 seconds to ensure the suspension is well- mixed.
2. From AFB-positive 7H11S and 7H11S+C plates:
   a. Test 2 to 4 week old growth.
   b. Add 200 µL of TBc ID extraction buffer, kit buffer, or sterile saline to a sterile cryovial, depending on the kit instructions.
   c. Using a sterile 10 μL loop, scrape a loopful of several colonies and mix with buffer/saline, avoiding any solid medium and/or contaminants present.
   d. Vortex the cryovial for 30 seconds to create a uniform suspension.
   e. Be sure the suspension turbidity is adjusted to approximately 0.5 McFarland. Insufficient density in the suspension can lead to false negative results.

Inoculation of Test Device:

1. If devices are refrigerated, bring to room temperature in the foil pouch prior to testing.
2. Place the device on a flat surface inside the BSC. Remove the rapid ID device from its foil pouch immediately before testing.
3. Label one device for each specimen to be tested with the specimen accession number.
4. Place 100 µL of sample, either MGIT culture or bacterial suspension from agar plate, into the specimen well of the test device. Change pipette tips between specimens.
5. Start timer for 15 minutes.

6. Examine the reading area of the test device after 15 minutes following the interpretation described below. Do not interpret test after 60 minutes.

Notes: The MPT64 test kit should be stored at 2°-30°C (preferably at 2°-8°C). Direct sunlight, excessive humidity, and high temperatures should be avoided. Foil pouches containing devices should not be opened until test is to be performed. Avoid touching the specimen well on the device with your hands.

Interpretation of Results:

1. Results from all kits are interpreted in the same manner.
   Positive: Pink/purple to red lines form on the reading areas labeled Test [T] and Control [C] of the device.
   Negative: A pink/purple to red line forms on the reading area labeled [C] of the device, but not [T].
   Invalid: If no line is observed on the reading area labeled [C], technical errors or product damage has occurred. In this case, the test should be considered invalid and repeated using a new device.
2. Record results on the laboratory's MGIT culture worksheet or the MPT64 antigen test worksheet.

NOTES

1. If the MPT64 Antigen test is negative, re-incubate the MGIT tube and repeat after 48 hours.
2. It is preferable to test pure cultures without contamination, although observations show that slight contamination with bacteria does not interfere with the test. If test is negative:
   - For MGIT cultures, decontaminate the culture and repeat testing.
   - For LJ cultures, attempt to sub-culture a few colonies well separated from the contaminants and repeat testing.
     Grossly contaminated cultures may cause interference; interpret with caution.
3. *Staphylococcus aureus* is known to produce protein A, which may interfere and/or cause false positive results.
4. A negative test does not always rule out *M. tb*, as some strains have mutations in the MPT64 antigen gene.
5. If *M. tb* is highly suspected, and test results continue to be negative, refer to additional testing algorithms (in Appendices) using a molecular assay (HAIN GenoType Mycobacterium CM or GenoType MTBDR*plus*).

Quality Control

A positive and a negative control must be tested with each new lot or new shipment of kits received and with each new batch of extraction buffer prepared. The positive control should be a positive MGIT culture which was inoculated with a suspension of H37Rv or the positive sputum processing control. The negative control should be a positive MGIT culture which was inoculated with a suspension of *M. kansasii*. Similarly, these controls mus be run weekly, or along with each batch of patient isolates, when tests are set up less frequently.

S. MGIT DRUG SUSCEPTIBILITY TESTING (DST)

PURPOSE

To determine susceptibility to streptomycin, isoniazid, rifampicin, ethambutol and pyrazinamide at critical concentrations using pure cultures of *Mycobacterium tuberculosis* and the BACTEC MGIT 960 System.

For the NC-005 study, drug susceptibility testing (DST) will be performed with positive cultures from a Baseline specimen (Day -2 or Day -1). Additionally, positive cultures from patients fulfilling the following criteria will be tested: 1) the last positive sputum from withdrawn subjects who have not converted to culture negative status, 2) subjects who are still culture positive at 8 weeks, and 3) the first positive sample after conversion to culture negative status for subjects who have relapsed.

PRINCIPLE

Drug susceptibility testing in the MGIT system is based on the same principles as isolation of mycobacteria from sputum. DST is performrd using an AST (antibiotic sususceptibility testing) set, which consists of a Growth Control tube and 1 tube for each drug, as well as a bar-coded tube carrier that holds the set. A known concentration of drug and a suspension of *M. tb* are added to a MGIT tube, and growth in the drug-containing tube is compared with the growth in the control tube containing the same inoculum. If the *M. tb* is susceptible, growth will be inhibited and fluorescence suppressed in the drug-containing tube; while the *M. tb* will grow in the drug-free control and show increasing fluorescence. If the *M. tb* is resistant, growth will occur in both the drug-free and drug-containing tubes, each will show increasing fluorescence. The MGIT instrument continually monitors tubes for increased fluorescence and automatically interprets results using predefined algorithms. *M. tb* is defined as being resistant if 1% or more of the test population grows in the presence of the critical concentration of the drug.

The first-line drugs available for the BACTEC MGIT DST are streptomycin, isoniazid, rifampin, ethambutol, and pyrazinamide (SIREZ). The BACTEC MGIT SIRE test is a 4 - 13-day protocol. The MGIT PZA kit is a 4 - 21-day protocol. For the MGIT PZA test, PZA medium must be used. This is a modified Middlebrook 7H9 broth with a reduced pH of 5.9, which is required for PZA activity.

PROCEDURE

Materials

Biological safety cabinet (BSC), Class II
Mycobactericidal disinfectant
Splash-proof waste container
Benchguard
Paper towels
Gloves
Biohazard bags
MGIT tubes and SIRE Growth Supplement
Test tube rack
MGIT SIRE Kit
MGIT PZA kit
PZA tubes and PZA Growth Supplement
AST Carrier sets, 5-tube and 2-tube

Sterile saline

Sterile distilled or deionized water
16.5 x 128 mm sterile tubes with screw cap
Sterile glass beads, 4 mm (if preparing inoculum from solid media)
7H9 broth (if preparing inoculum from solid media)
Sterile disposable loop or applicator stick (if preparing inoculum from solid medium)
Micropipettor
Aerosol-resistant (filtered) pipette tips
Sterile serological pipets (5 ml, 10 ml)
MGIT tubes with pure cultures of *M. tb* (O)

Procedure

Manipulations with positive MGIT cultures must be performed inside the biosafety cabinet (BSC) using BSL3 procedures and practices and appropriate PPE.

Preparation of Inoculum from Positive MGIT tube:

Preparing the test inoculum is a critical step in ensuring accuracy of the drug susceptibility test results. Once a positive culture is sampled for the ZN smear and BAP sub-culture, it must be returned immediately to the MGIT instrument for additional incubation and the time of incubation tracked. A positive MGIT tube should be used the day after it first becomes positive on the MGIT instrument (Day 1), up to and including the fifth day (Day 5) after instrument positivity. A tube which has been positive longer than 5 days must be sub-cultured to a fresh MGIT tube, incubated in the MGIT instrument until positive, and used from 1 - 5 days following positivity; see ‘Preparation of Inoculum from Seed Culture’.

1. Check the ZN and BAP results to be sure the culture is pure (MGIT CULTURE - Q).
2. If the tube is a Day 1 or Day 2 positive, proceed to ‘Drug-Containing Tube lnoculation’.
3. If the tube is a Day 3, Day 4, or Day 5 positive, dilute 1 ml of the positive broth in 4 ml sterile saline (1:5 dilution). Use the diluted suspension for the inoculation procedure. Proceed to ‘Drug-Containing Tube Inoculation’.

Preparation of Inoculum from Solid Media:

1. Add 4 ml of BBL Middlebrook 7H9 Broth to a 16.5 x 128 mm sterile tube with screw cap, containing 8-10 glass beads.
2. Use a sterile loop to scrape 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. The suspension should exceed in turbidity that of a 1 McFarland standard.
3. Vortex the suspension for 2-3 minute to break up the larger clumps.
4. Let the suspension sit for 20 minutes without disturbing.
5. Transfer the supernatant fluid to another 16.5 x 128 mm sterile tube with screw cap (avoid transferring any of the sediment) and let sit for another 15 minutes.
6. Transfer the supernatant fluid (it should be smooth, free of any clumps) to a third 16.5 x 128 mm sterile tube.
7. Adjust the suspension to a 0.5 McFarland standard by visual comparison to a 0.5 McFarland turbidity standard.

8. Dilute 1 ml of the adjusted suspension in 4 ml of sterile saline (1:5 dilution). Proceed to 'Drug-Containing Tube Inoculation'.

Preparation of Inoculum from Seed Culture:

1. If there is a delay in setting up the DST, the MGIT tubes should be stored at room temperature; do not re-incubate once the work-up (ZN, BAP, and MPT64 Ag ID) is complete.
2. Sub-culture to another MGIT tube or solid medium when ready to perform DST. This is referred to as the seed culture.
3. If using MGIT tubes, growth should be positive within 7-12 days and follow 'Preparation from a Positive MGIT Tube'. If positive sooner, the bacterial density is too high.
4. If using LJ or 7H11, fresh growth on solid medium should be used to prepare inoculum for DST; follow 'Preparation of the Isolate from Solid Media' above.

Preparation of Drug-Containing Tubes:

1. Reconstitute the drugs (in the BACTEC MGIT 960 SIRE and PZA kits) with 4 ml of sterile distilled/deionized water following manufacturer's instructions. The concentrations of the drug solutions are listed in the table below. Once re-constituted, the drug solutions may be frozen at -20^{o}C or -80^{o}C for up to 6 months, but not exceed the expiration date. Once thawed they must be used immediately. Discard any unused drug solution (do not re-freeze).
2. For SIRE, label 5 MGlT tubes for each test. Label these as GC (Growth Control), STR, INH, RIF and EMB.
3. For PZA, label 2 PZA tubes for each test. Label these as GC (Growth Control) and PZA.
4. Place the tubes in the correct sequence (i.e. GC, S, I, R, E) in the appropriate size AST set carrier, 5-tube carrier for SIRE and 2-tube carrier for PZA (i.e. GC, Z).
5. For SIRE, aseptically add 0.8 ml of BACTEC MGlT SIRE Supplement to each tube. Note use the correct supplement.
6. For PZA, aseptically add 0.8 ml of BACTEC MGlT PZA Supplement to each tube. Note use PZA tubes and PZA supplement as the pH of the medium is lower.
7. Aseptically pipette 100 µl of each drug into the respective tube tube. The final drug concentrations in the tubes are listed in the table below. No drug is added to the GC tubes.

Drug	**Concentration of Drug after Reconstitution***	**Volume Added to MGIT Tubes for Test**	**Final Concentration in MGIT Tubes**
MGIT STR	83 µg/ml	100 µl	1.0 µg/ml
MGIT INH	8.3 µg/ml	100 µl	0.1 µg/ml
MGIT RIF	83 µg/ml	100 µl	1.0 µg/ml
MGIT EMB	415 µg/ml	100 µl	5.0 µg/ml
MGIT PZA	8000 µg/ml	100 µl	100 µg/ml

* These drugs must be reconstituted using 4 ml sterile distilled/deionised water to achieve concentrations indicated.

SIRE Growth Control Tube Preparation and Inoculation:

1. Mix MGIT tube well and let stand for 10 minutes to settle.
2. Aseptically pipet 0.1 ml of the organism suspension into 10 ml of sterile saline to prepare the 1:100 Growth Control suspension.
3. Mix the Growth Control suspension thoroughly.

4. Using a sterile pipet place 0.5 ml of the 1:100 Growth Control suspension into the MGIT tube labeled GC. Mix well by gentle inversion of the tubes.

PZA Growth Control Tube Preparation and Inoculation:

NOTE: It is important to use a 1:10 diltion for the Growth Control tube when testing PZA in order to obtain an accurate result and avoid growth errors.

1. Mix MGIT tube well and let stand for 10 minutes to settle.
2. Aseptically pipet 1 ml of the organism suspension into 9 ml of sterile saline to prepare the 1:10 Growth Control suspension (alternatively, 0.5 ml organism suspension in 4.5 ml sterile saline).
3. Mix the Growth Control suspension thoroughly.
4. Using a sterile pipet place 0.5 ml of the 1:10 Growth Control suspension into the MGIT tube labeled GC. Mix well by gentle inversion of the tubes.

Drug-containing Tube Inoculation:

1. Aseptically pipette 0.5 ml of the organism suspension into each of the drug-containing tubes (STR, INH, RIF, EMB, and PZA).
2. Tightly recap the tubes and mix well by gentle inversion of the tubes.

Entering and Removing AST Carrier Sets:

1. Enter the AST set into the BACTEC MGIT 960 using the AST set entry feature. 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.
2. Open the desired MGIT drawer and follow the instructions below.

Function	**Directions**
1. Entering new AST sets	1. Press the <**tube entry**> soft key. 2. Scan the AST carrier's barcode label. The carrier barcode indicates that what you are entering is an AST set, how many tubes are in the set, and the set's sequence number. For PZA testing, select PZA as the drug in the 2-tube carrier definition as it is a longer protocol. 3. Scan the accession barcode if available; if not present, press the <**accession barcode not available**> soft key. 4. The display shows the default carrier set. Check that the AST set definition is displayed correctly for the AST set currently being entered. 5. The station LEDs of all the assigned stations for the set illuminate GREEN. Insert the tube set into the indicated stations, ensuring that the Growth Control tube is located in the leftmost indicated station. Make sure that all the tubes AND the carriers are fully seated in the drawer. 6. Repeat Steps 1 – 5 for each of the AST sets you want to enter. 7. When finished, close the drawer and wait a moment while the drawer performs a "quick scan" of drawer

	contents. If you have misplaced any AST sets, the quick scan can detect this and put the affected set into error status. Correct any errors before the MGIT 960 begins testing.
2. Removing completed AST sets (indicated by a red "**+**" on the drawer)	1. Open the desired drawer. Press the <**remove completed AST sets**> soft key. 2. The first completed AST set stations illuminate with FLASHING GREEN indicators. Remove the carrier, starting with the completed set closest to the front of the drawer, and scan its barcode label. The LEDs at this station extinguish. 3. Repeat steps 1 – 2 to remove additional AST sets. 4. Place completed AST sets in the AST tube rack.
3. Printing an "Unloaded AST Set" report	1. Press the <**printer**> soft key to access report selection. 2. Press <**unloaded AST sets report**> soft key. 3. Match the AST sets with the printed report, and resolve any discrepancies.

The 'Unloaded AST Report' form must be kept with the respective MGIT DST worksheet.

Interpretation of 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. When the growth unit (GU) of the growth control reaches 400 within 4-13 days (SIRE) or 4-21 days (PZA), the GU values of the drug-containing vials are evaluated.

S = Susceptible = the GU of the drug tube is less than 100
R = Resistant = the GU of the drug tube is 100 or more

Invalid tests are reported when certain conditions occur that may affect test results. X ### = Error or Indeterminate results; reported when certain conditions occur that may affect the test. If possible, determine the cause of error. In any case, repeat testing following the flow charts in Appendices. Alternatively, if a positive MGIT culture from a subsequent visit day is available (e.g. Day -1), this culture can be used.

X200 = System cannot detect sufficient indication of growth in the Growth Control tube in the specified protocol time, and does not provide an interpretation of the AST set results. Often a result of too little inoculum, non-viable organisms, or a slow growing drug-resistant strain. Refer to MGIT Flow Chart 4 – Invalid X200 Errors from MGIT DST (Appendix 10) for further instructions.

X400 = System detects indications of possible contaminated or overinoculated tube, and does not provide an interpretation of the AST set results. Check the tube for turbidity and sub-culture to a blood agar plate to rule out contamination of the specimen. Refer to MGIT Flow Chart 5 – DST Workup for Invalid X400 Errors from MGIT (Appendix 11) for further instructions.

Other conditions, such as power failure. Repeat DST using a seed culture from the original positive MGIT culture if there is power failure.

Observe all 'resistant' tubes visually for evidence of contamination when first removed from theinstrument.

Perform a ZN stain and sub-culture to a BAP on any suspicious tube to ensure that the growth is not due to contaminants or NTM.

Reporting of Study Results

Results for each drug tested are either recorded on the Mycobacteriology Results Report Form or into the laboratory database. Refer to Section F for more details.

The critical concentration tested must be recorded as well.

Select one option for each drug listed on the form:

- Susceptible, Resistant, Indeterminate, Not Done

If result is Indeterminate, indicate the reason by recording the approriate code:
Control tube failure, X200 error, X400 error, or Quality Control error

Quality Control

The validity of the drug susceptibility results is evaluated with each new lot of SIRE kit and PZA kit and monitored weekly by testing a drug-susceptible control strain with a batch of patient samples.

Frequency:

Upon receipt of a new lot number of BACTEC MGIT 960 SIRE kit or PZA kit
Once a week

Controls:

M. tuberculosis H37Rv ATCC27294
M. tuberculosis clinical isolate with known drug resistance (for testing new drug kits)

Procedure:

1. The control strain inoculum can be prepared from growth on solid media as described above or by inoculating a MGIT tube with a 1:500 dilution of a 0.5 McFarland suspension as described in Quality Control in MGIT Culture – O. However, the preferrable MGIT culture is one inoculated with the positive sputum processing control, if the timing of this culture coincides with the DST batch testing.
2. The quality control (QC) AST set must be prepared in the same manner as the patient samples.
3. Upon completion of the test, print the ‘Unloaded AST Report’ form and obtain results from this form.

Acceptance Criteria:

Drug-containing tubes – H37Rv is susceptible to each of the drugs; the drug resistant clinical isolate is resistant to the drugs to which it is known to be resistant
Drug-free control tube – the control strain/clinical isolate has GU $\geq$ 400

If the proper results are not observed, repeat the test. If, after repeating the test, the correct results are still not observed, report to the laboratory manager who will contact the manufacturer or supplier. Do not use the results obtained with a batch which failed QC or put into use a new drug kit which failed QC.

Documentation:

Record results from 'Unloaded AST Report' form for each drug on the laboratory' s QC forms.

References

1. Siddiqi SH and S Rusch-Gerdes. 2006. MGIT Procedure Manual, FIND Diagnostics.
2. BACTEC MGIT 960 User's Manual. Document Number MA-0117. 2004. Becton, Dickinson and Co., Sparks, MD.Barenfanger J. 1993. Making your laboratory safe against multi-drug resistant *Mycobacterium tuberculosis*. *Clin Microbiol Newsl* 15: 76-80.
3. Clinical and Laboratory Standards Institute. 2003. Approved Standard M24-A. Susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomycetes. CLSI, Wayne, Pa.

T. MEDIA PREPARATION

PURPOSE

To provide instructions on how to prepare Middlebrook 7H11S and 7H11S+ with 0.4% charcoal plates, Tween-saline, and 7H9 broth. The Middlebrook agar plates are used in the Quantitative Sputum Culture procedure (U). Tween-saline is used for diluting the Sputasol-treated sputum when setting up quantitative cultures. The use of 7H9 broth is described in multiple procedures, i.e. for preparing suspensions of *M. tb* to a specfic density for MGIT DST and MIC testing. Non-selective 7H11 medium can be prepared and used for growing pure cultures from liquid or solid media. Processing reagents used in preparing sediments from screening specimens are not described herein. The laboratory's routine procedures should be followed if not obtained commercially.

For the NC-005 study, the 7H11S+C medium will be used in conjunction with 7H11S. The charcoal (C) is added to absorb any residual bedaquiline in the sputum specimen. The antibiotics in the cocktail are added at twice the concentration (S+) in 7H11S to ensure there is drug activity to inhibit contaminant growth on the medium. The charcoal absorbs these drugs as well as the bedaquiline. The source of drugs for the cocktail can be either Selectatabs or in-house prepared antibiotic solutions.

PRINCIPLE

For both the liquid as well as solid media, glycerol and water is added to the powder and the medium is dissolved by heating or magnetic stirring. The media are sterilized by autoclaving. After autoclaving, media are cooled down and OADC is aseptically added. When selective agar medium is prepared, a cocktail of antibiotics is added at this stage. For Tween-saline, the NaCl is disolved in distilled/deionized water and autoclaved.

PROCEDURE

Materials

Distilled water
Glycerol
BBL 7H9 base
Middlebrook 7H11 base
Middlebrook OADC Enrichment
Selectatab (MAST)
Amphotericin B, Polymixin B, Trimethoprim lactate, Carbenicillin disodium salt - Sigma
    (if preparing antibiotic solutions)
Charcoal (Sigma # 05105)
Media flasks
Measuring cylinders
Magnetic stirrer
Magnets
25 ml disposable pipettes
Balance
Spatula
Weighing boat/scoop

Water bath
90 mm Petri dishes (single or 2-compartment)
Laminar flow cabinet
Bunsen flame
70% Ethanol
Pipetter

Procedures

When preparing antibiotic solutions and dispensing sterile media into plates and flasks work inside a laminar flow cabinet or biosafety cabinet.

Preparation of Antibiotic Stock Solutions for use in 7H11S Medium:

1. Calculate the volume of diluent and quantity of lyophilized antibiotic necessary to make stock solutions at the concentrations listed below:
    a. Amphotericin 10 mg/ml
    b. Carbenicillin 50 mg/ml
    c. Polymixin B 200,000 U/ml
    d. Trimethoprim 20 mg/ml
    2. Diluent may be added directly to the vial in which the antibiotic was shipped, in case measuring of the specific quantities of antibiotic might lead to error.
    3. Sterile distilled water may be used as the diluent for all of the antibiotics above. Ensure that the antibiotic is thoroughly dissolved before aliquoting.
    4. Sterilize all antibiotic stock solutions by membrane (0.22 µm pore size) filtration, unless otherwise recommended by the manufacturer.
    5. Place 1.2 ml aliquots of solubilized, sterile antibiotics into cryovials pre-labeled with antibiotic name, concentration (including units), date frozen, and expiration date.
    6. Stocks are stable for 6 months when frozen at -20°C; 1 year at -70°C.
    Once thawed, DO NOT re-freeze drugs. Thawed drugs can be kept in tightly capped cryo vials in the refrigerator at 2°-8°C for use for up to 1 week. Thereafter, discard all the unused aliquots.

Examples for preparing concentrated antibiotic stock solutions:

Be sure to verify the purity and potency for each lot number received.

1. Sigma Amphotericin B solubilized, catalog # A9528 - 100 mg/bottle
2. For *this* catalog number, solubilized Amphotericin B is ~45% amphotericin, ~35% sodium desoxycholate, and the balance is sodium phosphate.
    - Add 4.5 ml of sterile distilled water to the entire vial for a final stock concentration of 10 mg/ml.
3. Sigma Carbenicillin disodium salt, catalog # C1389 - 250 mg/bottle
    - Assume the potency of the lot number received is 814 ug/mg *(Potency varies by lot number)*
    - Add 4.07 ml of sterile distilled water to the entire vial for a final stock concentration of 50 mg/ml.
4. Sigma Polymixin B, catalog # P1004 - 5,000,000 units/bottle
    - For *this* catalog number, Polymixin B is in units/mg and vial contains 5,000,000 units.
    - Add 5 ml sterile distilled water to the entire vial for a stock concentration of 1,000,000 units/ml.
    - Dilute stock 1:5 in sterile distilled water for a final stock concentration of 200,000 U/ml.
5. Sigma Trimethoprim lactate, catalog # T0667 - 250 mg/bottle
    - Assume the potency of the lot number received is 900 ug/ml *(Potency varies by lot number)*.

- Add 12.38 ml sterile distilled water to the entire vial for a final stock concentration of 20 mg/ml.

Mycobacteria 7H11S Agar:

1. Suspend 19 g of the 7H11 powder in 900 ml of distilled water, containing 5 ml glycerol.
2. Add a magnet to the medium and stir to mix thoroughly.
3. Autoclave at 121°C for 15 minutes 10 minutes.
4. Place in water bath and cool down to 50°-55°C.
5. Bring OADC enrichment and Selectatab/antibiotic solutions to room temperature.
6. Aseptically add 100 ml of OADC.
7. If non-selective medium is being prepared, proceed to step 10.
8. When preparing selective medium with Selectatab, aseptically add 2 tablets (1 tablet per 500 ml medium).
9. When preparing with antibiotic stock solutions, use a micropipettor to add the appropriate volume of antibiotics as shown in the table below for a flask of 1L of selective medium.

Drug	**Concentration of Stock Solution**	**Volume of Stock Solution Added**	**Concentration Drug in Medium**
Amphotericin	10 mg/ml	0.5 ml	10 µg/ml
Carbenicillin	50 mg/ml	0.5 ml	50 µg/ml
Polymixin B	200000 U/ml	0.5 ml	200 units/ml
Trimethoprim	20 mg/ml	0.5 ml	20 µg/ml

10. Mix very well by magnetic stirring.
11. Work inside a laminar flow cabinet or BSC. Use a pipetting aid and 25 ml sterile, disposable pipets to dispense into Petri dishes – 20 ml per single 90 mm Petri dish or 10 ml per half of a 90 mm 2-compartment Petri dish.
12. Allow to stand undisturbed until properly set.
13. Seal in clean plastic bags.
14. Store in the dark at 2°-8°C for no longer than 4 weeks.

Mycobacteria 7H11S+C Agar:

1. Suspend 19 g of the 7H11 powder in 900 ml of distilled water, containing 5 ml glycerol.
2. Add 4 g of charcoal.
3. Add a magnet to the medium and stir to mix thoroughly.
4. Autoclave at 121°C for 15 minutes.
5. Place in water bath and cool down to 50°-55°C.
6. Bring OADC enrichment and Selectatab/antibiotic solutions to room temperature.
7. Aseptically add 100 ml of OADC.
8. Aseptically add 4 Selectatabs.
9. When preparing with antibiotic stock solutions, use a micropipettor to add the appropriate volume of antibiotics as shown in the table below for a flask of 1L of selective medium.

Drug	**Concentration of Stock solution**	**Volume of Stock Solution Added**	**Concentration of Drug in Medium**
Amphotericin B	10mg/ml	1 ml	20 µg/ml
Carbenicillin	50 mg/ml	1 ml	100 µg/ml

Polymixin B	200000 U/ml	1 ml	400 units/ml
Trimethoprim	20 mg/ml	1 ml	40 µg/ml

10. Mix very well by magnetic stirring.
11. Work inside a laminar flow cabinet or BSC. Use a pipetting aid and 25 ml sterile, disposable pipets to dispense into Petri dishes – 20 ml per single 90 mm Petri dish or 10 ml per half of a 90 mm 2-compartment Petri dish.
12. Allow to stand undisturbed until properly set.
13. Seal in clean plastic bags.
14. Store in the dark at 2°-8°C for no longer than 4 weeks.

Middlebrook 7H9 Broth:

1. Suspend 4.7 g of the 7H9 powder in 900 ml of distilled water, containing 2 ml glycerol or 0.5 g Tween 80.
2. Add a magnet to the medium and mix stir thoroughly.
3. Autoclave at 121°C for 15 minutes.
4. Cool down to 45°C.
5. Aseptically add 100 ml of Middlebrook OADC enrichment and mix very well by magnetic stirring.
6. If smaller volumes are desired, aseptically dispense in sterile bottles/flasks (e.g. 250 ml, 500 ml).

Saline-Tween Diluent:

Use commercially prepared 0.85% saline or prepare saline as follows:

NOTE: ***Be sure all glassware used in diluent preparations is absolutely clean and free of any reagent residue!***

Saline solution:
1. Weigh out 8.5 mg NaCl.
2. Dissolve completely in 1000 ml distilled water.

Saline-Tween 80 solution:
1. Add 2.5 ml Tween 80 to 997.5 ml 0.85% saline.
2. Rinse pipette several times to dislodge all Tween material.
3. Mix thoroughly.
4. Aliquot into 250 ml volumes, or alternatively use a 5 ml serological pipette and aliquot 4.5 ml of saline-Tween solution into small sterile capped tubes or vials. Label the containers with the reagent name, date of preparation, and date of expiration.
5. Sterilize by autoclaving for 15 minutes at 121°C, 15 psi.
6. Bring to room temperature before using to make dilutions.
7. If smaller volumes are desired, aseptically dispense in sterile bottles/flasks (e.g., 250 ml, 500 ml).
8. Store at 2°-8°C for up to 3 months.

Quality Control

7H9 Broth

Each batch of 7H9 broth must be tested for sterility and performance. Sterility check entails selecting 1-3% of the total volume prepared, incubating tubes at 37°C for 5-7 days, and checking visually for growth in tubes. Acceptable results are no growth in any of tubes. If any of the tubes are contaminated, investigate to resolve the problem, and prepare a new batch of media. Growth performance is tested using same number of tubes. For example, 2% of 1000 ml is 4, 5 ml tubes. Prepare 10^{-2} and 10^{-4} dilutions of a 0.5 MacFarland density suspension of H37Rv (same as MGIT QC, dilutions 2 and 4) and inoculate 0.1 ml of each dilution into each of 2 tubes. Incubate until growth is visable. Growth in all tubes must be consistent with the appearance of *M. tb* (granular, settled to bottom of tube). If any of the tubes do not grow M. tb, investigate to solve the problem, and prepare a new batch of media. Record results of the sterility and growth performance testing on the laboratory's Reagent/Media QC form.

Saline

Each batch of saline must be tested for sterility. Sterility check entails selecting 1-3% of the total volume prepared, incubating tubes at 37°C for 5-7 days, and checking visually for growth in tubes. Acceptable results are no growth in any of tubes. If any of the tubes are contaminated, investigate to resolve the problem, and prepare a new batch of saline. Record results of the sterility test on the laboratory's Reagent/Media QC form.

7H11S and 7H11S+C

Each batch of 7H11S and 7H11S+C media prepared in-house or purchased from a supplier is tested to ensure that the quality is of an acceptable standard. This entails checking sterility and growth performance of each new lot number of media purchased or each new batch of prepared media and each new lot number of Selectatab and antibiotic. The selective characteristics of the Mycobacteria 7H11 agar media is tested by comparing the growth of a stock *E coli* solution on selective media with non-selective media. The use of a positive control once a week with a processing batch monitors growth performance of the media as described in Internal Quality Control in Quantitative Sputum Culture (U).

Frequency:

Selectatab – on every new lot number of Selectatab or antibiotics received
Middlebrook medium – with every new batch of agar prepared or purchased

Controls:

Positive control – *M smegmatis* ATCC 14468
Negative control – Blank plate

Procedure:

For Selectatab:

1. Prepare a biplate with Middlebrook 7H11 in 1 half and selective Middlebrook 7H11 in the other.
2. Label the non-selective side of the plate as 'Non-selective' and the selective side of the plate with the batch number of the Selectatab tested.
3. Keep at 2°-4°C until needed.
4. Prepare a stock concentration of *E coli* as follows:

   a. From 24-hour growth on a blood agar plate, prepare a suspension to approximately No 1 McFarland standard in 20 ml of Mueller Hinton or other suitable broth.
   b. Freeze 20 x 1 ml aliquots in labelled cryovials at -80°C.
5. One day before inoculating selective Middlebrook 7H11 plates for selectivity QC streak 1 drop of thawed *E coli* stock suspension onto a blood agar plate and incubate overnight at 37°C.
6. On the day of inoculation for selectivity QC, prepare a slightly turbid suspension of *E coli* in sterile distilled water, using colonies picked from the blood agar plate inoculated the previous day.
7. Adjust turbidity to a 0.5 McFarland standard.
8. Dilute 100 fold by adding 10 µl to 1.0 ml of sterile distilled water. Mix well.
9. Repeat step 8, resulting in a 1 x 10^{-4} CFU/ml concentration for the final inoculum.
10. Place 50 µl of the dilution on each side of the selective / non-selective Middlebrook 7H11 bi-plate and streak for isolation.
11. Incubate at 37°C for 4 days.
12. Count the number of colonies on both sides of the bi-plate.
13. Calculate the percentage inhibition, by dividing the number of colonies on the selective side of the plate by the number of colonies on the non-selective side. Multiply this number by 100 and subtract the result from 100 to obtain the percentage inhibition.
14. If the acceptance criteria are not met, review all the specimen results that were obtained from the plates made with the same batch of Selectatab.

For Middlebrook Agar:

1. Record the batch number and expiry date of the agar, OADC and Selectatab used in the media preparation event.
2. After agar plates are made up and dried, pack plates in plastic bags and seal. Label the bags and incubate at 37°C for 24 hours.
3. Remove the plates from the incubator and check for contamination. Discard contaminated plates.
4. Remove 1 plate from the batch of prepared media for QC.
5. Store the rest of the plates at 2°-8°C until use; no longer than 4 weeks.
6. Prepare a fresh culture of *M smegmatis* ATCC 14468.
7. Adjust the suspension to turbidity equal to that of 0.5 McFarland standard.
8. Label the plate with the test date, date of preparation and mark as ‘Control’.
9. Inoculate 100 µl of the reference strain onto 1 half of the plate and leave the other half blank.
10. Incubate at 37°C for 48 hours before reading the results.
11. Inoculate 100 µl of the stock solution onto 1 half of the plate.
12. Spread the inoculum evenly with an L-shaped spreader over the surface of the agar.
13. Leave the other half without inoculum – negative control.
14. Incubate the plates at 37°C for 21 days.
15. Record growth of the reference strain and whether growth/contamination was observed on the negative control.

Acceptance Criteria:

Selectatab – the percentage inhibition must be higher than 80%.
There must be no contamination after overnight incubation.
The media must support growth of the *M smegmatis* ATCC 14468 reference strain.
There must be no growth/contamination on the negative control.

Documentation:

Record QC results on laboratory’s Media/Reagent QC form.

U. QUANTITATIVE SPUTUM CULTURE ON 7H11S and 7H11S+C

PURPOSE

To quantify the number of culture-viable *M. tuberculosis* contained in a sputum specimen. Sputum is collected during a 16-hour time period and pooled as one sample that is used to obtain an accurate assessment of the number of *M. tb* in the patient's lungs. This specimen is referred to as "Overnight Sputum". Quantification is expressed as a colony-forming unit (CFU) number per milliliter.

In the NC-005 study, supervised sputum will also be collected in the clinic and subjected to the same quantitative culture procedure. This specimen is referred to as "Coached spot sputum". The purpose is to compare the spot sputum with the overnight sputum for the quantitative assessment of *M. tb*.

In the NC-005 study, quantitative sputum cultures will be used to evaluate the change in number of *M. tb* CFU during the course of 8 weeks treatment in each study arm. Quantitative cultures will be performed on sputum specimens collected pre-treatment (Day -2, Day -1), on day treatment is initiated (Day 1), Days 3 and 7, and weekly thereafter (Days 14, 21, 28, 35, 42, 49, and 56).

If the sputum volume is less than 2 ml, less than 4 ml of DTT-digested sputum will be available for Quantitative Sputum Culture. To optimize MGIT culture results, digested specimens are handled according to the Flow Chart: Microbiology of Day -2, Day -1 and Treatment Coached Spot and Overnight Collected Sputa (E). Additional details are provided below in the procedure.

PRINCIPLE

The pooled sputum specimen is homogenized and digested using Dithiothreitol (DTT, Sputasol). Sodium hydroxide (NaOH), which is normally used to decontaminate sputum specimens, kills a significant number of organisms and is therefore not used in this procedure.
If sufficent volume allows, sputum specimens may be homogenized with a magnetic stirring bar and magnetic stirrer/mechanical shaker prior to adding Sputasol. This step is optional. If the pre-homogenization step is not used, the Sputasol is added directly to the specimen and mixed on a mechanical shaker.

Following DTT/Sputasol digestion, the specimen is serially diluted in Tween-saline and plated on two types of Middlebrook agar media: 7H11S and 7H11S+ with 0.4% charcoal (7H11S+C). Final concentrations of drugs in the selective (S) medium are the following.

200 units/ml polymixin B
10 μg/ml amphotericin B
50 μg/ml carbenicillin
20 μg/ml trimethoprim

The final concentrations of drugs in the S+ medium are twice that of the S medium. These are as follows.

400 units/ml polymixin B
20 μg/ml amphotericin B
100 μg/ml carbenicillin
40 μg/ml trimethoprim

In NC-005 the 3 experimental arms include bedaquiline. Due to the long half-life of bedaquiline there is a possibility that it will be in the sputum collected for culture. If present in the sputum and not diluted out, bedaquiline could inhibit the *M. tb* growth on the 7H11S agar. Thus, sputum specimens will be also inoculated on 7H11S+ with 0.4% charcoal. The charcoal binds to the bedaquiline making it inactive, i.e. not available to inhibit *M. tb* growth on the medium. The charcoal also will bind to the antibiotics in the selective cocktail, hence the double concentration of these drugs to maintain selective properties in inhibiting contaminant growth. One possible limitation of the 7H11S+C medium is the charcoal will bind some of the components in the medium resulting in competition for growth nutrients when a high number of *M. tb* are inoculated on the medium. If this were to happen, the CFU number would be inaccurate, i.e. number of colonies on the medium would be lower than what is in the sputum. Comparison of colony counts on 7H11S+C with those on 7H11S will enable monitoring for this possible occurence.

During collection of pooled samples, sputum must be refrigerated or kept on ice (in cooler box with ice packs) at bedside to prevent over-growth of contaminants and maintain an accurate viable count. Pooled and coached spot specimens must be refrigerated or kept in a cooler box with ice packs once collection is completed and during transport until the specimens reach the testing laboratory.

MATERIALS

Biological safety cabinet (BSC), Class II
Incubator, CO_2 incubator preferred
Vortex mixer
Platform rocker/shaker
Magnetic stirrer
Magnetic stirring bars
Timer
Disinfectant at sufficient concentration to kill *M. tb*
Waste receptacles (including a splash-proof container for liquids)
Benchguard
Paper towels
Biohazard bags
Dithiothreitol (Sputasol; Oxoid, UK)
Sterile glass beads, 4 mm (optional)
Sterile Tween-saline
Sterile capped-tubes with appropriately-sized racks for making dilutions (e.g., 13 x 100 mm tubes or 16 x 125 mm tubes)
Sterile, disposable, single-use, screw-capped, conical centrifuge tubes (50 ml)
Rack for 50 ml conical tubes
Micropipettors (20-100 ul, 200-1000 ul)
Micropipettor tips (aerosol resistant)
Autopipettor for serological pipets
Sterile serological disposable pipets, 1, 5, 10, or 20 ml
L-shaped or T-shaped sterile disposable spreaders
Gas permeable tape [3M #483 CL25 (Uline S-10239C) or 3M #1530-1 (3M Health Care)] or gas permeable sterile plastic bags
7H11S and 7H11S+C agar plates
PPE: gown, gloves, respirator, and shoe covers

FORMS

Sputum Processing Worksheet (lab's own worksheet)
Quantitative Culture Worksheet for 7H11S (Appendix 12)
Quantitative Culture Worksheet for 7H11S+C (Appendix 13)
Quantitative Culture Contamination Spreadsheet (Appendix 14)

PROCEDURE

Processing of sputum specimens, preparation of dilutions, and inoculation of plates must be done inside the biosafety cabinet using BSL3 procedures and practices and appropriate PPE. Reading of plates may be done outside the BSC as long as the plates are sealed with tape or remain in sealed plastic bags. Opening plates for observation, counting, or photographing must be done inside BSC.

Preparation of Tween-saline, 7H11S and 7H11S+C are described in a separate procedure (T).

Sputum Processing

1. For specimen registration, follow the login procedure as described in the laboratory's SOP manual.
2. Record all specimens processed in a batch using the laboratory's Sputum Processing Worksheet. Be sure to record the technician/technologist processing each batch.
3. Prepare the biological safety cabinet according to the laboratory's normal operating procedures.
4. Remove 7H11S and 7H11S+C agar plates from the refrigerator and allow to come to room temperature.
5. Remove the sputum specimen from the refrigerator and allow warming to room temperature. If not already in a sterile, 50 ml conical tube, label such tube with the subject ID number, visit interval, date, and lab accession number. Carefully decant the sputum from the wide-mouth container into the conical tube.

Pre-homogenization using mechanical stirring (optional step). If not used, proceed to step 7:

6. Homogenise the entire sputum specimen by adding a sterile 30 mm cylindrical magnet with pivot ring and stirring mechanically for 30 minutes. Other types of magnetic stirring bars can be used. A platform rocker/shaker can be used instead of stirring plate.
7. Estimate the total volume of the pooled sputum specimen according to the gradations on tube (preferably comparing to a 50 ml tube with gradations marked for every ml) or using a serological pipet, and record on the Quantitative Culture Worksheets, along with the subject ID number, visit interval, specimen accession number, and date of plating.
8. Prepare the Sputasol, (DTT; Oxoid, UK) at a 1:10 dilution (10%):
    - Aseptically add the contents of one Sputasol vial (7.5ml) to 92.5ml of sterile distilled water. Label the flask with the contents, date of preparation, and date of expiration.
    - Use the 10% DTT solution immediately or store at 2°-8°C for up to 48 hours only.
9. Add an equal volume of the 10% Sputasol solution to the specimen. Record the amount of Sputasol added to each tube on the Quantitative Culture Worksheet.
   Example: If the total sputum volume is 10 ml, add 10 ml of 10% DTT for a total volume of 20 ml and a final concentration of 5% DTT.
10. Vortex the specimen for 20 seconds and then incubate at room temperature for 20 minutes to digest. **Note: If sputum specimen was not mechanically homogenized, place on platform rocker to shake mechanically at 60-80 rpm for 15-20 minutes.** If the sputum appears not to be totally digested (i.e., not a homogenous suspension after shaking), add 5-10, 4mm sterile glass beads and shake mechanically another 10 minutes.
11. Since a portion of the digested sputum will be decontaminated and subsequently inoculated into MGIT medium, at this point refer to the Flow Chart: Microbiology of Day -2, Day -1 and Treatment Coached Spot and Overnight Collected Sputa (E) for guidance.

Preparation of Dilutions in Saline-Tween for Plating

1. Use commercially prepared or in-house prepared sterile Tween-saline.
2. Label 4 sterile test tubes with the numbers 1 through 4.
3. Place 4.5 ml of sterile Tween-saline into each tube or use tubes previously dispensed with 4.5 ml of diluent. Alternatively, place 9 ml of sterileTween-saline into each tube.
4. When using 4.5 ml diluent, add 500 µl of digested sputum (tube 0) to tube 1 using a 1 ml sterile serological pipet or a micropipettor and sterile aerosol-resistant tips. Rinse the pipet tip by repeatedly aspirating and expulsing the pipet. Cap the tube and vortex gently for 5-10 seconds to mix. When using 9 ml diluent, add 1 ml of digested sputum to tube 1 following the same instructions.
5. Changing the pipet tip, remove 500 µl from tube 1 and add it to tube 2 for the 4.5 ml diluent or 1 ml from tube 1 for the 9 ml diluent. Rinse the pipet tip by repeatedly aspirating and expulsing the pipet. Cap the tube and vortex gently for 5-10 seconds to mix.
6. Repeat step 5 for the remaining tubes. This procedure creates a set of serial dilutions of the digested sputum. *Note, for this study the* 10^{-5} *dilution is not prepared. If there is difficulty obtaining a colony count on a series of plates due to the lack of a -5 dilution, contact Kathy Eisenach or Sam Ogwang for guidance.*

Tube	Dilution
0	10^{-0}
1	10^{-1}
2	10^{-2}
3	10^{-3}
4	10^{-4}

Inoculation of Agar Media

Overnight and coached spot specimens at screening (Days -2 and –1) and soon after treatment is started (Days 1 and 3) will not be plated directly onto the plates, i.e. plating of undiluted sputum is omitted for these specific collection times. Sputum colony counts are generally high at these collection time points thus the undiluted plates are not useful, i.e. the plates have too much growth to count individual colonies.

If the digested sputum volume is 2 to 3.8 ml (original volume of sputum is 1 to 1.9 ml), you will have only about 1 to 2.8 ml to work with after removing 1 ml for MGIT culture. In such situations, use 0.5 ml to prepare first 1:10 dilution and plate 0.1 ml of undiluted, digested specimen onto each of 4 sides of 2 7H11S biplates (0.4 ml required). If there is any undiluted, digested specimen remaining use to inoculate 2 7H11S+C biplates (another 0.4 ml required). Indicate on Quantitative Culture Worksheet which plates were not inoculated, e.g., cross through boxes on worksheet where there are no plates for reading.

After pouring plates it is preferable to leave plates at room temperature overnight before placing in a refrigerator. Also, removing plates several hours before inoculating and allowing them to come to room temperature is preferable. These practices minimize the amount of moisture forming on the lid once incubated and inoculum on agar surface dries more quickly.

1. Label two 7H11S agar biplates and two 7H11S+C agar biplates with the respective dilutions, including two for the undiluted specimen; a total of 20 biplates. **For Days -2, -1, 1, and 3 the undiluted/neat specimen is not plated, thus there will be a total of 16 biplates for each of these time points.**

2. Due to the number of inoculations for each tube dilution, i.e. a total of 8 inoculations on 4 biplates, and the concern that the *M. tb* might settle in the tube and the inoculum might dry on the agar surface or adsorb into medium before spreading, each Tube will be inoculated onto 4 biplates and spread before proceeding to plate the next Tube.
3. One sterile aerosol-resistant tip and one spreader will be used per Tube. To avoid potential cross-contamination, start with the highest dilution and proceed to the lowest dilution or undiluted sputum (Tube 4 to Tube 0).
4. Starting with Tube 4 (highest dilution), mix gently by vortexing. Place 100 µl from the tube to each side of the 4 biplates using a micropipettor and sterile aerosol-resistant tip. **The order of inoculation should be: 1 biplate of 7H11S, 1 biplate of 7H11S+C, 1 biplate of 7H11S, and 1 biplate of 7H11S+C.** Dispose of the tip.
5. Spread each 100 µl inoculum with the same sterile spreader/hockey stick making sure not to spread it up to the edge of plate or middle division. Proceed quickly to avoid the inocula absorbing to the agar surface before spreading. Dispose of the spreader.
6. Proceed to Tube 3 and repeat steps 4 and 5.
7. For Days 7-56, mix the 50 ml tube (Tube 0) with the undiluted sputum by gently vortexing. Place 100 µl from the tube on each side of the biplates following the order specified in step 4, if there is sufficient sputum to inoculate all 4 plates. Eliminate 7H11S+C plates if there is not sufficient sputum volume. Repeat step 5.
8. Leave plates upright in BSC with lids on for about 30 minutes to allow inoculum to absorb. Then, invert plates and wrap with gas permeable tape or place in gas permeable bags. Tape is preferred over bags as it is easier to examine plates on weekly basis and minimizes contamination of plates. If bags are used, new bags must be used with each set of plate – DO NOT REUSE PLASTIC BAGS.
9. Place the plates in an incubator at 37°C. If using a CO_2 incubator, CO_2 concentration should be 5-10%.

Reading Plates and Counting Colonies

Recording colonies observed on each plate from the time colonies are visible until final reporting allows for monitoring quality. Specifically, following the progression of colony counts on plates of each dilution provides a means for: 1) monitoring growth rate and being assured the final count is accurate, b) examining serial dilutions to determine if the expected results for 10-fold dilutions are obtained, and c) obtaining colony counts in face of contamination should it occur.

1. Plates should be examined at Day 7 (for contamination) and weekly thereafter at Days 14, 21, 28, 35, and 42.
2. If a contaminant is present, attempt to count any mycobacterial colonies, even if they are small. Record the presence of contaminants on the Quantitative Culture Worksheet according to the dilution and day of incubation.
   Scenarios: When contaminants are present, a decision must be made as to whether or not the plate is reincubated. This is based on several factors: 1) the week of reading and if more incubation time is needed to obtain a final count, 2) if the contaminant is fungi and there is a possibility of overgrowth or the plate is already overgrown and you do not want to re-incubate, and 3) if *M. tb* growth is being inhibited by the presence of by contaminant growth thus the count is not going to be accurate.
   Action: 1) If the plate has incubated long enough to obtain a reliable count, discard plate, and report colony count and presence of contaminant(s) on Worksheet. 2 and 3) If plate is discarded before a final count can be obtained due to fungal growth or inhibition of *M. tb* growth, report as contaminated; do not report colony counts.
3. Colonies should first be counted when they are large enough to be seen (easily visible). In most cases

this will be on Days 14 or 21 for 7H11S and Days 28 or 35 for 7H11S+C, with growth on 7H11S+C being delayed. Some strains grow slowly, e.g. long exposure to anti-TB drugs or MDR, and will not have mature colonies until Days 35 and 42.

4. Mark the colonies counted on bottom of the 7H11S plates. If possible to see colonies through lid of 7H11S+C plates, making sure lid is in a fixed position with gas permeable tape, mark colonies counted on lid. If moisture in lid and unable to count colonies, follow one of the following alternatives, leaving lid off for shortest amount of time possible. One, remove tape, remove lid, use sterile gauze 2x2 to dry the lid inside, replace lid, retape and proceed to count marking colonies on lid. Alternatively, divide the plate into quadrants by marking on the lid and select a starting point for counting in each quadrant (mark these colonies on the lid – 4 reference points). Then remove tape and lid and count all the colonies in each quadrant (add colony counts from one side to obtain a total count for 1 side of biplate) holding the lid next to the plate or placing beside plate for orientation.
5. Record number of colonies for each side of the plate and each dilution having distinct colonies (even though small on initial read) on the respective day of reading on the corresponding Quantitative Culture Worksheets. Reincubate plates another week.
6. Each subsequent week of incubation, re-examine each of the plates with growth, mark the additional colonies observed, and add the number to the previous count. Record weekly the total number of colonies seen on all the plates with growth.
7. When majority of colonies are medium to large in size, growth is close to completion even if there are some small colonies. Refer to the counts on the Worksheets from the previous week. If the counts collectively are the same or close (within 10 colonies) to the previous counts, these are the final counts. If the counts collectively vary >10 colonies than the previous counts, continue to incubate, reading weekly, until the counts do not increase by more than 10 colonies.
8. Count colonies on all plates up to 200 colonies of *M. tb*. Plate may be divided and only a section counted providing the distribution of colonies is uniform across plate surface. If counting manually and more than 200 colonies are counted, record “TNTC”. If using an imager to visualize the plate and UVP software to assist in counting colonies, a count of 200-250 is accurate thus acceptable for reporting.
9. When selecting the counts for reporting, they must be taken from the plates of the same dilution. The dilution used for 7H11S may be the same or different from the dilution used for 7H11S+C. Ideally the reportable range is 50-150 colonies per side of biplate. However, it may be necessary to report as low as 20 and as high as 200. The higher the number the more likely the four counts will be consistent and provide an accurate number for calculating the CFU/ml.
10. Once counts have been recorded as final, all plates from this specimen can be discarded, except when the MGIT culture is contaminated and ID and DST are needed, then retain plates with growth.
11. A specimen is considered negative and plates are discarded if no growth is evident after 42 days of incubation on all the plates. If negative, record the result on the Quantitative Culture Worksheet (Appendix 12 and 13) in the Day 42 column.
12. If the colonies do not have classic *M. tb* morphology, check MGIT culture results for the same sputum specimen. It may be necessary to do a ZN stain on the colonies and if AFB positive tested with an identification method to confirm MTB Complex.

Photographic Documentation of Plates

Plates which are selected for final counts must be photographed with a digital camera. This provides a means for documenting the number of colonies on each plate as well as may facilitate counting, especially the charcoal containing media, if projected on the computer monitor or printed on regular paper. Organize the images in a file so that the examination of the images can be carried out in a logical manner, e.g., 7H11S biplates followed by 7H11S+C biplates; highest dilution to the lowest dilution.

1. Photograph each biplate individually. Perform inside BSC. If photographing plates inside the BSC is problematic, contact Kathy Eisenach or Sam Ogwang for guidance.
2. Remove the tape and lid. Minimize the time the medium is exposed (lid is off the plate).
3. If there is a question as to which dilution to select for counting, photograph both dilutions, e.g. the count on one set of biplates is high (~200) and the count on the next adjacent set of plates is too low (~20).
4. Attach copies of photos to respective Worksheets.

Study Data Reporting

1. Results are transferred from the 7H11S and 7H11S+C Quantitative Culture Worksheets (Appendices 12 and 13) to the Mycobacteriology Results Report Form (Appendix 1) or into the laboratory database. For each set of plates (7H11S and 7H11S+C) record the colony count for each side of the biplate, biplates are arbitrarily labeled A and B, and the sides are labeled 1 and 2. Thus, there are 4 colony counts reported for each media type, plate A, side 1 and 2; plate B, side 1 and 2. If using the plate reader and the whole plate is counted (not two sides), the count is recorded on side 1 for plate A and plate B. Also, record the dilution corresponding to the counts reported. If the plate is unreadable due to contamination, check this box. If there are no colonies on the plate, record 0.
2. The weekly CFU results on 7H11S and 7H11S+C with the H37Rv suspension must be monitored. When the CFU (average of 4 colony counts from one set of plates) for either media type varies more than +/- 0.5 $\log_{10}$, the laboratory manager should be consulted and guidance obtained from Kathy Eisenach or Sam Ogwang.

QUALITY CONTROL

7H11S and 7H11S+C Quality Control

Each batch of 7H11S and 7H11S+C media prepared in-house or purchased from a supplier is tested to ensure that the quality is of an acceptable standard. This entails checking sterility and growth performance of each new lot number of media purchased or each new batch of prepared media. Each new lot number of Selectatab and antibiotic is tested by comparing growth of a stock *E coli* suspension on selective with non-selective media. Procedures and criteria of acceptability are detailed in the SOP for preparing these media.

Internal Quality Control

To assess overall performance of the assay an internal control must be run at least weekly with either a sputum specimen or a batch of patient specimens. A suspension of a known amount of *M tuberculosis* H37Rv is serially diluted just as the patient specimens and plated on 2 7H11S bi-plates and 2 7H11S+C biplates. The suspension should consist of ~10^5 CFU/ml (1:500 dilution of bacterial suspension equivalent to 0.5 McFarland Standard) and can be prepared in bulk, frozen, and thawed for each use. Expected results (+/- 0.5 $\log_{10}$) should be obtained consistently. See Internal Quality Control in Sputum Processing for Decontamination for instructions how to make this positive control (M).

Quality Indicators

Serial 10-fold dilutions. Quality of the individual quantitative sputum culture is ensured by comparing colony counts of the neat sputum and serial dilutions. Expected results are serial 10-fold decreases starting from the neat sputum plates or the first countable dilution. This quality check can be performed by the technologist reading the plate when the final counting has been done and the plate counts (and corresponding dilution) are recorded on the worksheet. Alternatively this can be done by a reviewer (a second plate reader) who checks all colony counts recorded on the worksheet, verifies the appropriate set of plate counts (dilution) was selected, and re-counts 10% of plates with colonies. If time allows, this should be done when transcribing data from the Worksheets to the study report form. For the plate counts to be reported, a 10-fold decrease should be observed if colonies are observed on any of the dilution plates and the 4 counts from the biplates should be the same order of magnitude (within 0.5 $\log_{10}$). If this cannot be done in "real time", this should be carried out by the laboratory manager on a monthly basis.

Decrease in countable dilutions during treatment. An additional monitor of individual sputum culture is to compare results (countable dilution) with those from previous sputum cultures from the same study subject. As the *M. tb* load in the sputum decreases while the patient is on drug treatment, i.e. decrease in CFU from Day -2/Day -1 to later time points, the dilution with countable plates drops, e.g. 10^{-4} to neat/undiluted. There is no expected amount of decrease between collection time intervals. There are no acceptance criteria for this monitor, but one should question results if the colony counts are higher than counts from previous cultures (colony counts taken from higher dilution) .

Contamination rates of 7H11S and 7H11S+C. There are no acceptable contamination rates for quantitative sputum cultures. However, the amount of contamination and the loss of culture results due to overgrowth of contaminants are monitored by providing such data on the Quantitative Culture Contamination Spreadsheet (Appendix 14). The spreadsheet is a cumulative list of the number of contaminated quantitative cultures reported during a specified time period, e.g. every two weeks.

References

1. FA Sirgel, FJH Botha, DP Parkin, BW van de Wal, PR Donald, PK Clark, DA Mitchison. The early bactericidal activity of rifabutin in patients with pulmonary tuberculosis measured by sputum viable counts: a new method of drug assessment. *Journal of Antimicrobial Chemotherapy*, 1993; 32:867-875.
2. Sirgel FA, Venter A, Heilmann HD. Comparative *in vitro* activity of Bay y 3118, a new quinolone, and ciprofloxacin against *Mycobacterium tuberculosis* and *Mycobacterium avium* complex. *Journal of Antimicrobial Chemotherapy*, Feb 1995; 35(2):349-351.
3. Johnson J L, Hadad DJ, Boom WH, Daley CL, Peloquin CA, Eisenach KD, Jankus DD, Debanne SM, Charlebois ED, Maciel E, Palaci M, Dietze R. Early and extended early bactericidal activity of levofloxacin, gatifloxacin and moxifloxacin in pulmonary tuberculosis. *International Journal of Tuberculosis and Lung Disease*, 2006; 10 (6): 605-612.

V. Preparation, Storage, and Shipping of *M. tb* Isolates on selective 7H11 agar plates (7H11S)

PURPOSE

To prepare 7H11S agar plates for short-term storage of *M. tb* isolates recovered from a positive MGIT culture or 7H11S culture if a positive MGIT culture is not available. To provide instructions how to prepare the shipment of 7H11S agar plates to the CCTR laboratory.

PRINCIPLE

In this procedure pure cultures of *M. tb* are prepared for future testing, e.g. MIC determination. Plates are inoculated with broth from positive MGIT tubes that are ZN positive, BAP negative, and MPT64 antigen positive. Alternatively, several well-isolated colonies, confirmed as being *M. tb* by the MTB64 antigen test are picked from 7H11S biplates. Once a good amount of *M. tb* growth is obtained and there is no evidence of contaminant growth, these plates are stored in a cool, dark place.

MATERIALS

Biological safety cabinet (BSC), Class II
Incubator
Vortex mixer
Mycobactericidal disinfectant
Waste receptacles (including a splash-proof container for liquids)
Benchguard
Paper towels
Biohazard bags
Selective 7H11 agar plates (7H11S)
Sterile 1 ml disposable pipettes, single wrap
Sterile loops
Sterile Tween-saline
Sterile cryotubes
Parafilm
M. tb positive MGIT or 7H11S cultures
PPE: gown, gloves, respirator, and shoe covers

FORMS

TMTF NC005-004 Culture Isolate Transport Form (Appendix 15)
TMTF NC005-005 Culture Isolate Transport Log (Appendix 16)

PROCEDURE

Subculturing of MGIT tubes and 7H11S plates containing M. tb must be done inside the biosafety cabinet using BSL3 procedures and practices and appropriate PPE.

Labelling of 7H11S agar plates

1. Clearly label the 7H11S agar plates with the corresponding MGIT culture or 7H11S culture study label, which includes the information required to identify the isolate.

2. It is preferable to prepare cultures on the 7H11S agar off the positive MGIT or 7H11S culture. One agar plate can be retained at the laboratory while the other is shipped for MIC testing (if fewer than 3 months from time of subculture). Or having 2 agar plates may be useful if 1 becomes contaminated or 1 is needed for additional testing.

Sub-culturing from MGIT cultures

1. Match the MGIT culture and the 7H11S agar plate according to the study label.
2. Vortex the MGIT tube well and allow the aerosols to settle for a few seconds before removing the tube cap.
3. Using a sterile disposable 1 ml pipette, place 200 µl of MGIT broth onto the surface of the 7H11S medium.
4. Spread the inoculum over the centre surface of the plate with a 10 µl quadloop, or with a spreader.
5. Allow the plate to stand for 2 hours until dry.
6. Seal the plates in plastic bags (CO_2 permeable if a CO_2 incubator is used).
7. If possible, use an incubator with CO_2 but a regular incubator is acceptable.

Sub-culturing from 7H11S biplates

1. Pick several well-isolated *M. tb* colonies from the 7H11S biplates, and resuspend them in a sterile cryotube containing 500 µl of sterile Tween-saline.
2. Vortex the re-suspension and allow the aerosols to settle for a few seconds before inoculation.
3. Inoculate the surface of the 7H11S agar plate following the procedure described above.

Incubation of the 7H11S agar plates

1. Incubate the 7H11S plates for 4 to 6 weeks until abundant growth appears.
2. Once there is abundant, pure *M tuberculosis* growth on the plate, remove it from the incubator.
3. Open the containment bag inside the BSC and seal the plate with parafilm.

Storage of the 7H11S agar plates

1. Store all the 7H11S agar plates from each patient together in numerical order according to visit number and lab accession . -cultures were prepared, place them next to each other.
2. Store the 7H11S agar plates preferably in a refrigerator/cold-room if available or at room temperature until required to ship.
3. **Keep a log of stored 7H11S agar cultures which lists the date the plate was prepared, date the plate was stored, subject ID number, visit number, and laboratory accession number.**
4. Keep the agar cultures for no longer than 3 months. Ship or sub-culture again within 3 months of preparation.

Shipment of the 7H11S agar plates

1. If the 7H11S agar plate is contaminated at the time of shipment and there is no duplicate plate with pure *M. tb*, a 7H11S agar plate containing pure *M. tb* from an adjacent time interval is to be selected.
2. If the 7H11S agar plate has been stored for longer than 3 months, a fresh plate must be prepared from the original 7H11S agar plate, using the procedure described above. The new culture must be incubated for a sufficient time to obtain abundant growth on the plate prior to shipping.

3. Complete a TMTF NC005-004 (Culture isolate Sample Transport Form) for each 7H11S agar plate to be shipped; page 1 and top of page 2.
4. PATHPOUCH 95 kPa A4 with 4 or 6 Bay absorbent is to be used for shipment of the sealed 7H11S agar plates. The sealed 7H11S agar plates are to be individually placed into the pocket sleaves of the 4 or 6 Bay pouches, after which it is to be placed inside the PATHOPOUCH pathoseal bag. The primary and secondary contained 7H11S agar plates are then to be placed inside the packaging box, which is appropriately labelled.
5. Complete a TMTF NC005-005 (Culture Isolate Sample Transport Log) for all specimens being transported specimen per line on page 1 of the log. Details entered on page 2 and top half of page 3 apply to all isolates being shipped.
6. The bottom half of page 3 (Culture Isolate Sample Transport Log) is to be completed by the courier.
7. Once the specimen has been assigned to the Culture Isolate Sample Transport Log (NC-005 TMTF-005), make a copy of Form TMTF NC005-004 and email it to pnca@task.org.za. The original Culture Isolate Sample Transport Forms (TMTF NC005-004) should be placed in a sealed Ziploc bag and included with the Culture Isolate Sample Transport Log (TMTF NC005-005) in the shipment sent with the courier. Place the sealed forms and log on top of the specimens.
8. The IATA and/or UN shipping regulations must be strictly adhered to. The sponsor will assist with shipment of samples where required. If applicable, Material Transfer Agreements should be in place prior to shipment of isolates.
9. All 7H11S agar plates should be shipped at 2 – 8 °C range to the following address:

   Room **F561**
   Faculty of Medicine and Health Sciences
   Division of Molecular Biology and Human Genetics
   Department of Physiology
   Fisan Building, 5th Floor
   Francie van Zijl Drive
   Tygerberg, 7505
   Cape Town, South Africa

10. The sponsor will assist with arrangement of courier shipment of samples where required. If applicable, Material Transfer Agreements should be in place prior to shipment of isolates.

Removal of short-term stored LJs for additional tests

If the primary 7H11S agar short-term storage plate is removed to conduct additional testing, a new sub-culture must be prepared to ensure there is always a short-term storage plate.

Further use of Short-term isolates from 7H11S agar plates

The 7H11S agar cultures prepared can be used to prepare frozen isolates for long-term storage.

Disposal of 7H11S agar cultures at CCTR Laboratory

After 3 months of storage, 7H11S agar subcultures can be designated for disposal, provided that all necessary testing has been completed and all frozen stock aliquots have been appropriately stored. Approval to discard these 7H11S agar subcultures must be given by the Sponsor prior to disposal.

References

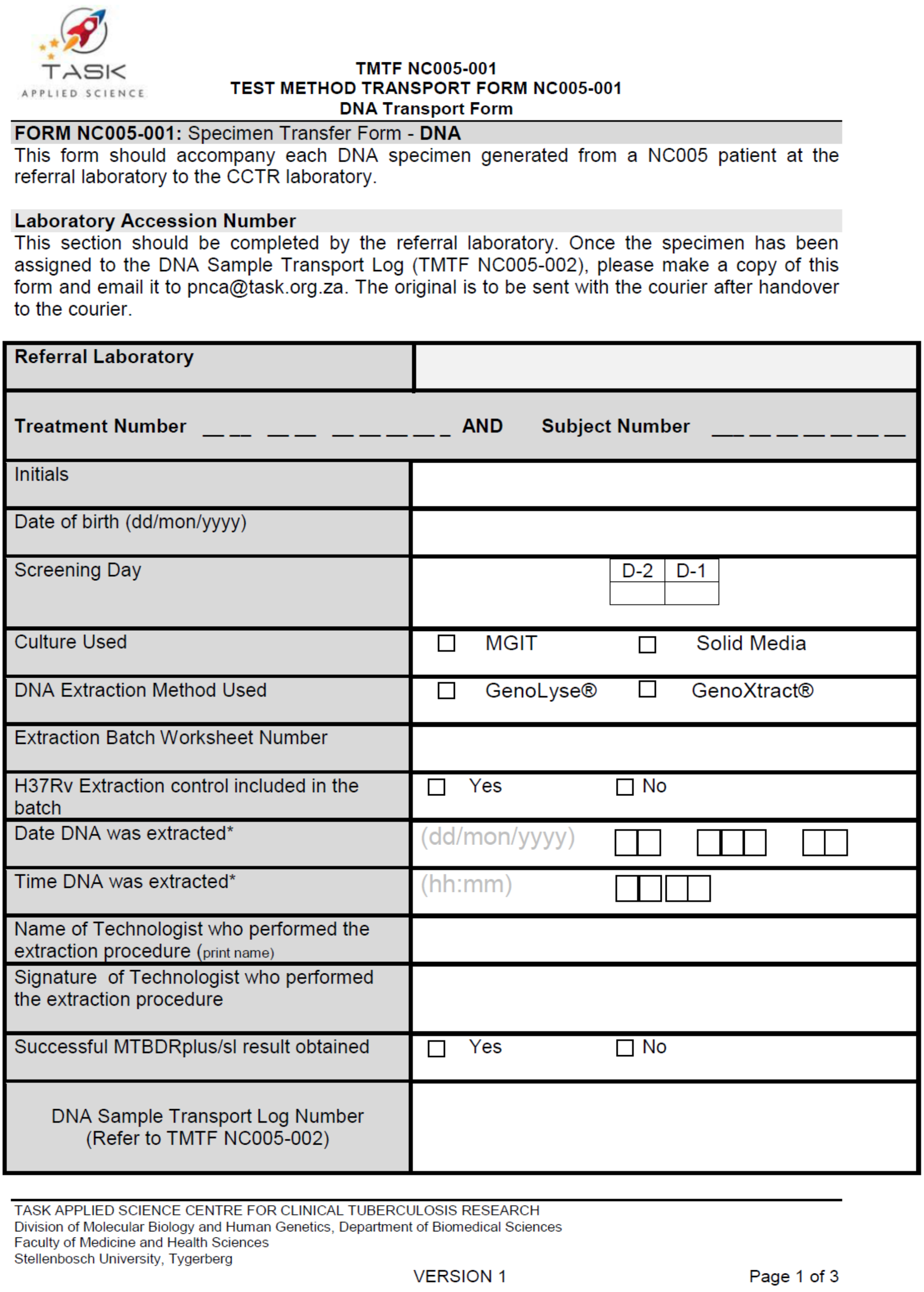
1. GLI Mycobacteriology Laboratory Manual, First Edition, April 2014, available at http://www.stoptb.org/wg/gli/assets/documents/gli_mycobacteriology_lab_manual_web.pdf

APPENDIX 1: MYCOBACTERIOLOGY RESULTS REPORT FORM

(Example)

NC005_Mycobacteriology_Results_Repo

APPENDIX 2: TMTF NC005-001 DNA Transport Form



TASK APPLIED SCIENCE

TMTF NC005-001
TEST METHOD TRANSPORT FORM NC005-001
DNA Transport Form

FORM NC005-001: Specimen Transfer Form - **DNA**

This form should accompany each DNA specimen generated from a NC005 patient at the referral laboratory to the CCTR laboratory.

Laboratory Accession Number

This section should be completed by the referral laboratory. Once the specimen has been assigned to the DNA Sample Transport Log (TMTF NC005-002), please make a copy of this form and email it to pnca@task.org.za. The original is to be sent with the courier after handover to the courier.

Referral Laboratory	
Treatment Number __ __ __ __ __ __ __ __ __ __ **AND** **Subject Number** __ __ __ __ __ __ __ __	
Initials	
Date of birth (dd/mon/yyyy)	
Screening Day	D-2 D-1
Culture Used	☐ MGIT ☐ Solid Media
DNA Extraction Method Used	☐ GenoLyse® ☐ GenoXtract®
Extraction Batch Worksheet Number	
H37Rv Extraction control included in the batch	☐ Yes ☐ No
Date DNA was extracted*	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time DNA was extracted*	(hh:mm) ☐☐☐☐
Name of Technologist who performed the extraction procedure (print name)	
Signature of Technologist who performed the extraction procedure	
Successful MTBDRplus/sl result obtained	☐ Yes ☐ No
DNA Sample Transport Log Number (Refer to TMTF NC005-002)	

TASK APPLIED SCIENCE CENTRE FOR CLINICAL TUBERCULOSIS RESEARCH
Division of Molecular Biology and Human Genetics, Department of Biomedical Sciences
Faculty of Medicine and Health Sciences
Stellenbosch University, Tygerberg

VERSION 1 Page 1 of 3

TASK
APPLIED SCIENCE

TMTF NC005-001
TEST METHOD TRANSPORT FORM NC005-001
DNA Transport Form

Date packaged DNA and the DNA Sample Log Sheet was handed over to courier**	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time packaged DNA and the DNA Sample Log Sheet was handed over to courier**	(hh:mm) ☐☐☐☐
Name of Technologist who handed over the packaged DNA and the DNA Sample Log Sheet to the courier (print name)	
Signature of Technologist who handed over the packaged DNA and the DNA Sample Log Sheet to the courier	
Referral laboratory Retention time of DNA sample after DNA extraction was done from the culture prior to shipment (** minus *)	☐☐ Days ☐☐☐☐ Hours

CCTR Laboratory Receipt

This section should be completed by the CCTR laboratory reception officer, or designated other, where the shipped DNA samples are received. Once a CCTR accession number has been generated, a *pncA* worksheet must be drawn up which will contain all the samples received from all referral laboratories on a specific day for *pncA* amplification. Once the *pncA* amplification worksheet has been created, the DNA specimens must be placed in order as they appear on the *pncA* amplification worksheet that was generated on that specific day. The worksheet and DNA is to be handed over to the molecular division to check that the DNA was placed in the correct order by the reception officer, prior to *pncA* amplification.

IATA Dangerous Goods Regulations and packaging instructions followed	☐ Yes ☐ No
Sample clearly labelled?	☐ Yes ☐ No
If no *please give details (detail problems, is this sample going to be processed? has another sample been requested?)*	
New CCTR Laboratory Accession number	ATTACH LABEL
Date that CCTR accession number was created by the reception officer	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time that CCTR accession number created by the reception officer	(hh:mm) ☐☐☐☐
Name of reception officer who created the CCTR accession number (print name)	

TASK
APPLIED SCIENCE

TMTF NC005-001
TEST METHOD TRANSPORT FORM NC005-001
DNA Transport Form

Signature of reception officer who created CCTR accession number	
***pncA* Amplification worksheet number** (The daily worksheet to which this sample has been added to)	

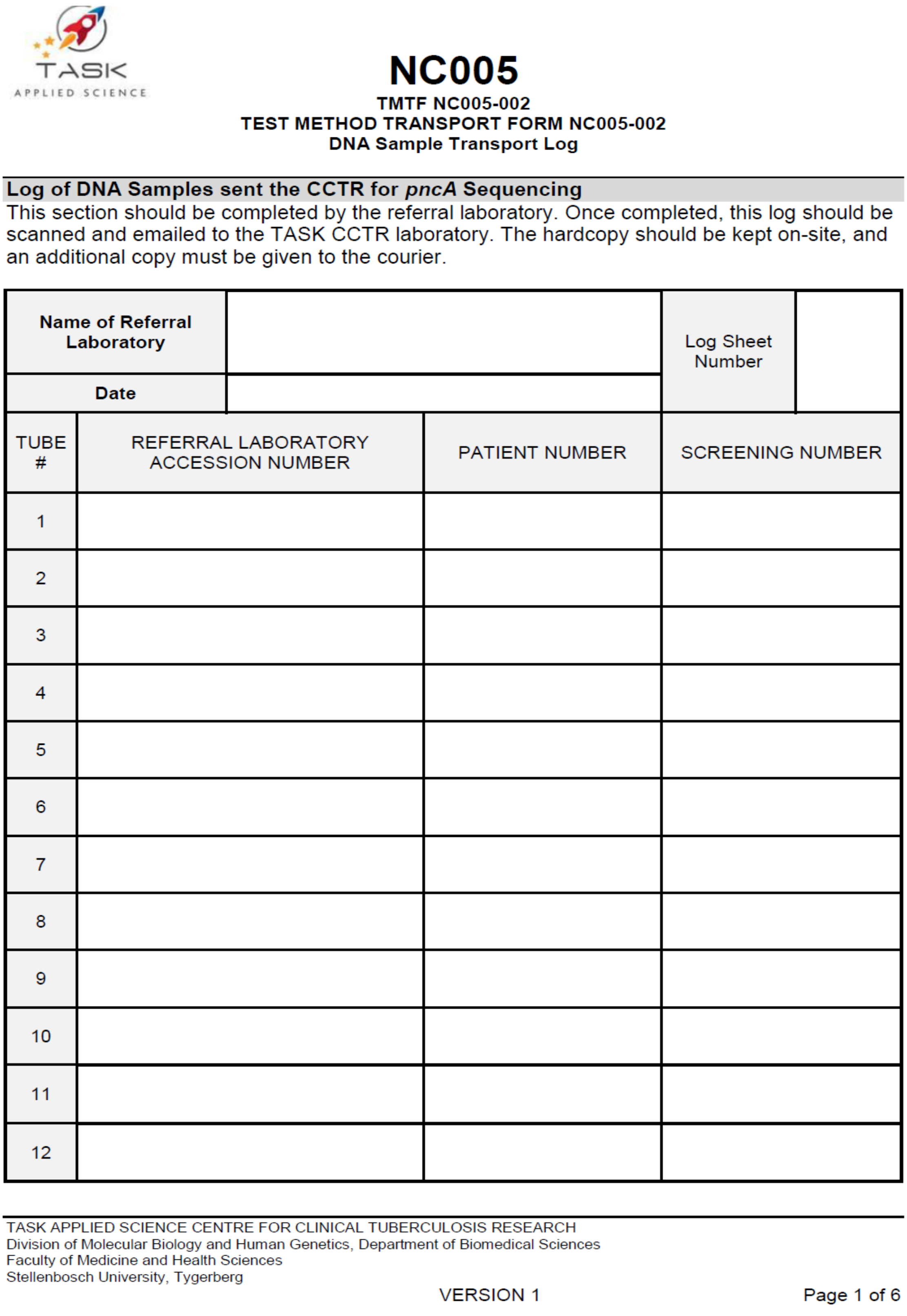
Molecular Division of the CCTR Laboratory

This section should be completed by the responsible technologist, or designated other, in charge of the molecular division of the CCTR laboratory. The *pncA* amplification worksheet created by the reception officer will be used for *pncA* sequencing purposes.

Date that DNA samples and *pncA* amplification worksheet that was received by the molecular division	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time that DNA samples and *pncA* amplification worksheet that was received by the molecular division	(hh:mm) ☐☐☐☐
Name of molecular division technologist who received the DNA samples and the *pncA* amplification worksheet (print name)	
Signature of molecular division technologist who received the DNA samples and the *pncA* amplification worksheet	
Date that *pncA* amplification was done	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time that *pncA* amplification was done	(hh:mm) ☐☐☐☐
Name of molecular division technologist who performed the *pncA* amplification (print name)	
Signature of molecular division technologist performed the *pncA* amplification	

TMTF NC005-003 (**Sample Transport Log for *pncA* Sequencing)** is to be used for the for the transport of the *pncA* amplicons.

APPENDIX 3: TMTF NC005-002 DNA Sample Transport Log



TASK
APPLIED SCIENCE

NC005

TMTF NC005-002
TEST METHOD TRANSPORT FORM NC005-002
DNA Sample Transport Log

Log of DNA Samples sent the CCTR for *pncA* Sequencing

This section should be completed by the referral laboratory. Once completed, this log should be scanned and emailed to the TASK CCTR laboratory. The hardcopy should be kept on-site, and an additional copy must be given to the courier.

Name of Referral Laboratory		Log Sheet Number	
Date			

TUBE #	REFERRAL LABORATORY ACCESSION NUMBER	PATIENT NUMBER	SCREENING NUMBER
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			

TASK APPLIED SCIENCE CENTRE FOR CLINICAL TUBERCULOSIS RESEARCH
Division of Molecular Biology and Human Genetics, Department of Biomedical Sciences
Faculty of Medicine and Health Sciences
Stellenbosch University, Tygerberg

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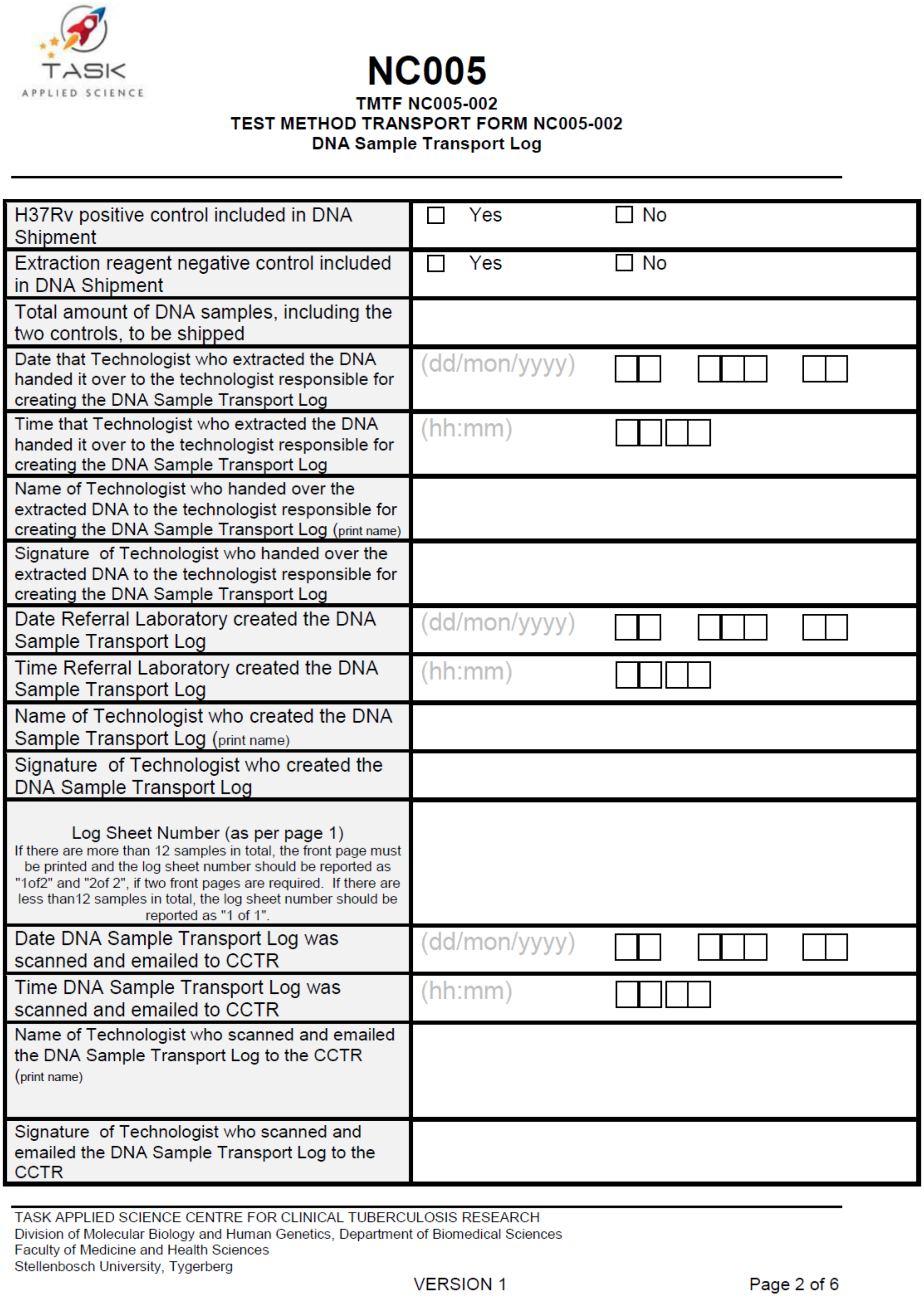

TASK
APPLIED SCIENCE

NC005

TMTF NC005-002
TEST METHOD TRANSPORT FORM NC005-002
DNA Sample Transport Log

H37Rv positive control included in DNA Shipment	☐ Yes ☐ No
Extraction reagent negative control included in DNA Shipment	☐ Yes ☐ No
Total amount of DNA samples, including the two controls, to be shipped	
Date that Technologist who extracted the DNA handed it over to the technologist responsible for creating the DNA Sample Transport Log	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time that Technologist who extracted the DNA handed it over to the technologist responsible for creating the DNA Sample Transport Log	(hh:mm) ☐☐☐☐
Name of Technologist who handed over the extracted DNA to the technologist responsible for creating the DNA Sample Transport Log (print name)	
Signature of Technologist who handed over the extracted DNA to the technologist responsible for creating the DNA Sample Transport Log	
Date Referral Laboratory created the DNA Sample Transport Log	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time Referral Laboratory created the DNA Sample Transport Log	(hh:mm) ☐☐☐☐
Name of Technologist who created the DNA Sample Transport Log (print name)	
Signature of Technologist who created the DNA Sample Transport Log	
Log Sheet Number (as per page 1) If there are more than 12 samples in total, the front page must be printed and the log sheet number should be reported as "1of2" and "2of 2", if two front pages are required. If there are less than12 samples in total, the log sheet number should be reported as "1 of 1".	
Date DNA Sample Transport Log was scanned and emailed to CCTR	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time DNA Sample Transport Log was scanned and emailed to CCTR	(hh:mm) ☐☐☐☐
Name of Technologist who scanned and emailed the DNA Sample Transport Log to the CCTR (print name)	
Signature of Technologist who scanned and emailed the DNA Sample Transport Log to the CCTR	

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NC005

TMTF NC005-002
TEST METHOD TRANSPORT FORM NC005-002
DNA Sample Transport Log

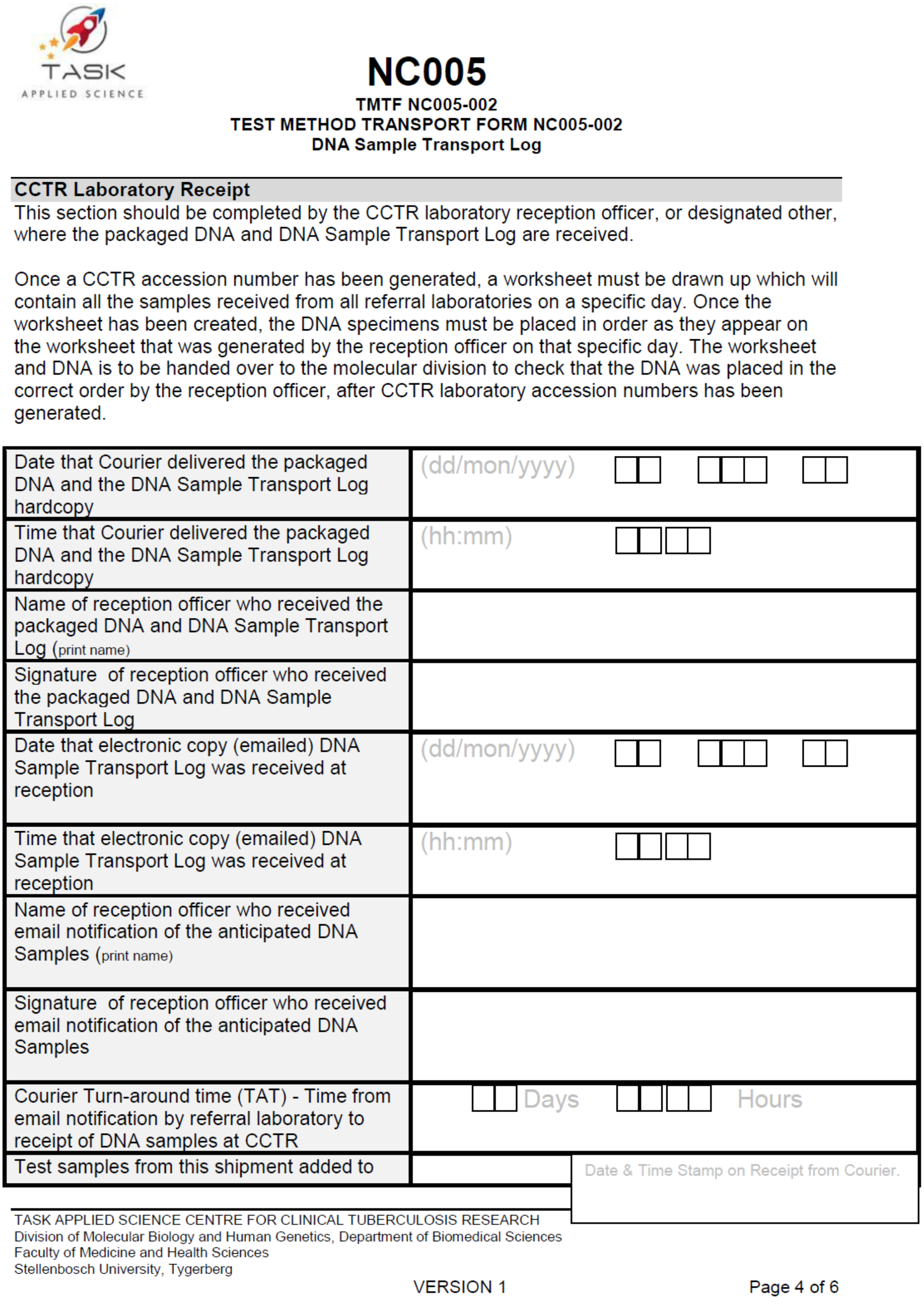
Date that both the DNA and the DNA Sample Transport Log was handed over to courier	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time that both the DNA and the DNA Sample Transport Log was handed over to courier	(hh:mm) ☐☐☐☐
Name of Technologist who handed over the packaged DNA and DNA Sample Transport Log to the courier (print name)	
Signature of Technologist who handed over the packaged DNA and DNA Sample Transport Log to the courier	

Courier Services
This section must be completed by the Courier **EXPORTING** the material.

Date that both the DNA and the DNA Sample Transport Log was received by the courier	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time that both the DNA and the DNA Sample Transport Log was handed over to courier	(hh:mm) ☐☐☐☐
Name of Courier who received the packaged DNA and DNA Sample Transport Log (print name)	
Signature of Courier who received the packaged DNA and DNA Sample Transport Log	

This section must be completed by the Courier **IMPORTING** the material.

Date that Courier delivered the packaged DNA and the DNA Sample Transport Log at the CCTR	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time that Courier delivered the packaged DNA and the DNA Sample Transport Log at the CCTR	(hh:mm) ☐☐☐☐
Name of Courier who delivered the packaged DNA and DNA Sample Transport Log (print name)	
Signature of Courier who delivered the packaged DNA and DNA Sample Transport Log	



TASK
APPLIED SCIENCE

NC005

TMTF NC005-002
TEST METHOD TRANSPORT FORM NC005-002
DNA Sample Transport Log

CCTR Laboratory Receipt

This section should be completed by the CCTR laboratory reception officer, or designated other, where the packaged DNA and DNA Sample Transport Log are received.

Once a CCTR accession number has been generated, a worksheet must be drawn up which will contain all the samples received from all referral laboratories on a specific day. Once the worksheet has been created, the DNA specimens must be placed in order as they appear on the worksheet that was generated by the reception officer on that specific day. The worksheet and DNA is to be handed over to the molecular division to check that the DNA was placed in the correct order by the reception officer, after CCTR laboratory accession numbers has been generated.

Date that Courier delivered the packaged DNA and the DNA Sample Transport Log hardcopy	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time that Courier delivered the packaged DNA and the DNA Sample Transport Log hardcopy	(hh:mm) ☐☐☐☐
Name of reception officer who received the packaged DNA and DNA Sample Transport Log (print name)	
Signature of reception officer who received the packaged DNA and DNA Sample Transport Log	
Date that electronic copy (emailed) DNA Sample Transport Log was received at reception	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time that electronic copy (emailed) DNA Sample Transport Log was received at reception	(hh:mm) ☐☐☐☐
Name of reception officer who received email notification of the anticipated DNA Samples (print name)	
Signature of reception officer who received email notification of the anticipated DNA Samples	
Courier Turn-around time (TAT) - Time from email notification by referral laboratory to receipt of DNA samples at CCTR	☐☐ Days ☐☐☐☐ Hours
Test samples from this shipment added to	

Date & Time Stamp on Receipt from Courier.

NC005

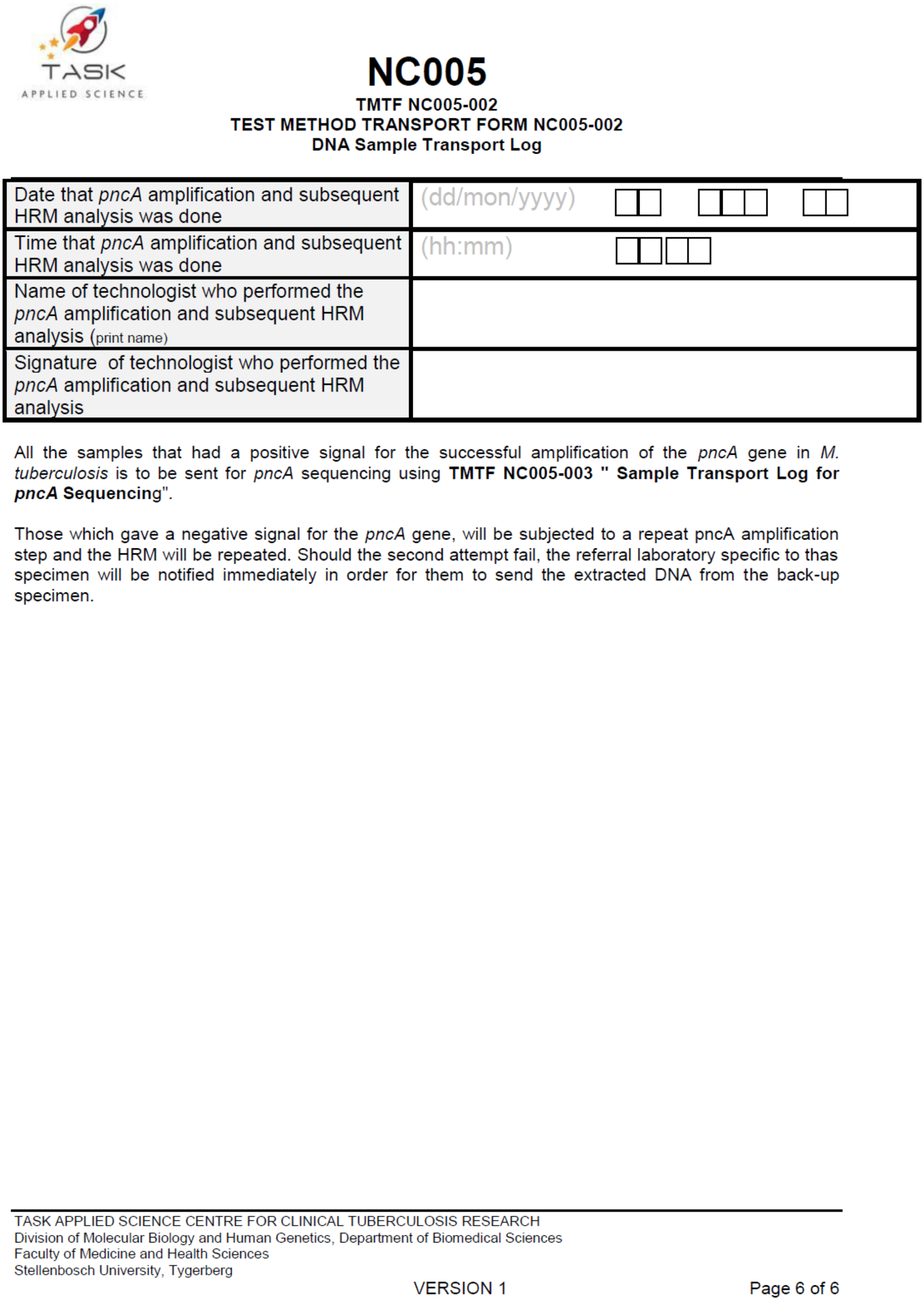
TMTF NC005-002
TEST METHOD TRANSPORT FORM NC005-002
DNA Sample Transport Log

other test sample shipments arriving on the same day and arranged in order to match the *pncA* amplification worksheet that was created from all the DNA samples received on this day	☐ Yes ☐ No
pncA Amplification worksheet number (The daily worksheet to which the samples of this shipment has been added to)	
Date that DNA samples and *pncA* amplification worksheet that was created were given to the molecular division by the reception officer	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time that DNA samples and *pncA* amplification worksheet that was created were given to the molecular division by the reception officer	(hh:mm) ☐☐☐☐
Name of reception officer who gave the DNA samples and the *pncA* amplification worksheet to the molecular division (print name)	
Signature of reception officer who gave the DNA samples and the *pncA* amplification worksheet to the molecular division	

pncA Sequencing

This section should be completed by the molecular division supervisor, or designated other, of the CCTR laboratory, where the packaged DNA and DNA Sample Transport Log are received.

Date that DNA samples were given to the molecular division for *pncA* amplification and subsequent HRM analysis	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time that DNA samples were given to the molecular division for *pncA* amplification and subsequent HRM analysis	(hh:mm) ☐☐☐☐
Name of technologist who received the DNA samples for *pncA* amplification and subsequent HRM analysis (print name)	
Signature of technologist who received the DNA samples for *pncA* amplification and subsequent HRM analysis	



TASK
APPLIED SCIENCE

NC005

TMTF NC005-002
TEST METHOD TRANSPORT FORM NC005-002
DNA Sample Transport Log

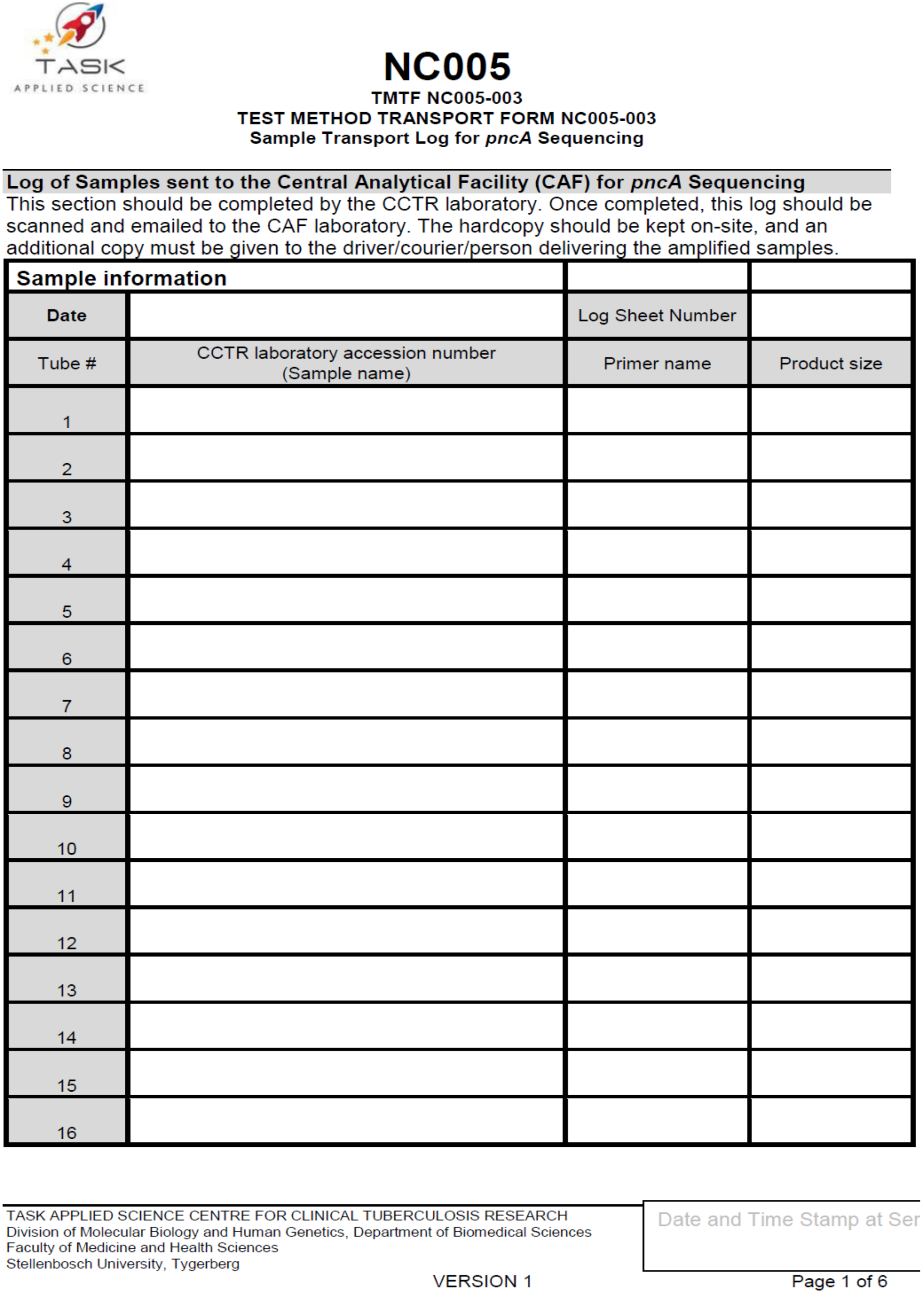
Date that *pncA* amplification and subsequent HRM analysis was done	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time that *pncA* amplification and subsequent HRM analysis was done	(hh:mm) ☐☐☐☐
Name of technologist who performed the *pncA* amplification and subsequent HRM analysis (print name)	
Signature of technologist who performed the *pncA* amplification and subsequent HRM analysis	

All the samples that had a positive signal for the successful amplification of the *pncA* gene in *M. tuberculosis* is to be sent for *pncA* sequencing using **TMTF NC005-003 " Sample Transport Log for *pncA* Sequencing**".

Those which gave a negative signal for the *pncA* gene, will be subjected to a repeat pncA amplification step and the HRM will be repeated. Should the second attempt fail, the referral laboratory specific to thas specimen will be notified immediately in order for them to send the extracted DNA from the back-up specimen.

APPENDIX 4: TMTF NC005-003 Sample Transport Log for pncA Sequencing

(CCTR Laboratory Only)



TASK
APPLIED SCIENCE

NC005

TMTF NC005-003
TEST METHOD TRANSPORT FORM NC005-003
Sample Transport Log for *pncA* Sequencing

Log of Samples sent to the Central Analytical Facility (CAF) for *pncA* Sequencing

This section should be completed by the CCTR laboratory. Once completed, this log should be scanned and emailed to the CAF laboratory. The hardcopy should be kept on-site, and an additional copy must be given to the driver/courier/person delivering the amplified samples.

Sample information			
Date		Log Sheet Number	
Tube #	CCTR laboratory accession number (Sample name)	Primer name	Product size
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			

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Faculty of Medicine and Health Sciences
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Date and Time Stamp at Ser

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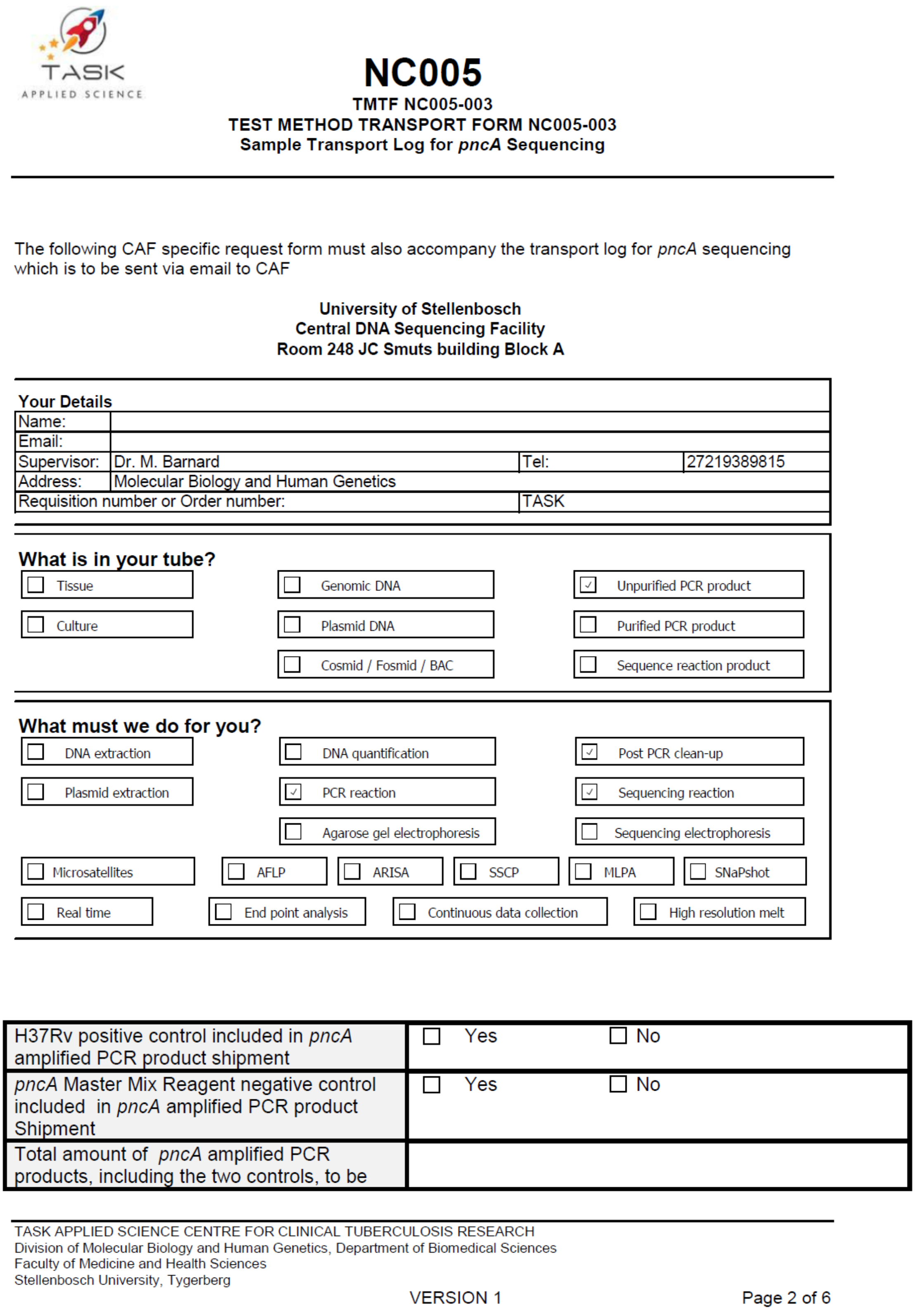

TASK
APPLIED SCIENCE

NC005

TMTF NC005-003
TEST METHOD TRANSPORT FORM NC005-003
Sample Transport Log for *pncA* Sequencing

The following CAF specific request form must also accompany the transport log for *pncA* sequencing which is to be sent via email to CAF

University of Stellenbosch
Central DNA Sequencing Facility
Room 248 JC Smuts building Block A

Your Details

Name:			
Email:			
Supervisor:	Dr. M. Barnard	Tel:	27219389815
Address:	Molecular Biology and Human Genetics		
Requisition number or Order number:		TASK	

What is in your tube?

☐ Tissue
☐ Culture
☐ Genomic DNA
☐ Plasmid DNA
☐ Cosmid / Fosmid / BAC
☑ Unpurified PCR product
☐ Purified PCR product
☐ Sequence reaction product

What must we do for you?

☐ DNA extraction
☐ Plasmid extraction
☐ DNA quantification
☑ PCR reaction
☐ Agarose gel electrophoresis
☑ Post PCR clean-up
☑ Sequencing reaction
☐ Sequencing electrophoresis
☐ Microsatellites
☐ AFLP
☐ ARISA
☐ SSCP
☐ MLPA
☐ SNaPshot
☐ Real time
☐ End point analysis
☐ Continuous data collection
☐ High resolution melt

H37Rv positive control included in *pncA* amplified PCR product shipment	☐ Yes	☐ No
pncA Master Mix Reagent negative control included in *pncA* amplified PCR product Shipment	☐ Yes	☐ No
Total amount of *pncA* amplified PCR products, including the two controls, to be		

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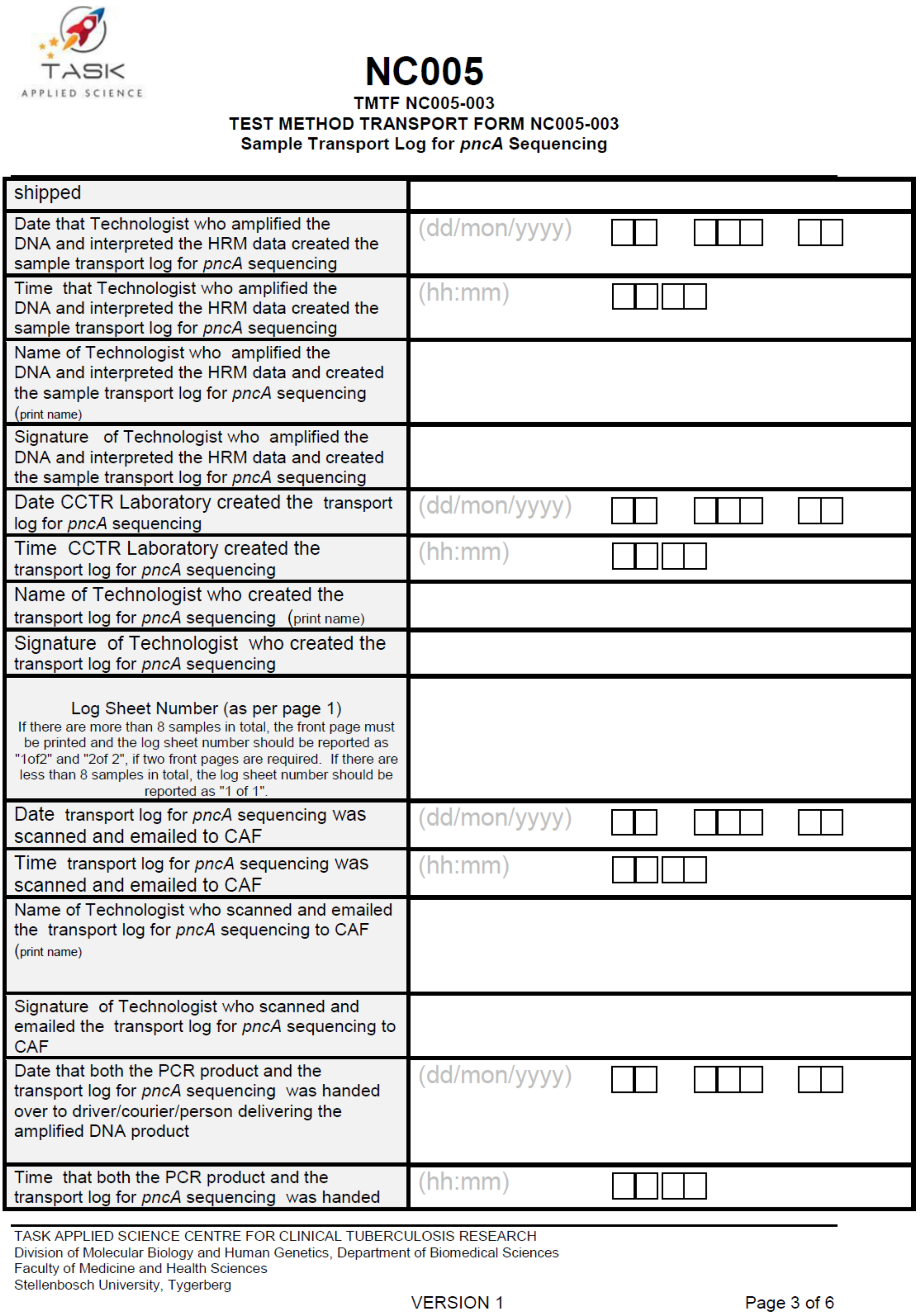

TASK
APPLIED SCIENCE

NC005

TMTF NC005-003
TEST METHOD TRANSPORT FORM NC005-003
Sample Transport Log for *pncA* Sequencing

shipped	
Date that Technologist who amplified the DNA and interpreted the HRM data created the sample transport log for *pncA* sequencing	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time that Technologist who amplified the DNA and interpreted the HRM data created the sample transport log for *pncA* sequencing	(hh:mm) ☐☐☐☐
Name of Technologist who amplified the DNA and interpreted the HRM data and created the sample transport log for *pncA* sequencing (print name)	
Signature of Technologist who amplified the DNA and interpreted the HRM data and created the sample transport log for *pncA* sequencing	
Date CCTR Laboratory created the transport log for *pncA* sequencing	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time CCTR Laboratory created the transport log for *pncA* sequencing	(hh:mm) ☐☐☐☐
Name of Technologist who created the transport log for *pncA* sequencing (print name)	
Signature of Technologist who created the transport log for *pncA* sequencing	
Log Sheet Number (as per page 1) If there are more than 8 samples in total, the front page must be printed and the log sheet number should be reported as "1of2" and "2of 2", if two front pages are required. If there are less than 8 samples in total, the log sheet number should be reported as "1 of 1".	
Date transport log for *pncA* sequencing was scanned and emailed to CAF	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time transport log for *pncA* sequencing was scanned and emailed to CAF	(hh:mm) ☐☐☐☐
Name of Technologist who scanned and emailed the transport log for *pncA* sequencing to CAF (print name)	
Signature of Technologist who scanned and emailed the transport log for *pncA* sequencing to CAF	
Date that both the PCR product and the transport log for *pncA* sequencing was handed over to driver/courier/person delivering the amplified DNA product	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time that both the PCR product and the transport log for *pncA* sequencing was handed	(hh:mm) ☐☐☐☐

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Faculty of Medicine and Health Sciences
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NC005

TMTF NC005-003

TEST METHOD TRANSPORT FORM NC005-003

Sample Transport Log for *pncA* Sequencing

over to driver/courier/person delivering the amplified DNA product	
Name of Technologist who handed over the PCR product and the transport log for *pncA* sequencing to the driver/courier/person delivering the amplified DNA product (print name)	
Signature of Technologist who handed over the PCR product and the transport log for *pncA* sequencing to the driver/courier/person delivering the amplified DNA product	

Driver/Courier/person delivering the PCR product and the transport log for *pncA* sequencing

This section must be completed by the Driver/Courier/designated other who delivers the above mentioned to CAF.

Date that both the PCR product and the transport log for *pncA* sequencing was received by the driver/courier/designated other.	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time that both the PCR product and the transport log for *pncA* sequencing was received by the driver/courier/designated other	(hh:mm) ☐☐☐☐
Name of driver/courier/designated other who received the PCR product and the transport log for *pncA* sequencing (print name)	
Signature of driver/courier/designated other who received the PCR product and the transport log for *pncA* sequencing	

CAF Laboratory Receipt

This section should be completed by the CAF laboratory reception officer, or designated other, where the PCR product , the transport log for *pncA* sequencing, and CAF specific request form are received.

The CAF laboratory uses the laboratory accession number created the CCTR laboratory and will use the transport log for *pncA* sequencing as a worksheet.

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APPLIED SCIENCE

NC005

TMTF NC005-003

TEST METHOD TRANSPORT FORM NC005-003

Sample Transport Log for *pncA* Sequencing

Date that driver/courier/designated other delivered the PCR product and the transport log for *pncA* sequencing in hardcopy	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time that driver/courier/designated other delivered the PCR product and the transport log for *pncA* sequencing in hardcopy	(hh:mm) ☐☐☐☐
Name of CAF reception officer who received the PCR product, the transport log for *pncA* sequencing and CAF request form (print name)	
Signature of reception officer who received the PCR product,the transport log for *pncA* sequencing and the CAF request form	
Date that electronic copy (emailed) transport log for *pncA* sequencing and request form was received at the CAF laboratory	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time that electronic copy (emailed) transport log for *pncA* sequencing and request form was received at the CAF laboratory	(hh:mm) ☐☐☐☐
Name of reception officer who received email notification of the anticipated *pncA* sequencing samples (print name)	
Signature of reception officer who received email notification of the anticipated *pncA* sequencing samples	
Date that *pncA* Sequencing,alignment and interpretation was done	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time that *pncA* Sequencing, alignment and interpretation was done	(hh:mm) ☐☐☐☐
Name of staff member who performed the *pncA* Sequencing, alignment and interpretation (print name)	
Signature of staff member who performed the *pncA* Sequencing,alignment and interpretation	
Date that *pncA* alignment was sent back to the CCTR for confirmation of interpretation	(dd/mon/yyyy) ☐☐ ☐☐☐ ☐☐
Time that *pncA* alignment was sent back to the CCTR for confirmation of interpretation	(hh:mm) ☐☐☐☐
Name of staff member who performed the *pncA* interpretation confirmation (print name)	

TASK APPLIED SCIENCE

NC005

TMTF NC005-003
TEST METHOD TRANSPORT FORM NC005-003
Sample Transport Log for *pncA* Sequencing

Signature of staff member who performed the *pncA* interpretation confirmation	
CAF turn-around time (TAT) - Time from *pncA* sequencing to CCTR laboratory reporting of pncA susceptibility	☐☐ Days ☐☐☐☐ Hours
Total turn-around time (TAT) - Time from DNA reception to reporting of pncA susceptibilities to back to the referral laboratory	☐☐ Days ☐☐☐☐ Hours

The Pyrazinamide susceptibility result will be reported as either exhibiting resistance to PZA or not.

I.e. PZA resistance detected, or
PZA resistance NOT detected.

APPENDIX 5: INTRODUCTION & MGIT FLOW CHART 0 – GENERAL ALGORITHM

MGIT 960 Culture Flow Charts: Introduction

Background

The following flow charts are based on the algorithms in the GLI Mycobacteriology Laboratory Manual and were further developed by the ACTG TB Laboratory Core Team (Kathy Eisenach and Anne-Marie Demers, co-chairs). They are guides for the work-up of MGIT cultures in the context of clinical TB trials. The abbreviations used in the flow charts are listed in Table 1. The various Hain tests available are listed in Table 2.

Note that there are 2 options for identification in Chart 1 Positive MGIT, depending what tests are available locally: either a non-tuberculous mycobacteria (NTM) amplification test (Hain CM test), or a MTBC amplification test (Hain MTBDR*plus* OR GeneXpert). Note that both lateral flow assays (also called immunochromatographic tests) for MPT64 antigen and the GenProbe Accuprobe MTBC identification test are not amplification tests.

Contamination

Although some TB laboratory resources list possible contaminants as: mycobacteria other than tuberculosis (MOTT or non-tuberculous mycobacteria -NTM), fungi, bacteria and yeasts, others refer to contamination only for bacteria or fungi. In the NC-005 study the AFB positive cultures, which are negative with the MPT 64 antigen test, are not further tested to confirm NTM and are classified as contaminants. If the laboratory decides to pursue the identification of NTM, that is acceptable however these results are not reportable in the study database. Also, there is no provision for reporting “mixed culture”, when more than one species of mycobacteria is recovered.

For the flow charts, a culture will be reported as “contaminated” if either growth of bacteria/fungi is observed on the blood agar plate and/or if non acid-fast bacteria are seen on the Ziehl-Neelsen stain. Note that not all contaminants grow on BAP.

Reporting

In the flow charts, the term “Report” refers to the final report for the study data. However, this applies only to the overnight collected sputum specimens where AFB positive growth in the MGIT culture is tested with the MPT 64 antigen identification test. The MPT 64 antigen cultures will not be further tested and are reported as “Contaminated”. No attempt will be made to distinguish *M. tb* complex from NTM in cultures from coached spot sputum specimens, unless the MGIT culture is negative or not done.

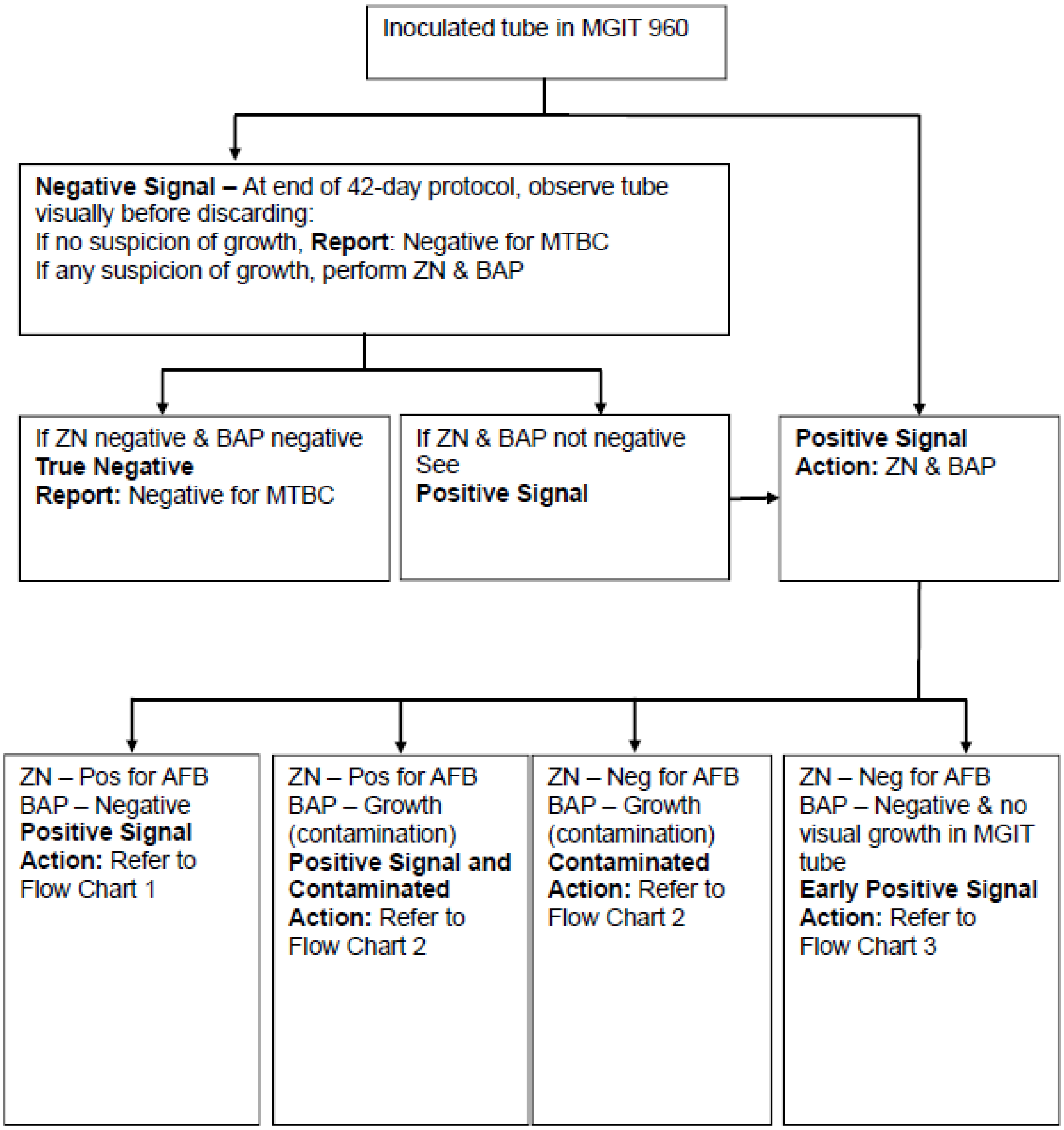
Table 1. Abbreviations

Abbreviation	Description
BAP	Blood agar plate
Rapid ID Test	Rapid Identification Test for MTBC: Lateral flow assays (also called immunochromatographic tests) for MPT64 antigen (ex:, Alere SD BIOLINE TB, BD TBcID, TAUNS Capilia TB-Neo)
LJ	Lowenstein-Jensen
MTBC	Mycobacterium tuberculosis complex
Neg	Negative
NTM	Non-tuberculous mycobacteria
Pos	Positive
TTP	Time to positivity as reported by the MGIT machine. The time to positivity is reported in days and hours.
ZN	Ziehl-Neelsen

Table 2. Available Hain Line Probe Assay Tests

Name	Genus control included	MTBC probes included
Hain GenoType MTBDRplus (detects MTBC and its resistance to INH and RIF)	No	Yes
Hain GenoType MTBDRsl (detects MTBC and its resistance to fluoroquinolones (FQ), aminoglycosides/cyclic peptides (AG/ CP), and ethambutol (EMB)	No	Yes
Hain GenoType Mycobacterium CM (permit simultaneous molecular genetic identification of the M. tuberculosis complex and 24 of the most common NTM species)	Yes	Yes
Hain GenoType Mycobacterium AS (permits the simultaneous molecular genetic identification of 19 additional NTM species)	Yes	No
Hain GenoType MTBC (for differentiation of the Mycobacterium tuberculosis Complex)	No	Yes

Flow Chart 0
General Algorithm



Inoculated tube in MGIT 960

Negative Signal – At end of 42-day protocol, observe tube visually before discarding:
If no suspicion of growth, **Report**: Negative for MTBC
If any suspicion of growth, perform ZN & BAP

If ZN negative & BAP negative
True Negative
Report: Negative for MTBC

If ZN & BAP not negative
See
Positive Signal

Positive Signal
Action: ZN & BAP

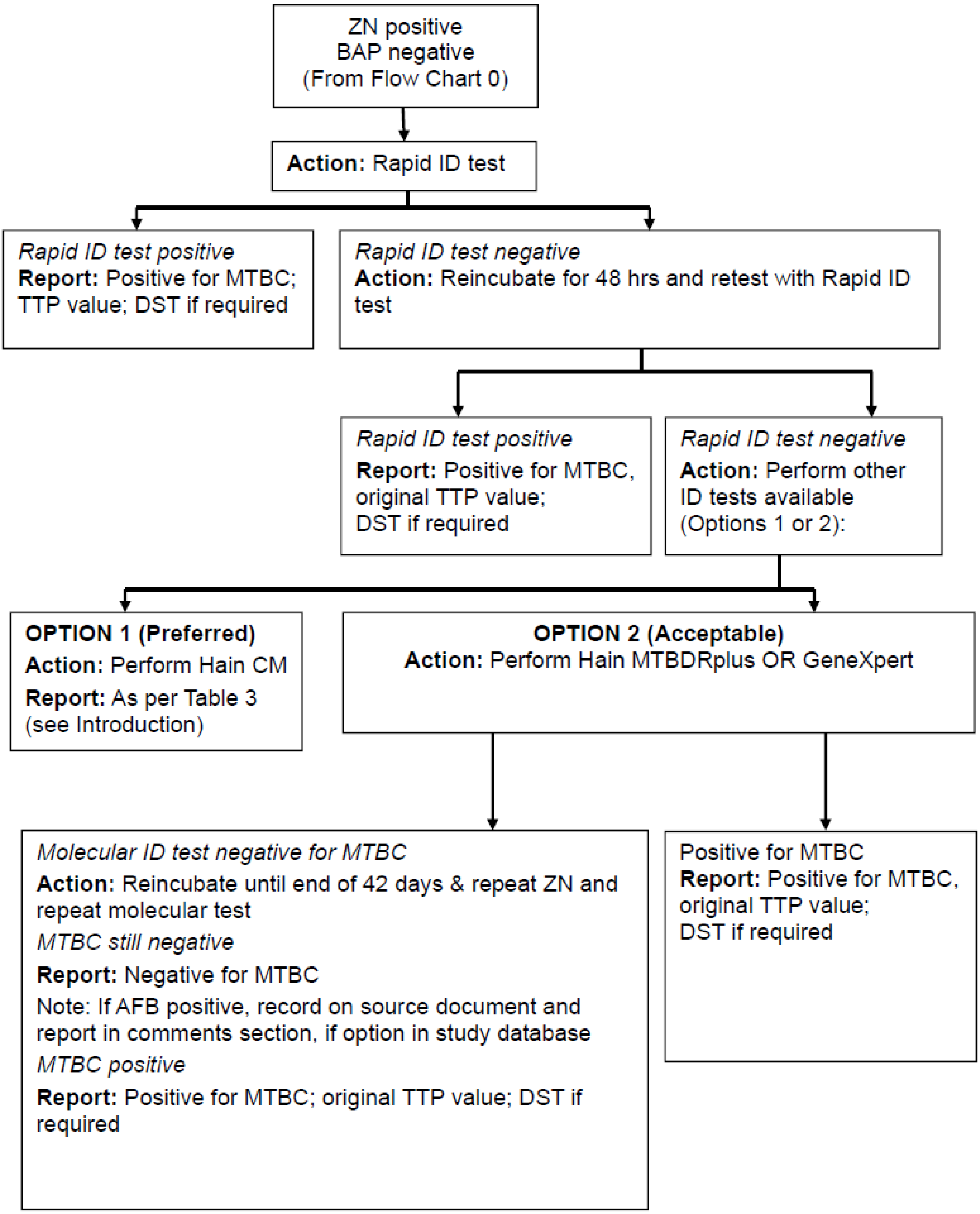
ZN – Pos for AFB
BAP – Negative
Positive Signal
Action: Refer to Flow Chart 1

ZN – Pos for AFB
BAP – Growth (contamination)
Positive Signal and Contaminated
Action: Refer to Flow Chart 2

ZN – Neg for AFB
BAP – Growth (contamination)
Contaminated
Action: Refer to Flow Chart 2

ZN – Neg for AFB
BAP – Negative & no visual growth in MGIT tube
Early Positive Signal
Action: Refer to Flow Chart 3

APPENDIX 6: MGIT FLOW CHART 1 – MGIT POSITIVE



ZN positive
BAP negative
(From Flow Chart 0)

Action: Rapid ID test

Rapid ID test positive
Report: Positive for MTBC; TTP value; DST if required

Rapid ID test negative
Action: Reincubate for 48 hrs and retest with Rapid ID test

Rapid ID test positive
Report: Positive for MTBC, original TTP value;
DST if required

Rapid ID test negative
Action: Perform other ID tests available (Options 1 or 2):

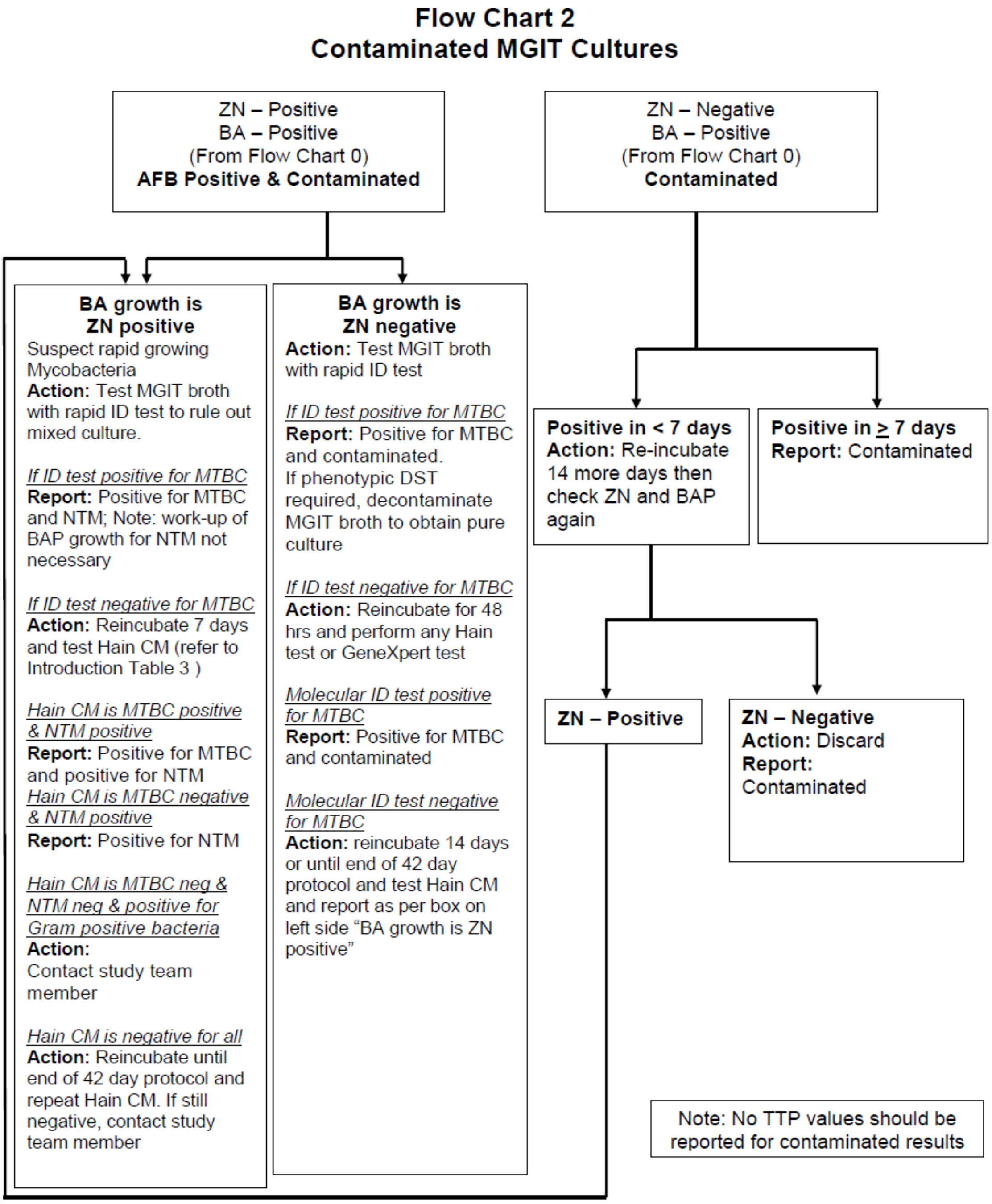
OPTION 1 (Preferred)
Action: Perform Hain CM
Report: As per Table 3 (see Introduction)

OPTION 2 (Acceptable)
Action: Perform Hain MTBDRplus OR GeneXpert

Molecular ID test negative for MTBC
Action: Reincubate until end of 42 days & repeat ZN and repeat molecular test
MTBC still negative
Report: Negative for MTBC
Note: If AFB positive, record on source document and report in comments section, if option in study database
MTBC positive
Report: Positive for MTBC; original TTP value; DST if required

Positive for MTBC
Report: Positive for MTBC, original TTP value;
DST if required

APPENDIX 7: MGIT FLOW CHART 2 – CONTAMINATED MGIT CULTURE



Flow Chart 2
Contaminated MGIT Cultures

ZN – Positive
BA – Positive
(From Flow Chart 0)
AFB Positive & Contaminated

ZN – Negative
BA – Positive
(From Flow Chart 0)
Contaminated

BA growth is ZN positive
Suspect rapid growing Mycobacteria
Action: Test MGIT broth with rapid ID test to rule out mixed culture.

If ID test positive for MTBC
Report: Positive for MTBC and NTM; Note: work-up of BAP growth for NTM not necessary

If ID test negative for MTBC
Action: Reincubate 7 days and test Hain CM (refer to Introduction Table 3)

Hain CM is MTBC positive & NTM positive
Report: Positive for MTBC and positive for NTM
Hain CM is MTBC negative & NTM positive
Report: Positive for NTM

Hain CM is MTBC neg & NTM neg & positive for Gram positive bacteria
Action:
Contact study team member

Hain CM is negative for all
Action: Reincubate until end of 42 day protocol and repeat Hain CM. If still negative, contact study team member

BA growth is ZN negative
Action: Test MGIT broth with rapid ID test

If ID test positive for MTBC
Report: Positive for MTBC and contaminated.
If phenotypic DST required, decontaminate MGIT broth to obtain pure culture

If ID test negative for MTBC
Action: Reincubate for 48 hrs and perform any Hain test or GeneXpert test

Molecular ID test positive for MTBC
Report: Positive for MTBC and contaminated

Molecular ID test negative for MTBC
Action: reincubate 14 days or until end of 42 day protocol and test Hain CM and report as per box on left side "BA growth is ZN positive"

Positive in < 7 days
Action: Re-incubate 14 more days then check ZN and BAP again

Positive in ≥ 7 days
Report: Contaminated

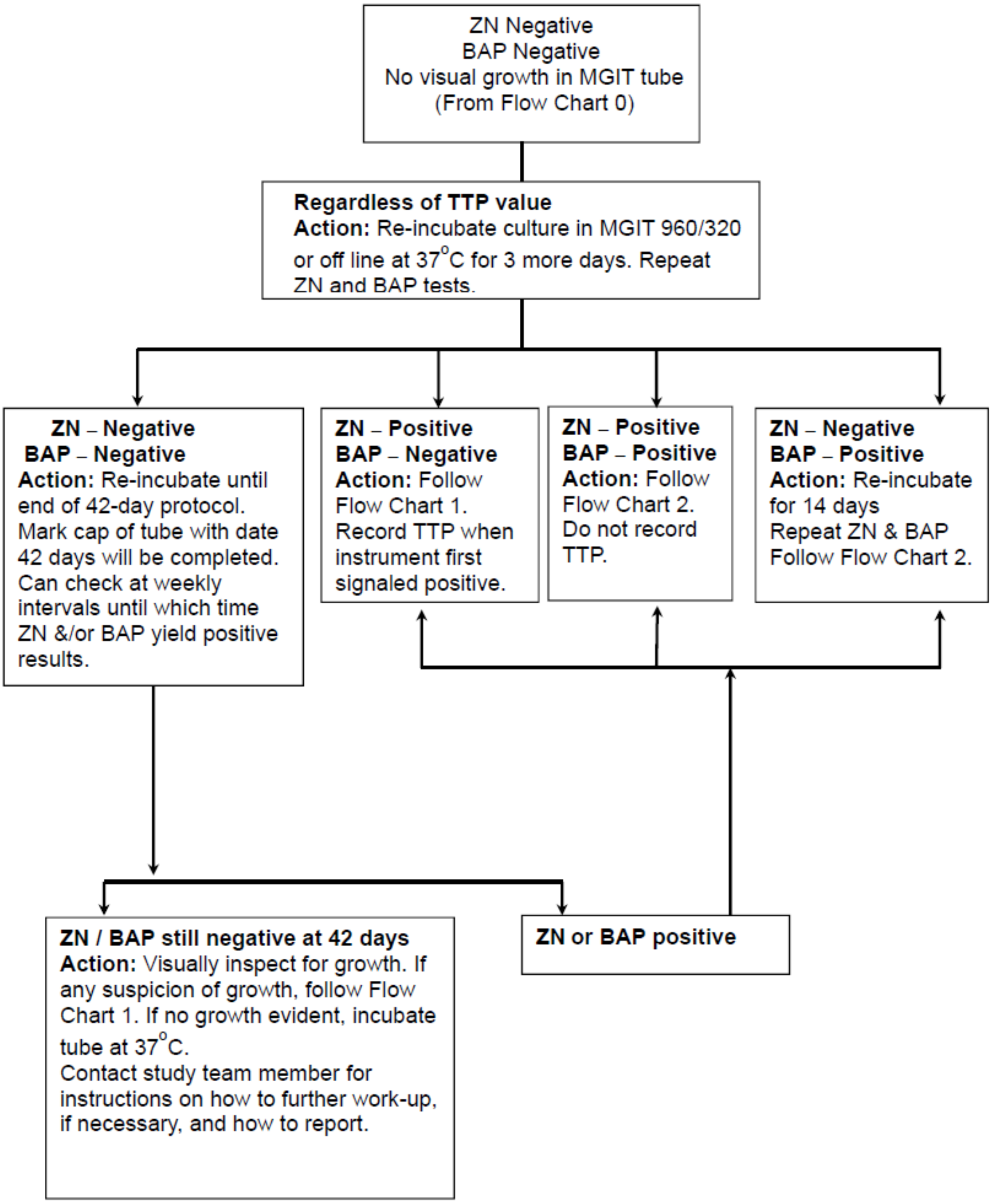
ZN – Positive

ZN – Negative
Action: Discard
Report:
Contaminated

Note: No TTP values should be reported for contaminated results

APPENDIX 8: MGIT FLOW CHART 3 – EARLY POSITIVE SIGNAL

Flow Chart 3
Early Positive Signal



APPENDIX 9: MGIT CULTURE CONTAMINATION SPREADSHEET

MGIT BI-WEEKLY

Clinical Site #:

MGIT CONTAMINATION BI-WEEKLY SUMMARY

Date of Bi-weekly Report DD/MM/YY	**Number Specimens Reported**	**Number Contam Specimens w/o MTB**	**Number Contam Specimens with MTB**

Date of Bi-weekly Report = date that data are entered on this spreadsheet; should be the same day of each week, every other week

Number of Specimens Reported = # specimens reported during the previous 2 weeks

Number of specimens with a contaminated MGIT culture and no MTB detected

Number of specimens with a MGIT culture haivng contaminants and MTB

MGIT BY SPECIMEN

Clinical Site #:

MGIT CONTAMINATION BY SPECIMEN

					MGIT				
Date of Bi-weekly Report DD/MM/YYYY	Inoculation Date DD/MM/YYYY	Patient Study ID #	Lab Accession Number	Visit #	TTP	ZN (P/N)	MTB ID (Y/N)	Decontam MGIT (Y/N)	Successful recovery of MTB from decontam

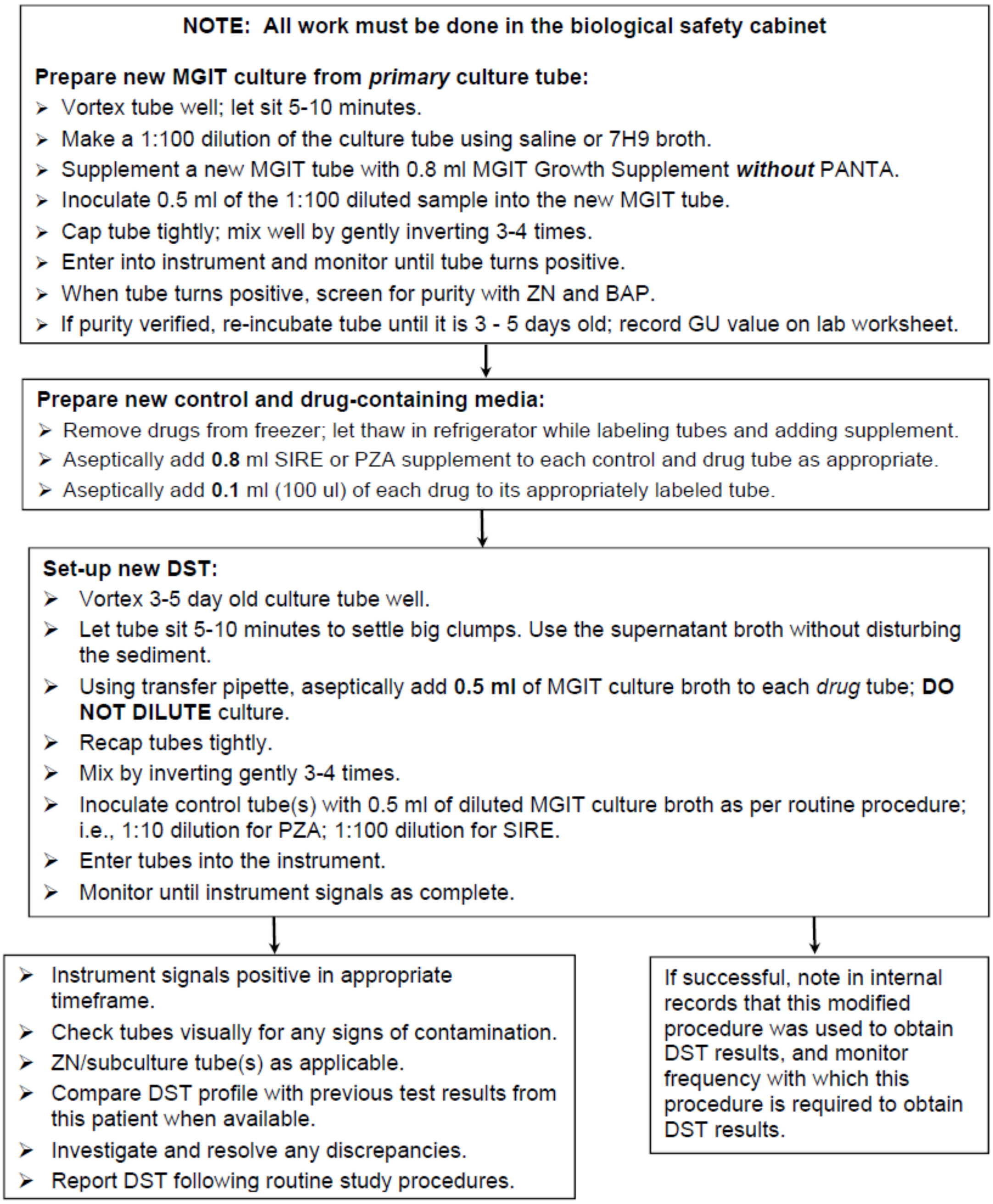
TTP = express in days, hours

Decontamination of MGIT = attemped to decontaminate MGIT culture to obtain MTB isolate for further testing

Successful recovery of MTB from decontamination = obtained pure MTB culture following MGIT decontamination

APPENDIX 10: MGIT FLOW CHART 4 – INVALID X 200 ERRORS FROM MGIT DST

Flowchart 4: Invalid x200 Errors from MGIT DST



NOTE: All work must be done in the biological safety cabinet

Prepare new MGIT culture from *primary* culture tube:

- Vortex tube well; let sit 5-10 minutes.
- Make a 1:100 dilution of the culture tube using saline or 7H9 broth.
- Supplement a new MGIT tube with 0.8 ml MGIT Growth Supplement ***without*** PANTA.
- Inoculate 0.5 ml of the 1:100 diluted sample into the new MGIT tube.
- Cap tube tightly; mix well by gently inverting 3-4 times.
- Enter into instrument and monitor until tube turns positive.
- When tube turns positive, screen for purity with ZN and BAP.
- If purity verified, re-incubate tube until it is 3 - 5 days old; record GU value on lab worksheet.

Prepare new control and drug-containing media:

- Remove drugs from freezer; let thaw in refrigerator while labeling tubes and adding supplement.
- Aseptically add **0.8** ml SIRE or PZA supplement to each control and drug tube as appropriate.
- Aseptically add **0.1** ml (100 ul) of each drug to its appropriately labeled tube.

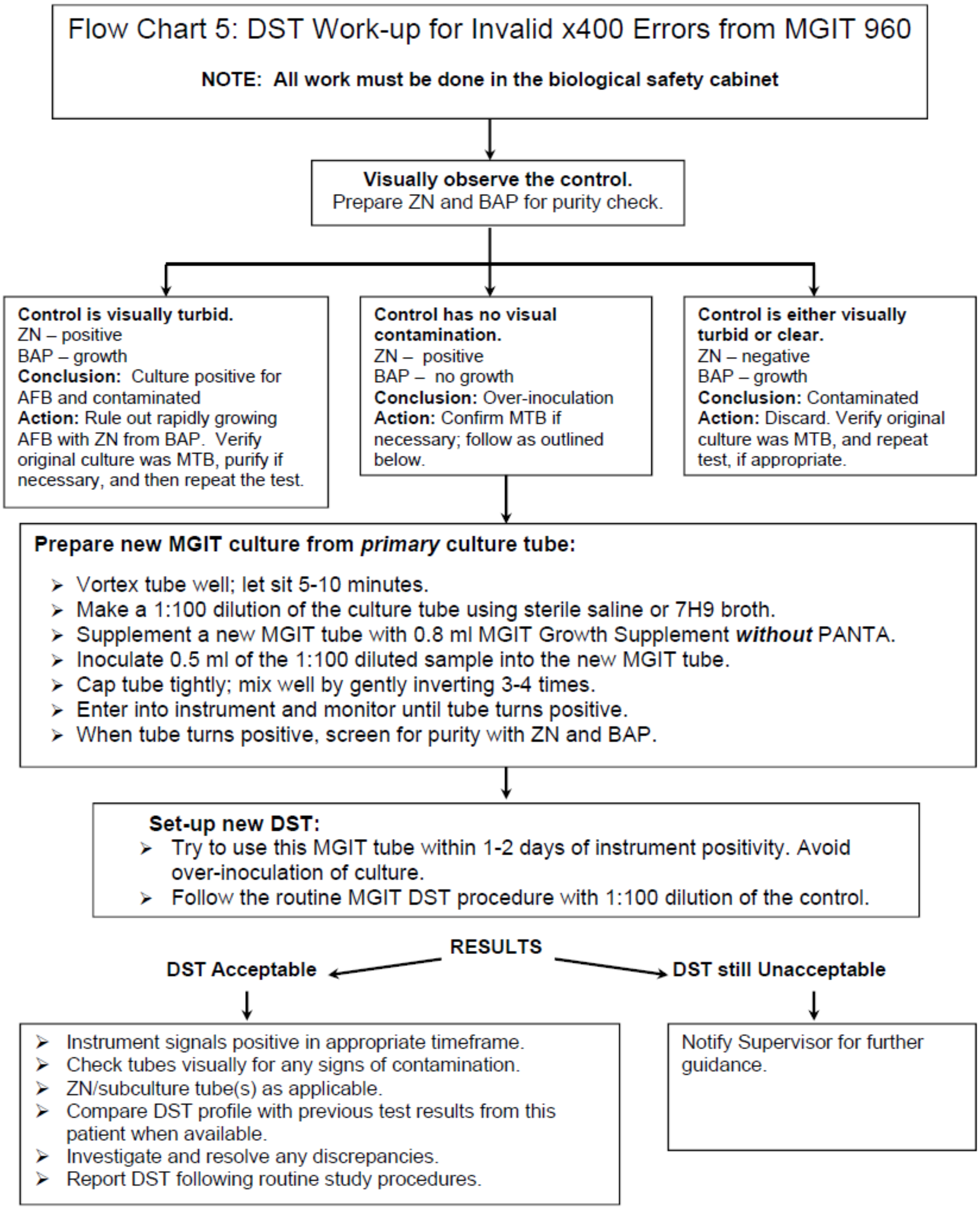
Set-up new DST:

- Vortex 3-5 day old culture tube well.
- Let tube sit 5-10 minutes to settle big clumps. Use the supernatant broth without disturbing the sediment.
- Using transfer pipette, aseptically add **0.5 ml** of MGIT culture broth to each *drug* tube; **DO NOT DILUTE** culture.
- Recap tubes tightly.
- Mix by inverting gently 3-4 times.
- Inoculate control tube(s) with 0.5 ml of diluted MGIT culture broth as per routine procedure; i.e., 1:10 dilution for PZA; 1:100 dilution for SIRE.
- Enter tubes into the instrument.
- Monitor until instrument signals as complete.

- Instrument signals positive in appropriate timeframe.
- Check tubes visually for any signs of contamination.
- ZN/subculture tube(s) as applicable.
- Compare DST profile with previous test results from this patient when available.
- Investigate and resolve any discrepancies.
- Report DST following routine study procedures.

If successful, note in internal records that this modified procedure was used to obtain DST results, and monitor frequency with which this procedure is required to obtain DST results.

APPENDIX 11: MGIT FLOW CHART 5 – DST WORKUP FOR INVALID X400 ERRORS FROM MGIT



Flow Chart 5: DST Work-up for Invalid x400 Errors from MGIT 960

NOTE: All work must be done in the biological safety cabinet

Visually observe the control.
Prepare ZN and BAP for purity check.

Control is visually turbid.
ZN – positive
BAP – growth
Conclusion: Culture positive for AFB and contaminated
Action: Rule out rapidly growing AFB with ZN from BAP. Verify original culture was MTB, purify if necessary, and then repeat the test.

Control has no visual contamination.
ZN – positive
BAP – no growth
Conclusion: Over-inoculation
Action: Confirm MTB if necessary; follow as outlined below.

Control is either visually turbid or clear.
ZN – negative
BAP – growth
Conclusion: Contaminated
Action: Discard. Verify original culture was MTB, and repeat test, if appropriate.

Prepare new MGIT culture from *primary* culture tube:

- Vortex tube well; let sit 5-10 minutes.
- Make a 1:100 dilution of the culture tube using sterile saline or 7H9 broth.
- Supplement a new MGIT tube with 0.8 ml MGIT Growth Supplement ***without*** PANTA.
- Inoculate 0.5 ml of the 1:100 diluted sample into the new MGIT tube.
- Cap tube tightly; mix well by gently inverting 3-4 times.
- Enter into instrument and monitor until tube turns positive.
- When tube turns positive, screen for purity with ZN and BAP.

Set-up new DST:

- Try to use this MGIT tube within 1-2 days of instrument positivity. Avoid over-inoculation of culture.
- Follow the routine MGIT DST procedure with 1:100 dilution of the control.

RESULTS

DST Acceptable

- Instrument signals positive in appropriate timeframe.
- Check tubes visually for any signs of contamination.
- ZN/subculture tube(s) as applicable.
- Compare DST profile with previous test results from this patient when available.
- Investigate and resolve any discrepancies.
- Report DST following routine study procedures.

DST still Unacceptable

Notify Supervisor for further guidance.

APPENDIX 12: QUANTITATIVE CULTURE WORKSHEET FOR 7H11S

TB Alliance Study NC-005

7H11S Quantitative Culture Worksheet

Lab ID:	

Date collection started:		**Time collection started:**		**Specimen Unique Number:**	
Sputum Volume collected:		**Sputum Volume Processed:**		**Patient Initials:**	
Date Inoculated:		**Technician initials:**			

ATTACH

	D7 **Date:** __________		**D14** **Date:** __________		**D21** **Date:** __________		**D28** **Date:** __________		**D35** **Date:** __________		**D42** **Date:** __________	
	Count Results		**Count Results**		**Count Results**		**Count Results**		**Count Results**		**Count Results**	
	Plate 1	**Plate 2**	**Plate 1**	**Plate 2**	**Plate 1**	**Plate 2**	**Plate 1**	**Plate 2**	**Plate 1**	**Plate 2**	**Plate 1**	**Plate 2**
10^{-0}												
10^{-0}												
10^{-1}												
10^{-1}												
10^{-2}												
10^{-2}												
10^{-3}												
10^{-3}												
10^{-4}												
10^{-4}												
10^{-5}												
10^{-5}												
	Tech initials:		**Tech initials:**		**Tech initials:**		**Tech initials:**		**Tech initials:**		**Tech initials:**	

Final Result

Dilution used for Colony Count (circle one):	10^{-0} 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5}	**Day used for Colony Count (circle one):**	D__

Comments:

Results Legend: **# of colonies; 0 = No growth; + = Growth (immature); TNTC = ≥ 200 col; CR/"n" = Contaminated but Readable/# of colonies; CU = Contaminated and Unreadable**

APPENDIX 13: QUANTITATIVE CULTURE WORKSHEET FOR 7H11S+C

TB Alliance Study NC-005

7H11S+ w/Charcoal Quantitative Culture Worksheet

Lab ID:	

ATTACH

Date collection started:		**Time collection started:**		**Specimen Unique Number:**			
Sputum Volume collected:		**Sputum Volume Processed:**		**Patient Initials:**			
Date Inoculated:		**Technician initials:**					

	D7 Date: ________		**D14** Date: ________		**D21** Date: ________		**D28** Date: ________		**D35** Date: ________		**D42** Date: ________	
	Count Results		Count Results		Count Results		Count Results		Count Results		Count Results	
	Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2
10^{-0}												
10^{-0}												
10^{-1}												
10^{-1}												
10^{-2}												
10^{-2}												
10^{-3}												
10^{-3}												
10^{-4}												
10^{-4}												
10^{-5}												
10^{-5}												
	Tech initials:		Tech initials:		Tech initials:		Tech initials:		Tech initials:		Tech initials:	

Final Result			
Dilution used for Colony Count (circle one):	10^{-0} 10^{-1} 10^{-2} 10^{-3} 10^{-4} 10^{-5}	**Day used for Colony Count (circle one):**	D__

Comments:

Results Legend: # of colonies; 0 = No growth; + = Growth (immature); TNTC = ≥ 200 col; CR/"n" = Contaminated but Readable/# of colonies; CU = Contaminated and Unreadable

APPENDIX 14: QUANTITATIVE CULTURE CONTAMINATION SPREADSHEET

QUANTITATIVE CULTURE BI-WEEKLY

Clinical Site #:

QUANTITATIVE CULTURE CONTAMINATION BI-WEEKLY SUMMARY

	Date of Bi-weekly Report DD/MM/YYYY	Number of Specimens Reported	Number of Specimens Contaminated

*Date of Bi-weekly Report = date that data are entered on this spreadsheet; should be the same day of each week, every other week

*Number of Specimens Reported = # specimens with completed CFU results reported during the previous 2 weeks

*Number of Specimens Contaminated = # specimens reported contaminated that had unreadable culture plates due to contaminant growth

QUANTITATIVE CULTURE BY SPECIMEN

Clinical Site #:

CONTAMINATED/UNREADABLE QUANTITATIVE CULTURES BY SPECIMEN

Date of Bi-weekly Report DD/MM/YYYY	Date of Inoculation DD/MM/YYYY	Patient Study ID #	Visit #	Specimen Lab Accession #	Date of Final Reading DD/MM/YYYY	NTM Present (Y/N)	MTB Present (Y/N)

Date of inoculation = date when plates were inoculated
Date of final reading = last date when plates were read
NTM present = colonies resembling NTM visable on plates
MTB present = colonies resembling MTB visable on plates

APPENDIX 15: TMTF NC005-004 Culture Isolate Transport Form

TASK
APPLIED SCIENCE

TMTF NC005-004
TEST METHOD TRANSPORT FORM NC005-004
Culture Isolate Transport Form

FORM NC005-004: Specimen Transfer Form - **Cultured Isolate on Solid Media (7H11S)**

This form should accompany each *M. tuberculosis* culture, isolated from a NC005 patient at the referral laboratory and sent to the CCTR laboratory for MIC analyses.

Laboratory Accession Number

This section should be completed by the referral laboratory. Once the specimen has been assigned to the Culture Isolate Transport Log (TMTF NC005-005), please make a copy of this form and email it to NC005@task.org.za. The original is to be sent with the courier after handover to the courier.

Referral Laboratory	

Subject Number __ __ __ __ __ __ **AND** **Treatment Number** __ __ __ __ __

Initials	
Date of birth (dd/mon/yyyy)	
Visit Day Number	see grid below
Visit Date	(dd/mon/yyyy) ☐☐ / ☐☐☐ / ☐☐☐☐
Culture Source	☐ D-2 / D-1 Screening specimen ☐ Non-conversion of withdrawal subject. (Last positive culture specimen) ☐ Subject is still culture positive at 8 weeks. ☐ Relapse participant. (Culture positive after culture conversion)
Date on which 7H11S agar plate was inoculated*	(dd/mon/yyyy) ☐☐ / ☐☐☐ / ☐☐☐☐
Culture Isolate Transport Log Number (Refer to TMTF NC005-005)	

D-2	D-1	D1	D3

D7	D14	D21	D28

D35	D42	D49	D56

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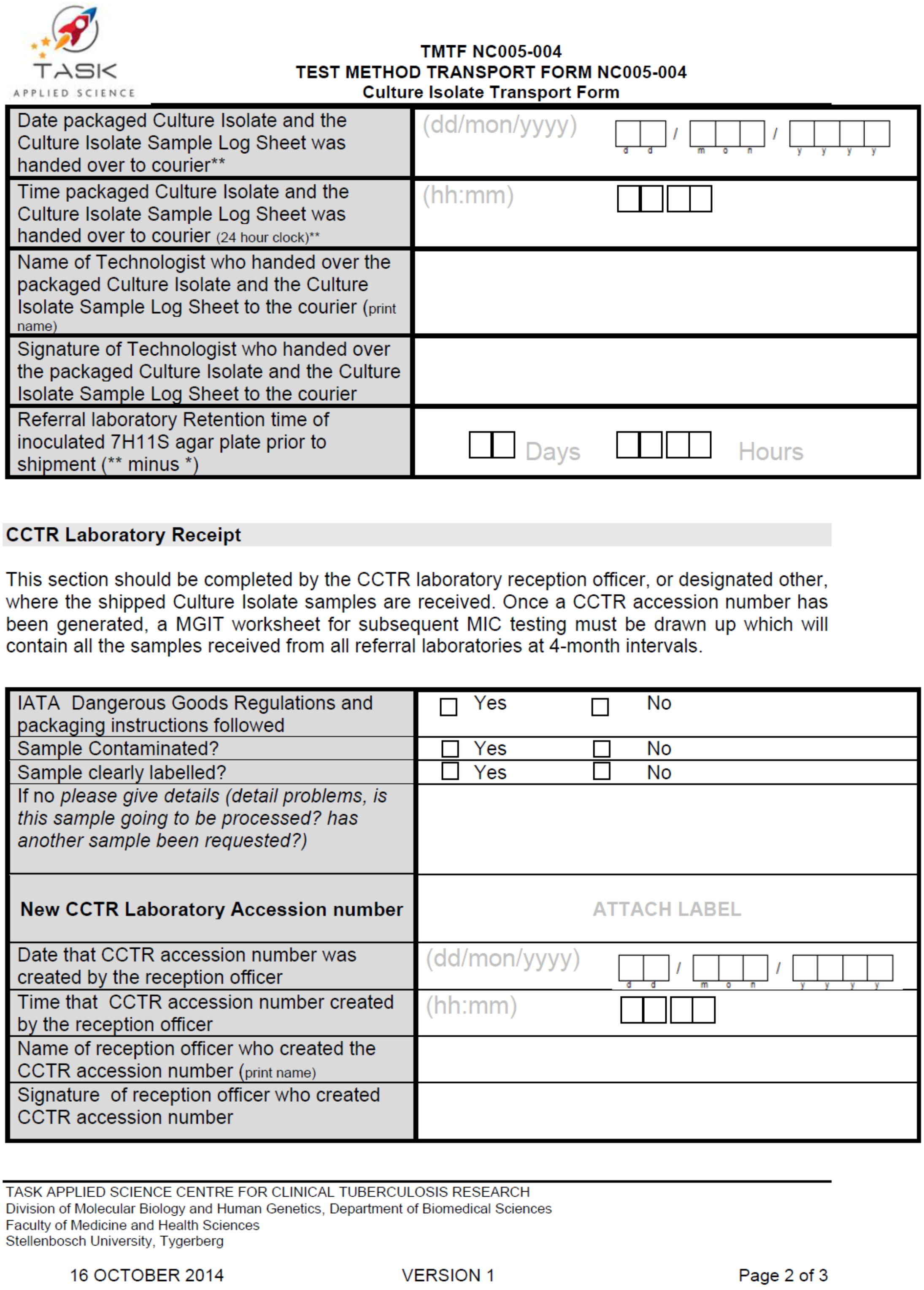

TASK
APPLIED SCIENCE

TMTF NC005-004
TEST METHOD TRANSPORT FORM NC005-004
Culture Isolate Transport Form

Date packaged Culture Isolate and the Culture Isolate Sample Log Sheet was handed over to courier**	(dd/mon/yyyy) ☐☐ / ☐☐☐ / ☐☐☐☐ d d m o n y y y y
Time packaged Culture Isolate and the Culture Isolate Sample Log Sheet was handed over to courier (24 hour clock)**	(hh:mm) ☐☐☐☐
Name of Technologist who handed over the packaged Culture Isolate and the Culture Isolate Sample Log Sheet to the courier (print name)	
Signature of Technologist who handed over the packaged Culture Isolate and the Culture Isolate Sample Log Sheet to the courier	
Referral laboratory Retention time of inoculated 7H11S agar plate prior to shipment (** minus *)	☐☐ Days ☐☐☐☐ Hours

CCTR Laboratory Receipt

This section should be completed by the CCTR laboratory reception officer, or designated other, where the shipped Culture Isolate samples are received. Once a CCTR accession number has been generated, a MGIT worksheet for subsequent MIC testing must be drawn up which will contain all the samples received from all referral laboratories at 4-month intervals.

IATA Dangerous Goods Regulations and packaging instructions followed	☐ Yes ☐ No
Sample Contaminated?	☐ Yes ☐ No
Sample clearly labelled?	☐ Yes ☐ No
If no *please give details (detail problems, is this sample going to be processed? has another sample been requested?)*	
New CCTR Laboratory Accession number	ATTACH LABEL
Date that CCTR accession number was created by the reception officer	(dd/mon/yyyy) ☐☐ / ☐☐☐ / ☐☐☐☐ d d m o n y y y y
Time that CCTR accession number created by the reception officer	(hh:mm) ☐☐☐☐
Name of reception officer who created the CCTR accession number (print name)	
Signature of reception officer who created CCTR accession number	

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TMTF NC005-004
TEST METHOD TRANSPORT FORM NC005-004
Culture Isolate Transport Form

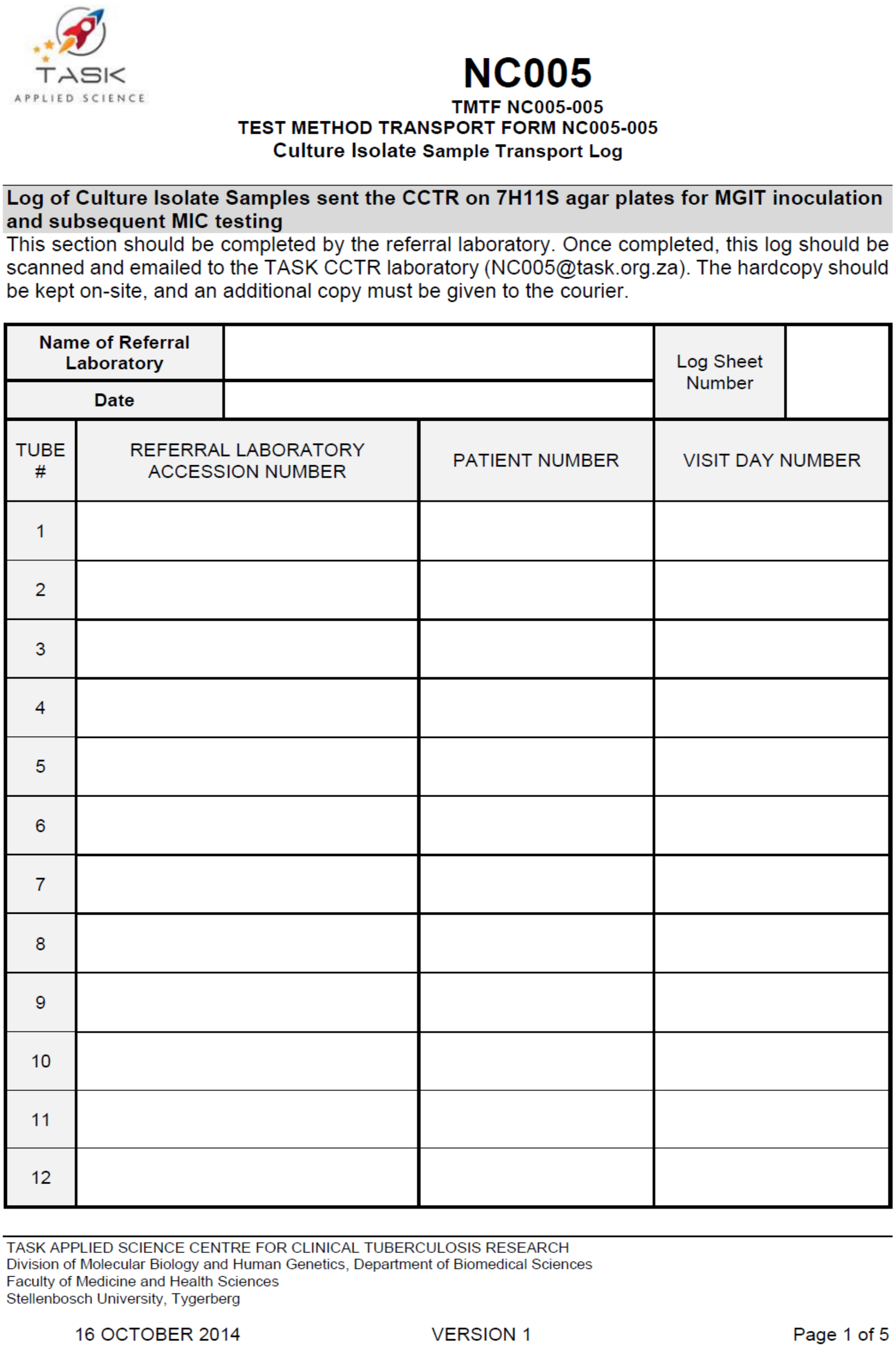
MGIT inoculation worksheet number (The worksheet to which this sample has been added to)	

DST Division of the CCTR Laboratory

This section should be completed by the DST supervisor, or designated other. The worksheet created by the reception officer will be used for MGIT inoculation for subsequent MIC determination.

Date that Culture Isolate samples and worksheet were received by the DST division	(dd/mon/yyyy) ☐☐ / ☐☐☐ / ☐☐☐☐
Time that Culture Isolate samples and worksheet were received by the DST division (24 hour clock)	(hh:mm) ☐☐☐☐
Name of DST division technologist who received the Culture Isolate samples and the worksheet (print name)	
Signature of DST division technologist who received the Culture Isolate samples and the worksheet	
Date that MGIT inoculation was done	(dd/mon/yyyy) ☐☐ / ☐☐☐ / ☐☐☐☐
Time that MGIT inoculation was done (24 hour clock)	(hh:mm) ☐☐☐☐
Name of DST division technologist who performed the MGIT inoculation (print name)	
Signature of DST division technologist who performed the MGIT inoculation.	

APPENDIX 16: TMTF NC005-005 Culture Isolate Transport Log



TASK
APPLIED SCIENCE

NC005

TMTF NC005-005
TEST METHOD TRANSPORT FORM NC005-005
Culture Isolate Sample Transport Log

Log of Culture Isolate Samples sent the CCTR on 7H11S agar plates for MGIT inoculation and subsequent MIC testing

This section should be completed by the referral laboratory. Once completed, this log should be scanned and emailed to the TASK CCTR laboratory (NC005@task.org.za). The hardcopy should be kept on-site, and an additional copy must be given to the courier.

Name of Referral Laboratory		Log Sheet Number	
Date			

TUBE #	REFERRAL LABORATORY ACCESSION NUMBER	PATIENT NUMBER	VISIT DAY NUMBER
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			

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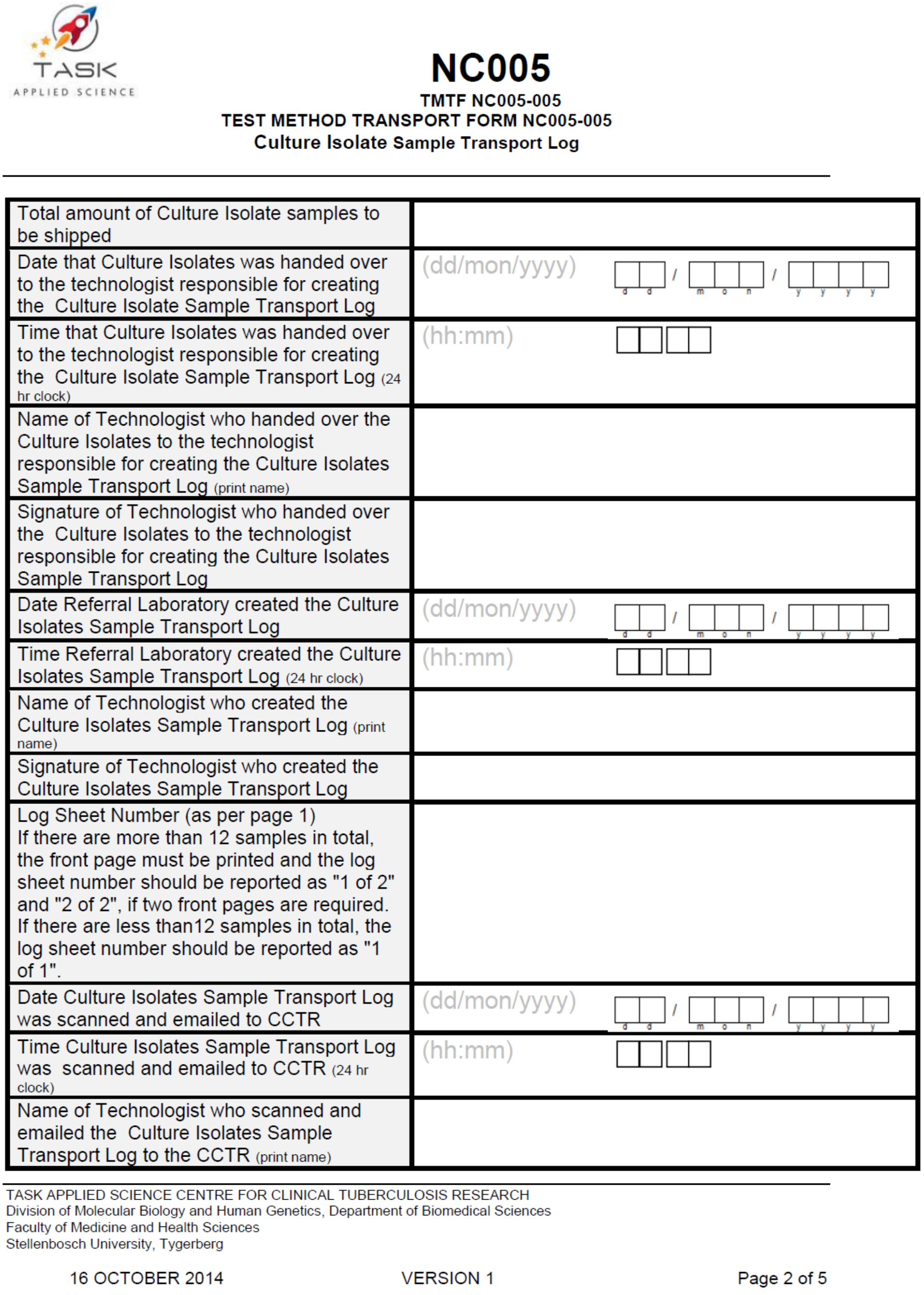

TASK
APPLIED SCIENCE

NC005

TMTF NC005-005
TEST METHOD TRANSPORT FORM NC005-005
Culture Isolate Sample Transport Log

Total amount of Culture Isolate samples to be shipped	
Date that Culture Isolates was handed over to the technologist responsible for creating the Culture Isolate Sample Transport Log	(dd/mon/yyyy) ☐☐ / ☐☐☐ / ☐☐☐☐
Time that Culture Isolates was handed over to the technologist responsible for creating the Culture Isolate Sample Transport Log (24 hr clock)	(hh:mm) ☐☐☐☐
Name of Technologist who handed over the Culture Isolates to the technologist responsible for creating the Culture Isolates Sample Transport Log (print name)	
Signature of Technologist who handed over the Culture Isolates to the technologist responsible for creating the Culture Isolates Sample Transport Log	
Date Referral Laboratory created the Culture Isolates Sample Transport Log	(dd/mon/yyyy) ☐☐ / ☐☐☐ / ☐☐☐☐
Time Referral Laboratory created the Culture Isolates Sample Transport Log (24 hr clock)	(hh:mm) ☐☐☐☐
Name of Technologist who created the Culture Isolates Sample Transport Log (print name)	
Signature of Technologist who created the Culture Isolates Sample Transport Log	
Log Sheet Number (as per page 1) If there are more than 12 samples in total, the front page must be printed and the log sheet number should be reported as "1 of 2" and "2 of 2", if two front pages are required. If there are less than12 samples in total, the log sheet number should be reported as "1 of 1".	
Date Culture Isolates Sample Transport Log was scanned and emailed to CCTR	(dd/mon/yyyy) ☐☐ / ☐☐☐ / ☐☐☐☐
Time Culture Isolates Sample Transport Log was scanned and emailed to CCTR (24 hr clock)	(hh:mm) ☐☐☐☐
Name of Technologist who scanned and emailed the Culture Isolates Sample Transport Log to the CCTR (print name)	

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TASK
APPLIED SCIENCE

NC005

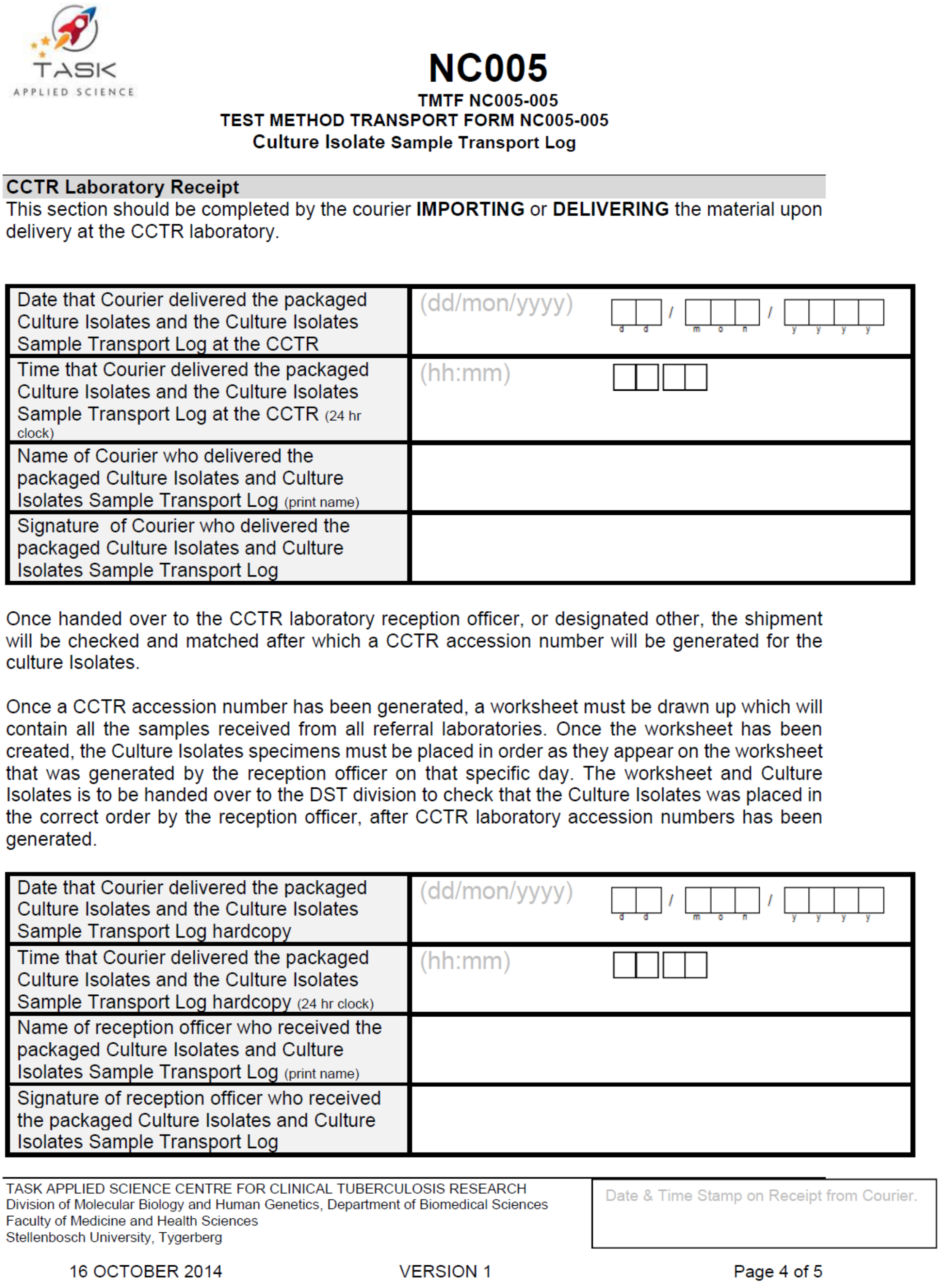
TMTF NC005-005
TEST METHOD TRANSPORT FORM NC005-005
Culture Isolate Sample Transport Log

Signature of Technologist who scanned and emailed the Culture Isolates Sample Transport Log to the CCTR	
Date that both the Culture Isolates and the Culture Isolates Sample Transport Log was handed over to courier	(dd/mon/yyyy) ☐☐ / ☐☐☐ / ☐☐☐☐ d d m o n y y y y
Time that both the Culture Isolates and the Culture Isolates Sample Transport Log was handed over to courier (24 hr clock)	(hh:mm) ☐☐☐☐
Name of Technologist who handed over the packaged Culture Isolates and Culture Isolates Sample Transport Log to the courier (print name)	
Signature of Technologist who handed over the packaged Culture Isolates and Culture Isolates Sample Transport Log to the courier	

Courier Services

This section must be completed by the Courier **EXPORTING** or **COLLECTING** the material from the Referral Laboratory.

Date that both the Culture Isolates and the Culture Isolates Sample Transport Log was received by the courier	(dd/mon/yyyy) ☐☐ / ☐☐☐ / ☐☐☐☐ d d m o n y y y y
Time that both the Culture Isolates and the Culture Isolates Sample Transport Log was handed over to courier (24 hr clock)	(hh:mm) ☐☐☐☐
Name of Courier who received the packaged Culture Isolates and Culture Isolates Sample Transport Log (print name)	
Signature of Courier who received the packaged Culture Isolates and Culture Isolates Sample Transport Log	



TASK
APPLIED SCIENCE

NC005

TMTF NC005-005
TEST METHOD TRANSPORT FORM NC005-005
Culture Isolate Sample Transport Log

CCTR Laboratory Receipt

This section should be completed by the courier **IMPORTING** or **DELIVERING** the material upon delivery at the CCTR laboratory.

Date that Courier delivered the packaged Culture Isolates and the Culture Isolates Sample Transport Log at the CCTR	(dd/mon/yyyy) ☐☐ / ☐☐☐ / ☐☐☐☐
Time that Courier delivered the packaged Culture Isolates and the Culture Isolates Sample Transport Log at the CCTR (24 hr clock)	(hh:mm) ☐☐☐☐
Name of Courier who delivered the packaged Culture Isolates and Culture Isolates Sample Transport Log (print name)	
Signature of Courier who delivered the packaged Culture Isolates and Culture Isolates Sample Transport Log	

Once handed over to the CCTR laboratory reception officer, or designated other, the shipment will be checked and matched after which a CCTR accession number will be generated for the culture Isolates.

Once a CCTR accession number has been generated, a worksheet must be drawn up which will contain all the samples received from all referral laboratories. Once the worksheet has been created, the Culture Isolates specimens must be placed in order as they appear on the worksheet that was generated by the reception officer on that specific day. The worksheet and Culture Isolates is to be handed over to the DST division to check that the Culture Isolates was placed in the correct order by the reception officer, after CCTR laboratory accession numbers has been generated.

Date that Courier delivered the packaged Culture Isolates and the Culture Isolates Sample Transport Log hardcopy	(dd/mon/yyyy) ☐☐ / ☐☐☐ / ☐☐☐☐
Time that Courier delivered the packaged Culture Isolates and the Culture Isolates Sample Transport Log hardcopy (24 hr clock)	(hh:mm) ☐☐☐☐
Name of reception officer who received the packaged Culture Isolates and Culture Isolates Sample Transport Log (print name)	
Signature of reception officer who received the packaged Culture Isolates and Culture Isolates Sample Transport Log	

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Date & Time Stamp on Receipt from Courier.

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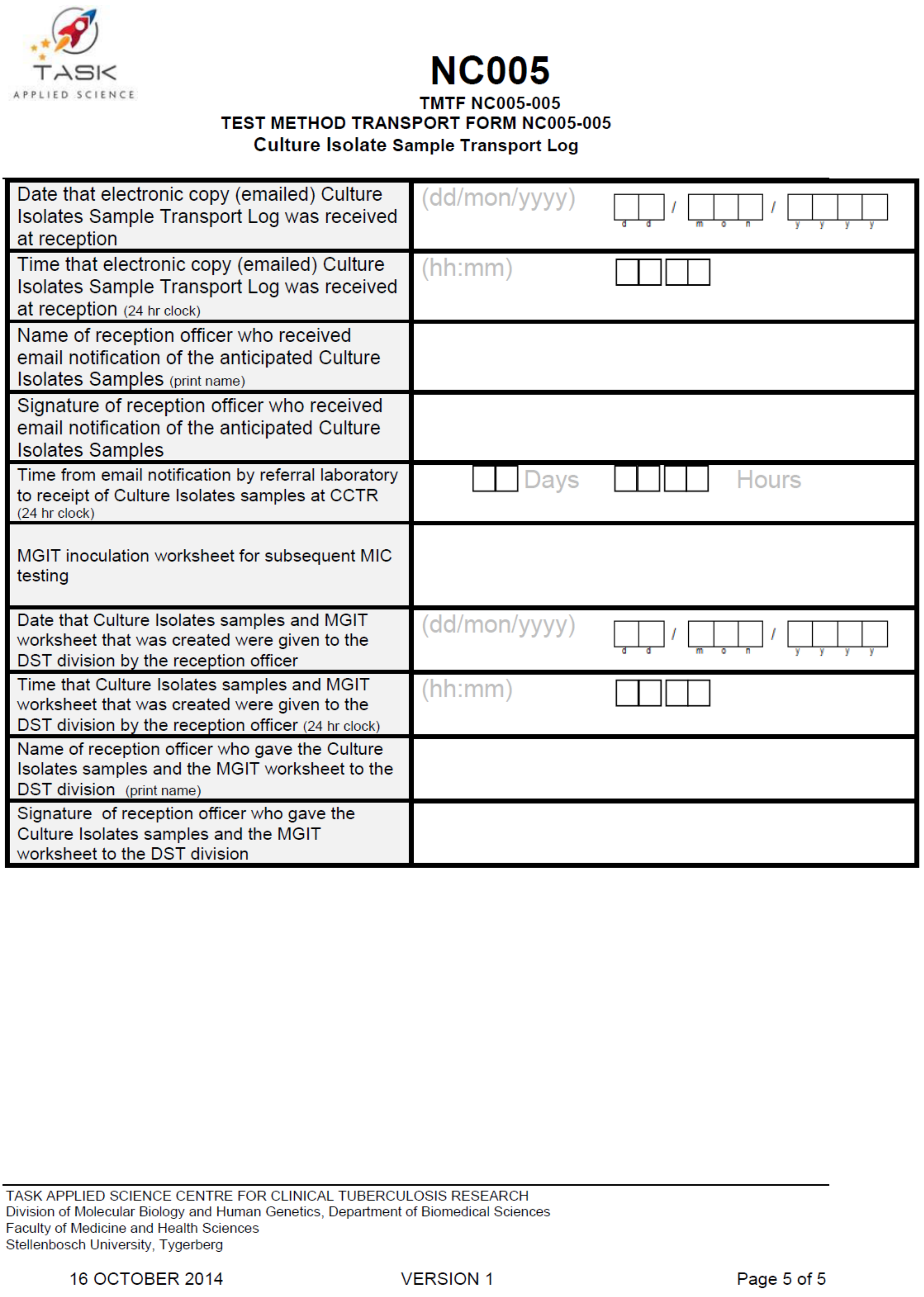

TASK APPLIED SCIENCE

NC005

TMTF NC005-005
TEST METHOD TRANSPORT FORM NC005-005
Culture Isolate Sample Transport Log

Date that electronic copy (emailed) Culture Isolates Sample Transport Log was received at reception	(dd/mon/yyyy) ☐☐ / ☐☐☐ / ☐☐☐☐
Time that electronic copy (emailed) Culture Isolates Sample Transport Log was received at reception (24 hr clock)	(hh:mm) ☐☐☐☐
Name of reception officer who received email notification of the anticipated Culture Isolates Samples (print name)	
Signature of reception officer who received email notification of the anticipated Culture Isolates Samples	
Time from email notification by referral laboratory to receipt of Culture Isolates samples at CCTR (24 hr clock)	☐☐ Days ☐☐☐☐ Hours
MGIT inoculation worksheet for subsequent MIC testing	
Date that Culture Isolates samples and MGIT worksheet that was created were given to the DST division by the reception officer	(dd/mon/yyyy) ☐☐ / ☐☐☐ / ☐☐☐☐
Time that Culture Isolates samples and MGIT worksheet that was created were given to the DST division by the reception officer (24 hr clock)	(hh:mm) ☐☐☐☐
Name of reception officer who gave the Culture Isolates samples and the MGIT worksheet to the DST division (print name)	
Signature of reception officer who gave the Culture Isolates samples and the MGIT worksheet to the DST division	

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ADDENDUM 1: SOP: MINIMUM INHIBITORY CONCENTRATION

(CCTR Reference Laboratory Only)

TM010 MINIMUM INHIBITORY CONCENTRATION (MIC)	
Issued by: CCTR Working Group	**Original date:** 11 July 2014
Signature:	**Version:** 1.0
Approved by: Dr. M. Barnard	**Date of Next Review:** 10 July 2015
Signature:	**Date withdrawn:**

PURPOSE

To determine the lowest concentration of a given drug that would inhibit 99% of a bacterial population of *Mycobacterium tuberculosis* in agar medium.

PRINCIPLE

The minimum inhibitory concentration (MIC) is defined as the lowest drug concentration that inhibits growth of more than 99% of a bacterial population of *Mycobacterium tuberculosis* on Middlebrook 7H10 agar medium within 21 days of incubation at 37°C. The proportion of bacilli resistant to a given drug is thus determined by comparing the number of colony forming units (CFUs) on a drug free control with those growing on drug containing medium.

PROCEDURE

Materials

Materials are listed under each sub-section of this document.

Methods

Methods are discussed under each sub-section of this document.

<u>Drug stock solutions</u>

1. Dissolve 0.1 g (100 mg) of drug powder (with a potency of 100% or 1 000 mg/g) in 10 ml of solvent. Use a solvent suitable for dissolving the particular test drug. This is the 10 000 µg/ml stock solution. If the potency of the drug is less than 100%, use the following calculation to obtain the correct weight of drug to be dissolved in 10 ml solvent:

   1 000 / drug potency x 100 = weight (mg)

2. Sterilise the stock solution by filtration through a filter with pore size 0.20 µm.
   NOTE: Be sure to use a filter with a PTFE membrane when dissolving the drug in DMSO and a hydrophilic PES membrane when using water as the solvent.
3. Prepare dilutions from the 10 000 µg/ml solution as needed for specific studies.
4. Store small volumes of sterile stock solution at -80° C in sterile cryogenic vials.

QUALITY CONTROL

Inclusion of a susceptible strain will give an indication of the accuracy of the test. Accuracy of results will be ensured by having two people do the interpretation of the cut-off point.

Frequency

A sensitive control will be included in every testing event

Controls

M tuberculosis H37Rv ATCC27294

Procedure

1. Process the control strain following the same procedure used for the test strains.
2. If the acceptance criteria are not met, review the validity of the results obtained from the specimens done in the same batch.
3. Repeat the test with fresh media and a new control.

Acceptance criteria

There should be >99% inhibition of the control strain on drug-containing media

Documentation

Record results on the laboratory source documents

References

1. Lee CN, Heifets LB. Determination of minimal inhibitory concentrations of antituberculosis drugs by radiometric and conventional methods. *Am Rev Respir Dis*. 1987 Aug;136(2):349-52.
2. Thomas Schön, Pontus Juréen, Christian G Giske, Erja Chryssanthou, Erik Sturegård, Jim Werngren, Gunnar Kahlmeter, Sven E Hoffner and Kristian A Ängeby. Evaluation of wild-type MIC distributions as a tool for determination of clinical breakpoints for *Mycobacterium tuberculosis*. *J Antimicrob Chemother*. 2009, 64:786-793.

Sub-sections to TM010

TM010A – Minimum inhibitory concentration of pretomanid (PA-824)
TM010B – Minimum inhibitory concentration of bedaquiline (TMC207)
TM010C – Minimum inhibitory concentration of moxifloxacin

TM010A: MINIMUM INHIBITORY CONCENTRATION (MIC) OF PRETOMANID

PURPOSE

To determine the lowest concentration of pretomanid (PA-824) which would inhibit 99% of a bacterial population of *Mycobacterium tuberculosis* in agar medium.

PRINCIPLE

M tuberculosis clinical isolates will be subjected to Middlebrook 7H10 agar medium which contains pretomanid (PA-824) at concentrations of 0.1, 0.2 and 0.4 µg/ml, respectively. Isolates which are found to be resistant to the suggested drug concentrations will be retested against increased drug concentrations.

PROCEDURE

Materials

Biological safety cabinet (BSC), Class II
Personal protective equipment (PPE) – PAPRs, gloves and shoe covers
Disinfectant
Sharps container for waste
Benchguard
Paper towels
Gloves
Biohazard bags
Vortex mixer
Middlebrook 7H10 agar base
OADC enrichment
Pretomanid powder
Solvent (Dimethyl sulfoxide [DMSO])
0.20 µm Filters (PTFE membrane for DMSO)
Sterile Tween/saline
Micropipette
Filtered pipette tips
13.5 ml disposable test tubes, sterile
Test tube rack for 13.5 ml tubes

Methods

<u>Inoculum medium</u>

1. Suspend 4.7 g dehydrated Middlebrook 7H9 broth base in 900 ml distilled water containing 0.05% (0.5 g) Tween 80 (Polysorbate 80).
2. Autoclave at 121°C for 10 minutes.
3. Let cool down to at least 40°C.
4. Add 100 ml Middlebrook OADC enrichment and mix well.
5. Dispense into 10 ml volumes into sterile 50 ml centrifuge tubes.
6. Store in a refrigerator at 2°-8°C until used.

Bacterial strains

1. Use two isolates from each patient for testing; one prior to treatment with bedaquiline and one obtained 24 hours after the last dosage of study drug was given.
2. *M tuberculosis* H37Rv (ATCC 27294) will be used as a sensitive control.

Inoculum

1. Prepare an inoculum by adding 0.5 ml of a fresh MGIT culture into 10 ml Middlebrook 7H9 broth.
2. Incubate at 37°C for 10 - 14 days.
3. Adjust each culture's turbidity to a McFarland standard #1 (≈10^8 cfu/ml).
4. Prepare 10^{-2} and 10^{-4} dilutions from this suspension in sterile 7H9 broth.
5. These two concentrations are used as the standard inoculum.

Drug stock solutions

1. Dissolve 0.1 g (100 mg) of pretomanid with potency of 100% in 10 ml DMSO. This is the 10 000 µg/ml stock solution.
2. Sterilise the stock solution by filtration through a 0.20 µm PTFE filter.
3. Store small volumes of sterile stock solution at -80°C in sterile cryogenic vials until needed.
4. Prepare working solutions as follows:
   - Dilute the 10 000 µg/ml stock solution 10-fold in filter sterilised DMSO to obtain a concentration of 1 000 µg/ml, i.e. 1 ml stock solution + 9 ml sterile DMSO = 1 000 µg/ml.
   - Dilute 1/5: 2 ml of 1 000 µg/ml solution + 8 ml sterile DMSO = 200 µg/ml.
   - Dilute ½: 2 ml of 200 µg/ml solution + 2 ml sterile DMSO = 100 µg/ml.
   - Dilute ½: 2 ml of 100 µg/ml solution + 2 ml sterile DMSO = 50 µg/ml.
   - Be sure to use filter sterilised DMSO for all dilution steps.

   Dilutions – Stock solution 10 000 µg/ml, dilute 1/10 = 1 000 µg/ml, dilute 1/5 = 200 µg/ml, dilute ½ = 100 µg/ml, dilute ½ = 50 µg/ml.

Agar medium

1. Prepare 200 ml volumes of Middlebrook 7H10 agar medium for each drug concentration and the drug-free medium as follows:
   - Suspend 3.8 g of dehydrated agar base in 180 ml distilled water containing 1 ml of glycerol.
   - Autoclave at 121°C for 10 minutes.
   - Let the agar cool to 56°-58°C in a water bath.
   - Add 20 ml of OADC enrichment and mix well.
   - See the table below to incorporate the correct volumes of pretomanid into the medium. Include drug-free medium for the controls.
   - Dispense medium into 90 mm 2-compartment Petri dishes by pouring 10 ml into each segment.
   - Allow agar to solidify at room temperature without exposure to daylight.
2. If needed, volumes of 1 litre (1 000 ml) of medium may be prepared.

Incorporating pretomanid into 200 ml (or 1 000 ml) 7H10 agar medium

Final drug concentration (µg/ml) in 7H10 agar medium	**Drug solutions (µg/ml) prepared from 10 000 µg/ml stock solution**	**Volume of drug solution to add to 200 ml (1 000 ml) medium**
0	0	400 µl (2 ml) DMSO
0.4	200	400 µl (2 ml)
0.2	100	400 µl (2 ml)
0.1	50	400 µl (2 ml)
0.05	25	400 µl (2 ml)
0.025	12.5	400 µl (2 ml)

Inoculation of 7H10 agar plates

1. Inoculate 100 µl of standard inoculum in duplicate onto drug containing and drug-free medium.
2. The control inoculum (10^{-4} dilution) is only plated onto drug-free medium.

Reading and interpretation of results

Read the MIC endpoint as the lowest concentration of antibiotic at which there is no visible growth or where the proportion of bacilli resistant to the specific drug concentration is less than 1% of the total population.

Recording of results

No colonies	0 or negative
Less than 200 colonies	actual number of colonies
200-500 colonies	1+
500-1 000 colonies	2+
>1 000 colonies	3+
Confluent growth	4+

Higher concentrations of pretomanid

Prepare working solutions as follows:

- Dilute the 10 000 µg/ml stock solution 5-fold in filter sterilised DMSO to obtain a concentration of 2 000 µg/ml, i.e. 2 ml stock solution + 8 ml sterile DMSO = 2 000 µg/ml.
- Dilute 1/1.25: 2 ml of 2 000 µg/ml solution + 0.5 ml sterile DMSO = 1 600 µg/ml.
- Dilute ½: 2 ml of 1 600 µg/ml solution + 2 ml sterile DMSO = 800 µg/ml.
- Dilute ½: 2 ml of 800 µg/ml solution + 2 ml sterile DMSO = 400 µg/ml.
- Make sure to use filter sterilised DMSO for all dilution steps.

Dilutions – Stock solution 10 000 µg/ml, dilute 1/5 = 2 000 µg/ml, dilute 1/1.25 = 1 600 µg/ml, dilute ½ = 800 µg/ml, dilute ½ = 400 µg/ml.

Incorporating pretomanid into 200 ml (or 1 000 ml) 7H10 agar medium

Final drug concentration (µg/ml) in 7H10 agar medium	**Drug solutions (µg/ml) prepared from 10 000 µg/ml stock solution**	**Volume of drug solution to add to 200 ml (1 000 ml) medium**
0	0	400 µl (2 ml) DMSO
3.2	1 600	400 µl (2 ml)
1.6	800	400 µl (2 ml)
0.8	400	400 µl (2 ml)

References

1. Sandeep Tyagi, E Nuermberger, T Yoshimatsu, K Williams, I Rosenthal, N Lounis, W Bishai, and J Grosset. Bactericidal activity of the nitroimidazopyran PA-824 in a murine model of tuberculosis. *Antimicrob Agents Chemother*. 2005 June; 49(6):2289-2293.
2. Anne J Lenaerts, Veronica Gruppo, Karen S Marietta, Christine M Johnson, Diane K Driscoll, Nicholas M Tompkins, Jerry D Rose, Robert C Reynolds, and Ian M Orme. Preclinical Testing of the nitroimidazopyran PA-824 for activity against *Mycobacterium tuberculosis* in a series of in vitro and in vivo models. *Antimicrob Agents Chemother*. 2005 June; 49(6):2294–2301.

TM010B: MINIMUM INHIBITORY CONCENTRATION (MIC) OF BEDAQUILINE

PURPOSE

To determine the lowest concentration of bedaquiline (TMC207) which would inhibit 99% of a bacterial population of *Mycobacterium tuberculosis* in agar medium.

PRINCIPLE

M tuberculosis clinical isolates will be subjected to Middlebrook 7H10 agar medium which contains bedaquiline (TMC207) at concentrations of 0.03, 0.06 and 0.12 µg/ml, respectively. Isolates which are found to be resistant to the suggested drug concentrations will be retested against increased drug concentrations.

PROCEDURE

Materials

Biological safety cabinet (BSC), Class II
Personal protective equipment (PPE) – PAPRs, gloves and shoe covers
Disinfectant
Screw capped bottle for waste
Benchguard
Paper towels
Biohazard bags
Vortex mixer
Middlebrook 7H10 agar base
OADC enrichment
Bedaquiline powder
Solvent (Dimethyl sulfoxide [DMSO])
0.20 µm Filters (PTFE membrane for DMSO)
Sterile Tween/saline
Micropipette
Filtered pipette tips
13.5 ml disposable test tubes, sterile
Test tube rack for 13.5 ml tubes

Methods

<u>Inoculum medium</u>

1. Suspend 4.7 g dehydrated Middlebrook 7H9 broth base in 900 ml distilled water containing 0.05% (0.5 g) Tween 80 (Polysorbate 80).
2. Autoclave at 121°C for 10 minutes.
3. Let cool down to at least 40°C.
4. Add 100 ml Middlebrook OADC enrichment and mix well.
5. Dispense into 10 ml volumes into sterile 50 ml centrifuge tubes.
6. Store in a refrigerator at 2°-8°C until used.

Bacterial strains

1. Use two isolates from each patient for testing; one prior to treatment with bedaquiline and one obtained 24 hours after the last dosage of study drug was given.
2. *M tuberculosis* H37Rv (ATCC 27294) will be used as a sensitive control.

Inoculum

1. Prepare an inoculum by adding 0.5 ml of a fresh MGIT culture into 10 ml Middlebrook 7H9 broth.
2. Incubate at 37°C for 10 - 14 days.
3. Adjust each culture's turbidity to a McFarland standard #1 ($\approx 10^8$ cfu/ml).
4. Prepare 10^{-2} and 10^{-4} dilutions from this suspension in sterile 7H9 broth.
5. These two concentrations are used as the standard inoculum.

Drug stock solutions

1. Dissolve 0.1 g (100 mg) of bedaquiline with potency of 100% in 10 ml DMSO. This is the 10 000 µg/ml stock solution.
2. Sterilise the stock solution by filtration through a 0.20 µm PTFE filter.
3. Store small volumes of sterile stock solution at -80°C in sterile cryogenic vials until needed.
4. Prepare working solutions as follows:
    - Dilute the 10 000 µg/ml stock solution 10-fold in filter sterilised DMSO to obtain a concentration of 1 000 µg/ml, i.e. 1 ml stock solution + 9 ml sterile DMSO = 1 000 µg/ml.
    - Dilute 1/10: 1 ml of 1 000 µg/ml solution + 9 ml sterile DMSO = 100 µg/ml.
    - Dilute ½: 2 ml of 100 µg/ml solution + 2 ml sterile DMSO = 50 µg/ml.
    - Dilute ½: 2 ml of 50 µg/ml solution + 2 ml sterile DMSO = 25 µg/ml.
    - Be sure to use filter sterilised DMSO for all dilution steps.

Dilutions – Stock solution 10 000 µg/ml, dilute 1/10 = 1 000 µg/ml, dilute 1/10 = 100 µg/ml, dilute ½ = 50 µg/ml, dilute ½ = 25 µg/ml.

Agar medium

1. Prepare 200 ml volumes of Middlebrook 7H10 agar medium for each drug concentration and the drug-free medium as follows:
    - Suspend 3.8 g of dehydrated agar base in 180 ml distilled water containing 1 ml of glycerol.
    - Autoclave at 121°C for 10 minutes.
    - Let the agar cool to 56°-58°C in a water bath.
    - Add 20 ml of OADC enrichment and mix well.
    - See the table below to incorporate the correct volumes of bedaquiline into the medium. Include drug-free medium for the controls.
    - Dispense medium into 90 mm 2-compartment Petri dishes by pouring 10 ml into each segment.
    - Allow agar to solidify at room temperature without exposure to daylight.
2. If needed, volumes of 1 litre (1 000 ml) of medium may be prepared.

Incorporating bedaquiline into 200 ml (or 1 000 ml) 7H10 agar medium

Final drug concentration (µg/ml) in 7H10 agar medium	**Drug solutions (µg/ml) prepared from 10 000 µg/ml stock solution**	**Volume of drug solution to add to 200 ml (1 000 ml) medium**
0	0	240 µl (1.2 ml) DMSO
0.12	100	240 µl (1.2 ml)
0.06	50	240 µl (1.2 ml)
0.03	25	240 µl (1.2 ml)

Inoculation of 7H10 agar plates

1. Inoculate 100 µl of standard inoculum in duplicate onto drug containing and drug-free medium.
2. The control inoculum (10^{-4} dilution) is only plated onto drug-free medium.

Reading and interpretation of results

Read the MIC endpoint as the lowest concentration of antibiotic at which there is no visible growth or where the proportion of bacilli resistant to the specific drug concentration is less than 1% of the total population.

Recording of results

No colonies	0 or negative
Less than 200 colonies	actual number of colonies
200-500 colonies	1+
500-1 000 colonies	2+
>1 000 colonies	3+
Confluent growth	4+

Higher concentrations of bedaquiline

Prepare working solutions as follows:
- Dilute the 10 000 µg/ml stock solution 10-fold in filter sterilised DMSO to obtain a concentration of 1 000 µg/ml, i.e. 1 ml stock solution + 9 ml sterile DMSO = 1 000 µg/ml.
- Dilute 1/1.25: 2 ml of 1 000 µg/ml solution + 0.5 ml sterile DMSO = 800 µg/ml.
- Dilute ½: 2 ml of 800 µg/ml solution + 2 ml sterile DMSO = 400 µg/ml.
- Dilute ½: 2 ml of 400 µg/ml solution + 2 ml sterile DMSO = 200 µg/ml.
- Make sure to use filter sterilised DMSO for all dilution steps.

Dilutions – Stock solution 10 000 µg/ml, dilute 1/10 = 1 000 µg/ml, dilute 1/1.25 = 800 µg/ml, dilute ½ = 400 µg/ml, dilute ½ = 200 µg/ml.

Incorporating bedaquiline into 200 ml (or 1 000 ml) 7H10 agar medium

Final drug concentration (µg/ml) in 7H10 agar medium	**Drug solutions (µg/ml) prepared from 10 000 µg/ml stock solution**	**Volume of drug solution to add to 200 ml (1 000 ml) medium**
0	0	240 µl (1.2 ml) DMSO
0.96	800	240 µl (1.2 ml)
0.48	400	240 µl (1.2 ml)
0.24	200	240 µl (1.2 ml)

References

1. E Huitric, P Verhasselt, A Koul, K Andries, S Hoffner, and D I Andersson. Rates and mechanisms of resistance development in *Mycobacterium tuberculosis* to a novel diarylquinoline ATP synthase inhibitor. *Antimicrob Agents Chemother*. 2010 March; 54(3):1022–1028.

TM010C: MINIMUM INHIBITORY CONCENTRATION (MIC) OF MOXIFLOXACIN

PURPOSE

To determine the lowest concentration of moxifloxacin which would inhibit 99% of a bacterial population of *Mycobacterium tuberculosis* in agar medium.

PRINCIPLE

M tuberculosis clinical isolates will be subjected to Middlebrook 7H10 agar medium which contains moxifloxacin at concentrations of 0.125, 0.25 and 0.5 µg/ml, respectively. Isolates which are found to be resistant to the suggested drug concentrations will be re-tested against increased drug concentrations.

PROCEDURE

Materials

Biological safety cabinet (BSC), Class II
Personal protective equipment (PPE) – PAPRs, gloves and shoe covers
Disinfectant
Screw capped bottle for waste
Benchguard
Paper towels
Biohazard bags
Vortex mixer
Middlebrook 7H10 agar base
OADC enrichment
Moxifloxacin powder
Solvent (Sterile H_2O)
0.20 µm Filters (hydrophilic PES membrane for water)
Sterile Tween/saline
Micropipette
Filtered pipette tips
13.5 ml disposable test tubes, sterile
Test tube rack for 13.5 ml tubes

Methods

<u>Inoculum medium</u>

1. Suspend 4.7 g dehydrated Middlebrook 7H9 broth base in 900 ml distilled water containing 0.05% (0.5 g) Tween 80 (Polysorbate 80).
2. Autoclave at 121°C for 10 minutes.
3. Let cool down to at least 40°C.
4. Add 100 ml Middlebrook OADC enrichment and mix well.
5. Dispense into 10 ml volumes into sterile 50 ml centrifuge tubes.
6. Store in a refrigerator at 2°-8°C until used.

Bacterial strains

1. Use two isolates from each patient for testing; one prior to treatment with moxifloxacin and one obtained 24 hours after the last dosage of study drug was given.
2. *M tuberculosis* H37Rv (ATCC 27294) will be used as a sensitive control.

Inoculum

1. Prepare an inoculum by adding 0.5 ml of a fresh MGIT culture into 10 ml Middlebrook 7H9 broth.
2. Incubate at 37°C for 10 - 14 days.
3. Adjust each culture's turbidity to a McFarland standard #1 ($\approx 10^8$ cfu/ml).
4. Prepare 10^{-2} and 10^{-4} dilutions from this suspension in sterile 7H9 broth.
5. These 2 concentrations are used as the standard inoculum.

Drug stock solutions

1. Dissolve 0.05 g (50 mg) of moxifloxacin with potency of 100% in 5 ml distilled water. This is the 10 000 µg/ml stock solution.
2. Sterilise the stock solution by filtration through a 0.20 µm hydrophilic PES filter.
3. Store small volumes of sterile stock solution at -80°C in sterile cryogenic vials until needed.
4. Prepare working solutions as follows:
    - Dilute the 10 000 µg/ml stock solution 10-fold in sterile distilled H_2O to obtain a concentration of 1 000 µg/ml, i.e. 1 ml stock solution + 9 ml sterile distilled H_2O = 1 000 µg/ml.
    - Dilute ¼: 2 ml of 1 000 µg/ml solution + 6 ml sterile distilled H_2O = 250 µg/ml.
    - Dilute ½: 2 ml of 250 µg/ml solution + 2 ml sterile distilled H_2O = 125 µg/ml.
    - Dilute ½: 2 ml of 125 µg/ml solution + 2 ml sterile distilled H_2O = 62.5 µg/ml.
    - Make sure to use sterilised distilled H_2O for all dilution steps.

Dilutions – Stock solution 10 000 µg/ml, dilute 1/10 = 1 000 µg/ml, dilute 1/4 = 250 µg/ml, dilute ½ = 125 µg/ml, dilute ½ = 62.5 µg/ml.

Agar medium

1. Prepare 200 ml volumes of Middlebrook 7H10 agar medium for each drug concentration and the drug-free medium as follows:
    - Suspend 3.8 g of dehydrated agar base in 180 ml distilled water containing 1 ml of glycerol.
    - Autoclave at 121°C for 10 minutes.
    - Let the agar cool to 56°-58°C in a water bath.
    - Add 20 ml of OADC enrichment and mix well.
    - See the table below to incorporate the correct volumes of moxifloxacin into the medium. Include drug-free medium for the controls.
    - Dispense medium into 90 mm 2-compartment Petri dishes by pouring 10 ml into each segment.
    - Allow agar to solidify at room temperature without exposure to daylight.
2. If needed, volumes of 1 litre (1 000 ml) of medium may be prepared.

Incorporating moxifloxacin into 200 ml (or 1 000 ml) 7H10 agar medium

Final drug concentration (µg/ml) in 7H10 agar medium	**Drug solutions (µg/ml) prepared from 10 000 µg/ml stock solution**	**Volume of drug solution to add to 200 ml (1 000 ml) medium**
0	0	400 µl (2 ml) distilled H_2O
0.5	250	400 µl (2 ml)
0.25	125	400 µl (2 ml)
0.125	62.5	400 µl (2 ml)

Inoculation of 7H10 agar plates

1. Inoculate 100 µl of standard inoculum in duplicate onto drug containing and drug-free medium.
2. The control inoculum (10^{-4} dilution) is only plated onto drug-free medium.

Reading and interpretation of results

Read the MIC endpoint as the lowest concentration of antibiotic at which there is no visible growth or where the proportion of bacilli resistant to the specific drug concentration is less than 1% of the total population.

Recording of results

No colonies	0 or negative
Less than 200 colonies	actual number of colonies
200-500 colonies	1+
500-1 000 colonies	2+
>1 000 colonies	3+
Confluent growth	4+

Higher concentrations of moxifloxacin

Prepare working solutions as follows:

- Dilute the 10 000 µg/ml stock solution 5-fold in sterile distilled H_2O to obtain a concentration of 2 000 µg/ml, i.e. 2 ml stock solution + 8 ml sterile distilled H_2O = 2 000 µg/ml.
- Dilute ½: 2 ml of 2 000 µg/ml solution + 2 ml sterile distilled H_2O = 1000 µg/ml.
- Dilute ½: 2 ml of 1000 µg/ml solution + 2 ml sterile distilled H_2O = 500 µg/ml.
- Make sure to use sterilised distilled H_2O for all dilution steps.

Dilutions – Stock solution 10 000 µg/ml, dilute 1/5 = 2 000 µg/ml, dilute ½ = 1000 µg/ml, dilute ½ = 500 µg/ml.

Incorporating moxifloxacin into 200 ml (or 1 000 ml) 7H10 agar medium

Final drug concentration (µg/ml) in 7H10 agar medium	**Drug solutions (µg/ml) prepared from 10 000 µg/ml stock solution**	**Volume of drug solution to add to 200 ml (1 000 ml) medium**
0	0	400 µl (2 ml) sterile distilled H_2O
4.0	2000	400 µl (2 ml)
2.0	1000	400 µl (2 ml)
1.0	500	400 µl (2 ml)

References

1. SH Gillespie and O Billington. Activity of moxifloxacin against mycobacteria. *J Antimicrob Chemother*. 1999, 44:393-395.
2. KA Ängeby, P Jureen, CG Giske, E Chryssanthou, E Sturegård, M Nordvall, AG Johansson, J Werngren, G Kahlmeter, SE Hoffner and T Schön. Wild-type MIC distributions of four fluoroquinolones active against *Mycobacterium tuberculosis* in relation to current critical concentrations and available pharmacokinetic and pharmacodynamic data. *J Antimicrob Chemother*. 2010, 65:946-952.

ADDENDUM 2: SOP: pncA SEQUENCING

(CCTR Reference Laboratory Only)

The following SOPs should be used by the Reference Laboratory to perform pncA sequencing as specified in the Study Protocol (Refer to the Flow Chart in the beginning of the Manual).

i. CCTR TM040: Amplification of the pncA gene
ii. CCTR TM041: HRM Analysis of the pncA gene
iii. CCTR TM042: PCR Product Cleanup Post HRM Analysis
iv. CCTR TM043: DNA Sequencing of PCR Amplicons using Sanger Sequensing Method
v. CCTR TM044: Dye Terminator Removal Post DNA Sequencing
vi. CCTR TM045: Capillary Electrophoresis of DNA Sequencing Products
vii. CCTR TM046: DNA Sequencing Analysis

i. CCTR TM040: AMPLIFICATION OF THE PNCA GENE

TM040 Amplification of the *pncA* gene	
Issued by: TB Working Group	**Date issued:** 14 July 2014
Signature:	**Version:** 1
Approved by: Dr. M. Barnard	**Date of Next Review:** 15 July 2015
Signature:	**Date withdrawn:** …………………………………….

PURPOSE

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

PROCEDURE

Due to the sensitivity of the polymerase chain reaction (PCR) it is required that different rooms are used for different procedures as the procedures in itself are incompatible (Refer to GL040 : Prevention of PCR contamination).

MASTER-MIX PREPARATION AREA

Equipment & Materials

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

Table 1. Gene, primers and target DNA sequences for genotypic Pyrazinamide drug susceptibility testing (DST).

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

4. RNA and DNA free water
5. HotStart Taq DNA polymerase (Qiagen)
6. PCR buffers
7. dNTP stock solutions
8. Intercalating agent (Syto-9)
9. Appropriate filter tips for the pipettes

10. 1.5 ml Eppendorf screw-cap tubes with O-ring
11. 1.5 ml tube racks
12. 0.2 ml PCR tubes
13. PCR tube racks
14. Disposable gloves (Small, Medium, Large)
15. Dedicated laboratory coat

Procedure

1. The master mix for a batch of 24 reactions will be prepared in a 1.5ml screw-cap tube with O-ring. The master mix reaction setup is shown in Table 2, and each reagent should be added into the 1.5ml screw-cap tube sequentially.

Table 2. *pncA* Master Mix Recipe

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

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

TEMPLATE (DNA) ADDITION AREA

Equipment & Materials

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

Procedure

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

4. Master mix Negative control (MNC) – add 2.5 µl of water (in place of DNA) after the ENC. This step is done to detect PCR reagent contamination.

AMPLIFICATION AREA

Equipment & Materials

1. Thermocycler (Veritas)
2. Foil

Procedure

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

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

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

DETECTION AREA

Amplification is confirmed by quantification of Amplification by High Resolution Melt (HRM) analysis by fluorescence detection. Please refer to SOP TM041 “Confirmation of pncA amplification by High Resolution Melt (HRM) Curve Analysis”.

LIMITATIONS

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

ii. CCTR TM041: HRM ANALYSIS OF THE PNCA GENE

TM041 **Confirmation of *pncA* amplification by High Resolution Melt (HRM) Curve Analysis**	
Issued by: TB Working Group	**Date issued:** 14 July 2014
Signature:	**Version:** 1
Approved by: Dr. M. Barnard	**Date of Next Review:** 15 July 2015
Signature: MM Barnard	**Date withdrawn:** …………………………………….

PURPOSE

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

Equipment & Materials

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

Procedures

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

HRM PCR

RotorGene 6000:

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

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

Bio-Rad CFX:

3. Keep samples covered in foil at all possible times, until loading them into the CFX

4. Load the samples into the CFX and conduct a melt at the following settings:

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

HRM Curve Analysis

<u>Positive *pncA* melt curve</u>

Below are typical melt profiles of the *pncA* PCR product. Amplification is confirmed by the detection of a distinct fluorescent melt curve signature consisting of two maxima (±92°C and ±93.5°C, respectively) using high-resolution melt (HRM) analysis (Fig. 2). Figure 3 represents multiplexed HRM curves.

Fig. 2: The melt curve of the normalised melt curve depicted in Fig.1

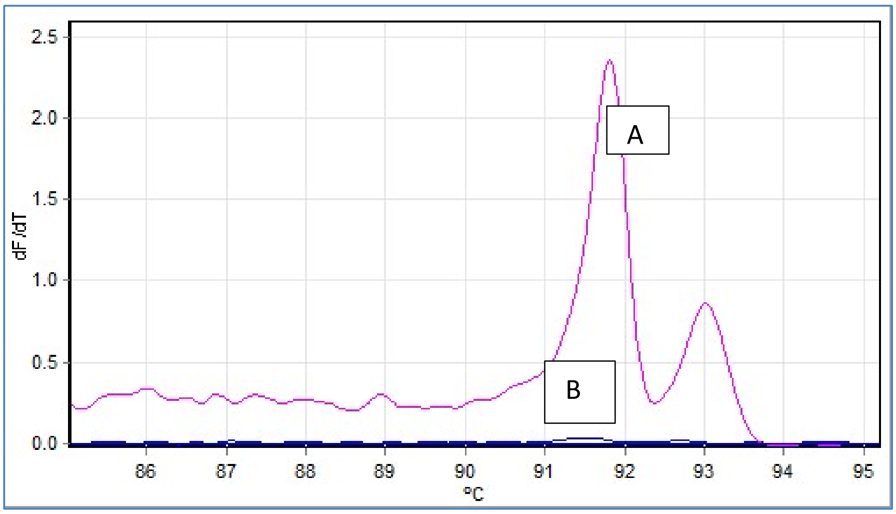
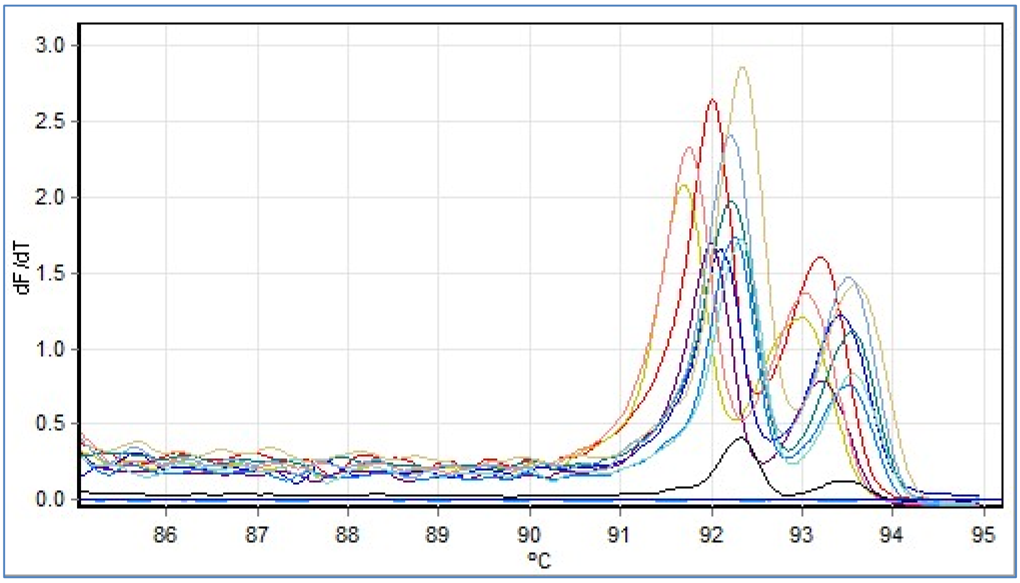


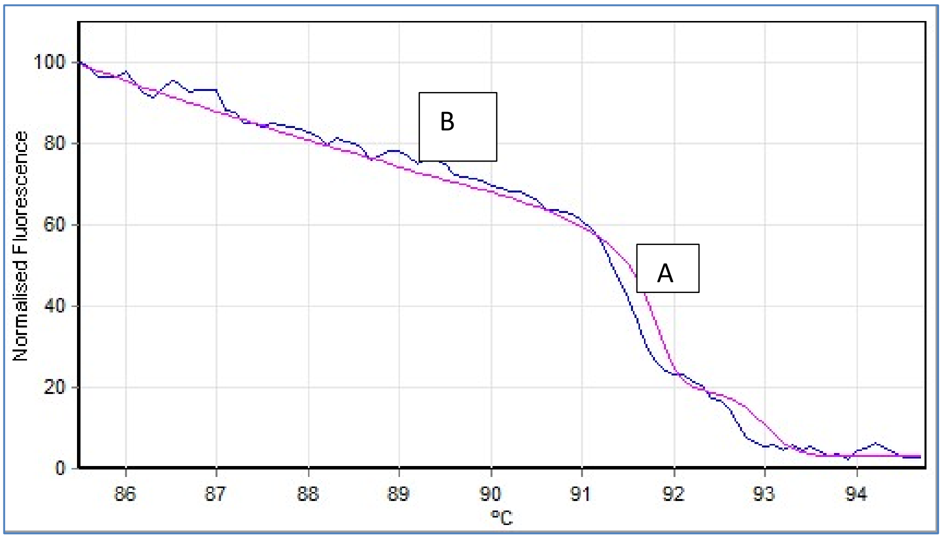
Fig. 3: A Fluorescence melt curve of positive *pncA* PCR products.



<u>Negative *pncA* melt curve</u>

1. In the case of an HRM revealing the absence of a PCR product, that sample is documented as not containing MTB, and therefore not sequenced.
2. Where the 1st peak of the pncA melt curve (dF/dT) is lower than 0.2 dF/dT, it is usually taken as negative, and not sent for sequencing. Sometimes it is ‘border line’ and is sent for confirmatory pncA sequencing.
3. The Normalised Fluorescence melt curve should also be taken into consideration. If the melt is not smooth, it is also taken as a negative PCR product (Fig. 1).

Fig.1: A normalised melt curve, representing both a positive (A) and a negative (B) *pncA* melt product.



GENOTYPIC PYRAZINAMIDE DST

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

iii. CCTR TM042: PCR PRODUCT CLEANUP POST HRM ANALYSIS

TM042 **Cleanup of *pncA* PCR products post HRM Curve Analysis**	
Issued by: TB Working Group	**Date issued:** 14 July 2014
Signature:	**Version:** 1
Approved by: Dr. M. Barnard	**Date of Next Review:** 15 July 2015
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PURPOSE

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

Cleanup for more than 10 Samples

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

Equipment & Materials

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

Procedure

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

1. Order the tubes containing PCR products generated in SOP TM041 "Confirmation of pncA amplification by High Resolution Melt (HRM) Curve Analysis", in the appropriate Eppendorf tube rack. If the PCR products are in a 96-well plate, place the plate in the appropriate Eppendorf rack.
2. Completely remove the lids from the tubes by cutting the stem holding the lid towards the tube.
3. Select the script entitled PCR_cleanupv4_1_var_tubes_to_tubes from the list generated from the control computer and click "OK".

4. Place the rack with PCR products (source tubes) on the Tecan in the position indicated in the script on the controlling computer.
5. Place the Nucleofast 96-well PCR clean-up plate on the Tecan vacuum station, again maintaining the orientation.
6. Place freshly filtered water in the indicated position on the Tecan.
7. Place a rack with empty 200µl PCR tubes (or empty 96-well flat no skirt PCR plate) in the indicated position (destination tubes).
8. Verify that all tubes, racks and containers are in the correct places and are properly seated.
9. Ensure that the correct disposable robotic tips are in the locations as indicated in the script.
10. Click "OK" to start the script.
11. Observe of the script to ensure that the procedure is being performed.
12. Monitor the progress of the script and ensure that the vacuum engages properly.
13. On completion of the script, remove source tubes with the original PCR products and verify that the PCR products have been removed properly. Store the empty tubes in the rack according to sequencing facility policy.
14. Remove the destination tubes and verify that the tubes contain the appropriate volume of sample (approximately 50µl).
15. If some samples were not transferred properly, immediately inform the laboratory manager of the script malfunction so that the source of the malfunction can be investigated and corrected. Identify the corresponding well positions in the Nucleofast 96-well PCR clean-up plate and transfer the sample manually.
16. Cover the rack and proceed to the NanoDrop to verify the concentration of the PCR products.
17. Follow the laboratory SOP for determining the concentration of the purified PCR product.

Cleanup for 10 or less Samples

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

Principle

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

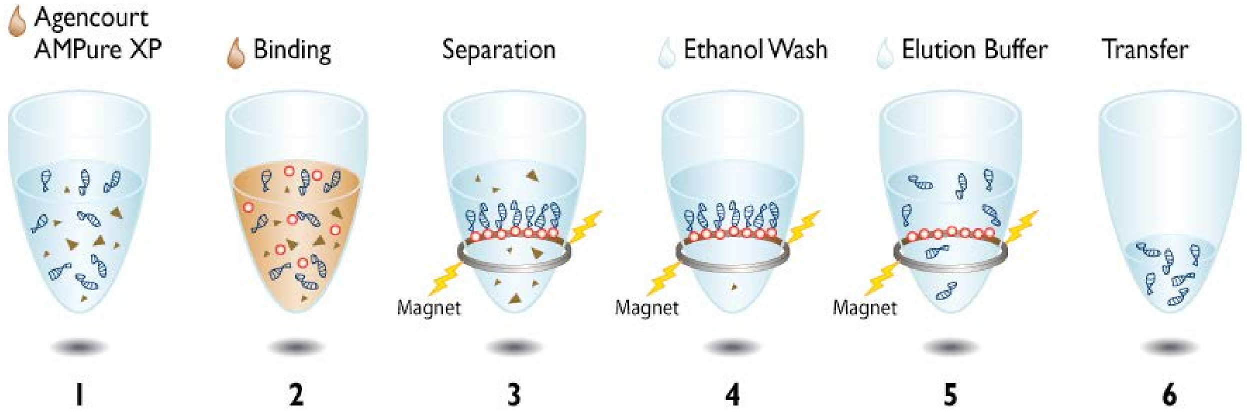
Equipment & Materials:

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

Procedure

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

Figure



iv. CCTR TM043: DNA SEQUENCING OF PCR AMPLICONS USING SANGER SEQUENSING METHOD

TM043 **DNA Sequencing of PCR Amplicons using the Sanger Sequencing Method**	
Issued by: TB Working Group	**Date issued:** 14 July 2014
Signature:	**Version:** 1
Approved by: Dr. M. Barnard	**Date of Next Review:** 15 July 2015
Signature:	**Date withdrawn:** …………………………………….

PURPOSE

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

Equipment & Materials

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

Procedure

Preparation of cycle sequencing mix:

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

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

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

Reaction set-up

3. Reaction set-up is to be done in either single 0.2ml PCR tubes, strips of eight 0.2ml tubes or in a 96-well Axygen plate
    a. Maintain sample order from Post PCR clean-up
    b. When using single tubes, or strips of eight tubes, use the tube holder for the GeneAmp 9700 thermal cyclers
    c. When working in 96-well plates, maintain the plate orientation
    d. Ensure that all plates and tubes are clearly marked
    e. DO NOT label part of tube that fits in PCR block
    f. DO NOT label the top of PCR tubes
    g. Use an electronic multi-dispensing pipet to dispense the sequencing primer and sequencing mix
    h. If a large number of reactions are performed a master-mix of primer and sequencing mix can be made. 6µl master-mix must then be dispensed into the strips of eight or 96-well Axygen plate and 4µl PCR product added
4. Add 3µl sequencing primer (at 1.1µM) to each well
    a. Tap the tubes/plate on the bench to collect the primer in the bottom of the plate
5. Add 3µl sequencing mix (mixture of BigDye and 5x Sequencing buffer)
    a. If multi-dispensing, ensure that the tip does not touch the primer drop
    b. Gently tap the tubes/plate on the bench to collect the mix in the bottom of the plate
6. Add 4µl purified PCR product (final concentration 5ng/µl) using a P10 pipette
    a. Pipette directly into the primer + sequencing mix in the bottom of the plate
    b. Mix by carefully pipetting up and down once WITHOUT introducing bubbles
7. Cap tubes tightly - either singles or those with strip of 8 caps or the 96-well plate with a cover plate with PCR rubber seal
8. Pulse spin the tubes/plates in a plate centrifuge to ensure that all reagents are mixed in the bottom of the tubes/plates
    a. Visually inspect to verify that all reagents are mixed in the bottom of the tubes/plates and that there are no bubles present at the bottom of the tubes
9. Place tubes/plate in GeneAmp 9700 thermal cycler
10. Select the following programme on the instrument: “seq55” to initiate the reaction run
11. Before starting the run, verify the following settings in the programme:
    1. Rapid thermal ramp to 94°C
    2. Denature at 94°C for 5 minutes
    3. Followed by 25 cycles
        a. 10 seconds at 94°C
        b. Rapid thermal ramp to 55°C for 5 seconds
        c. Rapid thermal ramp to 60°C for 4 minutes
    4. Rapid thermal ramp to 4°C and hold at 4°C
12. The run can now be initiated

v. CCTR TM044: DYE TERMINATOR REMOVAL POST DNA SEQUENCING

TM044 **Dye Terminator Removal Post DNA Sequencing**	
Issued by: TB Working Group	**Date issued:** 14 July 2014
Signature:	**Version:** 1
Approved by: Dr. M. Barnard	**Date of Next Review:** 15 July 2015
Signature:	**Date withdrawn: …………………………………..**

PURPOSE

To remove unincorporated dye terminators from cycle sequencing reactions.

Equipment & Materials

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

PROTOCOL

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

1. Purification of sequencing reactions

a. Pulse spin the tubes / plate in which the DNA was cycle sequenced (SOP TM 043 "DNA sequencing of PCR amplicons by the Sanger sequencing method", at 1500rpm in order to collect all liquid in the bottom of the tubes/plates.
b. Open the tubes by carefully removing the lids from the tubes.
c. Decant the 0.5% SDS into a disposable trough and pipette 4 µl of the 0.5% SDS to each cycle sequencing reaction by using a multi-channel pipette.
    i. Hold the tips just above the open tubes and dispense the 4 µl into the open tubes so that the tips do not come into contact with the sequenced amplicons. Should this happen a new set of tips must be loaded onto the multi-channel pipette.
d. Close the open tubes with new lids or rubber mat.
e. Return the tubes/plate to the thermal cycler.
f. Select the following program for SDS treatment of the sequenced amplicons: "SDS".
g. Ensure that the thermal cycler is set at 98°C for 5 minutes and 25°C for 10 minutes.
h. Initiate the reaction.
i. Once the reaction has completed, remove the plate from the instrument and keep it for later use in the Tecan Freedom Evo robotic system.

2. Preparation of the CENTRI SEP 96-Well plate

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

vi. CCTR TM045: CAPILLARY ELECTROPHORESIS OF DNA SEQUENCING PRODUCTS

TM045
Capillary Electrophoresis of DNA Sequencing Products

Issued by: TB Working Group	**Date issued:** 14 July 2014
Signature:	**Version:** 1
Approved by: Dr. M. Barnard	**Date of Next Review:** 15 July 2015
Signature:	**Date withdrawn: ……………………………………**

PURPOSE

To separate sequenced DNA fragments in order to sequence the electropherogram.

Equipment & Materials

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

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

PROTOCOL

DATA IMPORT

1. Import the Sample sheet containing information regarding the samples to be analyzed to the genetic analyzer. The format of the sample sheet is determined by the analyzer used.

2. Verify that the active spectral calibration matches your dye set and capillary array length for all scheduled runs. (See SOP 3130xl or 3730xl Genetic Analyzers for detailed operation.)

ELECTROPHORESIS OF SEQUENCING SAMPLES

1. Use the PCR system 9700 to denature the sequencing reactions by heating at 95°C for 1 minute. Hold at 4°C until ready to load Analyzer.

2. Assemble the plate “sandwich” by placing the plate into the plate base. Snap the plate retainer on top of the plate + plate base. Verify that the holes of the plate retainer and the septa strip are aligned.

3. Load the samples as indicated in the Analyzer SOP/user’s manual and make sure that the correct run module is selected for a specific run. See “Getting started guide” for Applied Biosystems 3130xl Genetic Analyzers p132 for sequencing run modules.

4. Close the doors of the Analyser.

5. To start a run, click the “Run” button – Run scheduler. The green LED light on the front panel of the Analyzer will flash while the run is in process. When the run is completed, the green light will stop flashing.

vii. CCTR TM046: DNA SEQUENCE ANALYSIS

TM046 **DNA Sequence Analysis**	
Issued by: TB Working Group	**Date issued:** 25 November 2014
Signature: N/A	**Version:** 1
Approved by: Dr. M. Barnard	**Date of Next Review:** 24 November 2015
Signature:	**Date withdrawn: ……………………………………**

PURPOSE

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

EQUIPMENT

PC Software: Variant Reporter™ v1 (Applied Biosystems, 2007)

Sample data path:
G:\<SAF07><"year"_Data><P><PZA_Project>
Tutorial data path:
G:\<SAF07><TB-Project><PZA><Results><2014_07_21>

PROCEDURE

1. Go to Desktop; double-click "Variant Reporter" (VR).
2. VR will open showing a table within the "Dashboard view".
3. Select the line with "PZA-Template" found in the Project Name column.
4. Now see the "Project View" pane that appeared vertically on the left. It contains Setup options, Quality control options as well as Results options.
5. Select the "Import" button found in the "Setup>Import and manage traces" pane that should be open already.
6. Go to the tutorial data folder in the path shown above.

7. Select "2014_07_22", and then click on "Add Selected Traces>>" (File type should be ".ab1 trace files".

8. A list of "Traces to Add" will appear on the right of the "Import Traces" window, whereafter just select "Okay".

9. The trace file names (ab1 file name) should already be assigned with strict nomenclature. Therefore just select "Auto Assign".

10. Keep the specimen name between the 1st and 2nd occurrence of "_" (underscore) and the amplicon name between the 2nd and 3rd occurrence of the "_". Then click "Okay".

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

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

12. Go to the the next Setup option in the "Project View" pane referred to as "Set Analysis Parameters" (Note: The template already has these parameters fixed and saved).

13. Leave "mobility file" unchanged.

14. Set "mixed base threshold" so that a mixed base is not assigned when the secondary peak height is ≤ 10%.

15. Keep analysed data scaling as "True Profile".

Trimming options

16. Trim using quality values: Mask each end until fewer than 2 bases in a window of 20 bases have QVs < 20.

17. Keep "Trim using base positions unchecked".

Filter options

18. Filter traces until the minimum trace score the less than 25.

19. Filter traces if the % Expected Trimmed Read Length (TRL) is less than 70%.

20. Go to the next option in "Project View" pane known as "Define Reference". Import the reference file i.e. "pncA_reference.txt". This should provide a reference sequence of 961 nt, starting with

"cacc" and ending with "cacg". The reference can be found in the path G:\<SAF07><TB-Project><PZA>

21. Now define your amplicon region by submitting the forward and reverse primer sequences. Select "Amplicon Primers/Known Variants". In the drop-down select "Edit primer sequences". Add the primer sequences, click on "Align", then "Okay". The primer sequences are as follow:

    - pncA-F: AGTCGCCCGAACGTATGGTG
    - pncA-R: CAACAGTTCATCCCGGTTCG

22. A yellow line representing the amplicon should be present at this point. When clicking on this line the corresponding sequence at the bottom should be highlighted in yellow.

23. Next, define the pncA gene sequence by highlighting nucleotide numbers 201 to 761, and then clicking on the "ROIs" drop-down. The gene starts with "5'-atgcgggc" and ends with "ctcctga-3'". In the "ROIs" drop-down window select "Create/edit ROI". A "Reference feature manager" window will appear.

24. Set the following:

    a. Name: set the name (e.g. PZase, pyramidase or PZA etc.).
    b. Type: gene
    c. Select the "Translatable" tick box

25. Now select the "Reference" drop-down window and click on "Change codon table".

26. Choose the "bacterial" codon table.

27. Now select the green "Analyze" button in the "Project View" pane. Now the software should successfully align all good-quality trace sequences to the reference sequences.

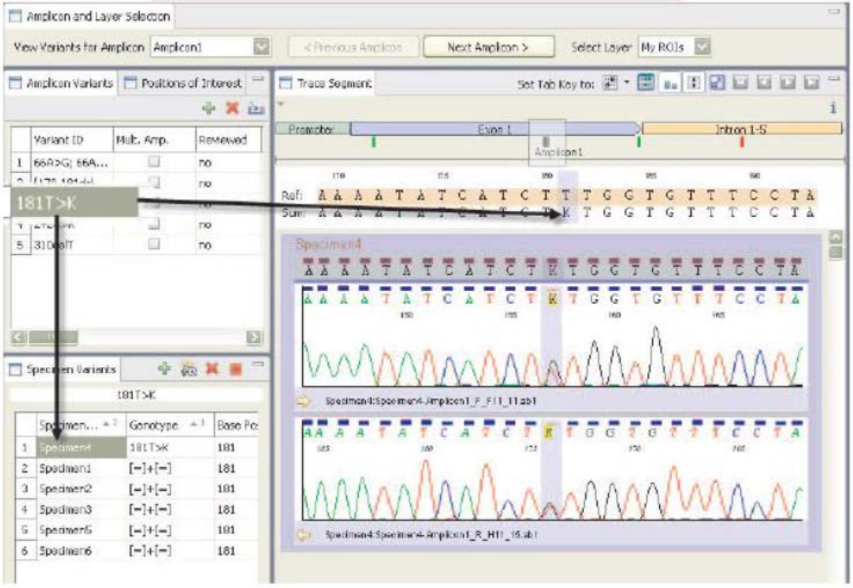
28. After analysis, the software opens the "Project Results Summary"" page if your project passed the quality threshold settings (Note: If your project does not meet the Quality Threshold settings during analysis, this page allows you to examine where the project failed in detail.

29. In the "Amplicon summary window" click to ℹ display the "Variant Legend" and "Amplicon quality legend".

30. Go to the "Variant Review" page in the "Project View" pane to see a full list of all variants, per amplicon, that the software detected during analysis.

31. Select the first variant in the Amplicon Variants table; all specimens containing the selected variant highlight immediately in both the Specimen Variants table and the Trace Segment pane (See figure below).

When you select a variant, the other panes update



Note: Click ▦ (Pane Action toolbar) to switch to a snippet view in the Trace Segment pane and view multiple trace segments at once.

To accept or reject selected specimen genotypes:

32. Select a variant that you want to review in the Variant ID column Amplicon Variants table

33. Review the specimens (extended trace view or snippet view) associated with each variant (Specimen Variants table).

34. Right-click to accept , or reject , the specimen genotype(s).

(Note: Click to accept or reject all specimen genotypes at once)
(Note: When you accept or reject a specimen genotype, both tables (Specimen Variants table, Amplicon Variants table) update simultaneously.

35. Save your project when you have completely reviewed all detected variants.

36. To create a report, click to open the Reports dialog box. Select the checkbox next to the report, or reports, you want to print and/or export.

(Note: When selecting a Specimen Report, you must also select the specimen from a drop-down list, then specify the file format).

37. Specify the file format as well as the target directory you want the report to export to, then click either “Print Preview”, “Print” or “Export”.

38. Results location:

    G:\<SAF07><”year”_Data><P><PZA_Project><Results><PZA_”year-month-day”>.

39. Once done, the results are to be mailed to the molecular division supervisor of TASK laboratory.