Protocol

This trial protocol has been provided by the authors to give readers additional information about their work.

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FIND
FOUNDATION FOR INNOVATIVE NEW DIAGNOSTICS

Evaluation Study Protocol

Xpert™ MTB Evaluation Study

Evaluation of the FIND/Cepheid Xpert™ MTB assay for the detection of pulmonary TB in sputum of symptomatic adults

Technical and Financial Agency:

Foundation for
Innovative New Diagnostics
71 avenue Louis-Casaï
P.O. Box 93
1216 Geneva, Switzerland
Tel: +41 22 710 05 90
Fax: +41 22 710 05 99
www.finddiagnostics.org

Study Coordinator: Dr Catharina Boehme
email: catharina.boehme@finddiagnostics.org

Xpert^{TM} MTB Evaluation Study
Protocol Version 3.0
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Confidentiality statement

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TABLE OF CONTENTS

TABLE OF CONTENTS 3
INVESTIGATORS 4
STATEMENT OF PRINCIPAL INVESTIGATORS 6
PROTOCOL SYNOPSIS 7
EVALUATION STUDY PROTOCOL 9
BACKGROUND 9
ENDPOINTS 11
STUDY DESIGN 11
BIOSTATISTICS: RATIONALE FOR SAMPLE SIZE FOR BETA TESTING 12
WORKFLOW FOR BETA TESTING PHASE 14
INCLUSION CRITERIA 18
COMPARATIVE GOLD STANDARD 18
SPUTUM SAMPLE RANDOMIZATION 19
DATA ANALYSIS 20
LABORATORY REQUIREMENTS 24
QUALITY CONTROL CHECK FOR INCOMING SHIPMENTS AND CHANGE CONTROL 24
TRIAL SITE CERTIFICATION AND PROFICIENCY TESTING 25
PROCESS CONTROLS TO EXCLUDE LABORATORY DNA CONTAMINATION 25
DATA MANAGEMENT 25
MINIMIZATION OF BIAS 26
ETHICAL CONSIDERATIONS 26
STUDY COORDINATION AND TRAINING 27
BUDGET 27
ANNEX 1: Case report form 28
ANNEX 2: Key to Case Report Form (CRF) 36
ANNEX 3: Sputum collection 43
ANNEX 4: Sputum decontamination for culture & microscopy (Kubica) 44
ANNEX 5: Semi-quantitative mycobacterial smear 46
ANNEX 6: Semi-quantitative mycobacterial culture 48
ANNEX 7: Speciation with Tauns Capilia TB 52
ANNEX 8: Drug susceptibility testing 54
ANNEX 9: Patient Informed Consent Form 60
ANNEX 10: Monitoring Plan 63
ANNEX 11: IQC check for incoming shipments and/or new lots 64
ANNEX 12: Change Control Form 67
ANNEX 13: Process Controls 68

INVESTIGATORS

Principal Investigators:

1. Dr. Eduardo Gotuzzo
Director, Instituto de Medicina Tropical "Alexander von Humboldt "
Universidad Peruana Cayetano Heredia (UPCH)
Av. Honorio Delgado 430, SMP, Lima 31, Perú
Phone: +511 482-3910
Fax: +511 482-3404
E-mail address: egh@upch.edu.pe

2. Dr. Roxana Rustomjee
Director Unit for Clinical and Biomedical TB Research
Medical Research Council Durban
491 Ridge Road, P.O. Box 70380
Overport, 4067, South Africa
Phone: +27 31 2034745
Direct Tel: +27 31 2034771; Mobile: +2772 2723 009
Fax: +27 31 2034702
Email: rrustomjee@mrc.ac.za

3. Dr. Gregory Hussey
Director, Institute of Infectious Diseases and Molecular Medicine (IIDMM)
Faculty of Sciences, University of Cape Town,
Wolfson Pavilion, Level 3
Anzio Road, Observatory 7925, Cape Town, South Africa
Phone: +27 21 406 6079
Fax: +2721 4066068
Email: ghussey@rmh.uct.ac.za

4. Dr. Camilla Rodrigues
Hinduja National Hospital and Medical Research Centre
400016 Mumbai, India
Phone: +912224447796
Phone 2: +912226441421
E-mail address: dr_crodrigues@hindujahospital.com; dr_crodrigues@yahoo.com.in

5. Dr. Sabine Ruesch-Gerdes
Forschungszentrum Borstel
German Reference Laboratory for Mycobacteria
Parkallee 18, 23845 Borstel, Germany
Phone: +49 4537 188-211
E-mail address: srueschg@fz-borstel.de

Co-Investigators:

1. Dr. Fiorella Krapp
Universidad Peruana Cayetano Heredia
Av. Honorio Delgado 430, SMP, Lima 31, Perú
Phone: +511 482-3910 / +511 7912479
E-mail: fiorellakrapp@yahoo.com.ar

2. Dr. Jenny Allen
Medical Research Council Durban
491 Ridge Road, P.O. Box 70380
Overport, 4067, South Africa
Phone: +511 482-3910 / +511 7912479
E-mail: Jenny.Allen@mrc.ac.za

3. Dr. Mark Nikol
Institute of Infectious Diseases and Molecular Medicine, University of Cape Town,
Wolfson Pavilion, Level 3
Anzio Road, Observatory 7925, Cape Town, South Africa
Phone: +27 21 406 6079
E-mail: Mark.Nicol@uct.ac.za

4. Dr. A. Mehta
Hinduja National Hospital and Medical Research Centre
400016 Mumbai, India
Phone: +912224447796
Phone 2: +912226441421
E-mail address: dr_amehta@hindujahospital.com

5. Dr. Rasim Tahirli
Special Treatment Institution for Detainees with Tuberculosis (STIDT)
Ministry of Justice – Republic of Azerbaijan
Tel: (994 12) 438 56 88
E-mail: tahirlir@justice.gov.az

STATEMENT OF PRINCIPAL INVESTIGATORS

In signing this page, I agree to conduct the study in accordance with the relevant, current protocol, applicable regulations and institutional policy.

I will ensure that the requirements relating to obtaining Institutional Review Board (IRB) review and approval are met. I will promptly report to the IRB all changes in the research activity.

I have understood that Xpert^{TM} MTB Assay is for investigational use only since the performance characteristics of this product have not been established.

I agree to notify FIND prior to making any changes in the protocol.

I will maintain confidentiality of protocol and investigational materials.

I agree to ensure that all colleagues and employees assisting in the conduct of the study are informed about their obligations in meeting the above requirements.

____

Dr. Eduardo Gotuzzo Date

____

Dr. Roxana Rustomjee Date

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Dr. Gregory Hussey Date

____

Dr. Camilla Rodrigues Date

____

Dr. Sabine Ruesch-Gerdes Date

____

Dr. Rasim Tahirli Date

PROTOCOL SYNOPSIS

Background

Commercial nucleic acid amplification tests (NAAT) for the detection of tuberculosis (TB) and rifampicin resistance have come into routine use in industrialized countries because of their great advantage of speed compared to culture. However, the complexity of existing commercial NAAT formats, the labor demands, and the need for a high degree of technical support and well-trained staff make it unsuitable for most developing country settings.

FIND has signed a development agreement with Cepheid to deliver a fully automated molecular platform for TB case detection and drug resistance testing for high-endemic countries. A prototype version of this assay has undergone clinical validation in Peru and Latvia. In the relatively small number of samples, Xpert™ MTB was highly specific and sensitive, detecting *M. tuberculosis* (MTB) DNA in almost all smear-positive sputum specimens and a high percentage of smear-negative, culture-positive specimens. Rifampicin resistance was detected with high accuracy. Since then, Cepheid finalized the design of the Xpert™ MTB assay. The product is now ready for a formal evaluation of its clinical performance in a statistically powered number of patients. It is designed to purify, concentrate, amplify and identify targeted *rpoB* nucleic acid sequences, and delivers answers from unprocessed samples in 120 minutes, with minimal hands-on technical time.

Study Design

This is a multi-center, blinded, mainly cross-sectional study to determine the performance of FIND/Cepheid Xpert MTB assay for TB and rifampicin resistance detection in patients with symptoms of pulmonary TB (PTB) and at risk of multi-drug resistant (MDR) TB in comparison to conventional methodologies. A clinical follow up after 8 weeks will be required to establish the diagnosis for smear-negative and culture-negative, but Xpert MTB-positive patients and a random control group. Of particular interest for the performance evaluation will be the smear-negative, culture-positive group and the rifampicin-resistant group.

Sample size

1. **Training and proficiency testing:** *All sites, 6 days*
   - 60 frozen sputum specimens <u>per site</u> from 20 TB suspects
   - 4 spiked specimens <u>per site</u> (provided by Cepheid)
2. **Beta testing:** *All Sites, 16 weeks enrolment*
   - 930 fresh specimens <u>per site</u> from 310 TB suspects

Trial sites

1. Universidad Peruana Cayetano Heredia, Lima, Peru (Site 01)
2. Medical Research Council, Durban, South Africa (Site 41)
3. Institute of Infectious Diseases and Molecular Medicine, Cape Town, South Africa (Site 18)
4. Hinduja National Hospital and Medical Research Centre (Site 42)
5. Forschungszentrum Borstel, Borstel, Germany / Special Treatment Institution, Baku, Azerbaijan (Site 15)

Timelines

Activity / Event	Date
IRB approvals	By Jan 08
Start collection of samples for training phase	Feb 08
Training site II-IV (one by one)	May 08
Enrolment for Beta testing	Jul-Oct 08
Intermittent data review milestone	End of Aug 08
Follow up phase & completion of culture/DST	Oct 08-Mar 09
Data & technical review milestone	Jan 09

EVALUATION STUDY PROTOCOL

BACKGROUND

Despite the enormous global burden of TB, approaches to TB diagnosis still rely on traditional methods that have major limitations. The disproportionate amount of smear-negative disease in sub-Saharan Africa, which shoulders two-thirds of the global burden of HIV/AIDS, has greatly complicated TB case detection and disease control. Multidrug-resistant (MDR) TB, defined as resistance to both isoniazid and rifampin, two of the most important "first-line" anti-TB drugs, is a rapidly growing problem, especially in Eastern Europe and countries of the former Soviet Union. Even more alarming is the recently documented phenomenon of XDR (or extensively drug-resistant) TB, defined not only as resistance to isoniazid and rifampin but also to one second line injectable agent and a fluoroquinolone antibiotic. XDR TB is often not curable with available drugs.

The low sensitivity of smear microscopy limits its impact on TB control. Culture methods are complex, slow and still scarcely available in high endemic-countries. Drug susceptibility testing is even slower and more technically complex. While patients await diagnosis, their disease progresses with an increased chance of dying from tuberculosis and they continue to transmit drug-resistant TB to others, especially family members.

Early case detection is essential to interrupt transmission and to prevent the further spread of tuberculosis and multidrug-resistant tuberculosis. A new diagnostic approach is therefore urgently needed.

Only a small fraction of the estimated 400,000 multi-drug resistant TB cases occurring each year have access to drug susceptibility testing (DST). Key obstacles to DST expansion over the past years were 1) the complexity of available tools and the laboratory infrastructure required for their implementation; 2) the unaffordable price of better (but equally complex) technologies; and 3) the unavailability of rapid and simple tools for identification of resistance.

Although nucleic acid amplification tests (NAAT) for the rapid detection of TB and rifampicin resistance exist, their complexity, along with the labor demands, including the need for a high degree of technical support and well-trained staff, make them unsuitable for most developing country settings.

Cepheid, in a joint development agreement with the Foundation for Innovative New Diagnostics (FIND), is developing a fully automated TB assay based on the GeneXpert real-time polymerase chain-reaction (PCR) platform which is designed to detect DNA directly from clinical specimens. The ultimate goal is to have a fully automated, rapid test that can reliably detect *Mycobacterium tuberculosis* and associated rifampicin resistance from human sputum samples with a sensitivity that is comparable to culture. The first prototype version of this assay has undergone Alpha trial testing in a relatively small number of patients. In these samples, Xpert™ MTB was highly specific and sensitive, detecting *M. tuberculosis* (MTB) DNA in almost all smear-positive sputum specimens and a high percentage of smear-negative, culture-positive specimens. Rifampicin resistance was detected with high accuracy. Since then, Cepheid finalized the design of the Xpert™ MTB assay. The product is now ready for a formal evaluation of its clinical performance in

a statistically powered number of patients. It is designed to purify, concentrate, amplify and identify targeted *rpoB* nucleic acid sequences, and delivers answers from unprocessed samples in 120 minutes, with minimal hands-on technical time. The GeneXpert system consists of a GeneXpert instrument, personal computer and disposable fluidic cartridges. The system combines cartridge-based sample preparation with amplification and detection in a fully integrated and automated nucleic acid analysis instrument. 4 Instrument sizes will be available and will contain between 1 and 72 individually accessible modules that are capable of performing separate real-time polymerase chain-reaction (PCR). Each module contains a syringe drive for dispensing fluids, an ultrasonic horn for lysing cells and a thermocycler.

The sample pre-treatment for the Xpert™ MTB assay is simple: Xpert solution is added to sputum samples and a defined volume of this mixture is then transferred to the sample chamber of the cartridge. The cartridge is then inserted in the GeneXpert. From this point on, all steps are automated.

ENDPOINTS

Primary Endpoints

1. **Performance characteristics of Xpert™ MTB compared to smear and culture**
- Determine sensitivity of Xpert MTB in smear-positive PTB patients
- Determine sensitivity of Xpert MTB in smear-negative, culture-positive PTB patients
- Determine specificity of Xpert MTB in smear- & culture-negative PTB suspects without prior TB treatment (Non-TB)

2. **Performance characteristics of Xpert™ MTB compared to conventional drug susceptibility testing (DST)**
- Determine sensitivity of Xpert MTB in PTB patients found to be rifampin resistant by culture
- Determine specificity of Xpert MTB in PTB patients found to be rifampin sensitive by culture

3. **Performance of Xpert MTB™ compared to standard NAAT (ProbeTec and Amplicor)**
- Determine sensitivity in smear- and culture-positive specimens compared to NAAT
- Determine sensitivity in smear-neg, culture-positive specimens compared to NAAT
- Determine specificity in smear- & culture-negative PTB suspects without prior TB treatment (Non-TB) compared to NAAT
- Determine operational feasibility compared to NAAT

Secondary Endpoints

1. **Technical fine tuning**
- Determine optimal cut-off points for semi-quantitation

2. **Operational feasibility**
- Identify potential barriers to implementation and determine appropriate use in the country's diagnostic algorithm

STUDY DESIGN

This mainly cross-sectional, multi-center study is aimed at evaluating the FIND/Cepheid Xpert™ MTB assay for TB and rifampicin resistance detection with regard to clinical and operational performance. The study will consist of the following phases:

Training & proficiency testing – all sites – 60 stored samples of 20 patients, 4 samples spiked with DNA, 6 days duration

During the initial on-site training phase, two lab technicians and the supervisor will receive training on the Xpert™ MTB assay from the FIND study coordinator. Upon arrival, the study coordinator will help the site in setting up the GeneXpert. Before the training begins, a quality income check will be performed to ensure that cartridges and GeneXpert are working properly. 2 spiked samples provided by Cepheid will be used. Results and .gxx files will be sent to Cepheid and the actual testing will only start once Cepheid has approved the QC income check. 64 tests (60 frozen samples with known smear, culture and DST results and of 4 spiked samples provided by Cepheid) will then be performed per site to gain practical experience and to determine the proficiency of the users. Proficiency will be assessed with the help of a questionnaire. The frozen samples will be collected and stored at the site before the study begins. Collection will follow the flow described in Figure 1, since this will allow the sites to practice the actual enrolment procedure.

Training will also comprise other study related aspects such as case report form completion, data management and data entry. At the end of the Xpert training, a certification record will be signed by all participants. Staff that did not pass the proficiency testing will not be able to participate in the study. Oral and written feedback on their overall impression of the assay (in the form of a questionnaire) will be provided by the lab technicians and supervisor.

Beta testing – all sites – 310 TB suspects per site, 16 weeks enrolment, completion of follow up 8 weeks after completion of enrolment and completion of cultures 16 weeks after completion of enrolment

For the beta testing phase, 310 patients meeting inclusion criteria will be enrolled in the study per site and 3 sputum samples collected per patient. Smear microscopy, culture (LJ, MGIT, DST) and Xpert™ MTB will be performed as described in Figure 1 below (2 specimens to be cultured, 3 tested with GeneXpert). Completion of solid culture will provide the final bacteriologic diagnosis for all patients enrolled in the evaluation study and will be available on day 56. Most DST results will also be obtainable by then.

BIOSTATISTICS: RATIONALE FOR SAMPLE SIZE FOR BETA TESTING

Group	Sensitivity or specificity target	Confidence interval (CI)	Required minimum group size	Average prevalence among TB suspects	Required TB suspects
s+, c+	95%	± 3%	203	18%	1128
s-, c+	90%	± 4.5%	171	11%	1554
Non-TB	98%	± 1%	335	46%	728
Rif- resistant	90%	± 7%	71	5%	1420
Rif-sensitive	95%	± 3%	203	22%	922

All values were calculated based on the estimation procedure. Based on these calculations, a total of **1550** patients with suspicion of PTB will be enrolled and tested according to the workflow described in Figure 1 and 2. Each site shall therefore aim to enroll **310** patients. Confidence intervals for *per specimen analysis* will be narrower than for *per patient analysis*. Calculations were based on the following assumptions:

1. Performance targets:

The primary objective of this study is to assess whether the product specifications for the Xpert™ MTB have been met. The critical performance targets are as follows:

Sensitivity in smear- and culture-positive patients:

Minimum >90%, optional >95%; since the feasibility study results showed a sensitivity >95%, the optional target was used for sample size calculation.

Sensitivity in smear-negative, culture-positive patients:

Minimum >60%, optional >90%; since we aim for a culture replacement test, the optional target has been used for sample size calculation.

The prevalence of smear-negative, culture-positive patients among TB suspects is expected to be in average only 11%, so that a confidence interval of 4-5% seemed a reasonable compromise between desirable and feasible. The amount of sputum required for the study flow automatically favors enrolment of smear-positive TB patients over smear-negative TB patients, since the latter group is known to have more difficulties with sputum production. With a same sample size of 171, the confidence interval for the minimum target would be wider, but still acceptable: For 171 patients: 60% +/- 7.3% CI.

Specificity in symptomatic non-TB patients:
Target >98%. The confidence interval for this group must be narrow to guarantee a low false positive rate and high positive predictive value for the assay.

Sensitivity for the detection of rifampin resistance
Minimum >90%; optional >95%; since we don't know if a sensitivity of 95% can be reached, samples size numbers have been calculated on the basis of 90%. The confidence intervals for Rif resistance determination do not have to be as narrow, since a lot of information can be gained from testing of strains.

Specificity for rifampin resistance
Minimum 95%, optional 98%; this group will be easier to enrol in sufficient numbers.

2. Prevalence of various study groups among TB suspects
The populations from which the 5 trial sites will recruit their TB suspects are quite different with respect to HIV prevalence, MDR prevalence, type of enrolment sites (primary care facilities, DOTS centres or tertiary care facilities), so that the prevalence for the various study groups will vary from site to site. The trial site in Peru for example is known to have a very high smear positive rate since patients are enrolled from DOTS centres; the rates of smear-negative, culture-positive patients and the MDR rate is comparatively low. The trial sites in South Africa have a high HIV prevalence and will therefore see more smear-negative, culture-positive patients and have an MDR rate that is higher than in Peru, but lower than for Eastern Europe. Due to the high HIV prevalence, it is expected that a higher number of patients will have to be excluded as indeterminate at these sites, since it will not be possible to exclude TB for some of the more immune-compromised patients. The patients enrolled for testing at the German trial site will come from Eastern Europe and will have the highest MDR rate among all participating sites. The trial site in India is a private tertiary care facility. MDR patients from Mumbai and surrounding areas are referred to this site for treatment. The HIV prevalence is overall low in India and the majority of TB patients smear positive. Based on discussions with the trial sites and experience from previous studies, the following prevalence can be expected:

Group	Highest estimated prevalence	Lowest estimated prevalence	Average
s+, c+	40% (Peru)	10% (3 sites)	18% (1x40%, 3x10%, 1x20%)
s-, c+	5% (Peru)	20% (Germany)	11% (1x5%, 3x10%, 1x20%)
Non-TB	60% (2 sites)	30% (2 sites with high exclusion rates)	46% (2x60%, 1x50%, 2x30%)

Rif- resistant	25% of 30% cul pos (Germany)	4% of 45% (Peru)	5% (1x7.5%, 1x6%, 1x5%, 1x4.5%, 1x1.8%)
Rif-sensitive	80% of 45% cul pos (Peru)	65% of 30% (Germany)	22%

3. Drop out rate

Participants will for example have to be excluded for the following reasons (complete list provided in chapter on data analysis:

Lost at follow up; cultures contaminated; growth of only non-tuberculous mycobacteria (NTM); smear-positive, but culture-negative patients; TB cannot be excluded with a high enough likelihood; enrolled by mistake without meeting inclusion criteria; missing data or incomplete CRF. Therefore, a relatively **high overall drop out rate of 25%** has been used as basis for prevalence calculations in the table above. In addition, it has been assumed that **2% of the 29% TB patients will not obtain a valid DST result**.

WORKFLOW FOR BETA TESTING PHASE

- Clinicians of the study team or of participating peripheral health facilities will screen patients to identify TB suspects, TB treatment failures and re-treatment cases meeting study inclusion criteria. These patients will be asked to participate and must be told that participation is voluntary and that they have the opportunity to ask questions individually. The consent forms (for participation and for HIV testing where applicable) must be signed by all participating patients. HIV testing is desirable at least in high prevalence settings.

- Eligible patients who signed the informed consent form will be instructed on how to produce sputum and will provide the first spot sputum. The medical history will be taken and a clinical examination performed. Participants will be asked to return the next day with a morning sputum sample and will provide a third sputum sample directly.

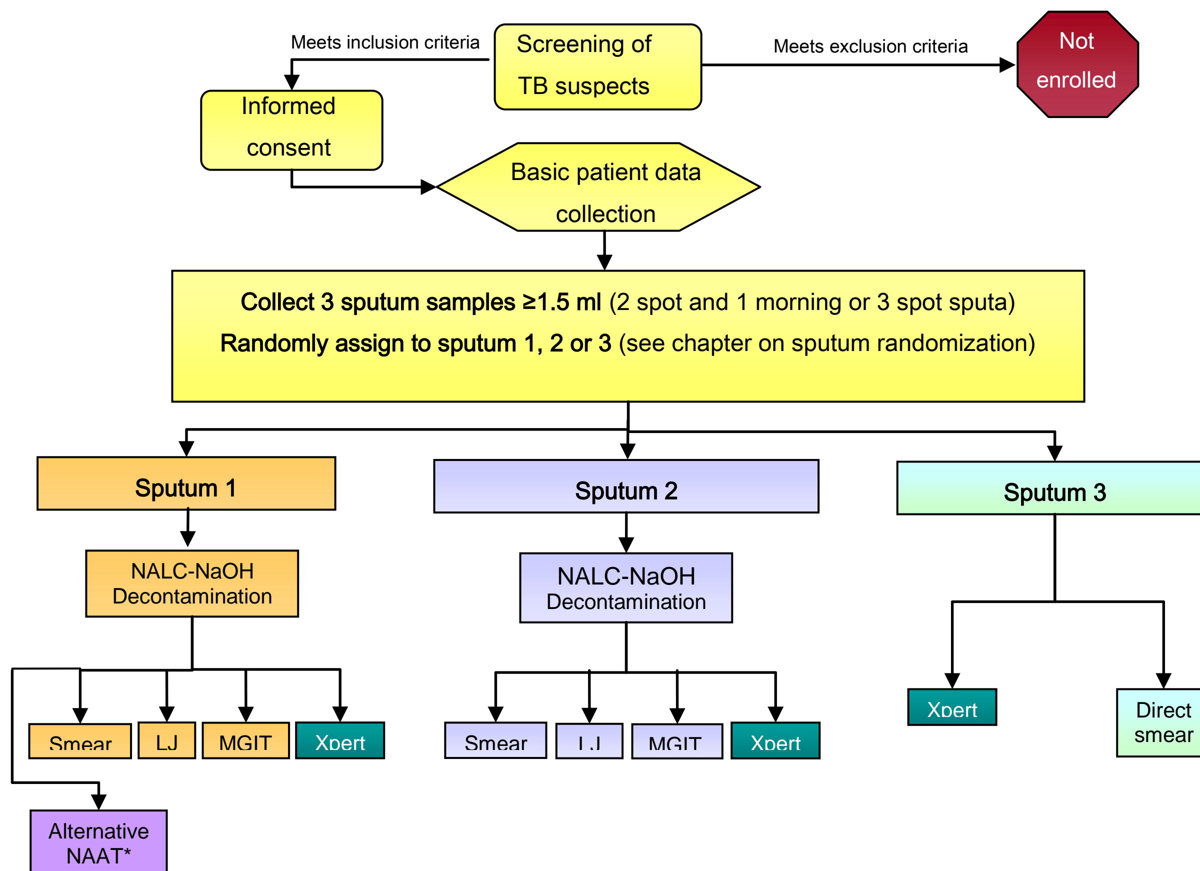
- Sample processing will only be done once all 3 patient specimens have arrived in the laboratory. Patients, who do not provide 3 specimens of sufficient quantity (1.5 ml), will not be included in the study. Complete sets of sputum samples will be relabelled according to instructions in chapter “sputum sample randomization”.

- Two sputum samples (minimum 1.5 ml) will undergo smear, culture, Xpert™ MTB examination and one of the two sputa will in addition undergo PCR /standard NAAT (Probetec or Amplicor) where applicable (smear, PCR and Xpert™ MTB will be done from the same pellet as culture). The third sputum will be tested by direct smear and Xpert™ MTB.

- Pellet leftovers from all participants and isolates of at least one MTB positive culture per culture-positive specimen (either LJ or MGIT) will be stored at -70°C to allow possible further analysis of discrepant cases. Xpert cartridges should also be stored after processing, if there is sufficient storage space, in order to allow resolution of discrepant cases by sequencing of amplicons in the cartridge.

- Drug susceptibility testing will be carried out for the first positive culture of each specimen. Only DST for first line drugs will be required for study purposes. A Genotype MTBDRplus test may be performed (and budgeted) for one of the two culture isolates.
- Blood/Serum will be drawn for HIV testing, where required as per local NTP guidelines. At least all smear-negative patients will undergo chest radiography. Smear-negative patients who are culture-positive should be contacted and referred for treatment by study personnel as soon as culture results are available.
- Follow-up evaluation is required after 8 weeks for smear-negative, culture-negative, but Xpert™ MTB –positive (or PCR/standard NAAT-positive) patients and the same number of randomly selected smear-negative, culture-negative and Xpert™ MTB-negative (or PCR/standard NAAT-negative) patientsThese patients with initial negative smear and culture examinations who are not thought to have TB and who are not placed on anti-tuberculous therapy require – during follow-up-1 additional smear and culture examination, 1 Xpert™ MTB test as well as a clinical work-up with CXR control (if the initial CXR was abnormal).
- Clinical follow-up only is required after 8 weeks for all smear-negative, culture-negative patients who are started on TB-treatment based on clinical findings. Clear clinical improvement is required to define such patients as group "CXR+".
- Follow-up lists containing patients ID numbers will be generated by FIND database and send to the sites on a weekly basis.
- To facilitate patient notification of positive culture results in smear negative-patients and follow-up procedures, study personnel should request permission from the study participant at the time of enrolment to visit their home or workplace should new information become available or should they fail to return for follow-up after 2 months.
- All patients will be assigned a final diagnosis that matches one of the categories outlined in the workflow below. All results must be recorded in the Case Report Form (CRF), which is to be entered in the database. Study participants with incomplete case report forms must be withdrawn and a new study subject enrolled instead.

Figure 1: Workflow at enrolment during Beta testing phase



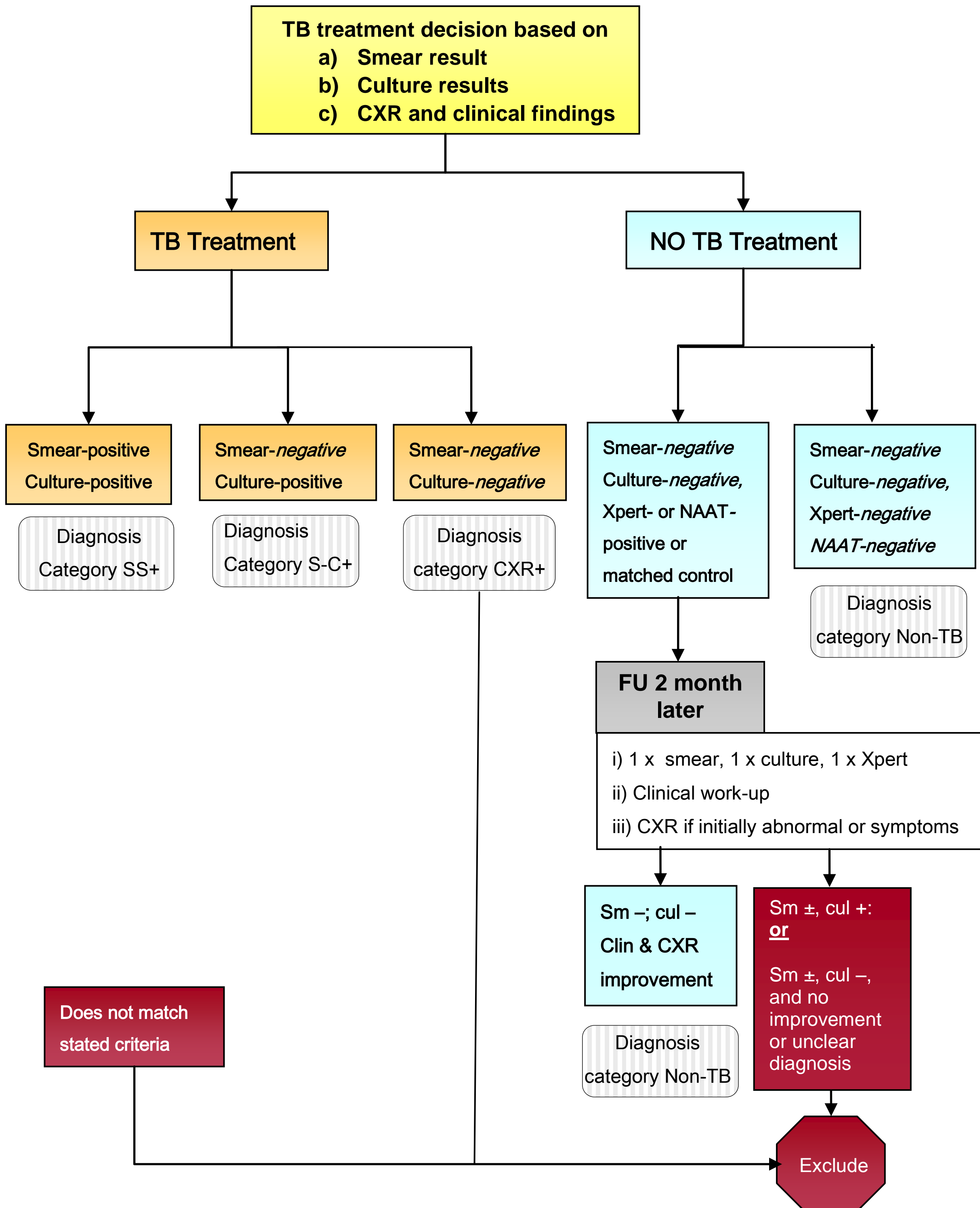
Store all remaining pellets and Xpert cartridges for sequencing of discrepant cases

MTB confirmation for all positive cultures: **Capilia rapid test**

<u>If MTB positive</u>
DST for **first line drugs** & storage of **1 isolate** from **first pos culture per specimen.**

*at least 2 sites (Roche Amplicor, Probetec, or Hain Genotype MTBDRplus); amount of pellet required: 0.5ml for Xpert, 0.5ml for PCR, 0.5ml for MGIT, 0.1-2 for LJ, 0.1ml for smear.

Figure 2: Workflow continued



INCLUSION CRITERIA

Case detection group

1. **Inclusion criteria for specimens of TB suspects:**
   - Clinical suspicion of pulmonary TB: persistent productive cough for ≥ 2 weeks
   - Patient volunteers to give 3 sputum specimens of at least 1.5 ml over the course of 2 days
   - Age 18 years and above

2. **Exclusion criteria for specimens of TB suspects:**
   - Inability of patient to produce 3 sputum samples of at least 1.5 ml over the course of 2 days
   - Patients receiving anti-TB medication in the 60 days prior to testing (except if suspicion of MDR, patient is then classified as MDR suspect)*
   - TB treatment started > 48 hours ago

MDR risk group

1. **Inclusion criteria for specimens of TB patients at high risk of MDR TB (MDR suspects):**
   - Re-treatment cases
   - Non-converting PTB cases (category I and category II failures)*
   - Patient volunteers to give 3 sputum specimens of at least 1.5 ml over the course of 2 days
   - Age 18 years and above

2. **Exclusion criteria for specimens of MDR suspects:**
   - Inability of patient to produce 3 sputum samples of at least 1.5 ml over the course of 2 days.

* TB suspects will only be eligible for the study if they have been on TB treatment for < 48 hours. However, PTB cases on TB treatment are eligible if they are suspected to be treatment failures. In this case, it does not matter how long TB treatment has been ongoing. All culture-negative study participants on TB treatment will be excluded from the analysis, even if they are smear-positive. In contrast to culture, smear microscopy and the Xpert™ MTB assay detect dead cells or DNA from dead cells. Including culture-negative patients on TB treatment in the analysis would thus negatively impair Xpert™ MTB specificity.

COMPARATIVE GOLD STANDARD

1. Smear microscopy: 2 specimens of concentrated sputum (after NALC/NaOH treatment), 1 direct smear
2. LJ and MGIT culture (decontaminated with 1-1.5% final NaOH); 1 each per specimen for 2 specimens per patient
3. Speciation of culture (rapid testing (molecular methods or Capilia))
4. MGIT SIRE or LJ proportion method 1st line drugs for all MTB culture-positive specimens (from 1 positive culture per specimen). For 1 of 2 positive specimens, a Hain Genotype MTBDRplus result would be desirable.

SPUTUM SAMPLE RANDOMIZATION

Purpose of randomization

A single Xpert^{TM} MTB result from sputum 3 will be evaluated against combined culture results of 2 sputum samples. In order to avoid a measurement bias in the results due to systematically lower quality of sputum 3, spot and morning sputum samples for each patient will be randomly assigned for processing as sputum 1, 2 or 3.

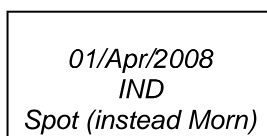
Sputum sample labeling

1. At collection sites

- Sputum containers will be labeled according to time of collection using the following labels provided by FIND: Core ID + Spot 1 or Morn or Spot 2.

Example of label composition for 1st spot sputum collected from patient 0001

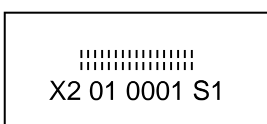
- The following information will be handwritten on every sputum container:
  - i. Date of collection.
  - ii. If sputum was obtained <u>with</u> induction ("IND"), please indicate.
  - iii. If instead of the morning sputum, a third spot sputum has been collected (not desirable but sample eligible), please indicate.



Handwritten information required on sputum container (3rd row only if applicable)

2. At the laboratory

- The person in charge of sputum reception ensures that sputum sample set is complete, i.e., that sputum samples "spot 1", "morn" and "spot 2" for each patient are available.
- Label all sputum containers <u>randomly</u> using the following labels provided by FIND: Core ID + S1 or S2 or S3. The Core ID of original sputum container label and the label added in the laboratory are identical. Ensure that the original label remains visible.



Example of barcode label for sputum 1 to be used in the laboratory for relabeling.

- Transfer the information from the sputum container in the respective fields of the CRF ("*sputum information"* sections): date of collection, time of collection (morning or spot) and whether obtained with induction.
- Continue with completion of CRF sections *"sputum information"*.

DATA ANALYSIS

The sensitivity, specificity, predictive values and diagnostic accuracy for the new methods in comparison with the gold standard will be calculated. Data analysis will be done *per patient* and *per specimen* applying the following rules:

Definitions for *per specimen* analysis

Diagnosis	Description
Smear-positive specimen	• ≥ 1+ smear (≥10/100 fields) • Smear positive specimen with 2 negative or contaminated cultures will be excluded from analysis. • A positive smear result in sputum 3 will only be valid if at least 1 culture in sputum 1 or 2 is positive. Otherwise, sputum 3 will be excluded from analysis.
Scanty-positive specimen	• 1-9 AFB per 100 fields • Scanty positive specimen with positive culture will be analysed 1) as smear-negative, culture-positive and 2) as separate group of scanty and culture-pos. • Scanty positive specimen with 2 negative or contaminated cultures will be excluded from analysis. • Scanty positive specimen in sputum 3 will be excluded from analysis if sputum 1 and 2 are culture negative or contaminated.
Culture-positive specimen	LJ and/or MGIT culture growth confirmed MTB complex, if considered trustworthy (not cross-contaminated). **Cross-Contamination:** A single LJ culture with < 20 colonies or a single MGIT culture with MTB growth >28 days per patient will be considered a questionable bacteriologic result (potential cross-contamination) and will be excluded from analysis. **NTM:** Specimens with growth of mycobacteria other than MTB complex only will be excluded from analysis.
Contaminated culture	• **LJ:** Cultures completely overgrown by bacterial or fungal contaminations within 3 weeks (discarded). In case of mixed cultures, isolated MTB colonies transferred to new LJ tube. • **MGIT:** Instrument positivity w/o detection of AFB. • Specimens with one contaminated culture will be regarded as interpretable • 2 contam cultures for same specimen will lead to exclusion of this specimen
Rifampicin resistant specimen	**<u>LJ proportion method</u>:** ≥1% growth in tube containing 40 µg/ml rifampicin compared to control tube. **<u>LJ concentration method:</u>** After 28 day of incubation, 20 or more colonies in the 40 µg/ml rifampicin containing tube. **<u>MGIT:</u>** The instrument interprets results at the time when the growth unit (GU) in growth control reaches 400 (within 4-13 days). At this point, the GU values of the drug vial are evaluated. If the GU of the Rif tube is 100 or more, the result is interpreted as resistant.
Rifampicin susceptible specimen	<1% growth compared to control tube or < 20 colonies after 28 days and GU of Rif tube <100.
Xpert™ MTB positive specimen	Any "MTB pos" result is considered positive.
Xpert™ MTB Rif resistant specimen	Any "Rif resistance detected" is considered positive. "Rif resistance indeterminate results" will be excluded from analysis for Rif resistance.
Xpert™ MTB invalid ("error"; "invalid", "no result")	For example as a consequence of BG failure, probe-check failure or pressure abort. Trial sites should repeat Xpert™ MTB for such specimens if 500 µl sputum or 250 µl pellet left. For calculation of sensitivity/specificity, specimens with invalid Xpert™ MTB results (also during repetition if done) will be excluded from analysis, as will specimens with 2 contaminated cultures. The rate of invalid results will be determined.
NAAT-pos specimen	Any MTB pos is considered pos, except if negative control of this run positive.
Contaminated NAAT	A false positive negative control will lead to the assumption that all samples in this run have been cross-contaminated.

Definitions for *per patient* analysis

Smear-positive Culture-positive case	≥ 1 ≥ 1+ smear or ≥ 2 scanty-positive of 3 smears & ≥ 1 of 4 MTB pos cultures. Smear-positive, culture-negative patients will be excluded from analysis.
Smear-negative, Culture-positive case	• 1 scanty positive smear or only negative smears & ≥ 1 of 4 MTB pos cultures. • See definition of cross-contamination in table above. Patients with more than 2 contaminated cultures are not included in analysis.
Non-PTB case	• Smear and culture-negative patient, who does not receive TB treatment on the basis of clinical criteria and chest x-ray. • If such patients are Xpert™ MTB - or PCR-positive, or have been selected as random controls, a follow-up with clinical and bacteriological work-up will be required to exclude PTB with the highest possible likelihood. Only if the patient has recovered or considerably improved without receiving TB treatment and the bacteriological work-up remains negative, the patient is called Non-PTB.
CXR+	• Smear and culture-negative patient, who receive TB treatment on the basis of clinical criteria and chest x-ray.
Indeterminate cases (excluded)	Excluded from analysis as indeterminate cases will be patients with less than 3 specimens examined; patients not meeting inclusion criteria or with incomplete case report forms; smear-positive, culture-negative cases; cases with >2 of 4 contaminated cultures; culture-negative TB-patients on TB treatment; cases with Mycobacterium other than tuberculosis (MOTT) only; cases with discrepant culture DST results; patients that require but do not get follow-up and initially smear- and culture-negative, but Xpert™ MTB - or PCR-positive cases, for which TB cannot be excluded or confirmed during follow-up.
Rifampicin resistant case	**LJ proportion method:** ≥1% growth (mean of colony growth in both tubes containing 40 µg/ml rifampicin) compared to control tube. **LJ concentration method:** After 28 day of incubation, 20 or more colonies in the 40 µg/ml rifampicin containing tube. **MGIT:** The instrument interprets results at the time when the growth unit (GU) in growth control reaches 400 (within 4-13 days). At this point, the GU values of the drug vial are evaluated. If the GU of the Rif tube is 100 or more, the result is interpreted as resistant. Only concordant results (interpreted as resistant or sensitive in both tubes) will be used for analysis. If there is any discrepancy between DST results for a patient (one DST result “Rif resistant” and one “Rif sensitive”), the case will be excluded from per patient analysis for Rif resistance.
Rifampicin susceptible case	Mean of growth in 2 tubes <1% growth compared to control tube or < 20 colonies after 28 days and GU of Rif tube <100. If there is any discrepancy between DST results for a patient (one DST result “Rif resistant” and one “Rif sensitive”), the case will be excluded from per patient analysis for Rif resistance.
Xpert™ MTB positive case	1-3 Xpert™ MTB results “MTB positive”. For each of the 2 Xpert™ MTB results for sputum 3, a separate per patient analysis will be carried out.
Xpert™ MTB Rif resistant case	If there is any discrepancy between Xpert™ MTB results for a patient (one Xpert™ MTB “Rif resistance detected” and one “Rif resistance not detected”), the case will be interpreted as Xpert™ MTB Rif sensitive, unless sequencing confirms the discrepancy, in which case the patient will be excluded from a per patient analysis.
Xpert™ MTB invalid case	3 out of 3 results “invalid” or “error” or “no result”. Participants with only 1-2 invalid Xpert results will not be excluded from *per patient* sensitivity/specificity calculations. Invalid cases with 3 invalid results will be excluded, but the number of such cases analysed and reported.
NAAT-positive case	1 NAAT MTB-positive test result

Handling of discrepant cases

Case	Analysis
Smear±, culture-positive, Xpert™ MTB negative	No further analysis (check for clustering in the lab to exclude culture cross-contamination).
Smear and culture-negative, Xpert™ MTB positive	Follow up after 2 months to exclude or confirm PTB. Gold-standard PCR of frozen pellet considered.
Rif resistant Xpert™ MTB Rif sensitive	Culture isolate and where available pellet and cartridge amplicons to be sequenced.
Rif sensitive Xpert™ MTB Rif resistant	Culture isolate and where available pellet and cartridge amplicons to be sequenced.
1 Xpert™ MTB Rif resistant, 1 Rif sensitive	Called Xpert sensitive unless pellets can be sequenced and confirm discrepancy.

DESCRIPTION OF Xpert™ MTB TEST

The Xpert TB assay performed on the Cepheid GeneXpert Dx system is a rapid test for the detection of *M. tuberculosis* (MTB) and rifampicin resistance.

The Xpert™ MTB test

The assay is based on multiplex, nested real-time PCR. A series of molecular beacons[1] is used to simultaneously detect the presence of MTB and to diagnose rifampicin resistance as a surrogate marker for MDR disease. Species-specific primers allow amplification of the MTB *rpoB* core region. Nested PCR is used in order to increase the sensitivity of the assay. Up to six target sequences can be detected simultaneously with the six color assay: one of the six molecular beacon probes was designed to detect DNA of the sample processing control *Bacillus globigii*; the other five molecular beacons are designed to hybridize to overlapping segments of the *rpoB* core region – the amplicon.

In wild-type *M. tuberculosis*, the *rpoB* core region is highly conserved but is mutant in 95% of rifampicin-resistant MTB. The presence of all five fluorescence signals indicates that rifampicin-susceptible MTB DNA has been detected. At least two but less than five fluorescence signals indicate the presence of rifampicin-resistant MTB. The presence of a single fluorescence signal (in addition to the signal for the sample processing control) is interpreted as an invalid result since experience has shown that even the most mutant MTB isolates produce at least two positive signals (David Alland; unpublished data). No fluorescence signal from one or fewer of the *rpoB* probes indicates absence of Mtb DNA (the positive signal for the internal process control indicates that the assay worked).

[1] Molecular beacons are single-stranded nucleic acid molecules that possess a stem-and-loop structure. The loop portion of the molecule serves as a probe sequence that is complementary to a nucleic acid target sequence. Short complementary "arm" sequences bind to each other to form the stem hybrid. A fluorophore is covalently linked to the end of one arm and a nonfluorescent quencher to the end of the other arm. When the molecular beacon hybridizes to its target, the stem helix is forced apart and the fluorophore is separated from the quencher, permitting the fluorophore to fluorescence when excited by light of an appropriate wavelength.

The GeneXpert platform

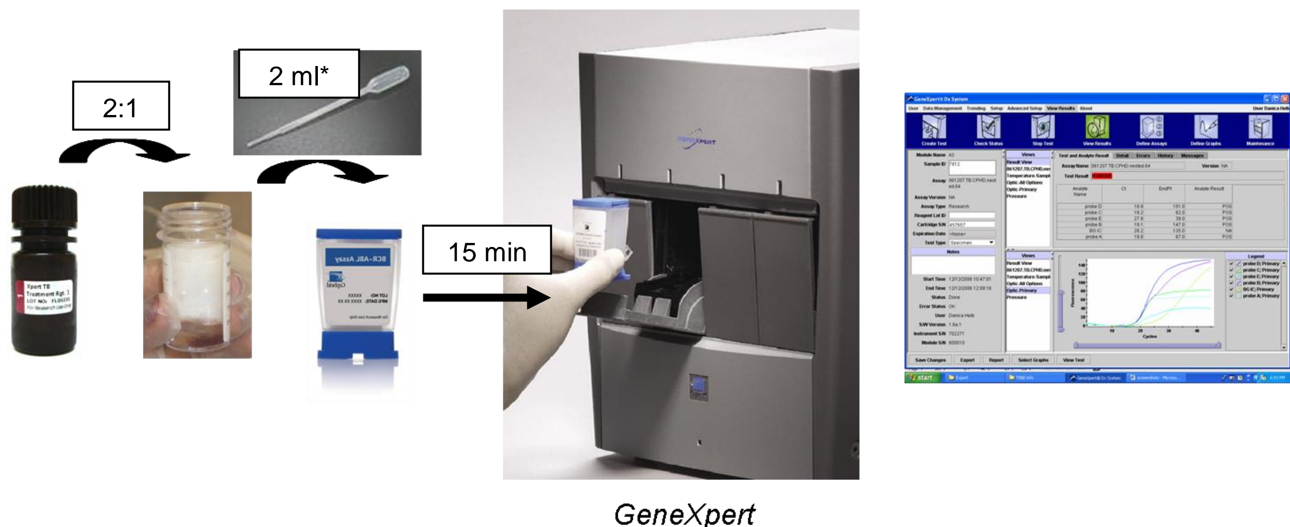
The GeneXpert system consists of a GeneXpert instrument, personal computer and disposable fluidic cartridges. The system combines cartridge-based sample preparation with amplification and detection in a fully integrated and automated nucleic acid analysis instrument. It is designed to purify, concentrate and identify targeted nucleic acid sequences, and delivers answers from unprocessed samples in less than 120 minutes with minimal hands-on time.

Each instrument contains 4 individually accessible modules that are capable of performing separate real-time polymerase chain-reaction (PCR) protocols. Each module contains a syringe drive for dispensing fluids, an ultrasonic horn for lysing cells, a thermocycler, and optical signal detection components.

The single-use cartridges contain i) chambers for holding sample and reagents, ii) a valve body composed of a plunger and syringe barrel, iii) a rotary valve system for controlling the movements of fluids between chambers, iv) an area for capturing, concentrating, washing and lysing cells, v) lyophilized real-time PCR reagents and wash buffers and vi) an integrated PCR reaction tube that is automatically filled by the instrument.

The sample treatment for the Xpert™ MTB assay is simple: Xpert sample treatment reagent (SR) is added to sputum samples and a defined volume of this mixture is then transferred to the sample chamber of the cartridge. From this point on, all steps are automated: GeneXpert first captures MTB organisms from the sputum sample on a filter membrane. Then inhibitors are washed from the captured cells with buffer. Finally the captured, washed cells are lysed with ultrasonic energy, and the released DNA eluted through the filter membrane. The DNA solution is finally mixed with dry PCR reagents and transferred into the PCR tube for real-time PCR and detection. To eliminate test-to-test contamination, all fluids, including amplicons, are contained within the disposable cartridge. Sample pretreatment steps are outlined below. A more detailed description of the sample pretreatment steps for the Xpert™ MTB assay will be provided in the training manual.

Figure 4: Xpert™ MTB assay procedure



LABORATORY REQUIREMENTS

Space

The sample processing for the Xpert™ MTB assay will be done in the biosafety cabinet which is also used for decontamination of specimens. Subsequently, the samples are not infectious anymore, so the GeneXpert can be set up in any convenient place, ideally in the smear microscopy room. In order to limit the risk of contamination, cultures should be processed in a different room.

Equipment and supplies

1. **Equipment & consumables supplied by manufacturer**
   - Test cartridges including reagents for sample preparation
   - GeneXpert prototype device (will not remain on site after the study)
   - Computer
   - Sterile plastic Pasteur pipettes (3-5 ml, with 0.5 ml graduation)
   - 2D barcode scanner

2. **Equipment needed but not supplied by manufacturer**
   - UPS 1500 VA
   - Adapter for American plug (GeneXpert and laptop)
   - Extension cables depending on set up of UPS, GeneXpert and laptop
   - USB memory stick
   - 4°C refrigerator & -70°C freezer
   - Autoclave
   - Facilities, reagents, consumable and equipment for LJ and MGIT culture (incubator, centrifuge, MGIT Bactec 960 or manual reader, tubes, PANTA, OADC, NALC-NaOH-Citrate, phosphate buffer, centrifuge tubes etc.) and microscopy (staining kits, slides)
   - Vortexer
   - 1 timer
   - Sterile sputum container (screw capped, leak proof, wide mouthed, 25 ml with graduation)
   - Cryotubes for storage of pellet and isolates (1.8 ml), cryorack, cryoboxes
   - Disinfectants, bleach
   - Gloves, N95 masks/respirator
   - Racks for cryotubes and centrifuge tubes
   - Sterile plastic Pasteur pipettes (1 ml and 5 ml, with graduation), required to split pellet, for culture etc.
   - Micropipette 1-200 µl and tips
   - Where applicable: Reagents and equipment for gold standard PCR.

QUALITY CONTROL CHECK FOR INCOMING SHIPMENTS AND CHANGE CONTROL

Each site will receive in total 2-3 reagent lots from Cepheid. Upon arrival of each new lot, the laboratories will have to conduct an Income Quality Check (IQC) following the instructions attached as Annex 11. Controls spiked with DNA will be provided by Cepheid and are to be used for testing. Test results (.gxx files and completed IQC form) are sent to Cepheid/FIND for approval. New lots may only be used after Cepheid and FIND have approved the results.

During the course of the study, all procedural changes (in enrolment or laboratory procedures or data management), including use of new lots, must be documented in the Change Control Form (Annex 12). Such changes shall not to be introduced without prior approval by FIND.

TRIAL SITE CERTIFICATION AND PROFICIENCY TESTING

Since the evaluation results are planned to be used for assay registration in several countries, the high quality of data must be ensured throughout the study. According to FIND's ISO standards, each trial site will need to undergo FIND certification of lab and clinic. This certification requires a trial site visit of a FIND inspector prior to study initiation. Should this visit show weaknesses in certain areas, a TB laboratory or clinical expert consultant will be sent on site for training. Participation in a proficiency testing for DST (rifampicin and isoniazid only) and smear microscopy will be part of this certification. The external quality assurance panels will be provided to the sites by FZ Borstel.

PROCESS CONTROLS TO EXCLUDE LABORATORY DNA CONTAMINATION

All participating sites that perform other *rpoB* amplifying NAA technologies such as line probe assays in the same laboratory must exclude *rpoB* amplicon contamination prior to start of the study and upon suspicion of a DNA contamination (cluster of identical positive results in Xpert). Such process controls will include 2 swabs and 2 negative controls. Laboratories that do not perform *rpoB* amplifying tests only have to include process controls upon suspicion of DNA contamination. Instructions will be provided in Annex 13.

DATA MANAGEMENT

Case report forms (CRFs) will be provided by FIND. The electronic data entry tool for the study will be connected to FIND's central database through secured VPN access, which will have the advantage that study monitors have continuous access to the electronic data. First data entry will have to be done as soon as results become available (real-time). Second data entry will be done based on completed CRFs. Xpert data files can be downloaded and will be imported in the database directly. The files will also be sent to Cepheid by e-mail on a monthly basis. This will help Cepheid to detect potentially occurring hard- or software problems while the study is ongoing. At the end of the enrolment phase, and once all preliminary Xpert results have been imported in the database, preliminary data sets will be provided to FIND and Cepheid for analysis. When final culture and DST results are available, each of the study sites will submit final data sets to FIND and Cepheid for analysis. FIND and Cepheid will be provided with copies of all CRFs for their internal documentation. The original CRFs must be stored on site for at least 3 years after study completion. For details on study monitoring, see study monitoring plan.

As a fundamental requirement from FIND's donor sponsors, all valid scientific data generated from FIND-sponsored projects has to be made available for dissemination in the form of presentations, abstracts and/or scientific articles in peer-reviewed journals. It is part of FIND's charter that FIND is responsible to ensure such dissemination and the results of this multi-center trial shall be published as a joint publication with co-authorship from all participating sites and will include at least 2 co-authors per site/partner. Site-specific data may be presented independently and in combination with other data, but only after the principal publication has been made available and/or accepted

for publication, and also in the case that the combined multi-site data are not of publishable quality. In this latter case FIND shall be the final arbitrator on the joint publication.

MINIMIZATION OF BIAS

Avoiding sampling and selection bias: Consecutive sampling method and community-based study

A consecutive series of patients with typical symptoms of TB will be included. The study group will consist of all subjects who satisfy the criteria for inclusion and are not disqualified by one or more of the exclusion criteria. The vast majority of samples will be collected from out-patients and not from the more severely ill hospitalized patients.

Measurement bias: Within-subject comparison and blinded interpretation of results

In order to increase the precision of the estimates for the accuracy of the Xpert™ MTB, the study will be based on a within-subject and on a within-sample comparison. Due to the variability of sputum samples within individuals and within samples themselves, a certain measurement bias cannot be avoided. In order to avoid a measurement bias in the results due to systematically lower quality of sputum 3, spot and morning sputum samples for each patient will be randomly assigned for processing as sputum 1, 2 or 3. The Xpert™ MTB data analysis is not done by the users, but real-time in the Xpert software, based on predefined mathematical algorithms. It will therefore not be necessary to blind the Xpert™ MTB users against smear or culture results. However, it will be necessary to blind the lab technicians performing smear microscopy against Xpert™ MTB results. The lab technicians involved in smear microscopy will therefore not have access to the GeneXpert software in order to ensure blinding between the gold standard and the method under investigation. The Xpert™ MTB results will be directly imported in the database by the supervisor and will not be documented on the same result form as the standard methods. Indeterminate or invalid GeneXpert results will be analyzed as described above.

ETHICAL CONSIDERATIONS

Each site will obtain local Institutional Review Board (IRB) approval or equivalent. The proposed project is a laboratory evaluation of a testing method and not a formal clinical trial. TB treatment decisions will not be made based on the result of the Xpert™ MTB assay under evaluation, but on the basis of the routine clinical and laboratory methods (smear, solid and liquid culture results, clinical work-up). Sputum specimens will be collected, as required for routine diagnostic evaluation, from patients who are suspected of having TB. No additional specimens will be requested. Follow-up visits will be required for smear- and culture-negative cases started on anti-TB treatment based on clinical findings, random controls and a limited number of discrepant patients to exclude TB with the highest possible likelihood. No sample bank will be created. The stored pellets and culture isolates will only be needed to resolve discrepant cases. An export permit may need to be obtained by sites if isolates of discrepant cases cannot be sequenced in the country. Patients will receive the best possible diagnostic work-up. Diagnosis will be substantially accelerated by the use of automated liquid MGIT culture for case detection and DST and the rapid speciation with Capilia. Patients may be compensated for travel and time according to the site's research standards. Patient identifiers will be encrypted. Barcode labels will be used. Only authorized medical personnel at the study sites will have access to patient names.

STUDY COORDINATION AND TRAINING

FIND is the study sponsor and is responsible for planning and managing the study. Dr. Catharina Boehme will be the FIND study coordinator. The study personnel will receive technical on-site training by FIND and Cepheid. Completion of case report forms and data management will be a substantial part of the initial training. Staff at clinical enrolment sites will be trained in checking study inclusion criteria, obtaining informed consent and will be familiarized with the SOP for sputum collection by the on-site study team. During beta testing, Catharina will be responsible for troubleshooting and logistical support. Cepheid will provide technical support. Several monitoring visits per site will be required to ensure highest standards for the study (see monitoring plan for details). Catharina will coordinate activities of the FIND monitors and will conduct the close out visits. Cepheid will be informed on the outcome of all monitoring visits and may send monitors from their side as required. Short progress reports will be provided after the training phase (by FIND) and then twice monthly to all partners (by the on-site study team who will fill a form provided by FIND). Sites will contact Catharina by e-mail or telephone if they have any difficulties or questions. She will intercede with Cepheid for Xpert™ MTB -related issues. Telephone conferences between FIND and the study sites will be scheduled twice a month, and between FIND and Cepheid on a monthly basis.

BUDGET

Costs for study sites will be covered by FIND and will include personnel costs, expenditures for consumables (LJ and MGIT reagents, slides, etc.), missing equipment (UPS). The Cepheid reagents and the GeneXpert (with desktop) will be shipped directly from Cepheid to the sites. The following number of Cepheid reagents will be required:

- Training: 70 cartridges per site.
- Beta testing phase: 1240 cartridges per site.
- Extra cartridges: 100 cartridges

Considering the daily sample workload, one GeneXpert will be sufficient for each site. The Xpert TB assay is a research use only test (thus not of further use for the site) and remaining Xpert TB assay materials will not remain on the site. Likewise, the GeneXpert instrument and computer will not remain on site.

ANNEX 1: Case report form

A. Eligibility

A.1. Patient group: ☐Case detection group (fill table 1) ☐MDR risk group (fill table 2)

Table 1: Inclusion criteria case detection group:	Yes	No
A.1.1. Persistent, productive cough ≥2 weeks	☐	☐
A.1.2. Clinically suspected to have TB	☐	☐
A.1.3. Volunteers to provide 3 sputum specimens of 2x ≥3ml and 1x ≥1.5 ml	☐	☐
A.1.4. ≥18 years	☐	☐
A.1.5. No TB treatment for past 60 days and not started on TB treatment >48 h	☐	☐

Table 2: Inclusion criteria MDR risk group:	Yes	No
A.2.1. Non-converting PTB case or re-treatment case or contact of MDR case	☐	☐
A.2.2. Clinically suspected or confirmed to (still) have TB	☐	☐
A.2.3. Volunteers to provide 3 sputum specimens of 2x ≥3ml and 1x ≥1.5 ml	☐	☐
A.2.4. ≥18 years	☐	☐

B. Patient Information

B.1. Enrolment Date: ____(day)/______(month)/________(year)

B.2. Sex: ☐ Male ☐ Female

B.3. Date of birth: __ __(day)/__ __(month)/__ __ __ __(year)

B.4. History of TB: ☐Yes (if yes, complete table 3) ☐No ☐Not known

Table 3: Most recent TB treatment
B.4.1. Date of most recent TB treatment ☐Ongoing, specify approx. start date __ __/__ __/_ _ _ _ ☐Stopped without completion, specify approx. end date __ __/__ __/_ _ _ _ ☐Completed, specify approx. start date __ __/__ __/_ _ _ _ end date __ __/__ __/_ _ _ _ ☐Completed, dates unknown
B.4.2. Patient considered cured by this TB treatment: ☐Yes ☐No ☐Unknown
B.4.3. Last Diagnostic Category DOTS Cat. I ☐ DOTS Cat. II ☐ DOTS Cat. III ☐ DOTS Cat. IV ☐ Unknown ☐ Other-Specify ____________________________ ☐
B.4.4. Anti-TB drugs administered ☐Unknown ☐Known, specify: [ ]INH [ ]RIF [ ]EMB [ ]SM [ ]PZA [ ]Thio [ ]KAN [ ]AMK [ ]CAP [ ]ETH [ ]Pt [ ]Oflox [ ]CIP [ ] Levo [ ]Moxi/Gati [ ]PAS [ ]Cs [ ]Other

C. *Chest X-ray*

C.1. ☐Done, specify date (C.1.1): __ __/__ __/__ __ __ __ ☐Not done

C.1.2. Normal: ☐Yes ☐No (if no specify in table 4)

Table 4: Abnormalities in chest X-ray
C.1.2.1. Localization of abnormality (tick one or several): ☐Left upper zone ☐Left mid zone ☐Left lower zone ☐Right upper zone ☐Right mid zone ☐Right lower zone ☐Diffuse
C.1.2.2. Picture (tick one or several): ☐ Infiltrate or consolidation ☐Cavitary lesion ☐Tuberculoma ☐Mediastinal/hilar lymphadenopathy ☐Micronodules (Miliar) ☐Pleural effusion ☐Other
C.1.2.3. Principal conclusion (tick one): ☐TB likely (pulmonary, pleural, pericardial) ☐Pneumonia or atypical TB ☐Pneumonia (TB unlikely) ☐Other: ____________________

D. *HIV results* ***(testing only to be performed by high prevalence sites)***

D.1. HIV status: ☐Positive ☐Negative ☐Not done

F. *Clinical Follow up after 2 months*

F.1. Status of follow up ☐Follow up ☐No follow up required ☐Lost to follow up ☐Passed away

F.1.1. Date of Follow up __ __/__ __/__ __ __ __

F.1.2. Started on TB treatment: ☐Yes, specify date of start (F.1.2.1.)__ __/__ __/__ __ __ __ ☐No

F.1.3. Symptoms: ☐Completely recovered ☐Improved ☐Same ☐Worse

F.1.4. CXR compared to baseline:
☐Normal ☐Improved ☐Same abnormal ☐Worse ☐Not done

F.1.5. Most probable diagnosis based on clinical findings during follow up:
☐TB ☐Non-TB ☐Unknown

Case Report Form – Laboratory at enrolment

Sp1 Sputum 1

Sp1.A. Sputum information

A.1. Date of collection: __ __/__ __/__ __ __ __

A.2. Time of collection: ☐Early morning ☐Spot

A.3. Induction: ☐W/o induction ☐W induction

A.4. Viscosity: ☐Very viscous ☐Viscous ☐Not viscous/watery

A.5. Purity: ☐Containing food or other particles ☐Not containing particles

A.6. Blood: ☐Very blood stained ☐Blood stained ☐Not blood stained

A.7. Date of receipt at laboratory: __ __/__ __/__ __ __ __

A.8. Date of culture inoculation: __ __/__ __/__ __ __ __

Sp1.B. ZN Microscopy

B.1. Score: ☐Neg ☐ Pos → B.1.1. Quantitative smear result ☐Scanty ☐1+ ☐2+ ☐3+

Sp1.C. Solid AFB Culture

C.1. ☐Done ☐Not done

C.1.2. Result: ☐Neg ☐Contaminated/lost

☐Pos → C.1.2.1. Date of AFB positivity: __ __/__ __/__ __ __ __

→ C.1.2.2. Colony count ☐<20, # col____ ☐1+ ☐2+ ☐3+

Sp1.D. Liquid AFB Culture

D.1. ☐Done ☐Not done

D.1.2. Result: ☐Neg ☐Contaminated/lost

☐Pos → D.1.2.1. Date of AFB positivity: __ __/__ __/__ __ __ __

Sp1.E. Speciation

E.1. Result: ☐ Not done/Not applicable ☐MTB complex ☐Not MTB complex

E.2. Result at species level: ☐Not done//Not applicable ☐Done: M.__________________

Sp1.F. Drug susceptibility testing

F.1. ☐Done, *specify date of inoculation (F.1.1.):*__ __/__ __/__ __ __ __ ☐Not done/Not applicable

Method	F.1.2. MGIT ☐Yes ☐No	F.1.3. LJ proportion ☐Yes ☐No	F.1.4. Other molecular ☐Yes ☐No
1. SM	☐S ☐R ☐I ☐ND	☐S ☐R ☐I ☐ND	
2. INH	☐S ☐R ☐I ☐ND	☐S ☐R ☐I ☐ND	☐S ☐R ☐I ☐ND F.1.4.2.1. Specify if R: ☐Kat G wt absent ☐Kat G mut present ☐ND F.1.4.2.2 Specify if R: ☐inhA wt absent ☐inhA mut present ☐ND
3. RIF	☐S ☐R ☐I ☐ND	☐S ☐R ☐I ☐ND	☐S ☐R ☐I ☐ND F.1.4.3.1. Specify if R: ☐*rpoB* wt absent ☐*rpoB* mut present ☐ND
4. EMB	☐S ☐R ☐I ☐ND	☐S ☐R ☐I ☐ND	
5. PZA	☐S ☐R ☐I ☐ND	☐S ☐R ☐I ☐ND	

S-sensitive, R-resistant, I-indeterminate, ND-not done

Sp1.G. NAAT

G.1. ☐Done, *specify date performed (G.1.1.):* __ __/__ __/__ __ __ __ ☐Not done

G.1.2. **Result case detection:**

☐TB neg ☐Indeterminate ☐No result ☐TB pos ☐Borderline

G.1.3. **Result in case of repetition:**

Repeated ☐Yes (if yes, fill G.1.3.1.) ☐No

G.1.3.1. ☐TB neg ☐Indeterminate ☐No result ☐TB pos ☐Borderline

Sp2 Sputum 2

Sp2.A. Sputum quality

A.1. Date of collection: __ __/__ __/__ __ __ __

A.2. Time of collection: ☐Early morning ☐Spot

A.3. Induction: ☐W/o induction ☐W induction

A.4. Viscosity: ☐Very viscous ☐Viscous ☐Not viscous/watery

A.5. Purity: ☐Containing food or other particles ☐Not containing particles

A.6. Blood: ☐Very blood stained ☐Blood stained ☐Not blood stained

A.7. Date of receipt at laboratory: __ __/__ __/__ __ __ __

A.8. Date of culture inoculation: __ __/__ __/__ __ __ __

Sp2.B. ZN Microscopy

B.1. Score: ☐Neg ☐ Pos → B.1.1. Quantitative smear result ☐Scanty ☐1+ ☐2+ ☐3+

Sp2.C. Solid AFB Culture

C.1. ☐Done ☐Not done

C.1.2. Result: ☐Neg ☐Contaminated/lost
☐Pos → C.1.2.1. Date of AFB positivity: __ __/__ __/__ __ __ __

→ C.1.2.2. Colony count ☐<20, # col____ ☐1+ ☐2+ ☐3+

Sp2.D. Liquid AFB Culture

D.1. ☐Done ☐Not done

D.1.2. Result: ☐Neg ☐Contaminated/lost

☐Pos → D.1.2.1. Date of AFB positivity: __ __/__ __/__ __ __ __

Sp2.E. Speciation

E.1. Result: ☐ Not done/Not applicable ☐MTB complex ☐Not MTB complex

E.2. Result at species level: ☐Not done//Not applicable ☐Done: M.________________

Sp2.F. Drug susceptibility testing

F.1. ☐Done, *specify date of inoculation (F.1.1):* __ __/__ __/__ __ __ __ ☐Not done/Not applicable

Method	**F.1.2. MGIT** ☐Yes ☐No	**F.1.3. LJ proportion** ☐Yes ☐No	**F.1.4. Other molecular** ☐Yes ☐No
1. SM	☐S ☐R ☐I ☐ND	☐S ☐R ☐I ☐ND	
2. INH	☐S ☐R ☐I ☐ND	☐S ☐R ☐I ☐ND	☐S ☐R ☐I ☐ND F.1.4.2.1. Specify if R: ☐Kat G wt absent ☐Kat G mut present ☐ND F.1.4.2.2 Specify if R: ☐inhA wt absent ☐inhA mut present ☐ND
3. RIF	☐S ☐R ☐I ☐ND	☐S ☐R ☐I ☐ND	☐S ☐R ☐I ☐ND F.1.4.3.1. Specify if R: ☐*rpoB* wt absent ☐*rpoB* mut present ☐ND
4. EMB	☐S ☐R ☐I ☐ND	☐S ☐R ☐I ☐ND	
5. PZA	☐S ☐R ☐I ☐ND	☐S ☐R ☐I ☐ND	

S-sensitive, R-resistant, I-indeterminate, ND-not done

Sp3 Sputum 3

Sp3.A. Sputum quality

A.1. Date of collection: __ __/__ __/__ __ __ __

A.2. Time of collection: ☐Early morning ☐Spot

A.3. Induction: ☐W/o induction ☐W induction

A.4. Viscosity: ☐Very viscous ☐Viscous ☐Not viscous/watery

A.5. Purity: ☐Containing food or other particles ☐Not containing particles

A.6. Blood: ☐Very blood stained ☐Blood stained ☐Not blood stained

A.7. Date of receipt at laboratory: __ __/__ __/__ __ __ __

A.8. Date of culture inoculation: __ __/__ __/__ __ __ __

Sp3.B. ZN Microscopy

B.1. Score: ☐Neg ☐ Pos → B.1.1. Quantitative smear result ☐Scanty ☐1+ ☐2+ ☐3+

Sp4 Sputum 4 *(sputum at follow-up)*

Sp4.FU.A. Follow-up

A.1. ☐Laboratory FU done ☐Laboratory FU not done

Sp4.FU.B. ZN Microscopy at FU

B.1. Score: ☐Neg ☐ Pos → B.1.1. Quantitative smear result ☐Scanty ☐1+ ☐2+ ☐3+

Sp4.FU.C. Solid AFB Culture at FU

C.1. ☐Done ☐Not done

C.1.2. Result: ☐Neg ☐Contaminated/lost

☐Pos → C.1.2.1. Date of AFB positivity: __ __/__ __/__ __ __ __

→ C.1.2.2. Colony count ☐<20, # col____ ☐1+ ☐2+ ☐3+

Sp4.FU.D. Liquid AFB Culture at FU

D.1. ☐Done ☐Not done

D.1.2. Result: ☐Neg ☐Contaminated/lost

☐Pos → D.1.2.1. Date of AFB positivity: __ __/__ __/__ __ __ __ __

Sp4.FU.E. Speciation

E.1. Result: ☐ Not done/Not applicable ☐MTB complex ☐Not MTB complex

E.2. Result at species level: ☐Not done//Not applicable ☐Done: M.__________________

Sp4.FU.F. Drug susceptibility testing

F.1. ☐Done, *specify date of inoculation (F.1.1):* __ __/__ __/__ __ __ __ ☐Not done/Not applicable

Method	F.1.2. MGIT ☐Yes ☐No	F.1.3. LJ proportion ☐Yes ☐No	F.1.4. Other molecular ☐Yes ☐No
1. SM	☐S ☐R ☐I ☐ND	☐S ☐R ☐I ☐ND	
2. INH	☐S ☐R ☐I ☐ND	☐S ☐R ☐I ☐ND	☐S ☐R ☐I ☐ND F.1.4.2.1. Specify if R: ☐Kat G wt absent ☐Kat G mut present ☐ND F.1.4.2.2 Specify if R: ☐inhA wt absent ☐inhA mut present ☐ND
3. RIF	☐S ☐R ☐I ☐ND	☐S ☐R ☐I ☐ND	☐S ☐R ☐I ☐ND F.1.4.3.1. Specify if R: ☐*rpoB* wt absent ☐*rpoB* mut present ☐ND
4. EMB	☐S ☐R ☐I ☐ND	☐S ☐R ☐I ☐ND	
5. PZA	☐S ☐R ☐I ☐ND	☐S ☐R ☐I ☐ND	

S-sensitive, R-resistant, I-indeterminate, ND-not done

G. Final Diagnosis

G.1. Diagnostic Category: ☐SS+ ☐S-C+ ☐CXR+ ☐Non-TB ☐F ☐I

G.2. Comments: ☐ Yes (G.2.1.) ☐No

G.2.1. ____

____

Transfer from source data to CRF Signature ____________________ Date of CRF completion: [__ __/__ __/__ __ __ __]

Investigator's Signature ____________________ Date of CRF approval: [__ __/__ __/__ __ __ __]

First data entry ______________________ Date of data entry completion: [__ __/__ __/__ __ __ __]

Second data entry ____________________ Date of data entry completion: [__ __/__ __/__ __ __ __]

Copy CRF sent ☐ Date [__ __/__ __/__ __ __ __]

FIND DIAGNOSTICS
FOUNDATION FOR INNOVATIVE NEW DIAGNOSTICS

Cepheid.

Xpert™ MTB EVALUATION STUDY RESULT SHEET

Patient ID	Sputum #	Result	Pre-/Post-performance check	Result requiring retesting	Retesting done from	Result in case of repetition	FU after 2 m required
X2 _ _ _ _ _ _	S1 ☐Done ☐ND	☐MTB neg ☐MTB pos ☐Other Rif resistance if applicable: ☐Not detected ☐Detected ☐ Other	☐Passed ☐Failed & reported to CB if ≥2 in a row	☐No ☐Yes	☐Pellet leftover w/o SR ☐Untreated sputum w/o SR ☐Pellet w SR for ___min ☐ Untreated sputum w with SR for ___min ☐Retesting **not done**	☐MTB neg ☐MTB pos ☐Other Rifampicin resistance: ☐Not detected ☐Detected ☐ Other	☐No ☐Yes
	S2 ☐Done ☐ND	☐MTB neg ☐MTB pos ☐Other Rifampicin resistance: ☐Not detected ☐Detected ☐ Other	☐Passed ☐Failed & reported to CB if ≥2 in a row	☐No ☐Yes	☐Pellet leftover w/o SR ☐Untreated sputum w/o SR ☐Pellet w SR for ___min ☐ Untreated sputum w with SR for ___min ☐Retesting **not done**	☐MTB neg ☐MTB pos ☐Other Rifampicin resistance: ☐Not detected ☐Detected ☐ Other	
	S3 ☐Done ☐ND	☐MTB neg ☐MTB pos ☐Other Rifampicin resistance: ☐Not detected ☐Detected ☐ Other	☐Passed ☐Failed & reported to CB if ≥2 in a row	☐No ☐Yes	☐Pellet leftover w/o SR ☐Untreated sputum w/o SR ☐Pellet w SR for ___min ☐ Untreated sputum w with SR for ___min ☐Retesting **not done**	☐MTB neg ☐MTB pos ☐Other Rifampicin resistance: ☐Not detected ☐Detected ☐ Other	
	S3_L ☐Done ☐ND	☐MTB neg ☐MTB pos ☐Other Rifampicin resistance: ☐Not detected ☐Detected ☐ Other	☐Passed ☐Failed & reported to CB if ≥2 in a row	☐No ☐Yes	☐Pellet leftover w/o SR ☐Untreated sputum w/o SR ☐Pellet w SR for ___min ☐ Untreated sputum w with SR for ___min ☐Retesting **not done**	☐MTB neg ☐MTB pos ☐Other Rifampicin resistance: ☐Not detected ☐Detected ☐ Other	
	S4 ☐Done ☐ND	☐MTB neg ☐MTB pos ☐Other Rifampicin resistance: ☐Not detected ☐Detected ☐ Other	☐Passed ☐Failed & reported to CB if ≥2 in a row	☐No ☐Yes	☐Pellet leftover w/o SR ☐Untreated sputum w/o SR ☐Pellet w SR for ___min ☐ Untreated sputum w with SR for ___min ☐Retesting **not done**	☐MTB neg ☐MTB pos ☐Other Rifampicin resistance: ☐Not detected ☐Detected ☐ Other	

Archived as .gxx & .csv file? ☐S1-S3_L File name (optional):____________________________ ☐S4 (if done) File name (optional):__________________

ANNEX 2: Key to Case Report Form (CRF)

Patient ID = as defined by FIND, composed as follows: X2 YY 0001 S1
X2=Study name; YY=Site ID as specified by FIND; 0001=Patient ID S1 = Sputum specimen 1

A. Eligibility

- Checklist with study inclusion criteria confirms eligibility of patients. Patients must either fulfill criteria for case detection group or MDR risk group. Patients are eligible to participate if "yes" has been checked for all statements.

B. Patient information

- **Enrolment date:** (day/month/year)
- **Sex:** (check box)
- **Date of birth:** (day/month/year) Estimate year of birth in case of uncertainty and use XX / YY / ZZZZ for day, month and year
- **History of TB:** (Check box) Checking YES implies prior tuberculosis (TB) for which the patient received or receives anti-tuberculous chemotherapy for at least one month. In this case, the table below should be completed.
  - **Table 3:** (Check box and enter dates (day/month/year)) If dates or treatment outcome cannot be remembered, fill according to best estimates or check unknown. If treatment is ongoing, fill start date and check ongoing instead of entering end-date. If treatment was stopped, because it was not successful or defaulted, tick "stopped before completion" and enter end-date. For patients belonging to the MDR risk group, "patient considered cured: No" should be checked. Treatment categories are defined according to WHO standards:

DOTS Cat. I	New sputum-positive PTB New smear-negative PTB with extensive parenchymal involvement New cases of severe extrapulmonary TB
DOTS Cat. II	Relapse, treatment failure, treatment default (tx interrupted)
DOTS Cat. III	Sputum-negative PTB with limited parenchymal involvement Less severe extrapulmonary TB
DOTS Cat. IV	Chronic cases
Unknown	[ ]
Other- Specify ____________________ (if not treated according to above listed criteria)	

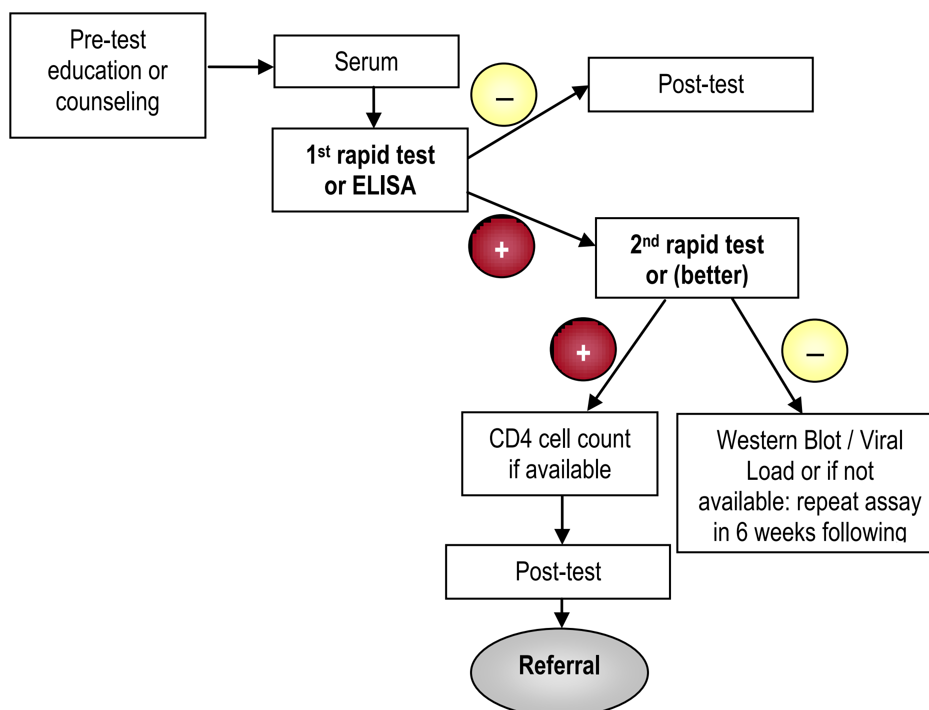
C. Chest X-ray

- Chest X-ray should be done at least for all smear-negative patients. Check box for C.1. Complete C.1.1 and C.1.2 if chest X-ray was done; then complete table 4 to provide details on radiological abnormalities if checked "no" in C.1.2.
- **Abnormalities in chest x-ray:** Localize the abnormality by checking at least one or several of the boxes in C.1.2.1. Define picture of abnormality by checking at least one of the description of

abnormalities in C.1.2.2. Provide the most likely conclusion with respect to TB in C.1.2.3 based on radiological and clinical findings. Especially in high HIV prevalence settings, TB does often not show the typical radiological characteristics, but rather mimics pneumonia, therefore the answer option "Pneumonia or atypical TB" has been included.

D. HIV result

- HIV testing will not be obligatory for the study, since it is not expected, that performance of the Xpert MTB assay is influenced by the HIV status or the level of immune-suppression. However, especially in high HIV prevalence settings, it is desirable to determine the HIV status of all enrolled patients, not only for study purposes, but - more importantly - for patient management. If HIV testing is done, the testing algorithm should follow WHO recommendations:



E. Clinical Follow up after 2 months

- **Status follow-up (FU):** tick category according to FU= FU visit will be required for the group of smear-negative and culture-negative patients with a Xpert-positive or PCR-positive result and a random control group of equal size; No FU required= FU visit is not required for all other groups; Lost to FU= the participant could not be found for a FU visit; Passed away= the participant died after enrolment.
- **Date of FU:** (day/month/year) specify date of FU visit
- **Received TB treatment:** if patient was started on TB treatment, specify date of start/first dose of anti-TB therapy received.
- **Symptoms:** Compared to the first visit at the time of enrollment.

- **CXR compared to baseline**: Compare the chest x-ray at enrolment to the chest x-ray at FU. If chest x-ray has not been repeated, check "Not done". If no abnormalities can be seen, check "normal". If abnormalities are seen, check "improved ", "same abnormal" or "worse".
- **Most probable diagnosis**: Based on clinical findings during FU, judge if TB, Non-TB or Unknown

Sputum 1

- **Date of collection** (enter day/month/year)
- **Sputum quality** (check one box per question)

  ☐Early morning ☐Spot (check one box; see explanation in annex on sputum collection)

  ☐W/o induction ☐W induction (check one box; normally sputum should be collected without induction)

  Since the assay performance of the test could potentially be influenced by the viscosity of specimens, we will collect information on whether specimens are: ☐very viscous **(entire specimen very sticky, high resistance to flow)**

  ☐viscous **(sticky or few very sticky clumps in specimen)**

  ☐not viscous/watery **(little resistance to flow as or similar to saliva)**

  Since particles could potentially clog the cartridge filter, we will collect information on whether specimens are: ☐Containing food or other particles ☐Not containing particles

  Since hemoglobin is a known PCR inhibitor, it will be important to know which samples contained blood: ☐very blood stained **(entire specimen brown/red)** ☐blood stained **(red/brown spots)** ☐not blood stained **(no red/brown spots)**
- **AFB** (check 1 box for processing, 1 box for result) Score positive slides using IUATLD scale.

  No AFB/100 fields = 0

  1-9/100 fields = record number

  10-99 AFB/100 fields = 1+

  1-10 AFB/field = 2+

  >10 AFB/field = 3+
- **Solid Culture:** (provide date of inoculation & positivity where applicable, check boxes). Use IUATLD scoring system. This is helpful not only in understanding the burden of infection, but also the chance that an individual culture may represent a laboratory cross-contaminant.

  No. AFB colonies = 0 (neg)

  1-19 AFB colonies = record exact number

  20-100 AFB colonies = 1+

  100-200 AFB colonies = 2+

  >200 AFB colonies = 3+

  A culture is reported as contaminated in the case of 1) complete overgrowth by non-mycobacteria within 3 weeks; or 2) color (pH) change; or 3) fungal contamination covering more than half of the culture surface (mycobacterial growth not possible anymore). For mixed cultures, isolated colonies are transferred to a new LJ tube.
- **Liquid Culture:** A semi-quantitative method will be used here as well, which will rely on the number of days from inoculation of liquid media until growth is detected, by any means, in order to estimate the burden of inoculates. Check result box and enter dates of inoculation and positivity. Instrument positive cultures for which no AFBs are detected by smear microscopy are to be reported as contaminated.

- **Speciation:** Each AFB positive culture per specimen should be speciated to the MTB complex versus NTM (non-tuberculous mycobacteria) level. This may be done using morphology + niacin/PNB or morphology + other (molecular, antigen detection) method, but should not rely on morphology alone. Not done or not applicable should only be ticked for negative or contaminated cultures. Identification at species level is not mandatory (check "not done or not applicable" if not performed).
- **Drug susceptibility testing:**
  - Check ND/NA if not done or not applicable (AFB negative culture). Enter date of inoculation (day/month/year) if done.
  - Table (check one or several methods used (at least one of the culture methods is needed for study purposes)). Fill the columns, for which method of use has been checked. S-sensitive, R-resistant, I-indeterminate, N-not performed.
- **PCR:**
  - Two trial sites will additionally perform other NAAT methods (ProbeTec and Amplicor) for sputum sample 1 as gold standard, in order to compare the Xpert performance to standard NAAT. Complete G.1 accordingly. Check done and enter date of performance (day/month/year) if applicable. Complete result case detection, any MTB pos is considered positive; except if negative control of this run is positive, In case of repetition, complete G.1.3.1 and G.1.3.2

Sputum 2 and 3 (see sputum 1)

Follow-up visit

- **For laboratory examinations, see sputum examinations at enrollment.**

F. Final diagnosis

- The final diagnostic category will be assigned by the local study coordinators according the definitions for data analysis and the table below. SS+ if smear and culture pos; S-C+ if smear neg and culture pos; CXR+ if chest radiograph abnormal and patient was stated on TB treatment based on clinical symptoms, suggestive of TB, but smear and culture examinations neg; Non-TB if smear and culture examinations were normal and patient was not stated on TB treatment based on clinical symptoms and CXR result; F if positive culture result during follow up; and I all for all indeterminate cases, that will be excluded from analysis (e.g. smear positive, culture negative patients, patients with 3 or more contaminated cultures, patients for which a clear diagnosis (TB yes or no) cannot be made after the follow up visit.
- **Comments** (text entry, not mandatory).
- **Transfer from source data to CRF signature and date of CRF completion:** each CRF should be completed and updated if new results are available by one (the same) staff member. When the laboratory data notation has been completed, signature and date of completion has to be recorded. This date may be 4 months after enrolment at the latest.
- **Investigator's signature and date of CRF approval:** Investigator should only sign and date after confirming that the CRF has been completed as required.

- **Signature and date of data entry completion** of staff conducting first and second electronic data entry: Data entry is done by local staff and to be completed 2 weeks after completion of CRF.
- **Copy CRF sent:** Tick and specify date when it has been sent to Cepheid/FIND

Diagnostic Categories for Case Report Form							
Category	**Diagnosis**	**Smear**	**Culture**	**Description**	**Initial CXR**	**Microbiological follow-up (FU)**	**Clinical & radiographic findings at FU**
SS+	TB, smear positive	pos	pos	On initial assessment at least 1 pos smear ≥1+ or ≥2 scanty pos smears ***and*** at least 1 MTB pos culture (see definition)	irrelevant	Not applicable (NA)	NA
S-C+	TB, smear negative, culture positive	neg	pos	On initial assessment 3 neg smears or 1 scanty smear and 2 neg smears and ≥1 MTB pos culture (see definition)	abnormal (except HIV pos)	NA	NA
CXR+	TB, culture negative (treated)	neg	neg	3 neg smears and only MTB neg cultures, pos CXR	abnormal	NA	NA
Non-TB	Non-TB (untreated)	neg	neg	3 neg smears and only MTB neg cultures on initial and FU assessment	irrelevant	Done for those with Xpert-pos MTB or PCR/NAAT-pos and randomcontrols: Smear and culture negative	for those with Xpert-pos MTB or PCR/NAAT-pos and for randomcontrols: Significant clinical improvement and CXR –if initially abnormal- not TB suggestive, normal or stable abnormal
F	TB, diagnosed during FU	irrelevant	at FU pos	Initially neg smears & cultures, but MTB pos culture during FU	irrelevant	Pos in culture or smear and culture	Clinical symptoms of TB
I	Indeterminate	Any other combination of results not matching other categories (see list in chapter per patient data analysis).					

Xpert^{TM} MTB Evaluation study result sheet (NO DATA ENTRY REQUIRED)

GeneXpert results will be imported from .csv file, so that no data entry is required.

Systematic check of Xpert^{TM} MTB results is required however, and should be registered for every enrolled patient:

- Done /Not done: Xpert^{TM} MTB should be done for 3 specimens per patient. For sputum 3, Xpert^{TM} MTB will be performed twice (once using 2 ml, once using 1 ml sample/SR mix as test volume) at least until the review meeting will be held July 08. An additional Xpert MTB will be performed for patients requiring follow up.
- Cartridge pre-performance check: Inspect visually as described in checklist provided by FIND. Contact Catharina Boehme if more than 2 cartridges in a row fail pre-performance check.
- Result (check box for case detection and for rifampin resistance). Results for case detection can be MTB negative, MTB positive or other (error, no result, invalid). Results for Rif resistance detection only needs to be completed if result for case detection MTB is positive. Answer options for Rifampicin resistance are Rifampicin resistance not detected, detected or indeterminate. Semiquantitative results do not have to be completed in this form.
- Post-performance check: Inspect visually as described in checklist provided by FIND. Contact Catharina Boehme if more than 2 cartridges in a row fail post-performance check. .
- Result requiring retesting (check box yes or no): A sample requires retesting if one of the following Xpert^{TM} MTB results is displayed: ERROR, INVALID, NO RESULT, Rif Resistance INDETERMINATE.
- Retest done from (tick and enter incubation time for sample treatment reagent where applicable): Retesting can be done from pellet leftover (this would typically be the case for S1, S2 and S4) or untreated sputum leftover (S3). In most cases, remaining pellet or sputum will already be pretreated with sample treatment reagent (SR). If this is the case, estimate the approximate SR incubation time for this sample in min. A minimal volume of 250 µl of pellet or 500 µl untreated sputum are needed to repeat the run. If not enough sputum or pellet were left for retesting, check retesting not done.
- Result in case of repetition (check results as explained above).
- Follow up after 2 months required (check box): Follow up will be required for all smear-negative, culture-negative patients with a positive Xpert^{TM} MTB result and a group of random controls, for which IDs will be provided by FIND. S4 (sputum 4) needs to be completed if follow up is done.

ANNEX 3: Sputum collection

- Specimens must be collected in appropriate sterile, screw-capped, leak-proof, wide-mouthed containers. Containers should be graduated.

- There are two basic types of sputum specimens

  - **<u>spot specimens</u>** - collected at a single time in the health facility; if the volume brought up from the lungs in a single cough is too small, the patient may collect sputum produced over a period of 1 hour in the same container; initial 'spot' specimen usually taken when the patient first presents with symptoms and another 'spot' specimen taken when the patient returns with the second (i.e., the early morning) specimen. Sputum 1 and 3 in this study are spot specimens.
  - **<u>morning specimens</u>** - these are spot specimens collected in the early morning when respiratory secretions that have gathered in the lower airways are cleared. If the volume brought up from the lungs in a single cough is too small, the patient may collect sputum produced over a period of 1 hour in the same container. Sputum 2 in this study is a morning specimen.

- Patients will be told that nasal secretions and saliva are not sputum. Subjects will be told that the desired sample is deep-cough sputum consisting of the thick mucoid white-yellow material from the lower airways and lung. Subjects will be instructed not to touch the inside of the specimen containers or lids.

- Labeling of the container will be done before it is used. Information written on the side, not on the lid must comprise patient ID, date of collection and sputum number 1, 2 or 3.

- Specimens will be collected in the following manner:

  - The subject will be preferably be seated or standing.
  - Whenever possible, subjects will be instructed to rinse the mouth twice with water.
  - Subjects will be instructed to inhale deeply, cough vigorously, and expectorate the material produced into collecting receptacle. The subject should be told "to cough the specimen from deep in the chest". If the subject does not cough spontaneously, instruct him/her to take several deep breaths and then hold their breath momentarily; repeating this step several times will often induce coughing.
  - After coughing, the subject will be instructed to hold the sterile specimen container to his/her lower lip and gently release the specimen into the container. Instruct the patient to avoid spills or soiling the outside of the container with the specimen. The lid should be carefully placed on the container without touching the inside of the lid.
  - Lids should be firmly screwed back onto specimen containers to prevent leakage.

- Specimens visibly contaminated with oral material, food particles or saliva will be discarded and the subject will receive a second sterile specimen container and be instructed to try again.
- Specimens to be taken to the laboratory for processing as soon as possible after collection.
- Throughout collection period and during transport to the laboratory, specimens should be held at 2-8 °C whenever possible.

ANNEX 4: Sputum decontamination for culture & microscopy (Kubica)

For study purposes, all participating laboratories will follow the sputum processing procedure described below.

Principle

The majority of clinical specimens sent to the mycobacteriology laboratory for cultural confirmation of suspected mycobacterial infection are contaminated by rapidly growing normal flora. To maximize the mycobacterial yield, contaminated specimens require treatment with a digestion and decontamination procedure. NALC-NaOH-Citrate-Solution (NALC-NaOH) is a gentle but effective decontaminating agent. NaOH (Sodium Hydroxide) can be used both as a digesting and decontaminating agent. As mucolytic agent, it is most effective at a final concentration of 2%. However, this concentration is not only toxic to contaminants, but also to some mycobacteria. NALC (N-Acetyl-L-cysteine) is a mucolytic agent. NALC loses its mucolytic activity on standing. Therefore, the reagents should be gently mixed before use and used within 24 h. Sodium citrate (Tri-sodium-citrate-dihydrate) is included in the NALC-NaOH-Citrate-Solution to bind heavy metal ions which could inactivate NALC. A phosphate buffer with a pH 6.8 decreases the activity of NALC-NaOH-Citrate-Solution and lowers the specific gravity of the specimen before the mycobacteria are recovered in a concentrated form by centrifugation. The resuspended pellets are used for semi-quantitative sputum smear and semi-quantitative culture onto solid media plus liquid culture.

Safety

- A safety cabinet biological class II is needed for the entire procedure.
- Working with *Mycobacterium tuberculosis* grown in culture requires biosafety level 3 practices.
- NaOH is alkaline and cause burns. In case of contamination, take off contaminated clothes immediately. Gloves must be worn. In the event of eye or skin contact, rinse for at least 15 min with tap water and seek medical advice. If ingested, drink milk and seek medical advice.

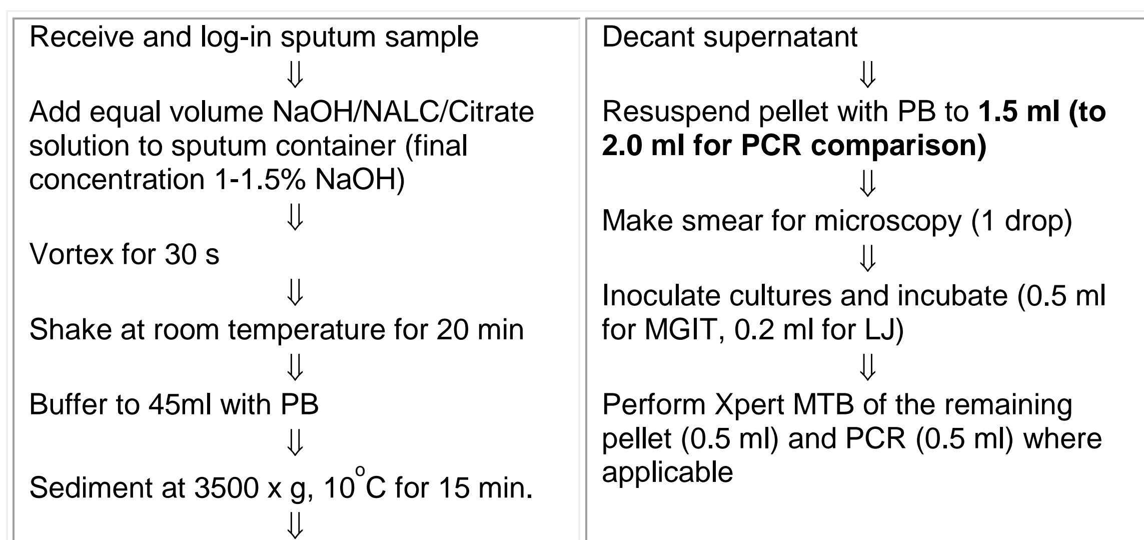
Materials

- N-Acetyl-L-cysteine, 0.5 g measured in sterile 100 ml Erlenmeyer flask
- 6 % Sodium hydroxide solution (NaOH), autoclaved, 50 ml
- 2.9 % Tri-sodium-citrate-dihydrate solution, autoclaved, 50 ml
- Phosphate buffer solution (Potassium-Di-hydrogen-phosphate) pH 6.8
- MGIT vials (7 ml)
- BBL MGIT PANTA and BACTEC MGIT Growth Supplement
- LJ-medium (Löwenstein Jensen medium)
- Safety cabinet biological class II, vortexer, shaker, centrifuge with employments for 50 ml centrifuge tubes and aerosol protection hoods, MGIT 960, Incubator 36 ± 1°C, refrigerator
- Multipette, timer, racks
- Graduated sterile cylinder 100 ml, sterile centrifuge tubes 50 ml, sterile transfer pipettes 5 ml sterile tips for multipette 10 ml
- Camping gas or Bunsen burner, microscopy slides, pencil, disinfectant, autoclave bag

Processing

- Sort the samples according to ascending sequence of numbers.
- Label all tubes, cultural media (MGIT, LJ) and slides with specimen numbers.
- Fill a report form for each investigation material arrived in the laboratory.
- Prepare NALC-NaOH-solution (use reagent within 24 hours).
- Add 50 ml 4-6% Sodium hydroxide solution (NaOH) and 50 ml 2.9 % Tri-sodium-citrate-dihydrate solution to 0.5 g N-Acetyl-L-cysteine to arrive at a NaOH concentration of 2-3%.
- Add 2-3% NALC-NaOH-Citrate to the sputum container in a volume that is equal to the specimen volume (final NaOH concentration after 1:1 dilution with specimen 1-1.5%).
- Vortex the alkaline suspension briefly and set at room temperature for 15 minutes. Start timer when NaOH is added to the first specimen. Place the specimens on a shaker to improve homogenization.
- When 15 minutes have passed, add phosphate buffer to the 50 ml mark on the centrifuge tube. Do not touch the centrifuge tube with the buffer container when dispensing buffer.
- Mix suspension by inverting the tubes several times or by vortexing.
- Place tubes in centrifuge in a balanced arrangement. Subject tubes to centrifugation at 3500 times g (= 4000 rpm) for 15 minutes. If possible, use a refrigerated centrifuge to maintain centrifugation temperature at 8-10°C.
- Remove tubes from centrifuge and decant supernatant into splash-proof container in the biosafety cabinet, leaving only sample pellet in the tube. If necessary, swab lip of tube with disinfectant-soaked gauze.
- Add phosphate buffer to the centrifugation pellet to adjust volume to **1.5 ml**. For the pellets, which will be used for an Xpert/PCR comparison **2.0 ml** will be required. Recap and vortex briefly to resuspend the pellet.
- Spread a drop of the sediment (20-30µl) onto a labeled glass microscope slide. Place slides on slide rack to dry for about 20-30 min.
- Add 0.5 ml into the MGIT tube, close the tube tightly and shake it twice upside-down. Leave the tube in the rack for 30 min and insert it then into the MGIT 960.
- Add 0.2 ml (5-10 drops) to LJ-medium.
- Transfer 0.5 ml into the PCR tube where needed and use the remaining 0.5 ml for Xpert MTB.

Summary



Receive and log-in sputum sample ⇓ Add equal volume NaOH/NALC/Citrate solution to sputum container (final concentration 1-1.5% NaOH) ⇓ Vortex for 30 s ⇓ Shake at room temperature for 20 min ⇓ Buffer to 45ml with PB ⇓ Sediment at 3500 x g, 10°C for 15 min. ⇓	Decant supernatant ⇓ Resuspend pellet with PB to **1.5 ml (to 2.0 ml for PCR comparison)** ⇓ Make smear for microscopy (1 drop) ⇓ Inoculate cultures and incubate (0.5 ml for MGIT, 0.2 ml for LJ) ⇓ Perform Xpert MTB of the remaining pellet (0.5 ml) and PCR (0.5 ml) where applicable

ANNEX 5: Semi-quantitative mycobacterial smear

Principle

The goal of acid-fast microscopy is to detect mycobacteria in clinical samples and to give a semi-quantitative estimation of their number, which serves as a rough guide to the bacterial burden of disease and the infectiousness of the diseased patient. Mycobacteria and related organisms retain carbolfuchsin and other dyes despite washing with acid/alcohol. This feature is exploited to differentiate mycobacteria from the background of cellular material and other organisms in clinical specimens.

Materials

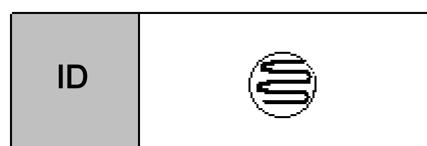
- Microscope slides w/ one frosted end, pencil
- Binocular microscope with 20, 40, 100x objectives
- Ziehl-Neelsen staining kit
- Camping gas or Bunsen burner and staining rack

Procedure

- Briefly flame fix after air dry.
- Stain for acid-fast microscopy using Ziehl-Neelsen technique:

Ziehl-Neelsen
Cover sample area with carbolfuchsin/phenol
Flame heat until steam rises, do **not** boil. Leave 2 min
Rinse lightly with water
Flood slide with acid-alcohol, leave to sit for 2 min
Rinse lightly with water
Flood slide with Methionine blue for 3 min
Rinse lightly with water

- Examine the slide using visual light (Ziehl-Neelsen, ZN).
- Begin examination with low-power screen of entire sample area to identify thick areas, areas that have been lost during staining and washing process, and other problem areas.
- Read several representative sections of the slide. At least 100 fields should be read. Slides should be read in a sweeping pattern, avoiding repeated examination of the same area (see figure below).



- Examine the slide using the 100x oil-immersion objective. Score the smear using the table below.

Number of acid fast Bacilli	**Result**
0	negative
1-9/100 fields	record number of AFB
10-99/100 fields	+
1-10/field	++
> 10/field	+++

ANNEX 6: Semi-quantitative mycobacterial culture

Principle

The goal of mycobacterial culture is to detect viable mycobacteria in clinical samples. Mycobacteria grow on specific media after processing. Factors such as the rate of growth, the colony morphology, and the microscopic appearance of culture isolates help distinguish mycobacteria from contaminating flora. Liquid culture provides increased sensitivity and speed, while solid culture yields information about morphology and purity of culture. The higher the bacterial load in a sample, the greater the number of colonies in solid culture and the faster growth detection in liquid culture.

Materials

- Commercial LJ and MGIT media
- Sterile plastic pipettes (5 ml)
- Materials for microscopic culture confirmation and speciation of culture isolates

Bactec MGIT 960

Please refer to the FIND MGIT Manual for detailed information (the manual can be downloaded from the FIND Web site: www.finddiagnostics.org).

Reconstitution of PANTA

- Dissolve PANTA in 15 ml MGIT Growth Supplement.
- Add 0.8 ml of the resultant enrichment to each MGIT tube just prior to inoculation (amount for manual MGIT is 0.1 ml of PANTA and 0.5 ml OADC).
- To maintain the CO_2 concentration in the media open tubes one at a time and for as short a time as possible. Do not leave multiple MGIT tubes uncapped at the same time.
- Perform these steps in biosafety cabinet class 2.

Inoculation of MGIT Medium

- Perform all inoculation steps in the class II biosafety cabinet.
- Mark each MGIT tube with patient study ID number label.
- Using the pipette used for adding the buffer (or a fresh, sterile pipette or transfer pipette for each specimen) or adjustable pipettor with filter plugged tips, add 0.5 ml of a well mixed processed/concentrated specimen to the appropriately labeled MGIT tube.
- To reduce the risk of cross-contamination and to maintain the CO_2 concentration in the media, open tubes one at a time and for as short a time as possible. Do not leave multiple MGIT tubes uncapped at the same time.
- Tightly recap the tube and mix by inverting the tube several times.
- Wipe tubes and caps with a mycobactericidal disinfectant.
- Leave inoculated MGIT tubes at room temperature for 30 min.

Incubation

- Open the desired MGIT 960 drawer and press the “tube enter” key. The barcode scanner will light up.
- Scan the inoculated MGIT tube and load into the slot identified by the MGIT 960. Perform these steps in biosafety cabinet class 2.
- Be sure that the cap is tightly closed.

- Do not to shake the tube during the incubation.
- Check MGIT 960 daily for indicator lights flagging positive and negative cultures.
- Incubate MGIT tubes until the instrument flags them as positive or negative.
- Positive and negative tubes will be displayed on the screen and outside indicator of the drawer. Positive will be followed by continuously beeping sound signal.
- Inside of the drawer after pushing the proper button:
- Positive tubes will be displayed by the indicator light changing from red to green at the exact location of the tube in the instrument drawer.
- Negative tubes will be displayed by a green indicator light at the exact location of the tube in the instrument drawer.
- If capacity of the MGIT 960 becomes an issue, remove negative tubes at 4 weeks and transfer to incubator, checking manually (with Wood's lamp or UV transilluminator) for growth daily.

Work-up of Positive MGIT Cultures

- Open the desired drawer and press the "positive" key.
- This will be displayed by a green indicator light showing the exact location of the positive tube in the instrument drawer.
- Remove the positive tube and scan.
- Visually inspect MGIT tube for potential mycobacterial growth.
- Mycobacterial growth typically appears granular with only slight turbidity.
- *M. tuberculosis* growth settles at the bottom of the tube.
- *Usually the growth time is more then 3 days*
- Proceed with inoculation of a blood agar plate, AFB smear and LJ subculture (required for DST in case of MGIT contamination), in that order and freeze aliquot of pos culture.
- Incubate blood agar plate at 37°C for 48 hours, checking for growth of contaminants at 48 hours.
- Species identification is to be carried out on the first positive tube for each specimen (MGIT or LJ) using the Tauns Capilia test or analogous rapid system according to SOP provided below.

Ziehl-Neelsen Smear of Positive MGIT Cultures

- Mix the broth by vortexing and then remove a small aliquot, using a sterile pipette. Place 1-2 drops of this on the slide and spread it on a small area (approximately 2 x 1 cm).
- If the smear is negative for AFB and the tube does not appear to be contaminated, (broth is clear) re-enter the tube into the instrument for further monitoring. (See MGIT 960 System's User Manual, Section 4.6.3, for returning positive tubes to instrument for further testing).
- After 3 days, visually inspect MGIT tube for growth and repeat AFB smear as above. If AFB smear remains negative, contamination is not found on the blood agar, and the LJ slants are not positive by 8 weeks, the culture is considered to be negative.

Work-up of Negative MGIT Culture

- Open the desired drawer and press the "negative" key.
- Remove the negative tube and scan.
- Visually inspect MGIT tube for potential mycobacterial growth.
- If there is suspicion of mycobacterial growth in a "negative" tube, proceed with AFB smear, inoculation of blood agar plate, and subculture on LJ.

Dealing with MGIT Contamination

Isolation of Mycobacteria from Contaminated or Mixed Cultures whenever DST is indicated and no other Positive, Non-Contaminated Cultures are available for that Patient

- If contamination is confirmed with negative AFB smear from the broth, discard the specimen and report as contaminated.
- If contamination is confirmed with a positive AFB smear from the broth but other specimens collected from a patient are not contaminated, it is not necessary to attempt to salvage a contaminated culture.
- If contamination is confirmed with a positive AFB smear from the broth and no other non-contaminated cultures are available, complete the following procedures:
- Transfer the entire tube of MGIT broth into a 50 ml centrifuge tube.
- Add an equal volume of sterile 4% NaOH solution.
- Mix well and leave at room temperature 20 minutes, mixing and inverting the tube periodically.
- Add sterile phosphate buffer pH 6.8 up to 40 ml mark and mix well.
- Centrifuge at 3500 x g for 20 minutes.
- Carefully pour off the supernatant fluid into a suitable container with mycobactericidal disinfectant.
- Re-suspend the pellet in 1.0 ml of buffer and mix well.
- Inoculate 0.5 ml into a fresh MGIT tube supplemented with MGIT Growth Supplement/PANTA.
- Additionally inoculate one LJ slant with 0.1-0.2 ml of reprocessed culture.
- Wipe tubes and caps with a mycobactericidal disinfectant.
- Leave inoculated MGIT tubes at room temperature for 30 min.
- Load tube into MGIT 960 and observe for growth of mycobacteria as before.

LJ Primary Culture

Inoculation

- Using the pipette used for adding phosphate buffer to the pellet (or a new sterile pipette), inoculate 0.2 ml of the resuspended processed sputum sample onto an egg slant (1 LJ or 1 LJ-PACT slant).
- Lay slants with medium face up for 30 min to allow the bacteria to adhere to the surface of the medium.
- Incubate at 37°C for up to 8 weeks.
- Examine for growth weekly for up to 8 weeks.
- Continue examinations until the first of the following three events occurs: (a) colonies are sufficiently large to count, (b) contamination is apparent, or (c) no growth is apparent after 8 weeks.
- Any egg culture with growth believed to be *Mycobacteria* has to be confirmed by Ziehl-Neelsen smear microscopy.
- Cultures completely overgrown by bacterial or fungal contamination are discarded and the result recorded as “contaminated” if occurred within 3 weeks. In case of mixed LJ culture, pick isolated colonies and re-culture for purity.
- Species identification is to be carried out on the first positive tube for each specimen (MGIT or LJ) using the Tauns Capilia test or analogous rapid system according to SOP provided below.
- Record the semiquantitative growth result according to table below.

Smear from LJ

- Place 2 clean loops of sterile water on the slide. Use of 10 mcl volume loops. Pick up several pieces from several colonies on slant. Put material onto the slide mix with water and spread it on a small area (approximately 1 x 1 cm).
- If microscopy test reveals AFB with reliable color this may mean the culture belongs MTB complex.
- If the smear is negative for AFB or it appears blue or semi violet-crimson color bacteria this may mean the tube is contaminated.

Inoculation and reporting

<table>
<tr><th>LJ</th><th>MGIT</th></tr>
<tr><td>Place 5-7 drops (~30µl each) onto the solid media (~0.2 ml).</td><td>Inoculate MGIT tube with 0.8 ml of antibiotics (PANTA) and growth supplement and then with 0.5 ml of resuspended pellet.</td></tr>
<tr><td>Leave plate face-up until inoculum is absorbed. Then incubate LJ slants at $37^{\circ}C$.</td><td>Only insert into instrument after 30 min to avoid oxygen consumption by still living bacteria.</td></tr>
<tr><td>Solid media are examined weekly for 8 weeks.</td><td>Incubate liquid media vials at $37^{\circ}C$ for 6 weeks.</td></tr>
<tr><td>Cultures completely overgrown by bacterial or fungal contamination are discarded and the result recorded as “contaminated” if occurred within 3 weeks. In case of mixed LJ culture, pick isolated colonies and re-culture for purity.</td><td>see above for details</td></tr>
<tr><td>Colonies on LJ suspected to be mycobacteria are examined by Ziehl-Neelsen staining.</td><td>Positive liquid cultures are confirmed by Ziehl-Neelsen staining and contamination excluded by inoculation of blood agar plates and LJ subculturing.</td></tr>
<tr><td colspan="2">Results are reported semi-quantitatively:</td></tr>
<tr><td><table>
<tr><th># of AFB colonies</th><th>Result</th></tr>
<tr><td>0</td><td>negative</td></tr>
<tr><td>1-19</td><td>record number</td></tr>
<tr><td>20-100</td><td>+</td></tr>
<tr><td>101-200</td><td>++</td></tr>
<tr><td>201-500</td><td>+++</td></tr>
<tr><td>>500</td><td>++++</td></tr>
</table></td><td>The date of inoculation and the date of positivity are recorded and will provide the time to positivity in days.</td></tr>
<tr><td colspan="2">Positive mycobacterial cultures must at least be speciated to the level of MTB-complex vs. NTM using Tauns Capilia test (at least from the first positive tube (MGIT or LJ) for each specimen).</td></tr>
</table>

ANNEX 7: Speciation with Tauns Capilia TB

Assay Principle

This immunochromatographic assay is based on a double antibodies sandwich technique, in which (i) antibody labeled by colloidal gold particles reacts with target antigens to form an antigen-antibody complex, (ii) this complex migrates across a chromatographic carrier such as a filter paper and (iii) the complex is captured by second antibody readily fixed in the middle of the chromatographic carrier. If the target antigens are present in the test specimen, a color reaction caused by the labeled colloidal particles is observed at the site on the chromatographic carrier where the second antibody is fixed, and the specimen is interpreted as positive. This kit employs colloidal gold-labeled MPB64 monoclonal antibody (mouse).

Capilia TB Test Procedure

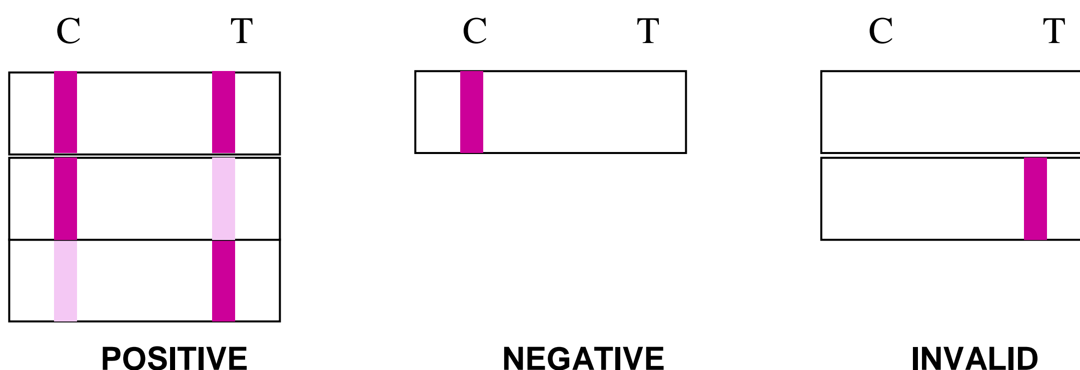
Capilia identification test should only be done on **AFB positive** cultures.

Preparation for LJ tubes
Dispense **0.2 ml** of Tauns extraction buffer into an Eppendorf tube.
Pick **1µl** of bacteria (equivalent to the amount of a 1mm-diameter platinum micro-loop) from the bacterial colony that grew on the solid medium.
Suspend the collected bacteria in the buffer solution in the tube.
Close the tube with a lid and fully suspend with a vortex mixer.

For AFB positive MGIT tubes, no preparation is required.

Capilia TB testing
Apply **100µl** of the AFB positive MGIT media or the LJ colony suspension to the sample well on the Capilia TB specimen placing area of the test plate directly without any manipulation.
Observe the reading area of the test plate for **5 minutes** and for presence or absence of red to deep purple (or faint) bands both for the control and the test bands. (*all results should be read within 1 hour after dispensing the sample into the sample well)*
Interpret the results as explained below and report result.
For specimens with a negative or invalid test at 5 min, read the test again at 1 hour.

Interpretation of Capilia TB test results



Positive for Mycobacterium tuberculosis complex

Red-purple colour appears in both the T and C reading area.

The result is read as positive if area T shows red-purple colour that is lighter than or the same as, or darker than the colour in area C.

Negative for Mycobacterium tuberculosis complex

A test is negative if red-purple colour appears in area C but **not** in area T.

Invalid test

A test is invalid if red-purple colour does not appear in area C.

If a test is invalid, repeat test using a new test plate, preferably from a newly opened foil pouch. If the repeated test result is valid, enter this result on the case report form.

ANNEX 8: Drug susceptibility testing

LJ proportion method

Principle

Enables precise estimation of the proportion of mutants resistant to a given drug; several 10-fold dilutions of inoculum are inoculated in both, control media and drug–containing media; at least one dilution should yield isolated countable (50 -100) colonies. When these numbers are corrected by multiplying by the dilution of inoculum used, the total number of viable colonies observed on the control medium, and the number of mutant colonies resistant to the drug concentrations tested can be determined. The proportion of bacilli resistant to a given drug is then determined by expressing the resistant portion as a percentage of the total population tested.

Preparation of drug containing media

Drug concentrations are as follows:

Rifampicin 40 µg/ml

Isoniazid 0.2 µg/ml

Ethambutol 2 µg/ml

Streptomycin 4 µg/ml, (dihydro-streptomycin sulfate, concent. corresponding to 4 mg/ml base)

Preparation of drug stock solution is done along with preparation of drug free LJ media. For each prepared batch, quality must be assured by inoculation of a wild strain. One set consist of one LJ slope each for two 10^{-2} drug free slopes, two 10^{-4} drug free slopes, eight LJ drug containing slopes, two each for drugs H, R, E & S (one each for 10^{-2} and 10^{-4} suspensions), total 12 LJ slopes. Each LJ slope requires approximately 5 ml of LJ fluid. The following table gives the number sets to be prepared is calculated in the following manner;

Number of sets required to be prepared	Total volume of LJ solution required for each set for proportion method (ml)
10	600
20	1200
30	1800
40	2400
50	3000
60	3600
70	4200
80	4800
90	5400
100	6000

Drug-containing LJ slopes are made by adding appropriate amounts of drugs asceptically to LJ fluid before inspissation. A stock solution of the drugs is prepared based on the potency of the drug in sterile distilled water for streptomycin, isoniazid and ethambutol and rifampicin is dissolved in absolute methanol. The solutions are sterilized by membrane filtration. Suitable working dilutions are made in sterile distilled water and added to the LJ fluid, dispensed in 5 ml amounts and inspissated once at 85°C for 50 minutes.

Drug stock solutions should be prepared fresh on the day of drug media preparation.

The medium can be stored in the cold for 3-4 weeks.

Isoniazid (H):

Drug potency = 1g to 1g substance.

Stock solution preparation:

Weigh out 20mg of isoniazid in 40ml of sterile distilled water (500μg/ml). Label with date of preparation.

Working solution:

Prepare the working solution on the day of drug media preparation (Sterilize by filtering through a membrane filter.)

2 ml of stock solution (500μg/ml) + 48ml of sterile distilled water (=50ml of 20μg/ml). **Do not store this solution.**

Number of sets required to be prepared	Number of bottles of 'H' drug media required (each bottle with ~5ml of LJ fluid)	Ml of working solution of H (20μg/ml)	Amount of LJ fluid to be added (ml)	Final concentration of H in LJ (μg/ml)
5	10	0.5	49.5	0.2
10	20	1	99	0.2
15	30	1.5	148.5	0.2
20	40	2	198	0.2
25	50	2.5	247.5	0.2
30	60	3	297	0.2

Ethambutol (E):

Drug potency = 1g to 1g substance

Stock solution preparation:

Weigh out 20mg of ethambutol and dissolve in 100ml of sterile distilled water to get 200μg/ml of stock solution. Sterilize by filtering through a membrane filter. **Stock solution of E should not be stored.**

Number of sets required to be prepared	Number of bottles of 'E' drug media required (each bottle with ~5m of LJ fluid)	Ml of stock solution (200μg/ml)	Amount of LJ fluid to be added	Final concentration of E in LJ (μg/ml)
5	10	0.5	49.5	2
10	20	1	99	2
15	30	1.5	148.5	2
20	40	2	198	2
25	50	2.5	247.5	2
30	60	3	297	2

Dihydro Streptomycin Sulphate (S):
Preferred substance Sigma 7253
Correction for potency required.
Stock solution preparation:
Weigh out 20mg / Potency of dihydro streptomycin sulphate and dissolve in 50 ml SDW to obtain 400μg/ml of stock solution. For example, if the potency is 0.731, the required amount of active drug is 20/0.731 = 27.35 mg. 27.35 mg is dissolved in 50 ml of SDW to obtain 20 mg of active drug. Always look for the potency mentioned on the drug bottle.
Do not store this solution.

Number of sets required to be prepared	Number of bottles of 'S' drug media required (each bottle with ~5m of LJ fluid)	Ml of stock solution (400μg/ml)	Amount of LJ fluid to be added	Final concentration of S in LJ (μg/ml)
5	10	0.5	49.5	4
10	20	1	99	4
15	30	1.5	148.5	4
20	40	2	198	4
25	50	2.5	247.5	4
30	60	3	297	4

Rifampicin:
Preferred substance Sigma R3501
Correction for potency required.
Stock solution preparation:
Weigh out 40mg / Potency of rifampicin and dissolve in 5 ml of absolute methanol, followed by addition of 5 ml of 99% ethanol to get 4000μg/ml of stock solution. **Do not store this solution.**

Number of sets required to be prepared	Number of bottles of 'R' drug media required (each bottle with ~5m of LJ fluid)	Ml of stock solution (4000μg/ml)	Amount of LJ fluid to be added in ml	Final concentration of R in LJ (μg/ml)
5	10	0.5	49.5	40
10	20	1	99	40
15	30	1.5	148.5	40
20	40	2	198	40
25	50	2.5	247.5	40
30	60	3	297	40

MGIT Drug Susceptibility Testing

Perform DST according to the following scheme:

- Positive, non-contaminated MGIT and positive LJ from this specimen:
  → Perform DST from MGIT
- Positive, non-contaminated MGIT and negative LJ
  → Perform DST from MGIT
- Negative or contaminated MGIT and positive LJ
  → Perform DST from LJ
- Positive, contaminated MGIT and negative LJ
  → Redecontaminate MGIT tube and perform DST from MGIT if successful.
- Negative, contaminated MGIT and negative LJ
  → No DST
- Negative, non-contaminated MGIT and negative LJ
  → No DST

Standard Drug Concentrations for Use in MGIT (µg/ml)

STR:	1.0
INH:	0.1
RIF:	1.0
EMB:	5.0
PZA:	100.0

- Perform all drug reconstitution/addition steps in the class II biosafety cabinet.
- Label each MGIT tube with relevant drug, concentration, and patient study ID number. Tubes and drug concentrations are listed below:
- SIRE GC: MGIT Tube (= SIRE growth control and will not contain any of the SIRE drugs)
- STR 1.0: MGIT tube
- INH 0.1: MGIT tube
- RIF 1.0: MGIT tube
- EMB 5.0: MGIT tube
- Where done:
- PZA GC: PZA medium MGIT tube (=PZA medium growth control and will not contain PZA)
- PZA 100: PZA medium MGIT tube
- Using a sterile pipette or transfer pipette, reconstitute each of the SIRE drug vials with 4 ml of sterile distilled/deionized water:
- Use separate pipette for each drug.
- Reconstitute PZA drug vial with 2.5 ml of sterile distilled/deionized water.
- Add 0.8 ml MGIT SIRE Supplement to each SIRE tube and the SIRE growth control tube.
- Add 0.8 ml MGIT PZA Supplement to each PZA medium tube.
- Add 0.1 ml (100 µl) of the appropriate reconstituted drug solutions into each of the corresponding labeled BACTEC MGIT 960 tubes.
- Do not add drug solution to the GC tubes

Preparation of Inoculum from MGIT Tube:

- The day a MGIT tube is positive by the instrument is considered **Day 0**
- The tube should be kept incubated for at least one more day (**Day 1**) before drug susceptibility testing (may be incubated in a separate incubator at 37ºC ± 1ºC).
- A positive tube may be used up to and including the fifth day (**Day 5**) after it becomes instrument positive.

- A tube that has been positive for more than 5 days should be subcultured in a fresh MGIT tube supplemented with MGIT 960 growth supplement and should be tested in MGIT 960 instrument until it is positive. Use this tube from one to five days of instrument positivity.
- If growth in a tube is of Day 1 or Day 2, mix well to break up clumps (vortex). Leave the tube undisturbed for about 5-10 minutes to allow large clumps to settle to the bottom. Use the supernatant undiluted for inoculation of the drug set.
- The best way is to use the vortex or shaker and sterile 3 mm glass beads in the volume of 3-4 ml. For this purpose do use it in 20 mm thick wall glass tube. Perform vortex 15-20 min.
- If growth is on Day 3, 4, or 5, mix well to break up the clumps. Let the large clumps settle for 5-10 minutes and then dilute 1.0 ml of the positive broth in 4.0 ml of sterile saline. This will be a 1:5 dilution. Use this well–mixed, diluted culture for inoculation.

Inoculation from Positive MGIT Tube Specimen

SIRE Growth Control Tube
- Using sterile pipettes, dilute 0.1 ml of MGIT specimen in 10 ml sterile saline and mix well.
- This is the 1:100 Growth Control Suspension for a 1-2 day culture and 1:500 for a 3-5 day culture
- Inoculate 0.5 ml of this suspension into the GC-labeled tube
- Immediately recap the tube tightly and mix by inverting the tube several times.

PZA Growth Control Tube
- Dilute 0.5 ml of the MGIT specimen prepared in section 8.2 into 4.5 ml sterile saline and mix well.
- This is the 1:10 PZA Growth Control Suspension for a 1-2 day culture and 1:50 for a 3-5 day culture
- Inoculate 0.5 ml of this suspension into the PZA GC-labeled tube
- Immediately recap the tube tightly and mix by inverting the tube several times.

Drug-Containing Tubes
- Inoculate each labeled, drug-containing tube with 0.5 ml of the MGIT specimen prepared in section.
- Immediately recap the tube tightly and mix by inverting the tube several times.
- Wipe all tubes and caps with a mycobactericidal disinfectant.

Preparation of Inoculum from LJ Media

- Colonies from solid media may be used if they are no more than 15 days from the first appearance of positive growth. (Do not use very young culture because the growth rate for sensitive and resistant colonies may be different).
- Using a sterile loop or proper applicator (spatula), scrape as many colonies as possible trying not to remove any of the solid medium.
- Transfer the growth into a sterile tube (approximately16x128 mm) containing 4 ml 0.85% saline (or BBL Middlebrook 7H9 broth) and 3-4 ml of 3 mm sterile glass beads.
- Tighten the cap and Vortex the tube for 2-3 minutes to break up any large clumps. Repeat it as many as necessary. The turbidity of the suspension should be greater than the McFarland number 1 standard.
- Let the suspension stand for 20 minutes undisturbed.
- Using a sterile pipette, carefully transfer the supernatant suspension into another sterile tube. Avoid taking any growth that has settled on the bottom.
- Let this tube stand for another 15 minutes undisturbed.
- Using a sterile pipette carefully transfer the supernatant suspension out with a pipette without disturbing the sediment and transfer into a third sterile tube.
- Again avoid taking any growth that has settled on the bottom.
- The turbidity of this suspension should be greater than McFarland 0.5 standard.

- Adjust the turbidity of this suspension to McFarland 0.5 standard by adding sterile saline and adjusting by visual comparison.
- The turbidity should not be less than McFarland 0.5.
- Dilute 1.0 ml of this suspension in 4.0 ml of sterile saline and mix well.
- This 1:5 dilution will be used as the inoculum for DST.

Inoculation from Solid Media-Derived Specimen

SIRE Growth Control Tube

- Using sterile pipettes, dilute 0.1 ml of MGIT specimen prepared in Section 8.4 into 10 ml sterile saline and mix well.
- This is the 1:500 Growth Control Suspension
- Inoculate 0.5 ml of this suspension to the GC-labeled tube
- Immediately recap the tube tightly and mix by inverting the tube several times.

PZA Growth Control Tube

- Dilute 0.5 ml of the MGIT specimen prepared in section 8.4 into 4.5 ml sterile saline and mix well.
- This is the 1:50 PZA Growth Control Suspension
- Inoculate 0.5 ml of this suspension into the PZA GC-labeled tube
- Immediately recap the tube tightly and mix by inverting the tube several times.

SIRE and PZA Drug-Containing Tubes

- Inoculate each labeled, drug-containing tube with 0.5 ml of the MGIT specimen prepared.
- Immediately recap the tube tightly and mix by inverting the tube several times.
- Wipe all tubes and caps with a mycobactericidal disinfectant.

Incubation

- Enter the inoculated set of DST specimens into the BACTEC 960 instrument using the AST set entry feature.
- Be sure that the tubes are loaded according to the order specified for the AST set entry feature
- Be sure that the caps are tightly closed
- Do not to shake the tube throughout the incubation
- The BACTEC 960 instrument will monitor the inoculated media and will indicate once the test is complete within 4-21 days (Growth Control reaches GU 400 or more). At this point the susceptibility set can be taken out after scanning and a report can be printed out.
- The susceptibility report will indicate "**S**" (susceptible) or "**R**" (resistant).
- The instrument interpretation of results is based on GU values as described for SIRE drugs
- If the GC tubes become positive in less than 4 days or remain negative up to 21 days or some other conditions occur which may affect the test results, the instrument report will be as an Error ("**X**"). In such situations, the test needs to be repeated.

FOUNDATION FOR INNOVATIVE NEW DIAGNOSTICS

ANNEX 9: Patient Informed Consent Form

Dear Participant,

You are invited to participate in this research study of the **(NAME OF TRIAL SITE)** looking at **new tests to diagnose tuberculosis (TB)**.

You have been invited because your doctor thinks that you may have **tuberculosis of the lungs**. Your samples will be used both to determine if you have tuberculosis and to help develop new and better tests to diagnose TB.

The tests that are used today to diagnose tuberculosis are slow and difficult to use. They require a laboratory technician to examine several sputum samples, with a microscope, to look for the germs that cause tuberculosis. The technician may spend more than an hour looking at your samples.

The **Foundation for Innovative New Diagnostics (FIND)** is working with **(NAME OF TRIAL SITE)** to develop new tests to make diagnosis of tuberculosis easier for both patients and health workers.

FIND is a leading non-profit organization dedicated to the development of **diagnostic tools for poverty-related diseases**. By fostering the production of **effective and affordable TB tests** and by ensuring access to these products by developing country health systems, FIND will fundamentally contribute to the decrease in global health inequities.

FIND has signed a development agreement with Cepheid to deliver **a fully automated test for TB case detection and drug resistance testing**. This new sputum test is now ready to undergo the first evaluation during a study.

If you agree to join this project, all you will have to do is to provide 3 sputum samples as for routine laboratory examination. The sputum samples will be examined in the laboratory with the best available TB tests and, in addition, with the new TB test. The results will be given to the clinic doctor. He/she will make a decision about treatment. You will receive the standard medical care services in your area for the disease you have. With the exception of the treatment costs, the cost of laboratory tests will be covered by the research study. A follow-up visit might be required and the study personnel will contact you to arrange an appointment in that case. During the follow-up visit you will be asked questions about your health status and to provide 1 sputum sample for analysis.

There are no risks for you through this study and you will benefit by receiving the best possible diagnostic work-up.

All information that you provide will be considered **confidential**, and no mention of your name or any other identifying information will appear on the stored samples or in any publication in

connection with this study. NO personal information will be stored together with the samples. No persons other than the health care workers overseeing your care will have access to any information that identifies you personally. Only professional technicians will have the key to link the samples and the information attached to your name.

You may also choose not to participate in this study and you may refuse to participate at any time without penalty or loss of benefits to which you would otherwise be entitled. You do not have to explain why you do not wish to participate.

If you have questions during the course of this study, you can directly contact the investigators (Name and address of the investigators, Telephone).

Certificate of consent

Purpose:
The purpose of this study is to assess the performance of a new and promising test to diagnose TB in comparison to the best currently available TB tests.
Procedures:
If I participate in this study I will give 3 sputum samples. I will provide 2 of the samples at the health facility and 1 at home as it needs to be an early morning sample. I might be asked to return for a follow-up visit after 2 months and will give 1 sputum sample at this point.
Risks:
None.
Benefits:
Best possible diagnostic work-up.
Confidentiality:
All information that I provide will be considered confidential. No persons other than the health care workers overseeing my care will have access to any information that identifies me individually.
Contact information:
If I have any questions, or if any problems arise, please contact (NAME, ADDRESS, and PHONE.)

Participant's Statement:
I have read the foregoing information/The foregoing information has been read to me. I have had the opportunity to ask questions about it and all questions have been answered to my satisfaction. I consent voluntarily to participate as a subject in this study and understand that I have the right to withdraw from the study at any time without this in any way affecting my further medical care.

	If participant is illiterate
______________________________	______________________________
Name of Participant	Name of Literate Witness
______________________________	______________________________
Signature of Participant	Signature of Literate Witness
______________________	______________________
Date	Date

Interviewer's Signature: Date: ________________________________

ANNEX 10: Monitoring Plan

<table>
<tr><td colspan="8">Study Title / #: Evaluation Xpert™ MTB / #7210 2/1</td><td colspan="9">Study coordinator: Catharina Boehme</td></tr>
<tr><th rowspan="2">Site / Investigator</th><th rowspan="2">Monitor</th><th colspan="2">Site Certification</th><th colspan="2">Initial Visit</th><th colspan="2">Site Training</th><th colspan="2">Monitor. Visits</th><th colspan="3">Periodic Contacts</th><th colspan="2">Cl.-Out Visit</th></tr>
<tr><td>yes</td><td>no</td><td>yes</td><td>no</td><td>pers</td><td>call</td><td>yes</td><td>no</td><td>calls</td><td>e-mails</td><td>other</td><td>yes</td><td>no</td></tr>
<tr><td>UPCH, Peru (#01) /
E. Gotuzzo</td><td>Pamela Nabeta (PN)</td><td></td><td>x</td><td>x
PN</td><td></td><td>x
PN</td><td></td><td>x
PN</td><td></td><td>x</td><td>x</td><td></td><td>x
PN</td><td></td></tr>
<tr><td colspan="15">1. <u>Site initiation visit including site training and – where required- site certification.</u>
<u>Site certification:</u> Has been done for this site.
<u>Study specific training and study initiation:</u> PN (4-6 days). Report to be shared with Cepheid and David Alland's team.
- Training on GeneXpert and Xpert TB assay (participants: 2 lab techs, 2 biologists and on site study coordinator; theoretical part (background, required software functions, basic curve interpretation, trouble shooting); practical part (and proficiency testing) with 75 frozen, pre-characterized specimens.
- Training on study specific tasks (e.g. clinical and laboratory workflow, blinding, other documentation (CRF, change control etc.), data entry, GeneXpert data management.
- Study initiation: Study workflow as outlined in protocol, testing of fresh samples.
2. <u>Monitoring visits:</u> 2 visits during enrolment. 5 % of data entry fields to be compared with CRFs for QA of data management.
3. <u>Calls</u>: Once monthly: CB & PN (FIND) & on site study coordinator (UPCH). Cepheid and David Alland's team will be asked by CB to participate in case of GeneXpert related technical changes, problems or unexpected events/observations.
4. <u>E-mails:</u> Xpert data to be mailed to Cepheid as .gxx files on a monthly basis. Progress reports will be sent at least on a monthly basis (database will allow to generate those automatically). Cepheid will be copied on all emails with Xpert related questions/topics.
5. <u>Close out visit:</u> 2 months after end of enrolment. Preliminary excel data sheets to be provided by site at the end of enrolment (culture and DST results pending) and final excel sheet after completion of culture/DST results. CRF copies to be provided to Cepheid and FIND. Final study report to be provided by CB.</td></tr>
<tr><td>South African sites</td><td>Pamela Nabeta (PN)</td><td>x
HA</td><td></td><td>x
CB</td><td></td><td>x
CB</td><td></td><td>x
HA</td><td></td><td>x</td><td>x</td><td></td><td>x
CB</td><td></td></tr>
<tr><td>Hinduja Hospital, India</td><td>TBD</td><td>X
CNP</td><td></td><td>x
CB</td><td></td><td>x
CB</td><td></td><td>x
TBD</td><td></td><td>x</td><td>x</td><td></td><td>x
TBD</td><td></td></tr>
<tr><td>FZ Borstel</td><td>Catharina Boehme (CB)</td><td></td><td>x</td><td>x
CB</td><td></td><td>x
CB</td><td></td><td>x
PN</td><td></td><td>x</td><td>x</td><td></td><td>x
CB</td><td></td></tr>
</table>

ANNEX 11: IQC check for incoming shipments and/or new lots

1. Purpose

To ensure high quality of all reagents used for Xpert™ evaluation study. Two positive controls (provided by Cepheid) and 1 negative control (sterile water) will be processed upon arrival of each shipment and prior to starting to use a new reagent lot.

2. Quality control procedure

1. Take 3 cartridges and 3 bottles of sample treatment reagent (SR) from the shipment or the new lot.
2. Remove the cartridges from the packaging. Examine each cartridge according to pre-performance checklist. Record all observations.
   a. Do not run the cartridge if there is obvious moisture in the pouch.
   b. Do not run the cartridge if any of the fluid volumes is less than ¾ of the fluid volume shown in the “Before Run” cartridge pictures.
   c. For condensation at the seal, record observation only.
   d. Do not run the cartridge if the white pre-filter has come loose from the filter housing.
3. Examine each SR bottle for leaks or other obvious defects and record all observations.
4. Prepare 1 negative sample as follows:
   a. Label sterile tube NC1. The tube must have a capacity of at least 5 ml and a leak-proof cap.
   b. Add 1 ml of sterile water to the sterile tube.
   c. Add 2 ml of SR.
   d. Mix by shaking 10 times and repeat after 15 min.
   e. Let stand for a total 15 minutes (using a timer).
   f. Go to step 6. below.
5. Use 2 positive controls (**TB control B**) provided by Cepheid:
   a. Label these tubes PC1 and PC2.
   b. Add 2 ml of SR directly to each cell suspension. Use a separate bottle of SR for each sample.
   c. Securely close lids.
   d. Mix by shaking 10 times and repeat after 15 min.
   e. Let stand for a total of 15 minutes (using a timer)
   f. Go to step 6. below.
6. Preparation of the cartridge for testing
   a. Label the cartridges NC1, PC1, PC2.
   b. Open the cartridge lid of the first cartridge.
   c. Using a separate sterile transfer pipette for each sample, transfer between 2.0 and 2.5 ml of sample to chamber 3 of each of the cartridges labeled NC1, PC1, PC2.
   d. Close each cartridge lid.
   e. Run each cartridge using the correct GX protocol (“MTB Beta”). The sample ID in GeneXpert should have the following format: NC1, PC1, PC2.
7. Post – run observations and run file exporting.
   a. Examine each cartridge according to post-performance checklist (top of cartridge dry; plunger at top, fluid levels correct, prefilter in housing) and record observations.

b. Complete yellow fields of below attached form. Send completed form along with .gxx files for respective tests to catharina.boehme@finddiagnostics.org, who will obtain green light from Cepheid. IQC stands for incoming quality control check and means the results obtained at trial sites for PC1, PC2 and NC. OQC stands for outgoing quality control check and means QC results obtained by Cepheid prior to release of product. OQC will be completed by Cepheid and OQC documents provided to FIND.

c. Start using the new reagents only after having received green light from Catharina Boehme by email.

d. Complete Change Control Form when starting to use a new lot or a new shipment.

8. Specifications
   a. Negative samples (NC1): Test Result must be negative (analyte CF1 Ct < 40)
   b. Positive samples (PC1 and PC2): Test Result must be positive (analytes CF3, CF5, FAM, CF4 and CF6 all with Ct < 40).

FIND FOUNDATION FOR INNOVATIVE NEW DIAGNOSTICS

Document type: **report** Confidentiality: confidential	**IQC LOT RELEASE**	**MS E**

Study name: Xpert evaluation study Study protocol #: 7210_2/1 Project #: Cepheid, 7210

Trial site short name: Trial site ID code:

Name of investigational product: Xpert[Tm] MTB FIND cooperation partner (manufacturer): Cepheid

IQC lot results and lot release

Investigational product	* Lot #	* Date of analysis	QC result of control PC1	QC result of control PC2	QC result of control NC	***Release (yes or no)	***Signature (manufacturer)	***Date of signature
Xpert[Tm] MTB			* **IQC**: ☐MTB neg ☐Error ☐Invalid ☐No result ☐MTB pos Semiquantitation: ☐very low ☐low ☐medium ☐high Result Rifampicin resistance: ☐ Rif resistance **not** detected ☐Rif resistance detected ☐Rif resistance indeterminate ☐no result	* **IQC**: ☐MTB neg ☐Error ☐Invalid ☐No result ☐MTB pos Semiquantitation: ☐very low ☐low ☐medium ☐high Result Rifampicin resistance: ☐ Rif resistance **not** detected ☐Rif resistance detected ☐Rif resistance indeterminate ☐no result	* **IQC**: ☐MTB neg ☐Error ☐Invalid ☐No result ☐MTB pos Semiquantitation: ☐very low ☐low ☐medium ☐high Result Rifampicin resistance: ☐ Rif resistance **not** detected ☐Rif resistance detected ☐Rif resistance indeterminate ☐no result			
			*****OQC**: ☐ Matching IQC results ☐ Not matching IQC results ☐ OQC documents provided to FIND	*****OQC**: ☐ Matching IQC results ☐ Not matching IQC results ☐ OQC documents provided to FIND	*****OQC**: ☐ Matching IQC results ☐ Not matching IQC results ☐ OQC documents provided to FIND			

Observations at trial site:

Pre-performance check: ☐passed ☐failed for following reason:____

Post-performance check: ☐passed ☐failed for following reason:____

Other observations:

* To be filled by trial site *** To be filled by manufacturer

--- ---

Study coordinator **Date**

ANNEX 12: Change Control Form

Study Title / #: Xpert TB Evaluation Study / #7210 2/1	**Trial site:**

Describe old procedure (Indicate old lot # if change cartridges)	Describe required change (Indicate new lot # if change cartridges)	Date of receipt of instructions for new procedure (if applicable)	Date of implementation	Signature study site coordinator

ANNEX 13: Process Controls

Why
- To exclude *rpoB* amplicon contamination that affects the Xpert^{Tm} MTB performance.

Who
- Laboratories using other *rpoB* amplifying assays such as line probe assays (Hain Genotype MTBDRplus)
- Laboratories with suspicion of DNA contamination based on clustering of positive Xpert results (especially if with identical pobe dropout) or based on line probe results (uncommon band combinations).

When
- Prior to starting enrolment (for laboratories using rpoB amplifying assays)
- Whenever Xpert or line probe results arouse suspicion of DNA contamination

What
- Test 2 negative controls by Xpert (using 2 ml of distilled water per test)
- Test 2 wet swabs by Xpert

How
- Take 4 cartridges and 4 bottles of sample treatment reagent (SR).
- Prepare 2 negative samples as follows:
  a. Label sterile tube NC1. The tube must have a capacity of at least 5 ml and a leak-proof cap.
  b. Add 1 ml of sterile water to the sterile tube.
  c. Add 2 ml of SR.
  d. Mix by shaking 20 times and repeat after 5-10 min.
  e. Let stand for a total 15 minutes (using a timer).
  f. Go to step 6. below.
- Prepare 2 swabs as follows:
  a. Label these tubes Swab1 and Swab2.
  b. Add 2 ml of SR directly to each sterile tube. Use a separate bottle of SR for each sample.
  c. Dip the tip of 2 cotton swabs in sterile water. Cotton swabs should only be moist, not wet.
  d. Swab 1x the inner surface of a GeneXpert module and 1x the bench where sample preparation is carried out.
  e. Dip the swabs in the SR containing, labeled tubes and and turn them several times while touching the tube all to wring out potential DNA.
  f. Securely close lids.
  g. Mix by shaking 20 times and repeat after 5-10 min.
  h. Let stand for a total of 15 minutes (using a timer)
  i. Go to step below.

Preparation of the cartridge for testing

  a. Label the cartridges NC1, NC2, Swab1, Swab2.
  b. Open the cartridge lid of the first cartridge.
  c. Using a separate sterile transfer pipette for each sample, transfer between 2.0 and 2.5 ml of sample to chamber 3 of each of the cartridges.

d. Close each cartridge lid.
e. Run each cartridge using the correct GX protocol ("MTB Beta"). The sample ID in GeneXpert should have the following format: NC1, NC2, Swab1, Swab2.

Send .gxx files for respective tests to catharina.boehme@finddiagnostics.org