Canadian Tuberculosis Standards

7th Edition

Appendix D: Tuberculosis and Mycobacteriology Laboratory Standards: Services and Policies

Public Health Agency of Canada
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APPENDIX D

TUBERCULOSIS AND MYCOBACTERIOLOGY LABORATORY STANDARDS: SERVICES AND POLICIES

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INTRODUCTION

The diagnosis of tuberculosis (TB) is a collaborative effort involving physicians and other health care providers, the public health department and mycobacteriology and clinical laboratories. Before offering mycobacteriology services, each laboratory should assess the level of services required and the capacity and capability for the provision of these services.[1,2] A complete questionnaire for the assessment of a laboratory's capacity for handling *Mycobacterium tuberculosis* complex (MTBC) organisms can be found in the publication *Mycobacterium Tuberculosis: Assessing Your Laboratory,* 2009 edition*,* produced by the Association of Public Health Laboratories.[1,2] This appendix addresses some specific standards for the Canadian mycobacteriology laboratory.

LABORATORY REQUIREMENTS

BIOSAFETY REQUIREMENTS

Compared with the general population, laboratory personnel have a 3- to 9-fold greater risk of acquiring latent TB infection.[3,4] Laboratories that handle human pathogens and microbial toxins in Canada must comply with the *Human Pathogens and Toxins Act* (http://lois-laws.justice.gc.ca/eng/acts/H-5.67/index.html) and the corresponding operational and physical biosafety requirements outlined in the Government of Canada's *Canadian Biosafety Standards and Guidelines* (CBSGs) (http://canadianbiosafetystandards.collaboration.gc.ca/cbsg-nldcb/index-eng.php?page=0). The pathogens found within the MTBC are examples of Risk Group 3 pathogens, for which biosafety Containment Level (CL) 3 is required for research and other higher risk activities, but for which certain diagnostic activities can be conducted safely at CL2 with additional practices, as specified in the new MTBC Biosafety Directive. This Directive is a comprehensive overview of the activities and MTBC sample types that can be handled with derogated containment requirements (CL2 with additional physical containment and operational practices). The *MTBC Biosafety Directive* is to be used in conjunction with the Public Health Agency of Canada's CBSG.

REPORTING CRITERIA AND TURNAROUND TIMES

The following are suggested for each laboratory reporting system:

- Established turnaround times and reporting parameters for each testing methodology (Table 1) should be readily available in the laboratory standard operating procedures.
- Reports should be date stamped and signed by the reporting technician.
- Reported information should be disseminated by secure telephone, facsimile or e-mail within 24 hours of test completion and the original hard copy mailed within the following 24 hours.
- Whenever possible, reported results should not be transcribed, in order to avoid transcription errors. Original reports should be forwarded to the appropriate personnel.
- Anticipated delays should be communicated to the client by a preliminary report.
- Reports on non-standardized testing (such as antimicrobials not recommended by the Clinical Laboratory Standards Institute [CLSI] for susceptibility testing) should indicate these limitations.
- Turnaround times should be monitored periodically (monthly) to check compliance and evaluated annually.

Table 1. Summary of standard turnaround times (refer to individual section for more information)[2]

Procedure	Turnaround time to completion/report
Specimen collection and arrival at the laboratory	24 hours
Acid-fast bacteria (AFB) smear microscopy	24 hours from specimen receipt
Nucleic acid amplification testing (NAAT) for MTBC detection	24 hours from smear result or 24 hours from receipt of specimen
Bacteriological diagnosis – culture	Up to 6 weeks for broth cultures and 8 weeks for solid media cultures from specimen receipt
Identification of mycobacterial species	Maximum 21 days from specimen receipt
Primary phenotypic susceptibility testing	15 to 30 days from receipt of specimen in a primary laboratory[5] 7-15 days from a positive culture in reference laboratories
Reporting of all test results (electronically)	24 hours from test completion
Reporting of all test results (mailed hard copy)	48 hours from test completion

QUALITY ASSURANCE AND PROFICIENCY TESTING

All laboratories should be accredited by a recognized national/international accrediting organization and should participate in internal and external quality assurance/quality control activities in conjunction with a reference laboratory. These programs will assess the reproducibility and the inter-laboratory variability of the methods used and adherence to standardized testing procedures.

All laboratories should have a document control system in operation that will detect and correct significant clerical or analytic errors that could affect patient management.[6,7]

LABORATORY SERVICES

RECEIVING AND TRANSPORTING SPECIMENS

Most specimens submitted for mycobacterial culture originate from the respiratory tract, but tissue, sterile body fluids, urine and gastric aspirates are also commonly submitted (Table 2) (see Chapter 3, Diagnosis of Active Tuberculosis and Drug Resistance). If a laboratory does not have processing facilities, specimens should be referred to a laboratory that does. This should be done within 24 hours of specimen collection to avoid overgrowth by other microorganisms or deterioration of the sample. Specimens should be kept refrigerated at 4 °C (except blood culture and cerebrospinal fluid specimens) if not transported immediately.

All types of clinical specimens are potentially contagious and therefore should be handled with the same procedures. However, cultures of MTBC are much more hazardous than clinical specimens or cultures of nontuberculous mycobacteria (NTM) and require specific procedures for packaging and shipment. Laboratories are required to adhere to the *Transportation of Dangerous Goods Act* Canada) and the *International Air Transport Association's Dangerous Goods Regulations* (for transport by air) when submitting clinical specimens or cultures to another facility. The accepting facility is required to accept and process the incoming specimens according to the relevant acts and regulations. The most current information, legislation and regulations are available from the Pathogens Regulation Directorate: http://www.phac-aspc.gc.ca/lab-bio/about-apropos-eng.php.

Table 2. Ideal specimens for submission to the mycobacteriology laboratory[8,9]

Specimen type	Ideal specimen submissions	Unacceptable specimens
Abscess contents, other aspirated fluid	As much as possible in sterile plastic container.	Dry swab Swabs in anerobic transport medium.
Blood (for culture)* *See section interferon-gamma release assays	• 7 mL SPSSPS (yellow top) or • 7 mL heparin (green top) blood collection tube or • 10 mL isolator tube or • 5 mL inoculated directly into Myco/F Lytic medium.	Blood collected in EDTA, which greatly inhibits mycobacterial growth even in trace amounts; coagulated blood; serum or plasma.
Body fluids (pleural, pericardial, peritoneal, etc.)	As much as possible (10–15 mL minimum) in sterile container.	
Bronchoalveolar lavage or bronchial washing	≥5 mL in sterile container.	
CSF	≥2 mL in sterile container.	<0.5 mL
Gastric lavage fluid	5-10 mL in gastric lavage container. Collect in the morning soon after patient awakens in order to obtain sputum swallowed during sleep.	Specimen in which the acidity has not been neutralized.
Sputum (spontaneous or induced)	5-10 mL in sterile, wax-free, disposable container. Do not pool specimens. Where feasible, three sputum specimens (either spontaneous or induced) can be collected on the same day, a minimum of 1 hour apart.	24-hour pooled specimens; saliva.
Tissue biopsy sample	1 g of tissue, if possible, in sterile container without fixative or preservative. Normal saline is acceptable.	Specimen submitted in formalin. Inappropriate because of inability to culture and degradation of DNA for molecular tests.
Urine	Catheter or midstream urine as much as possible (minimum 40 mL) of first morning specimen. For suprapubic tap, as much specimen as possible with needle removed and Luer Lock cap in place. Aspirate can be sent in sterile container.	24-hour pooled specimens; urine from catheter bag; specimens of <40 mL unless larger volume is not obtainable. Urine specimens should only be tested if renal or urinary tract TB is suspected and should not used as a routine screen.

SPS = Specimen Preparation System, EDTA = ethylene diamine tetraacetic acid, CSF = cerebrospinal fluid

DETECTING AND IDENTIFYING *MYCOBACTERIUM* SPECIES

Mycobacteriology laboratories should have the capability to detect MTBC and NTM using rapid molecular methods. Table 3 illustrates the types of specimens and samples encountered by the mycobacteriology laboratory and the suggested method for detection and identification of AFB in those specimens as well as the resulting cultures.

Table 3. Recommended methods for detection and identification of AFB from clinical samples and cultures[2,9,10,11]

Mycobacterium species	Clinical sample/culture	Detection/identification method
Mycobacterium tuberculosis	Sputum	• AFB staining and smear microscopy • culture • NAAT (commercial or in-house)
Mycobacterium tuberculosis	Tissue (fresh or paraffin embedded) or fluids	• AFB staining and microscopy • NAAT • Culture (if possible; not performed for formalin-fixed or paraffin-embedded specimens)
Mycobacterium tuberculosis	Culture	• Commercial DNA probes/NAAT • gene sequencing (e.g. 16s rRNA, *gyr*B) • line-probe assays
Nontuberculous mycobacteria	Sputum	• AFB staining and smear microscopy • culture • NAAT
Nontuberculous mycobacteria	Culture	• Commercial DNA probes (*M. avium* complex, *M. gordonae, M kansasii*) or commercial NAAT kits • Gene sequencing (e.g. 16s rRNA , *hsp*65 gene , *rpo*B gene) • line-probe assays

NAAT = nucleic acid amplification tests

DIGESTION, DECONTAMINATION AND CONCENTRATION OF SPECIMENS

Digestion, decontamination and concentration of a clinical specimen are commonly performed using the established N-Acetyl-L-Cysteine–sodium hydroxide (NALC-NaOH) procedure.[11] All specimen concentrates should undergo acid-fast smear microscopy and be inoculated to both liquid and solid media.

ACID-FAST SMEAR AND MICROSCOPY

The early and rapid diagnosis of TB still relies on the traditional AFB smear. For rapid results some laboratories perform a "direct smear" from the specimen, without digestion, decontamination and concentration steps. Direct smears are discouraged because of the inherent lack of sensitivity. If direct smears are performed, the result should always be considered as a preliminary step before transfer of the specimen to a reference laboratory where a concentrated (more sensitive) smear can be performed for confirmation. Overall, smears have a reported sensitivity of 20%-80%, depending on many factors including the type of specimen, stain used and the experience of the technologist.[12-15] A minimum of 5,000 to 10,000 bacteria/mL are needed in a sputum sample to obtain a positive result from concentrated smear, as compared with culture, which can detect a bacillary load as low as 10 bacteria/mL.[12]

The following guidelines should be observed:[1,2,10,16,17]

- Slides should be individually stained to prevent cross-contamination.
- Control slides that contain known acid-fast and non-acid fast organisms should be run with each batch of smears prepared.
- All primary specimen smears should be stained and reviewed using the fluorochrome method. Laboratories should confirm new AFB positive smears by a second reader. Smears that are questionable should be repeated or can be stained using a carbol-fuschin method for review.
- Fluorochrome stain performance should be confirmed with each new lot of reagents by reviewing AFB positive and AFB negative control slides prior to reading patient smears.
- For purposes of quality control, 10% of negative slides should be examined by a second qualified person.
- Smears should be reported following an established grading system (see Chapter 3 Diagnosis of Active Tuberculosis and Drug Resistance).
- Laboratory technologists should read a minimum of 15 smears/week for proficiency.[2]
- Laboratories should participate in an approved proficiency program that includes acid-fast smears.[2]

The American Thoracic Society, U.S. Centers for Disease Control and Prevention (CDC) and the Canadian Thoracic Society recommend that laboratories not performing a minimum of 15 AFB smears/week should refer specimens to another laboratory or reference laboratory.[1,7]

MOLECULAR DETECTION OF MYCOBACTERIA DIRECTLY FROM CLINICAL SAMPLES

Nucleic acid amplification (NAA) tests, which amplify target sequences of DNA or RNA from the MTBC, have several important advantages over smear microscopy and culture.[18,19] They are rapid, have excellent specificity and provide results within 2 to 24 hours. Additionally, they are more sensitive than AFB smears, although less sensitive than TB cultures. They are currently recommended for use only on airway secretion specimens, excluding pleural fluid, although upon special request they can be used on other specimens (e.g. CSF). At least one respiratory sample should be tested with a Health Canada approved or validated in-house NAAT in all new, smear-positive cases. In addition, NAA testing may be performed in smear-negative patients upon request by the physician or the TB control program. NAAT results should not be used for monitoring TB treatment response (see Chapter 3 on Diagnosis of Active Tuberculosis and Drug Resistance) or for infection control purposes (e.g. removal of patient from isolation).

There are many commercially available options that provide rapid, molecular tests for the identification of MTBC in clinical samples (see Medical Devices Active License Listing online query website at http://www.hc-sc.gc.ca/dhp-mps/md-im/licen/mdlic-eng.php).

Health Canada has approved assays from Roche (COBAS® Taqman® MTB; real-time polymerase chain reaction [RT-PCR]), Becton Dickson (BD ProbeTec®, strand displacement amplification), Gen-Probe (Amplified Mycobacterium tuberculosis Direct [AMTD], transcription mediated amplification), Hain Lifescience (GenoType® Mycobacteria Direct, PCR) and Cepheid (Xpert MTB/RIF®, automated, cartridge-based nested PCR). The COBAS® Taqman® MTB, AMTD, and Xpert MTB/RIF tests are approved for direct testing on sputum specimens. The Xpert MTB/RIF (Cepheid, Sunnyvale, CA) system was recently approved by Health Canada, and recommendations for the use of this new assay are provided in Chapter 3 . None of the NAA tests can be used to the exclusion of culture and phenotypic drug susceptibility testing (DST), which are required for confirmation of all direct molecular detection testing.[9,20]

False-positive and false-negative rates should be monitored, as the rates can be very high without careful attention to proper technique by highly trained and closely supervised laboratory staff.

In some cases, results may be "indeterminate" because of inhibitors in the specimen or a very low bacterial load. Appropriate controls should be included when applicable to rule out inhibition by the specimen. Special care should be taken to avoid cross-contamination of NAA specimens because of the sensitive nature of these tests. Laboratories should ensure that there is a clean environment and should follow proper molecular testing hygiene in the preparation of solutions used in NAA tests. There should be a physical separation of the laboratory areas used to prepare solutions, to add DNA template and to conduct post-amplification detection.

“In-house” PCR methods targeting the IS*6110* element in the genome of MTBC[21] are less costly than commercially available methods but are less reproducible, are non-standardized and require advanced technical skill. Such methods can be used for detection of MTBC in specimens not recommended for testing with a commercial kit, such as formalin-fixed tissue blocks. The analytical limitations (i.e. limits of sensitivity and processing) of such tests should be reported with the results. Before using an in-house or a “home-brew” molecular assay, laboratories should consult the Clinical and Laboratory Standards Institute (CLSI) guideline, *Molecular Diagnostic Methods for Infectious Disease*[21] for guidance on the validation and implementation of a new molecular diagnostic test. Validation of any new or adapted test methods should be completed to evaluate the performance characteristics and technical competence of the test. All test methods should be verified as being appropriate and adequate before being undertaken.[22]

The design of validation studies should include the following:[21]

- comparison of the new method with a “gold-standard” test;
- evaluation of both inter- and intra-laboratory reproducibility;
- number of samples/isolates determined by a mathematical model or according to validation guidelines where they exist;
- testing of reference strains and isolates exhibiting a range of known, characterized values;
- performance characteristics to be evaluated and the statistical analysis to be used.

Results from NAA testing should be reported as soon as they are available and within 24 hours of a smear- positive result or receipt of the specimen. At a minimum the report should include information on the organism tested, the target of the NAA test and an interpretation of the results. The CLSI MM3-A2 guideline can be consulted for further information.

MYCOBACTERIAL CULTURE

Culture remains the gold standard for a positive laboratory diagnosis of TB.[1,2,20] As outlined in the section on digestion, decontamination and concentration (section 3.2.1), at least one solid and one liquid medium should be inoculated from each clinical specimen for culturing of AFB. Cultures should be kept an average of 6-8 weeks for observation of growth. Positive cultures should be retained for at least 1 year should additional testing be required.[2,10]

It is important to remember that occasionally cultures can be falsely positive for MTBC, primarily because of cross-contamination within the laboratory, although specimen contamination and "mix-up" by the submitter has been documented.[22,23] A report of a single positive culture from a patient with a low clinical suspicion for TB, particularly if the culture has taken much longer than the average time (8-12 days) to become positive, should be reviewed and investigated as a potential false-positive. Laboratories should have an established process in place to investigate possible incidents of cross-contamination or other false-positive cases.

IDENTIFICATION OF MYCOBACTERIAL SPECIES FROM CULTURE

Mycobacterial identification based on biochemical and/or physical characteristics is labour-intensive and slow, and may not adequately identify the organism.[24,25] DNA sequence analysis, such as 16S rDNA gene sequencing, provides rapid, accurate and highly reproducible data and can be used in the absence of organism propagation. Rapid, accurate species identification is a necessity for public health and clinical reasons.[21]

Mycobacteriology laboratories should have the capability to differentiate *M. tuberculosis* from *M. bovis* and *M. bovis* BCG in view of the intrinsic resistance of the latter two organisms to pyrazinamide (PZA), and for public health reporting and investigation.. Laboratories not differentiating MTBC organisms should refer to a reference laboratory. Current molecular approaches available for MTBC differentiation include analysis of polymorphisms of the *gyrB* gene,[24] identification of regions-of-difference[25,26] and spoligotyping, and commercial assays.[27-29]

Similar criteria used for identification of the MTB complex should be used for the NTM species. For CL2 and CL3 laboratories that can perform identification tests of the MTBC and other NTM, identification of the *M. avium* complex, *M. kansasii* and *M. gordonae* can be accomplished by the use of commercial DNA probe assays; other mycobacteria can be identified by molecular sequencing targets, such as the 16S rDNA, *rpoB,* the ITS region and *hsp*65 genes.[30-32]

Accurate sequence analysis requires that both the positive and negative strand of DNA be sequenced and analyzed for single nucleotide polymorphisms. For quality control of sequence data, consistent use of a reference sequence should be included in the test procedure. Culture identification should be completed before other testing, such as susceptibility testing, is carried out to ensure that the most appropriate testing method is used and that tests are interpreted accurately.

- **The time frame for culture identification of *M. tuberculosis* complex is dependent on the growth rate of the organism. Culture identification should make use of rapid, state-of-the art technologies such as molecular-based techniques. In the absence of such resources, culture specimens should be sent to a reference laboratory for identification.[33]**

SUSCEPTIBILITY TESTING FOR ANTITUBERCULOUS DRUGS

Agar proportion is still considered the gold standard for MTBC antibiotic DST.[1,5] However, because of the labour-intensive nature and lengthy incubation time for the assay, the more rapid liquid media detection methods using continuous monitoring systems are now recommended.[5,10] The most current CLSI guideline[5] should be consulted for testing parameters.

- Laboratories should perform DST of first-line antibiotics or ensure that this is available for all MTBC cases. First-line antibiotics are
  - isoniazid (INH)
  - rifampin (RMP)
  - ethambutol (EMB),
  - pyrazinamide (PZA)
- Second-line DST should be limited to accredited reference laboratories. In laboratories where such DST is not available, culture specimens should be referred to a reference laboratory for testing if resistance to one or more of the first-line antibiotics is detected.
- Second-line DST should be set up when resistance to first-line antituberculous drugs is detected, regardless of whether the first-line DST is repeated.
- Second-line antituberculous drugs for which there are standards[5,34] for DST in Canada include the following:
  - injectable agents (streptomycin, amikacin, kanamycin, capreomycin)
  - fluoroquinolones (ciprofloxacin, ofloxacin, levofloxacin, moxifloxacin)
  - rifabutin
  - ethionamide,
  - *p*-aminosalicylic acid[5,10]
  - linezolid
- Laboratories should test at least one drug from each class;[6] in particular, at least one fluoroquinolone should be tested, and the selection of which fluoroquinolone to test is based on consultation with the physicians who manage most of the patients with drug-resistant TB. Note that streptomycin, ciprofloxacin and ofloxacin are *no longer* recommended for use in the treatment of TB in Canada.[5]
- Although cycloserine is a viable treatment option, the **CLSI does not recommend testing of cycloserine.**[5]

MOLECULAR DETECTION OF ANTITUBERCULOUS DRUG RESISTANCE

The molecular detection of antituberculous drug resistance in MTB has become an important tool in the rapid identification of multidrug-resistant TB. These molecular methods can decrease the time it takes to detect resistance using phenotypic methods and can guide therapy. Molecular detection of MTB and determinants of drug resistance is considered presumptive, and the use of these tests does not eliminate the need for conventional culture and DST. Culture and DST are required to confirm initial results and also detect resistance to drugs other than RMP and INH (see Chapter 3).

These methods should be validated just as any other method would be and used only in conjunction with phenotypic susceptibility testing. The methods include in-house PCR and sequence- based assays, approved commercial line-probe and real-time PCR-based assays.

DNA sequencing is the only technology option to identify both known and novel insertions, deletions or mutations and remains the gold standard for molecular work.[7] Table 4 lists the genes that should be sequenced in order to identify the most commonly encountered molecular determinants of resistance.

Reporting of molecular gene sequence data for antibiotic resistance should include the genetic region tested, nucleotide and amino acid mutation, and the limitations of the testing.[30] **In the absence of a mutation, a statement should be included in the report explaining that the lack of a mutation does not exclude the possibility of phenotypic resistance.[5,35,36]**

Table 4. Genes to be sequenced for the molecular detection of first-line antibiotic resistance[35]

Antibiotic	Gene(s) to sequence for detection of resistance
INH	*inh*A *kat*G
RMP	*rpo*B
EMB	*emb*B
PZA	*pnc*A

GENOTYPING OF *M. TUBERCULOSIS*

The gold standard for genotyping of *M. tuberculosis* remains IS*6110* restriction fragment-length polymorphism (RFLP) analysis.[37,38] In the majority of cases, the technique has the highest discriminatory power, although this power is limited in cases in which fewer than six copies of the IS*6110* insertion element are present in the genome.

There are many factors that make this method less than ideal:[37,38]

- The technique requires large amounts of DNA and therefore requires weeks of culture growth.
- Strict adherence to the standardized protocol is required for accurate comparisons to be made both between and within laboratories.
- Interpretation of banding patterns is subject to observer bias.

The currently accepted international standard for PCR-based genotyping of MTBC is mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) genotyping.[37,39] This methodology requires very small amounts of DNA and provides a numerical output for ease of comparison.[39] The Public Health Agency of Canada, the Public Health Ontario Laboratories, the US CDC and many European countries have implemented the MIRU-VNTR method as the first-line genotyping test, in conjunction with spoligotyping.[40] Reporting of MIRU-VNTR results should include the order of the loci as they are presented, as this order is not standardized among laboratories. It is essential to be able to re-order the loci for accurate comparison.

MIRU-VNTR genotyping requires a high level of technical expertise and has higher accuracy when capillary-electrophoresis is used, which can be costly. Laboratories should establish technical competency and proficiency with MIRU-VNTR genotyping before embarking on in-house testing. In laboratories where technical expertise is lacking, or where through-put is low and expertise is hard to maintain, specimens should be referred to a reference laboratory for testing. Alternatively, commercially standardized kits are available, which rely on specialized capillary electrophoresis equipment, but they are costly and still require a high level of aptitude with the technique.[40] A proposal for standardization of optimized MIRU-VNTR typing of *M. tuberculosis* has been published.[37,39]

Spoligotyping,[27] another commonly used PCR-based genotyping method, lacks the individual discriminatory power of the MIRU-VNTR, but in conjunction with MIRU-VNTR genotyping it can provide reasonable discriminatory power approaching that of RFLP.[39]

INTERFERON-GAMMA RELEASE ASSAYS (IGRA)

IGRA are tests that have been developed for identifying latent TB infection (LTBI). They detect cell-mediated immune responses to specific antigens found in MTBC that are absent from *M. bovis* and *M. bovis* BCG, and most nontuberculous mycobacteria. Detection of a response to these antigens indicates infection with MTB. There are two assays currently approved for use in Canada, the QuantiFERON-TB Gold In-Tube assay (QFT-GIT) (Cellestis/Qiagen, Carnegie, Australia) and the T-SPOT.*TB* (T-SPOT) (Oxford Immunotec, Abingdon, UK).

IGRA use whole blood samples and may be performed by any licensed laboratory in Canada. They do not require specialized TB and mycobacteriology laboratory expertise or a CL3 laboratory facility. The assays do, however, require specific technical expertise in specimen collection and transportation, and performing the assay. These skills are available within most laboratories that perform serum, plasma and whole-blood assays for various biological and other markers, but the two IGRAs require specific technical training.

Laboratories should also ensure that specimen collection and transportation, two critical components of the assay performance, can be provided appropriately. As well, standardization of pre-analytical procedures is required, such as tube shaking, time interval between blood draw and incubation, and exact duration of incubation. If portable incubators are used, it is important to make sure that such incubators can accurately stabilize the temperature at 37 °C. Laboratories should avoid manual entry of results, utilizing laboratory information systems where possible to achieve optimal data entry and decrease the risk of data-entry errors. Test kits should be transported and stored in optimum conditions to prevent exposure to excessive heat. Strict quality assurance is necessary to detect unusual patterns in results (such as a spike in the number of indeterminate results due to low mitogen response or high negative control responses), and it is important to run both positive and negative controls with each assay.[41-47]

ASSAY PERFORMANCE, QUALITY ASSURANCE AND RESULTS INTERPRETATION – KEY TECHNICAL INFORMATION

*NOTE: for technical accuracy, the use of the word "must" indicates a requirement that must be followed when obtaining specimens and performing the assays. Please refer to the product inserts (referenced below or as supplied by the kit manufacturers) for specific details.

QFT-GIT[41,42]

Specimen collection

- QFT-GIT has special collection tubes consisting of the Nil Control (grey cap), TB Antigen (red cap) and Mitogen Control (purple cap). Tubes must be kept at room temperature (17-25 ^{o}C).
- The TB antigens are dried onto the inner wall of the tubes, so the tube contents, after blood draw, *must be mixed thoroughly.*
- Ensure that a volume of 1 mL is collected into each tube (to the black mark on the tube).
- Tubes must be shaken immediately after blood is collected approximately 10 times, such that the entire inner surface of each tube is coated with blood. Thorough mixing dissolves the heparin in the tubes, preventing clotting, and re-solubilizes the stimulating antigens. Do not shake over-vigorously as gel disruption in the tubes could lead to aberrant results.

Specimen transportation, incubation and processing (pre-analytical)

- According to the product insert, blood tubes must be incubated at 37 ^{o}C within 16 hours of collection. However, studies show that immediate incubation is optimal, as this reduces indeterminate results. Thus, incubation within 4 hours would be optimal where feasible.[43,44]
- Before incubation, tubes must be maintained at ambient temperature (22 ^{o}C $\pm$5 ^{o}C). Do not refrigerate or freeze blood samples.
- If tubes are not incubated immediately after collection, they must be re-mixed by inverting 10 times immediately before incubation.
- Tubes must be incubated upright at 37 ^{o}C for 16-24 hours in ambient air.
- After incubation, tubes may be held for *up to 3 days* at 4-27 ^{o}C prior to centrifugation.

- Centrifugation of incubated tubes is performed to obtain plasma – the gel plug in the tubes will separate the cells from the plasma; if this does not occur, tubes must be centrifuged again at a higher speed.
- Avoid any mixing of plasma prior to harvesting, and do not disturb material on the surface of the gel plug.
- Only harvest plasma samples using a pipette.
- Plasma samples may be loaded immediately into the QFT-GIT ELISA plate or can be stored for *up to 28 days* at 2-8 ^{o}C, or harvested plasma samples may be stored at –70 ^{o}C for extended periods

Testing (analytical)

- Plasma samples and reagents (except conjugate 100x concentrate) must be brought to room temperature (22 ^{o}C $\pm$5 ^{o}C), equilibrating with room temperature for at least 60 minutes.
- During the assay performance, thorough washing is key – each test well must be completely filled with wash buffer for each wash cycle. *An automated plate washer is recommended.*

Quality control

- QFT-ITG has analysis software available from Cellestis that can be used to analyze the raw data and calculate results; use of the software is recommended.
- The QFT analysis software performs a quality control check of the assay, generates the standard curve and provides a test result for each subject.
- Accuracy of the test results depends on the *generation of an accurate standard curve.*
- The standard curve must be examined before interpretation of the test sample result to determine whether the results meat the expected values.[41]
- If the standard curve criteria are not met, the run is considered invalid and must be repeated.
- If the “zero standard” has a mean optical density that is high (>0.15), then plate washing must be investigated.
- Laboratories should include external quality control samples for testing with patient samples; quality control samples can consist of pooled patient sera for specimens that are known mitogen negative or TB antigen positive and negative, or diluted assay standards.[42]

Result reporting and interpretation (post-analytical), taken from manufacturer’s package insert[41]

Table 5.

Nil [IU/mL]	TB Antigen minus Nil [IU/mL]	Mitogen minus Nil [IU/mL][1]	QFT Result	Report/Interpretation
≤ 8.0	< 0.35	≥ 0.5	**Negative**	*M. tuberculosis* infection NOT likely
	≥ 0.35 and < 25% of Nil value	≥ 0.5		
	≥ 0.35 and ≥ 25% of Nil value	Any	**Positive**[2]	*M. tuberculosis* likely
	< 0.35	< 0.5	**Indeterminate**[3]	Results are indeterminate for TB Antigen responsiveness
	≥ 0.35 and < 25% of Nil value	< 0.5		
> 8.0[4]	Any	Any		

Responses to the Mitogen positive control (and occasionally TB Antigen) can be commonly outside the range of the microplate reader. This has no impact on test results.

Where *M. tuberculosis* infection is not suspected, initially positive results can be confirmed by retesting the original plasma samples in duplicate in the QFT ELISA. If repeat testing of one or both replicates is positive, the individual should be considered test positive.

Refer to Trouble Shooting section for possible causes.

In clinical studies, less than 0.25% of subjects had IFN-γ levels of >8.0 IU/mL for the Nil Control.

- While the QFT assay cut-off is interferon(IFN)-gamma 0.35 IU/mL, it is important to provide to clinicians who have requested this test the actual numerical value of the result (quantitative value) as well as the interpretation (positive, negative, indeterminate). This information is critical to the interpretation in individuals. Because of recent studies on high rates of IGRA conversions and reversions, and emerging literature on reproducibility, it is recommended that IFN-g values of 0.20-1.00 IU/mL for QFT be interpreted cautiously, as nonspecific variation can result in false conversions and reversions if the initial value falls within this borderline zone (see Chapter 4. Diagnosis of Latent Tuberculosis Infection).

- Reports should include information for the clinician to consider interpretation of the results in light of epidemiologic and clinical findings when assessing the probability of TB infection and disease.

- Guidance should be provided for an indeterminate result related to the following:
  - high Nil (high background interferon production) – does not allow an interpretation to be made
  - low Mitogen (lack of response to antigen stimulation) – does not allow an interpretation to be made and may indicate immunosuppression

Interpretation issues

- Unreliable or indeterminate results may be due to
  - technical failure, including improper protocol
  - excessive levels of circulating IFN-gamma or the presence of heterophile antibodies
  - greater than 16 hours between time of blood draw and incubation at 37 ^{o}C
  - storage of blood outside ambient temperature range (22 ^{o}C $\pm$5 ^{o}C)
  - insufficient mixing of blood collection tubes
  - incomplete washing of the ELISA plate.
- If an indeterminate result is suspected as a result of technical protocol issues (e.g. plate washing), repeat testing.
- Laboratories may consider a repeat test if the result is close to assay cut-off:
  - 0.35-1.0 for positives
  - 0.20-0.34 for negatives.[42]

T-SPOT[45-47]

- The T-SPOT assay uses the ELISPOT technique, which involves incubating peripheral blood mononuclear cells (PBMC) with antigens specific for *Mycobacterium tuberculosis*.

Specimen collection

- Does not require special collection tubes. Blood may be collected in sodium citrate, sodium heparin or lithium heparin containers.
- If T-Cell *Xtend* product will be used, DO NOT use cell preparation tubes (CPT).
- EDTA tubes are NOT acceptable.
- If blood is collected by a syringe and needle, the needle must be removed prior to transferring the blood into a blood collection tube to avoid cell lysis.
- CPT have anticoagulant, separation gel and density gradient liquid, which allow blood collection and PBMC separation to be conducted in one tube.
- Invert tubes 8-10 times to ensure that whole blood is mixed thoroughly with the anticoagulant, and store at room temperature (18-25 ^{o}C) before processing; do not refrigerate or freeze.
- For immunocompetent adults, one 8 mL tube or two 4 mL tubes should be sufficient to obtain enough cells.

Specimen transportation, incubation and processing

- Blood specimens must be processed on the day of blood collection (within 8 hours).
- If using the T-Cell *Xtend* product, whole-blood specimens collected in lithium heparin tubes and stored at room temperature (18-25 ^{o}C) may be processed within 0-32 hours of specimen collection; a gradient separation method (Ficoll) is required for processing.

- Centrifugation is an extremely important step to ensure that enough cells are obtained for the assay; the centrifuge must be able to maintain samples at room temperature.
- After centrifugation, the PBMCs must be isolated immediately using a large-bore pipette tip; if using a CPT, avoid transferring any of the separation gel, which may block the pipette.
- PBMCs must be washed twice in serum-free media (e.g. GIBCO™ AIM-V) and immediately resuspended and mixed in the media that will be used for the overnight incubation
- Cells must be counted to determine numbers of viable cells available prior to incubation with test wells.

Testing

- T-SPOT requires 2.5 x 10^5 viable PBMCs per test well, and a total of four wells are required for each patient sample (for a total of 1 x 10^6 viable PBMCs per patient):
  - Nil Control
  - Panel A (ESAT-6 antigen)
  - Panel B (CFP-10 antigen) and
  - Positive Control (phytohaemagglutinin [PHA]), which tests for PBMC functionality.
- A new pipette tip must be used for every addition of each patient's cells to avoid cross-contamination between wells.
- Test plates must be incubated at 37 ^oC with 5% CO_2 in a humidified incubator for 16-20 hours; plates must not be stacked in the incubator as this may lead to uneven temperature distribution and ventilation.
- After incubation, plates must be washed with phosphate buffered saline (PBS) and developer reagents added; pipette tips must not touch the wells, or artifacts may be produced and misinterpreted as spots.
- Medium is removed from the plates by inverting the plate and shaking contents out into an appropriate container; DO NOT remove well contents by pipetting.
- Avoid the use of detergents (e.g. Tween™) in the PBS as this can cause high background counts in the test wells.
- Plates must be allowed to dry completely either in an oven at up to 37 ^oC for a minimum of 4 hours or overnight at room temperature.
- Counting cells (distinct dark blue spots on the membrane of each well) should be performed by visualizing with a magnifying glass, plate microscope or an ELISPOT plate reader instrument.

Quality control and test result interpretation
(See Figure 1)

- Typical results have few or no spots in the Nil Control.
- A Nil Control spot count in excess of 10 spots should be considered as "Indeterminate".
- If a high numbers of spots or a dark background is observed in the Nil Control wells, the assay reagents and culture media should be checked for contamination.
- Greater than 20 spots should be counted in the Positive Control.
- When the Positive Control is less than 20 spots, it is considered "Indeterminate" (unless panel A or B are "Reactive" as per the Result Reporting below); check to ensure that recommended incubation conditions were used. Weak PHA responsiveness may reflect anergy in the patient.

Result reporting

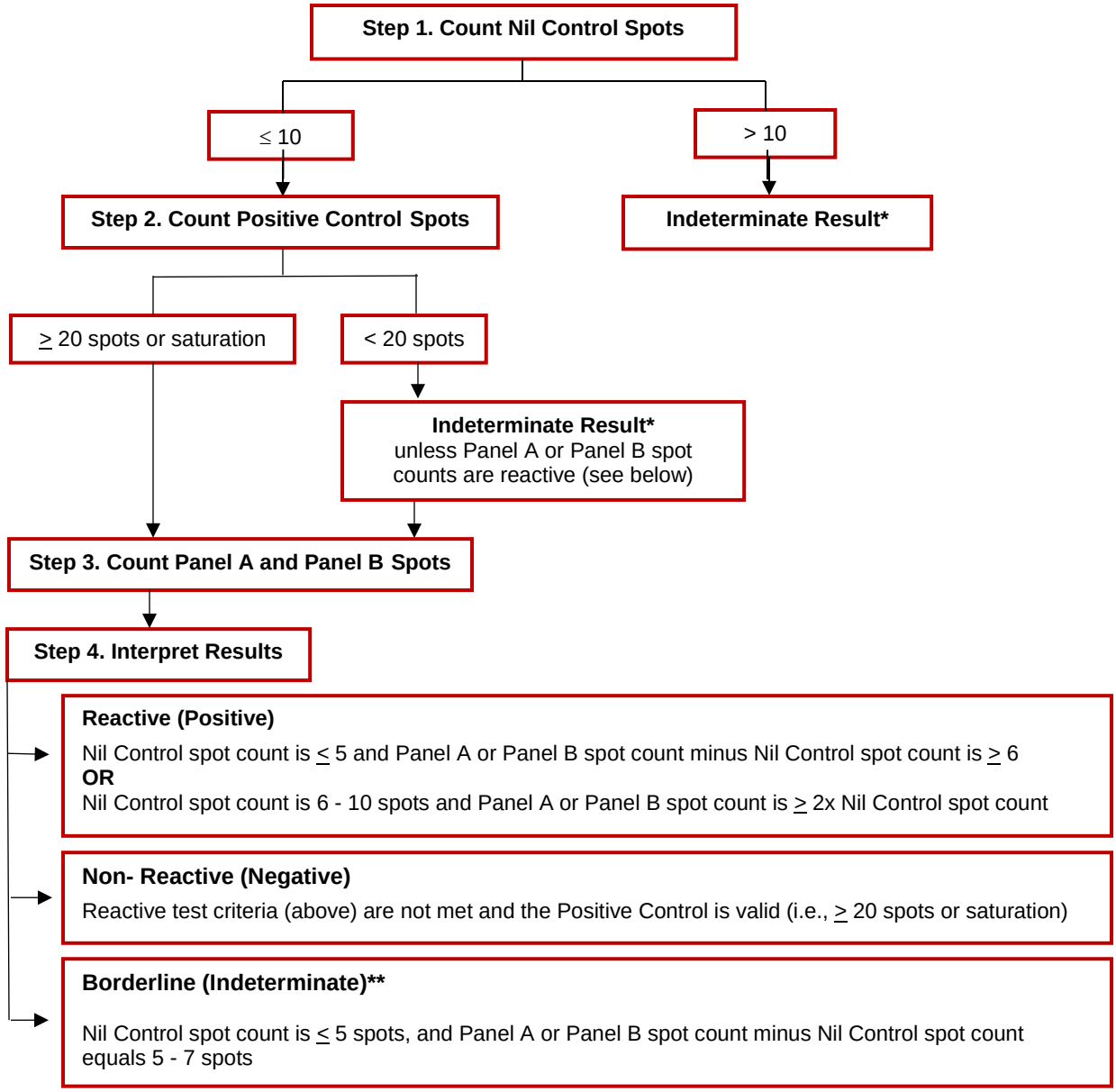
- A test is considered *Reactive*[*] *Positive* if either or both Panel A and Panel B show the following:
  - Nil Control has 0 – 5 spots and (Panel A or Panel B spot count) – (Nil Control spot count) ≥ 6;
  - Nil Control has 6 – 10 spots and (Panel A or Panel B spot count) ≥ 2x (Nil control spot count).

- A test is considered *Non-Reactive* if the above criteria are not met and the Positive control is valid.

- A test is considered *Indeterminate* if:
  - the Positive Control is "*Indeterminate*" and both Panel A and Panel B are "*Non-reactive*" and should be repeated;
  - the Nil Control has 0 – 5 spots and (Panel A or Panel B spot count) – (Nil Control spot count) = 5 – 7.

[*] It is possible that a "Reactive" result may be due to infection with non-tuberculous mycobacteria (M. *kansasii*, M. *szulgai*, M. *marinum* or M. *gordonae*). Alternative tests are required if infection with these organisms is suspected.

Figure 1. Algorithm for interpretation of T-SPOT® .*TB* assays



*Refer to the T-SPOT®.*TB* Technical Handbook for possible causes (may be downloaded from www.oxfordimmunotec.com). It may be necessary to collect a further sample and re-test the individual.

**Result should be considered in conjunction with all available clinical information. It may be necessary to collect a further sample and re-test the individual.

The T-SPOT®.*TB* Package Insert[45] interpretation guide provides an algorithm for interpretation; please refer to package insert for more details and tables (www.oxfordimmunotec.com/CANpageinsert).

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