PROVIDENCE
Sacred Heart
Medical Center &
Children's Hospital

High Performance Liquid Chromatography
Sherlock Mycobacteria Identification System

Department of Microbiology

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1.0 Purpose and Principle

The use of high performance liquid chromatography (HPLC) as a method for identifying mycobacteria was first standardized by the Centers for Disease Control. This method relies on the analysis of cell wall mycolic acids, which are species specific. The chromatogram patterns that are obtained from HPLC analysis permit the identification of test isolates by comparing the isolate's chromatogram to patterns of reference strains. MIDI, Inc. has created automated pattern recognition software that performs the matches to facilitate identification.

There are two MIDI assays. One is intended for acid-fast bacilli (*Mycobacterium* spp.) and the other assay is for partially acid-fast bacteria (*Gordonia*, *Nocardia, Rhodococcus,* and *Tsukamurella*). Test samples are first saponified with potassium hydroxide to cleave the mycolic acids bound to the cell wall. The mycolic acids are then converted to their free acid forms by acidification followed by an extraction with chloroform. After conversion into fluorescent derivatives by esterification with 4-bromomethyl-6, 7-dimethoxycoumarin and cis-dicyclohexyl-18-crown-6 ether, the mycolic acids are analyzed using HPLC with fluorescence detection. A gradient of methanol and isopropanol generated by microprocessor-controlled pumps is used to separate the mycolic acid esters. As the compounds pass through the fluorescence detector, the fluorescent tags are excited by 345-nm wavelength light, in turn causing light emission at 425-nm that is quantitatively detected by a photo-multiplier tube in the detector. The amount of light emitted is related to the concentration of the tagged compound, while the time of elution is related to the structure of the compound. The signals are integrated to create a pattern of different diagnostic peaks, called the chromatogram. The column retention time, peak width, and response of each peak are then transmitted from the HPLC hardware to the analysis software for processing. Peaks in the chromatogram are identified by mycolic acid Equivalent Carbon Length (ECL) value (name). When peak naming is complete, the HPLC analysis software searches its library to compare the peak name and peak amount values of the sample pattern to reference profiles of known mycobacterial species. Each library entry is a computer-generated composite of the reference strains of each species or subspecies group of organisms, taking into consideration strain-to-strain and experimental variability. Following the library search, the computer prints the Composition Report and a Chromatographic Report. The Composition Report includes the peak naming and library matching results. The Chromatographic Report shows the chromatogram, a visual plot or trace of the electronic signal generated by the fluorescence detector as mycolic acids of the sample elute from the column. An additional feature of the Sherlock Software allows the user to perform a visual comparison of the HPLC pattern to a known "reference" chromatogram. The chromatogram will be aligned using the internal standard peak times and the reference chromatogram will be scaled to the same area as the current analysis. The reference chromatogram is printed as a mirror image immediately below the current analysis for easy visual comparison.

2.0 Clinical Significance

Many species within the genus *Mycobacterium* are prominent pathogens, above all the members of *Mycobacterium tuberculosis* complex. Apart from *M. tuberculosis* complex, there is a growing number of nontuberculous mycobacteria (NTM) species, some of which are sources of important diseases in humans. A rapid and reliable method for the identification of *M. tuberculosis* complex and NTM is essential to patient care and infection control.

3.0 Scope

This procedure is classified under CLIA as Highly Complex. It should be carried out by technical personnel familiarized and trained on all levels of the operation of the HPLC testing platform. Testing includes but is not limited to: instrument start up, shutdown, routine maintenance, performance checks, basic troubleshooting, QC checks, administrative tasks and record keeping of information vital to verification of instrument and technical proficiency. Records of continued competence and proficiency on the equipment are to be kept within the employee's record in the department. Performance reviews of technical personnel are to be carried out annually.

4.0 Safety & Personal Protective Equipment

Performance of this procedure will expose testing personnel to biohazardous material. All isolates must be handled as potentially infectious material as outlined in the Providence Sacred Heart Microbiology Safety Guidelines.

The reagents and/or chemicals that are used in this procedure may be hazardous to your health if handled incorrectly. A brief listing of precautions for each chemical hazard is included in the reagent section of this procedure.

More extensive information concerning the safe handling of the reagents and/or chemicals used in this procedure, as well as other important safety information may be obtained by consulting the Material Safety Data Sheet (MSDS). Before performing any part of this procedure, the technologist must take any and all precautions and adhere to all prescribed policies.

This procedure may expose you to:

- Bloodborne pathogens
- Airborne pathogens
- Hazardous reagents

To perform this procedure, you must use:

- Gloves – must be worn when handling cultures and reagents.
- Safety glasses – must be worn when handling reagents.
- Laboratory Coat – must be worn when handling cultures and reagents.
- N95 respirator – must be worn when manipulating cultures.
- Biological safety cabinet – All work with active cultures must be performed within a biosafety cabinet using biosafety level-3 practices.
- Fume hood – must be used when handling organic solvents.

Disinfectant following procedure:

- Diluted bleach (10% solution made fresh daily)

Reference for spill/decontamination:

- MSDS
- Chemical hygiene plan

5.0 Specimen Requirements

5.1 Mycobacteria

Reliable identifications can be achieved using growth obtained from solid media, such as Lowenstein-Jensen (LJ) or Middlebrook 7H11. Alternatively, growth can be harvested from broth culture, such as the Middlebrook 7H9 broth in MGIT tubes or BacT/ALERT MP Culture Bottles. While most test isolates are obtained from these media in cultures incubated at 35 ± 2 °C, some species of mycobacteria may normally be grown under different conditions. For example, *M. marinum* and *M. haemophilum* grow best at 30 °C, with the latter being grown on chocolate agar rather than Middlebrook. Test isolates should be actively growing. Rapid growers can often be tested within a week of culture incubation, while slow growing species may require two weeks or longer.

5.2 Aerobic Actinomycetes

Suspected aerobic actinomycetes, such as *Nocardia*, should be grown on TSA with 5% sheep blood at 35 ± 2 °C until sufficient log phase growth is present (generally 24-48 h).

6.0 Materials Required

6.1 Equipment and/or Testing System

- Autoclave
- Heat evaporator block for 13 mm tubes
- Vortex type mixer
- Pipet-Aid
- 10-100 µL pipette and tips
- Agilent Technologies 1200 Series HPLC Hardware
- ChemStation (Version A.06.03 or above), Agilent Corporation
- Windows® 2000-based computer loaded with the MIDI Sherlock® sample analysis software
- Sherlock® Mycobacteria Identification System software

6.2 Consumables

- Test tubes, 13 x 100 mm borosilicate glass tube with Teflon-lined screw caps
- Glassware for reagent preparation and storage - borosilicate, amber bottles with Teflon-lined screw caps
- Pipette, 5.75 inch glass Pasteur
- Wood applicator or disposable, sterile, plastic bacteriological loop
- Repeating dispensers: resistant to acids organic reagents
- Glass 1 mL serological pipette with suction bulb
- Auto sampler vials
    - 2 mL amber borosilicate glass
    - 2 mL clear borosilicate glass
- Vial caps with Teflon-faced septa
- 1,000 µL and 200 µL micropipetters, with corresponding tips

6.3 Chemicals & Reagents

6.3.1 Chemicals

- Methanol (HPLC grade)
  Shelf life: 3 years in unopened container (1 year, if opened).
- Isopropanol (HPLC grade)
  Shelf life: 3 years in unopened container (1 year, if opened).
- Hydrochloric acid (ACS grade)
- Chloroform (HPLC grade)
  Shelf life: 1 year in unopened container (6 months, if opened).
- Potassium hydroxide (ACS grade)
- Potassium Hydrogen Carbonate (ACS grade)
- 4-(Bromomethyl)-6,7-dimethoxycoumarin (F.W. = 299.1)
- cis-dicyclohexyl-18-crown-6 ether (F.W. = 264.3)

Note: Except when noted, all reagents and solvents must be American Chemical Society (ACS) reagent grade or HPLC grade. Use ACS or United States Environmental Protection Agency (USEPA)-type II or equivalent reagent-grade water. All flammable reagents should be stored in the flammable cabinet.

6.3.2 Reagents

Reagent 1: Saponification Reagent (200 mL) – shelf life 1 year.

- Potassium Hydroxide (KOH): 100 g
- Deionized Water (DI): q.s. to 200 mL with continuous stirring

Danger: Reagent is extremely caustic. Wear eye protection and observe splash precautions. Substantial heat is released during reagent preparation that may cause the mixture to boil, bump or steam. Prepare reagent only in borosilicate glass inside a chemical fume hood. Prepare by slowly adding KOH pellets to about 75 mL of DI until totally dissolved. Continue adding DI until the final volume is 200 ml. Note: reagent will absorb CO_2. Protect from air.

Reagent 2: Acidification reagent (1 L) – shelf life 1 year

- Hydrochloric acid (stored in Chemistry): 500 mL
- Deionized Water (DI): 500 mL

Danger: Reagent is extremely corrosive. Wear eye protection and observe splash precautions. Substantial heat is released during reagent preparation that may cause the mixture to boil, bump, or steam. Prepare reagent ONLY in borosilicate glass inside a chemical fume hood. Slowly add acid to water with continuous stirring. ***Never*** add water to acid. Reagent should be prepared in the Chemistry department close to where the HCL is stored.

Reagent 3: Chloroform – shelf life: 1 year in unopened container, 6 months if opened.

- HPLC-Grade Chloroform

Danger: Chloroform is toxic and should only be manipulated inside a chemical fume hood. Wear protective gloves and lab coat to prevent skin exposure.

Reagent 4: Derivatizing reagent (50 mL) – shelf life 6 months.

- 4-bromomethyl-6, 7-dimethoxycoumarin: 0.05 g
- cis-dicyclohexyl-18-crown-6 ether: 0.05 g
- Chloroform: 50 mL

Prepare by dissolving 4-bromomethyl-6, 7-dimethoxycoumarin and cis-dicyclohexyl-18-crown-6 ether in chloroform.

Danger: This reagent is a toxic, lacrimator. Wear eye protection and work within a chemical fume hood.

Reagent 5: 2% Methanolic Potassium Bicarbonate reagent (100 mL) – shelf life for reagent: 1 year. Shelf life for coated vials: 6 months.

- Potassium bicarbonate: 2 g
- Methanol: 50 mL
- Deionized water (DI): 50 mL

Dissolve potassium bicarbonate in 50% aqueous methanol (50 mL methanol; 50 mL DI). Prepare vials by adding 100 µl of Reagent 5 to each amber, 2-mL auto sampler vial. Dry the contents of the vial completely at 60°C on the evaporator. The coated vials may be prepared in batches and stored at room temperature in a dried covered condition.

Reagent 6: Sample Extract Diluent – shelf Life: 3 years (unopened container); 1 year in opened container

- HPLC-Grade Isopropyl Alcohol (2-propanol)

6.4 Controls and Standards

6.4.1 Mycobacteria Assay

6.4.1.1 Mycobacteria Controls

Mycobacterium gordonae ATCC 14470 and *Candida albicans* ATCC 60193 cultures grown on Middlebrook 7H11 agar at 35 ± 2°C. New stock cultures should be subbed from the -70°C freezer at the beginning of each month.

6.4.1.2 Mycobacteria Calibration Standard

Vial inserts (MIDI Item # 1500-A, box of 5). The Calibration Standard is shipped dry in glass inserts. The expiration date of the Calibration Standard is listed on the package. It should be stored dry, in the dark and at 4 ± 2°C. The Calibration Standard should be solubilized by addition of 80 µL of isopropanol. If the Calibration Standard is not completely used in a sequence of analyses, it may be recapped with a fresh cap and stored in the dark at 4 ± 2°C for up to one month. Before reuse, the Calibration Standard should be brought to ambient temperature to assure complete solution of all mycolic acids.

6.4.1.3 Mycobacteria Internal Standard

Vial inserts (MIDI Item # 1600-A, box of 100). One Internal Standard vial insert is used for each control and each patient isolate.

6.4.2 Mycolic Acid Bacteria (Actinomycete) Assay

6.4.2.1 Mycolic Acid Bacteria Controls

Gordonia sputi ATCC 29627 culture. Maintain the control strain by subbing a monthly stock culture from the -70°C freezer as outlined in the QC Organism Maintenance Procedure. The control strain should be grown on TSA with 5% sheep blood at 35 ± 2°C and subcultured as necessary for log-phase growth. For a negative control, prepare the extract using the reagents only. Do not use the *Candida albicans* extract used with the MYCOLC1 library.

6.4.2.2 Mycolic Acid Bacteria Calibration Standard

Vial inserts (MIDI Item # 1800-A, box of 5). The Calibration Standard is shipped dry in glass inserts. The expiration date of the Calibration Standard is listed on the package. It should be stored dry, in the dark and at 4 ± 2°C. The Calibration Standard should be solubilized by addition of 80 µL of chloroform. If the Calibration Standard is not completely used in a sequence of analyses, it may be recapped with a fresh cap and stored in the dark at 4 ± 2°C for up to one month. Before reuse, the Calibration Standard should be brought to ambient temperature to assure complete solution of all mycolic acids.

6.4.2.3 Mycolic Acid Bacteria Internal Standard

Vial inserts (MIDI item # 1900-A, box of 100). One Internal Standard vial insert is used for each control and each patient isolate.

6.5 Software Instructions

Refer to the Mycobacteria Identification System Operating Manual for complete instructions.

7.0 Procedure

7.1 Harvesting Cultures and Saponification

1. Obtain a sufficient number of 13 x 100 mm screw cap tubes for the sample batch. Carefully inspect each tube for defects and proper cap fit. Discard any defective tubes/caps.
2. Label tubes with appropriate identifier using a laboratory-marking pen that will withstand autoclave conditions.
3. Manipulate cultures within biological safety cabinet while wearing an N95 respirator.

7.1.1 Mycobacteria Growing on 7H11 or Lowenstein-Jensen Agar

4. Add 1 mL of Saponification Reagent (Reagent 1: KOH) to each tube.
5. Transfer sample into the saponification reagent. Only a very small amount of cells is needed therefore an amount of cells barely visible on the end of a sterile applicator stick or disposable plastic bacteriological loop is sufficient. It is better to work with isolated colonies.
6. Securely tighten a Teflon-lined screw cap onto each tube.
7. Vortex each tube for 5-10 s.
8. Place autoclave indicator tape on the rack containing the tubes.
9. Autoclave for 30-60 min at 121°C.

7.1.2 Mycobacteria Growing in MGIT Broth (growth from MP Broth should only be used if cording morphology is evident microscopically)

4. Vortex the bottle or tube to stir up sediment.
5. Remove 4 mL of broth.
6. Transfer the broth to a 13 x 100 mm tube and securely tighten a Teflon-lined screw cap onto each tube.
7. Place tubes into 50-mL adapters and centrifuge broth at 3,000 x *g* for 15 min.
8. Using a sterile transfer pipette, transfer supernatant to a discard container. Once the supernatant has been removed, add 1 mL of Saponification Reagent (Reagent 1: KOH). Securely tighten a Teflon-lined screw cap onto each tube.
9. Vortex each tube for 5-10 s.
10. Place autoclave indicator tape on the rack containing the tubes.
11. Autoclave for 30-60 min at 121°C.

7.1.3 Aerobic Actinomycetes Growing on TSA with 5% Sheep Blood

4. Add 1 mL of Saponification Reagent (Reagent 1: KOH) to each tube.
5. Transfer sample into the saponification reagent. Only a very small amount of cells is needed therefore an amount of cells barely visible on the end of a sterile applicator stick or disposable plastic bacteriological loop is sufficient. It is better to work with isolated colonies.
6. Securely tighten a Teflon-lined screw cap onto each tube.
7. Vortex each tube for 5-10 s.
8. Place autoclave indicator tape on the rack containing the tubes.
9. Autoclave for 30-60 min at 121°C.

7.2 Extraction

1. Remove tubes from autoclave and allow them to cool to ambient temperature. The organisms are no longer viable, and the tubes are safe to work with in the chemical fume hood.
2. Add 1.8 mL of Acidification Reagent (Reagent 2: HCL) with the repeat dispenser. Cap the tubes, and vortex briefly (1 s) to mix thoroughly. Allow the tubes to stand at room temperature for 5 min.
3. Uncap tubes, and add 1.5 mL of chloroform (Reagent 3) using the repeat dispenser.
4. Recap each tube, and vortex vigorously for 30-60 s. Visually inspect each tube to insure complete mixing.
5. After phase separation, transfer the bottom (chloroform) layer to a clean 13 x 100 mm borosilicate glass tube. Note: It may be necessary to briefly centrifuge the tube if an emulsion is present. The tube must be labeled with the corresponding label number from the original 13 x 100 mm tube.
6. Evaporate the chloroform from each tube, using the heat block/evaporator unit.
7. Remove the tubes, and cool to ambient temperature.

7.3 Derivitization

1. With a pipette, add 200 μl Derivatizing Reagent (Reagent 4) to each tube containing an extract.
2. Gently swirl the tube to dissolve its contents.

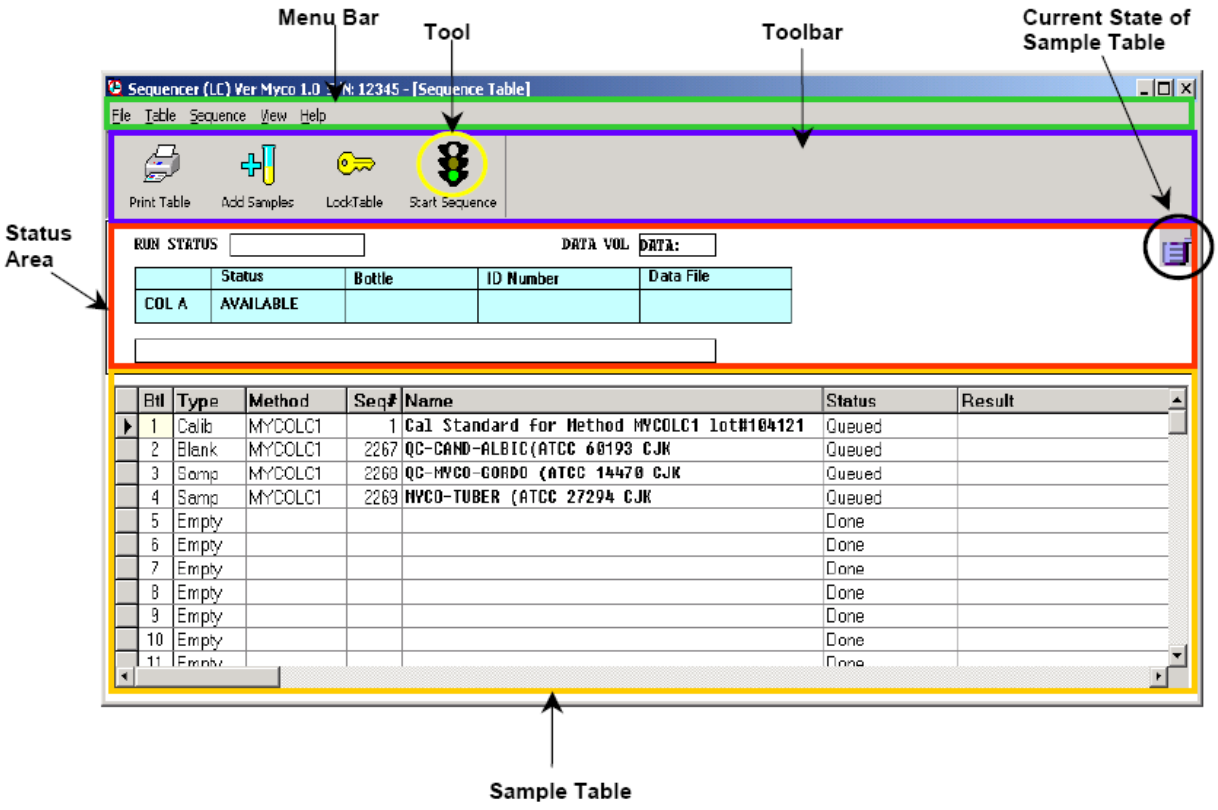
3. Transfer the mixture from each tube by pouring the contents into a correspondingly labeled 2-mL amber vial which has had the interior pre-coated with 100 µl of 2% Methanolic Potassium Bicarbonate (Reagent 5).
4. Swirl the vial gently by hand to assure mixing of the contents.
5. Heat the vials uncapped for 10 min at 60 ± 2°C.
6. Slowly evaporate the contents of each vial.
7. Remove the vials and cool to ambient temperature.
8. With a pipette, add 500 µL of isopropyl alcohol (Reagent 6) and cap the vial.
9. Swirl to totally dissolve the contents and mix the sample extract.
10. Using a pipette, remove 50 µL of each sample extract and add it to a separate internal standard vial insert. Dispense the extract along the walls of the insert.
11. Once dispensed, thoroughly mix the sample by slowly pumping the solution up and down, dispensing against the wall of the insert to ensure that the dried internal standards are totally solubilized.
12. After capping the vials, the samples are ready for HPLC analysis**.**

7.4 Entering Sample Information into the Sequencer

The Sequencer is accessed from the *Sequencer* Icon on the desktop. Sherlock uses the sequencer as a Sample Table and as a link with the Agilent ChemStation. A view of the Sherlock Sequencer Screen can be seen on the following page. The top menu bar and toolbars allow manipulation of the Sample Table. The middle windowpane gives Sherlock and ChemStation status information, currently running sample status and the data storage location. The bottom section is the Sample Table, where the Calibration Standard and sample identification information is logged. Before putting a sample bottle into the sample tray, the proper identification information must be logged into the corresponding bottle number in the Sample Table. The sample vial type can be a calibration sample (**Calib**) or a sample extract (**Samp**) to be processed for identification. In addition, the vial type can be labeled as **Stat**, which indicates a priority sample that will be run next, **Blank** for the negative control, or **Empty**.

To program a run:

1. Clear any information left in the table from the previous run. On the Menu Bar, select Table/Clear If Done. This will remove samples that have already been analyzed (bottles with a status of DONE).
2. Click on the Add Samples icon on the toolbar.
3. The Sample Table editor automatically advances to the first empty bottle position. The first bottle position should be occupied by the Calibration Standard. The sample type, method, and status will be entered automatically by the system based on the previous sample's information, while the user will enter sample name and pertinent sample information. The Seq # is automatically incremented and displayed.
4. In the Name column on the Sample Table, enter the positive run control followed by the negative run control and then the patient sample accession numbers. The negative control must be designated as Blank under the Type column. The positive control and patient samples should be designated as Samp in the Type column. Information can be typed by hand or scanned from a barcode. When typing information, hitting the Enter key will advance the Sample Table editor to the next bottle position. When scanning a barcode, the Sample Table editor may automatically advance to the next position. If an extra position is queued at the end of the run, be sure to change the Status of that position to Done.
5. When all of the information has been entered into the Sample Table, click on the Done Adding icon.



7.5 Loading the Automatic Liquid Sampler & Starting the Sequence

Place the capped vials into the HPLC sample tray according to the sample order entered into the MIDI Sherlock® Sequencer Table/Sample Addition screen of the HPLC software. The first position in the sample tray (numbered "1") is always occupied by a vial containing the Calibration Standard. Positions with higher numbers should contain sample vials that correspond to their numbers in the sequence table. There must not be empty spaces in the sample tray unless those positions are designated as "Empty" in the Sherlock sequence table.

Prior to starting the sample sequence, verify the following:

- The printer has sufficient paper.
- Each sample has a unique sequence number, and the information is logged into the Sample Table.
- The correct method is selected for each sample. For mycobacteria, the MYCOLC1 method should be used. For the aerobic actinomycetes, the MAB1 method should be used.
- The sample tray is loaded with the samples and the Calibration Standards corresponding to the Sample Table entries.
- Confirm that ChemStation is not open or running.
- All instrument modules are on and in a ready state.
- There is sufficient solvent in the solvent bottles.
- There is sufficient room in the waste container.

When the above steps have been taken, the system is ready to start. To start a sequence, press on the *Start Sequence* tool in the Toolbar. If prompted, make any necessary adjustments to the Sample Table. If no adjustments are needed, click on Start Sequence. The system will prompt the user to warm up the system. Select "Yes." The system will prompt the user to warm up the system. Select "Yes."

7.6 Instrument Warm-up

Start the HPLC analysis via the Sherlock® Sequencer software. The system will begin a conditioning process that includes a warm up period and a "blank run", or analysis in which no sample is injected. As a result, the solvent gradient is performed. This conditioning is helpful in enabling the system to equilibrate so that the analysis of the calibration is not only successful, but provides optimal times for the peak naming table to use in naming peaks in subsequent samples.

7.7 Calibration

At the completion of the blank run, the robotic arm will pick up the vial in Calibration Standard in position 1 and move it to a space between the injection syringe and the syringe seat. The syringe will then pick up the amount of sample designated by the Sherlock method. The sample vial is then returned to its original position and the syringe moves down into the syringe seat, causing injection of the sample into the flowing stream of carrier solvents. The entire sample is put into the analysis and the syringe is continuously flushed by solvent during the entire analysis. This virtually eliminates carry-over of sample to the next analysis. The Calibration Standard is used for the first two injections of a sequence and is reanalyzed after every 10th sample injection to ensure system stability.

7.8 Analysis

Once the sample is injected, the ChemStation plots the signal from the fluorescence detector of the HPLC, creating the chromatogram. Mycolic acids in the sample are separated by the column and identified by the retention time of each peak. Retention times are measured to a resolution of 0.001 min. The peak retention time, width and height data from the ChemStation are transmitted to Sherlock data files at the end of each run. The data are processed, peaks are assigned names, the unknown is compared to the library and a report is printed.

Extracts that are too dilute will not result in valid searches since the smaller mycolic acid peaks will not be detected. Usually this occurs when the isolate is a very slow grower and too few cells were harvested. If the sample injected results in a "Total Response" of less than 20,000, the "Minimum Response" warning message will be printed on the report. The system will automatically reanalyze the sample injecting four times the original injected amount (20 μL). The report will be labeled with the letters "RR" (rerun) in the parenthetical portion of the name field.

Peaks with heights greater than 450,000 Luminosity Units (LU) cause saturation of the fluorescence detector and thus the height assigned to them will be inaccurate. This is detected by the Sherlock software and the sample is automatically reanalyzed by injecting only 2 μL.

7.9 Shutdown

After all samples have been analyzed, the software automatically shuts off the pumps, detector, and heater column modules of the HPLC, and closes the ChemStation software.

8.0 Calibration Reports & Performance Qualification Table

The calibration standard contains 16 compounds prepared by MIDI for use in the calibration standard. The first and last peaks of the chromatographic profile of the calibration standard are the same as the internal standards. The calibration standard is used to:

1. assign the ECL values of peaks in the unknown samples,
2. check the chromatographic performance of the system and
3. allow the "LC Adjust" algorithm to make all systems using the MIDI HPLC Mycobacteria Identification System software achieve highly similar results.

The "ECL" values of the calibration standard are those by which peaks in subsequent analyses are named. The system calculates how much the calibration analysis has deviated from the expected relative retention times and reports the Root Mean Square (**RMS**) fit error. If a calibration run is invalid due to a high RMS fit error; Sherlock will print a message to warn the user and then will repeat the calibration analysis. If the system fails to calibrate after two consecutive attempts, the error message will be repeated and the sample sequence aborted.

The user should refer to the Mycobacteria Identification System Operating Manual, Chapter 7 Troubleshooting.

The key parameters on the calibration report that should be recorded on the Performance Qualification Table are the **40.000** and **97.000 ECL Peak Retention Time (RT)**, the **Similarity Index (Sim Index)**, **Total Response**, and **Peak Position Matching Error (RMS)**. Tracking of these parameters will give an indication of the acceptable performance of the system. The expected range for each parameter is given in parenthesis in the corresponding column on the Performance Qualification table. Parameters that are out of the expected range, give an indication that action may need to be taken to continue proper performance. The user should refer to the Mycobacteria Identification System Operating Manual, Chapter 7 Troubleshooting.

9.0 Quality Control

Control organisms must be extracted and carried through the entire procedure with each run. Run controls should be loaded so that the positive control is analyzed first followed by the negative run control in order to detect potential carryover.

9.1 Mycobacteria Assay Quality Control

9.1.1 Control Organisms

M. gordonae ATCC 14470 should be used for the positive control and *C. albicans* ATCC 60193 for the negative control. *C. albicans* does not produce mycolic acids and is primarily used to measure reagent purity. Incubate both on Middlebrook 7H11 agar at 35 ± 2°C. The same plates can be repeatedly harvested for up to 1-2 months. Stocks should be routinely subcultured at the beginning of each month to maintain working cultures.

9.1.2 Acceptable Quality Control

The positive control must name as *M. gordonae* at a similarity index (SI) value > 0.600. A SI value of < 0.600 for *M. gordonae* suggests improper processing of samples or possibly a mixed culture. Record the SI result on the Performance Qualification Table. The Total Named response and the Percent Named results should also be recorded on the Performance Qualification Table. These results are for reference only. The result for negative control *C. albicans* should not match any library entry and must have the Total Response < 1000. If the negative control has a total response > 1000, reagent contamination should be suspected. If the *M. gordonae* SI < 0.600 or the *C. albicans* Total Response > 1000, the source of the problem should be identified and corrected. Isolates from patient cultures should be re-extracted, along with the positive and negative controls, and re-analyzed.

9.2 Aerobic Actinomycete Assay Quality Control

9.2.1 Control Organisms

Use *Gordonia sputi* ATCC 29627 as a positive control. Maintain the control strain by subbing a monthly stock culture from the -70°C freezer as outlined in the QC Organism Maintenance Procedure. The control strain should be grown on a TSA with 5% sheep blood at 35 ± 2°C and subcultured as necessary for log-phase growth. For a negative control, prepare the extract using the reagents only. Do not use the *Candida albicans* extract used with the MYCOLC1 library.

9.2.2 Acceptable Quality Control

The positive control must name as *Gordonia sputi* at a similarity index (SI) value > 0.600. A SI value of < 0.600 for *Gordonia sputi* suggests improper processing of samples or possibly a mixed culture. Record the SI result on the Performance Qualification Table. The Total Named response and the Percent Named results should also be recorded on the Performance Qualification Table. These results are for reference only. The result for negative control should not match any library entry and must have the Total Response < 1000. If the negative control has a total response > 1000, reagent contamination should be suspected. If the *Gordonia sputi* SI < 0.600 or the negative control Total Response > 1000, the source of the problem should be

identified and corrected. Isolates from patient cultures should be re-extracted, along with the positive and negative controls, and re-analyzed.

10.0 Interpretation and Reporting Results

10.1 Sherlock Reports

The Sherlock system reports analysis results in two forms.

- **Chromatographic Report**
  The chromatogram is a visual plot or trace of the electronic signal generated by the fluorescence detector as mycolic acids of the sample elute from the column.
- **A Sherlock Composition Report & Library Search**
  The Sherlock Composition Report comprises a Mycolic Acid Composition Report, a Library Search Report, and a Comparison Chart.
  - The **Composition Report** contains the mycolic acid composition of the organism. Refer to the Mycobacteria Identification System Operating Manual for detailed explanation of the information provided on the Composition Report.
  - The **Library Search Report** lists the results of comparing the mycolic acid composition to the Sherlock Library. The Sherlock Library Search Report lists the most likely matches to the unknown composition, and provides a similarity index (SI) for each match. If the search results in more than one possible match, the suggested identities are listed in order of descending similarity index. Isolates with a SI of 0.500 or higher with a separation of 0.200 between the first and second choice SI are considered good library comparisons. Isolates that have an SI below 0.500 or with an unacceptably close second choice should be considered indeterminate, and confirmatory tests should be used. Consult with Rounds.
  - The **Comparison Chart** is a plot comparing the mycolic acid composition of the unknown to the most similar library entry. This chart provides a visual comparison of the test isolate chromatogram against the reference chromatogram.

10.2 Sherlock® Mycobacteria Library

Note: Not all library entries have been validated by the PSHMC Department of Microbiology. A sufficient number of test isolates are needed to validate the accuracy of each library entry. Those entries that have been validated are indicated in bold font. These identifications may be reported if the Similarity Index and separation is acceptable (see Reporting section below). Isolates that produce identifications that have not been validated must be sent to a reference laboratory for identification. Data will be gathered prospectively and used to validate additional library entries.

- ***Mycobacterium-abscessus/chelonae***
- *Mycobacterium-asiaticum*
- *Mycobacterium-aurum/vaccae*
- *Mycobacterium-bovis* BCG (not 35737)
- *Mycobacterium-celatum*
- ***Mycobacterium-chelonae/abscessus***
- *Mycobacterium-flavescens*
- ***Mycobacterium-fortuitum/peregrinum* (report as *Mycobacterium fortuitum* complex)**
- ***Mycobacterium-gordonae***
- *Mycobacterium-haemophilum* (grown at 30ºC, on chocolate agar)
- *Mycobacterium-interjectum*
- *Mycobacterium-intermedium*
- ***Mycobacterium-kansasii***
- *Mycobacterium-lentiflavum/triplex*
- ***Mycobacterium*-MAC A (report as *Mycobacterium avium-intracellulare* complex)**
- ***Mycobacterium*-MAC B (report as *Mycobacterium avium-intracellulare* complex)**
- ***Mycobacterium*-MAC C (report as *Mycobacterium avium-intracellulare* complex)**

- ***Mycobacterium*-MAIS (report as *Mycobacterium avium-intracellulare* complex/*Mycobacterium scrofulaceum*)**
- *Mycobacterium-malmoense*
- ***Mycobacterium-marinum* (grown at 30ºC)**
- ***Mycobacterium-mucogenicum***
- ***Mycobacterium-neoaurum***
- *Mycobacterium-nonchromogenicum/terrae*
- ***Mycobacterium-peregrinum/fortuitum* (report as *Mycobacterium fortuitum* complex)**
- ***Mycobacterium-simiae***
- *Mycobacterium-szulgai*
- *Mycobacterium-terrae/nonchromogenicum*
- *Mycobacterium-thermoresistible*
- *Mycobacterium-triviale*
- ***Mycobacterium-tuberculosis* complex**
- ***Mycobacterium-xenopi***

10.3 Sherlock® Mycolic Acid Bacteria (Actinomycete) Library

Note: Not all library entries have been validated by the PSHMC Department of Microbiology. A sufficient number of test isolates are needed to validate the accuracy of each library entry. Those entries that have been validated are indicated in bold font. *Gordonia, Nocardia, Rhodococcus, and Tsukamurella* have been validated to the genus level only. These identifications may be reported if the Similarity Index and separation is acceptable (see Reporting section below). Isolates that produce identifications that have not been validated must be sent to a reference laboratory for identification. Data will be gathered prospectively and used to validate additional library entries.

- *Corynebacterium*: Several species of *Corynebacterium* are included in the library. However, HPLC is not a reliable method for differentiating *Corynebacterium* species and alternate methods are available when clinically warranted.
- *Dietzia-maris*
- ***Gordonia* species:**
  Gordonia aichiensis, Gordonia bronchialis, Gordonia rubropertincta, Gordonia sputi, Gordonia terrae **(report as *Gordonia* species)**
- *Mycobacterium-abscessus*
- *Mycobacterium-aurum*
- *Mycobacterium-avium complex*
- *Mycobacterium-boenickei*
- *Mycobacterium-brisbanense*
- *Mycobacterium-canariasense*
- *Mycobacterium-chelonae*
- *Mycobacterium-cosmeticum*
- *Mycobacterium-flavescens*
- *Mycobacterium-fortuitum-acetamidolyticum*
- *Mycobacterium-fortuitum-fortuitum*
- *Mycobacterium-immunogenum*
- *Mycobacterium-mucogenicum*
- *Mycobacterium-neoaurum*
- *Mycobacterium-neworleansense*
- *Mycobacterium-scrofulaceum*
- *Mycobacterium-smegmatis*
- *Mycobacterium-vaccae*
- ***Nocardia* species:**
  Nocardia abscessus, Nocardia africana, Nocardia asteroides, Nocardia brasiliensis, Nocardia brevicatena, Nocardia carnea, Nocardia farcinica, Nocardia nova, Nocardia otitidiscaviarum,

Nocardia paucivorans, Nocardia pseudobrasiliensis, Nocardia transvalensis, Nocardia veterana **(report as *Nocardia* species)**

- ***Rhodococcus* species:**
  Rhodococcus equi, Rhodococcus erythropolis, Rhodococcus rhodnii, Rhodococcus rhodochrous **(report as *Rhodococcus* species)**
- ***Tsukamurella* species:**
  Tsukamurella inchonensis, Tsukamurella paurometabola, Tsukamurella tyrosinosolvens, Tsukamurella wratislaviensis **(report as *Tsukamurella* species)**

10.4 Reporting Acceptable Results

Only genus/species which have been validated in-house should be reported. Refer to the library information above for a list of validated identifications and how they should be reported (by genus, species, and/or complex). Isolates that are identified with a SI of 0.500 or higher with a separation of 0.200 between the first and second choice SI can be reported. All results should be correlated with the morphologic characteristics of each test isolate.

Results should be reported with the name of the isolate identified along with the method used. For example: "***Mycobacterium tuberculosis* identified by High Performance Liquid Chromatography.**" The Sherlock® Mycobacteria Identification System is FDA-cleared for use in the identification of *M. tuberculosis.* For reporting nontuberculous mycobacteria, the following comment must be added to the report. "**This test uses a reagent or kit designated by the manufacturer as "for research or investigational use." The performance characteristics of this test were validated by PAML/PSHMC Division of Laboratory Medicine. The U.S. Food and Drug Administration (FDA) has not approved or cleared this test. The results are not intended to be used as the sole means for clinical diagnosis or patient management decisions. PAML/PSHMC is authorized under Clinical Laboratory Improvement Amendments (CLIA) to perform high-complexity testing.**" **[RUO1 + RUO2]**

For non-tubercular *Mycobacterium* species isolated from respiratory specimens, the following comment should also be added to the report. "**The American Thoracic Society clinical criteria for non TB mycobacterial lung disease requires the presence of pulmonary symptoms and lung nodules or cavities, with the exclusion of other diagnoses. Microbiological diagnosis requires at least 1) two sputa or one bronchial wash or lavage with a positive culture or 2) a lung biopsy showing granulomatous inflammation or AFB, with one or more sputum or bronchial washings that are culture positive (Am J Respir Crit Care Med. 175:367 to 416, 2007).**" **[NTBLD1 + NTBLD2]**

10.5 Indeterminate Results

Isolates that produce no library match, have a SI below 0.500, or have a SI with an unacceptably close second choice must be referred to a reference laboratory for further testing for identification. Broth cultures should be subcultured to 7H11 agar and scrutinized for purity. Testing can be repeated from the 7H11 plate. In some cases, it may be appropriate to report an isolate as "***Mycobacterium* species other than *Mycobacterium tuberculosis***" **[MOTT]** until the identification from the reference laboratory is available. Consult on Rounds prior to reporting.

When an identification of *Mycobacterium avium-intracellulare-scrofulaceum* (MAIS) is produced, the isolate should be reported as "***Mycobacterium* species other than *Mycobacterium tuberculosis***" **[MOTT].** An overnight urea broth test for AFB should be performed. If the overnight urea test is negative, the isolate can be reported as MAI. If the overnight urea test is strongly positive, the isolate should be reported as *M. scrofulaceum.* Refer to the Urea Broth for AFB Procedure for instructions. Consult Rounds if results are weak or ambiguous.

10.6 Chromatogram Controls

Patterns for each validated organisms are available for visual reference in a notebook kept in the MIDI testing area. These patterns were obtained from ATCC strains or clinical strains that have been characterized by DNA sequencing.

10.7 Purity Check

All isolates tested from broth culture must be subcultured to a 7H11 agar plate and incubated to evaluate the culture for purity. If the subculture produces more than one morphotype, isolated colonies should be tested to rule out the possibility of a mix culture. Growth characteristics and pigmentation should correlate with the original identification. Discordant results should be reviewed during Rounds.

11.0 Instrument Maintenance

11.1 Routine Maintenance

Routine maintenance of the Sherlock HPLC Mycobacteria identification system is comprised of data backup and changing the purge valve frit. To prevent loss of data in the event of a computer hard drive crash, it is important to regularly back up data to a more permanent storage system. Sherlock provides a Windows-based program to move sequence data out of the Sherlock DATA directories. Refer to the Mycobacteria Identification System Operating Manual, Chapter 6 Routine Maintenance for instructions. Data should be backed up at least monthly.

The purge valve frit should be changed:

- When the pump piston seals are replaced or when contaminated or blocked. Blockage may be indicated by increased back-pressure of the system during routine operation.
- After each series of about 500 analyses or approximately every 3 months.

Refer to the Mycobacteria Identification System Operating Manual, Chapter 6 Routine Maintenance for instructions.

11.2 Preventative Maintenance

A MIDI technician trained by Agilent Technologies should perform all other maintenance tasks. The annual preventative maintenance (PM) procedure performed by a MIDI technician is designed to ensure the operational performance of the Sherlock Mycobacterial Identification System. The MIDI representative will document all results of the PM protocol. These documents are kept in the testing area and include:

- System verification documents
- System information
- Pre-maintenance test run(s)
- Pump leak test
- Autosampler pressure test
- Column compartment thermostat test
- Detector intensity test
- Detector dark current test
- Post-maintenance test run(s) for peak verification.

When necessary, the column is replaced by a MIDI application specialist. New columns are conditioned and verified by MIDI.

12.0 Limitations

Sherlock can identify only those microorganisms for which mycolic acid composition profiles of a representative number of correctly named reference strains have been determined and entered into the Mycobacteria Library. The library entries have been determined by analyzing reference strains grown under controlled culture conditions. These culture conditions and sample preparation procedures must be followed.

Unusual or infrequently encountered species of mycobacteria that are not in the Sherlock Mycobacteria Library are often misidentified as *Mycobacterium-nonchromogenicum/terrae*. When this identification is encountered, the isolate should be sent to a reference laboratory for confirmatory testing.

Refer to the Validation section below for testing details for each species.

13.0 Verification & Validation Information

13.1 Accuracy

13.1.1 Mycobacterium tuberculosis Complex

The Sherlock® Mycobacteria Identification System is FDA-cleared for use in the identification of *M. tuberculosis.* Verification testing was performed using a total of 29 isolates previously identified by DNA hybridization using the AccuProbe® *Mycobacterium tuberculosis* Complex Culture Identification Test by GEN-PROBE. Isolates were tested from both solid and liquid media. This included 2 isolates grown in MGIT broth, following inoculation with previously frozen positive specimen concentrates, 17 isolates seeded into MGIT broth and incubated until they were instrument-positive, 2 isolates received in MP vials, 5 isolates grown on 7H11 agar, and 7 isolates grown on Lowenstein-Jensen agar. The isolates included *M. tuberculosis* ATCC 2517, *M. tuberculosis* ATCC 27294, CAP isolate E-11 from 2011, CAP isolate E-04 from 2013, and 25 clinical isolates. All 29 (100%) of the isolates were correctly identified and produced Similarity Indexes (SI) well above the acceptable cutoff.

13.1.2 Nontuberculous Mycobacteria (NTM)

The system is also capable of identifying several other species of NTM. To validate which species the system could accurately identify, a total of 240 NTM isolates were tested.

Photochromogens

- ***Mycobacterium kansasii***

  A total of 12 *Mycobacterium kansasii* isolates were tested using the Sherlock® system. These isolates were previously characterized by DNA sequencing performed by ARUP Laboratories. Isolates were tested from both solid and liquid media. This included 3 isolates seeded into MGIT broth and incubated until they were instrument-positive, 1 isolates received in a MP vial, and 8 isolates grown on 7H11 agar. The isolates included *M. kansasii* ATCC 12478, CAP isolate E-09 from 2010, CAP isolate E-09 from 2011, CAP isolate E-11 from 2012, CAP isolate E-03 from 2013, and 7 clinical isolates. All 12 (100%) of the isolates were correctly identified by the Sherlock® system with acceptable SI and > 0.200 separation between from any secondary choices. One of the three isolates harvested from broth culture, initially produced unacceptable identifications due to less than 0.200 separation between *M. kansasii* and other species. This isolate was subcultured to 7H11 agar and retested. The repeat testing from growth on 7H11 agar produced correct identification with SI with > 0.200 separation from any secondary choices. One MGIT seed tube that was prepared from a clinical MGIT broth culture had both *M. kansasii* and MAI. However, the Sherlock® system produced an acceptable identification for *M. kansasii*.

- ***Mycobacterium marinum***

  A total of 7 *Mycobacterium marinum* clinical isolates have been tested using the Sherlock® system. Two of these isolates were previously characterized by DNA sequencing performed by ARUP Laboratories. The other five isolates were previously identified by conventional methods. Isolates were tested from both solid and liquid media. This included 2 isolates seeded into MGIT broth and incubated until they were instrument-positive and 5 isolates grown at 30°C on 7H11 agar. Four (57%) of the isolates were correctly identified by the Sherlock® system with acceptable SI and > 0.200 separation from any secondary choices. The other 3 (43%) of the isolates also produced the correct identification, but the SI was below 0.500 and/or there was < 0.200 separation from secondary choices. The Sherlock® system may erroneously identify *M. haemophilum* isolates as *M. marinum*. However, the Chocolate agar requirement of *M. haemophilum* would preclude reporting the erroneous identification.

- ***Mycobacterium simiae***

  A total of 11 *Mycobacterium simiae* clinical isolates were tested using the Sherlock® system. Five of these isolates were previously characterized by DNA sequencing performed by ARUP Laboratories and six of the isolates were identified by conventional methods. Isolates were tested from both solid and liquid media. This included 1 isolate seeded into MGIT broth and incubated until instrument-positive and 10 isolates grown on 7H11 agar. Eight (73%) of the isolates were correctly identified by the Sherlock® system with acceptable SI and > 0.200 separation from any secondary choices. The isolate harvested from broth culture produced the correct identification, but the SI was a slightly low at 0.428. Two of the isolates grown on 7H11 agar produced identifications with secondary choices that were less than 0.200 of separation.

Scotochromogens

- ***Mycobacterium gordonae***

  A total of 30 *Mycobacterium gordonae* isolates were tested using the Sherlock® system. These isolates were previously characterized by DNA hybridization with the AccuProbe® *Mycobacterium gordonae* Culture Identification test. Isolates were tested from both solid and liquid media, including 22 isolates seeded into MGIT broth and incubated until they were instrument-positive, 1 isolate received in a MP vial, and 9 isolates grown on 7H11 agar. The isolates included *M. gordonae* ATCC 14470, CAP isolate E-08 from 2012, and 28 clinical isolates. Twenty-nine (97%) of the isolates were correctly identified by the Sherlock® system. The CAP isolate produced an incorrect identification of *M. fortuitum/peregrinum* with an acceptable SI. However, the isolate produced colonies with obvious yellow pigmentation that were not consistent with the identification produced by the Sherlock® system. Two (7%) of the isolates from broth culture initially produced unacceptable identifications due multiple species listed with less than 0.200 separation. One of these isolates was from a seeded MGIT tube, and the other isolate was received in a MP vial. Both of the isolates were subcultured to 7H11 agar and retested. The repeat testing from growth on 7H11 agar produced correct identifications with no other choices.

- ***Mycobacterium neoaurum***

  A total of 7 *Mycobacterium neoaurum* isolates were tested using the Sherlock® system. This included 5 clinical isolates and 2 CAP isolates (E-08 from 2010 and E-11 from 2012). One of the clinical isolates was identified by the WA Public Health laboratory by unknown methods. Four of the clinical isolates were identified by ARUP Laboratories, one by DNA sequencing and 3 by MALDI-TOF. Isolates were tested from both solid and liquid media. This included 5 isolates grown in MGIT broth, and 4 isolates grown on 7H11 agar. All 7 (100%) of the isolates were correctly identified by the Sherlock® system with acceptable SI and > 0.200 separation between any secondary choices. One of the clinical isolates from a MGIT tube produced a SI of 0.446. The isolate was subcultured to 7H11 agar and retested. The repeat testing from growth on 7H11 agar produced a correct identification with no other choices.

- ***Mycobacterium scrofulaceum***

  No clinical isolates of *Mycobacterium scrofulaceum* were available for testing with the Sherlock® system. *M. scrofulaceum* ATCC 19981 was grown on 7H11 agar and tested. This produced an identification of *Mycobacterium* – MAIS complex (*avium/intracellulare/scrofulaceum*). Since clinical isolates are rare, it may not be feasible to prospectively evaluate the Sherlock® system for identifying this organism. However, MAI colonies are typically buff to pale yellow, while strains of *M. scrofulaceum* have bright yellow to orange colonies. Isolates identified as MAI or MAIS that subsequently produce bright yellow/orange colonies on 7H11, should be brought up on Rounds for review.

- ***Mycobacterium szulgai***

  A total of 4 *Mycobacterium szulgai* isolates were tested using the Sherlock® system. The clinical isolates were previously characterized by DNA sequencing performed by ARUP Laboratories. Isolates were tested from growth harvested from 7H11 agar. The isolates included CAP isolate E-10 from 2011, and 3 clinical isolates. Three (75%) of the isolates were correctly identified by the Sherlock® system with acceptable SI and discrimination. One of the clinical isolates produced the correct identification with an acceptable SI, but there were other possible species listed with < 0.200 separation. Data will need to be gathered prospectively in order to validate the Sherlock® system for identifying this species.

- ***Mycobacterium flavescens***

  Only 1 *Mycobacterium flavescens* isolate was available for analysis during the system validation. This isolate had been previously identified using conventional methods. The isolate was initially seeded into a MGIT broth tube and grown until instrument-positive. Testing with the Sherlock® system produced an unacceptable identification due to multiple choices with insufficient discrimination. However, testing using growth on 7H11 agar produced a correct identification with an acceptable SI and no other choices. Data will need to be gathered prospectively in order to validate the Sherlock® system for identifying this species.

- ***Mycobacterium lentiflavum***

  Two *Mycobacterium lentiflavum* clinical isolates were tested using the Sherlock® system. The isolates were previously characterized by DNA sequencing performed by ARUP Laboratories. Isolates were tested from growth harvested from 7H11 agar. Both (100%) of the isolates were correctly identified as *M. lentiflavum/triplex* by the Sherlock® system with acceptable SI and no secondary choices. Data will need to be gathered prospectively in order to validate the Sherlock® system for identifying this species.

Nonphotochromogens

- ***Mycobacterium avium* complex**

  A total of 45 *Mycobacterium avium* Complex (MAI) isolates were tested using the Sherlock® system. These isolates were previously characterized by DNA hybridization with the AccuProbe® *Mycobacterium avium* Complex Culture Identification test. Isolates were tested from both solid and liquid media. This included 9 isolates grown in MGIT broth, following inoculation with previously frozen positive specimen concentrates, 28 isolates seeded into MGIT broth and incubated until they were instrument-positive, 3 isolates received in MP vials, 8 isolates grown on 7H11 agar, and 1 isolate grown on Lowenstein-Jensen agar. The isolates included CAP isolate E-02 from 2013 and 44 clinical isolates. All 45 (100%) of the isolates were correctly identified by the Sherlock® system. Four (10%) of the isolates from broth culture initially produced unacceptable identifications due to less than 0.200 separation between MAI and other species. Two of these isolates were from MGIT tubes inoculated with specimen, 1 isolate was seeded into a MGIT tube, and the other isolate was received in a MP vial. All 4 isolates were subcultured to 7H11 agar and retested. The repeat testing from growth on 7H11 agar produced correct identification for MAI with SI > 0.200 separation between MAI and any other choices.

- ***Mycobacterium haemophilum***

  A total of 3 *Mycobacterium haemophilum* isolates have been tested with the Sherlock® system. This included one clinical isolate and two CAP isolates (E-08 from 2011 and E01 from 2013). All three isolates were grown on chocolate agar incubated at 30°C. Testing of the clinical isolate produced the correct identification with an acceptable SI and no other choices. However, testing of both CAP isolates produced identifications of *M. marinum*, with acceptable SI. Misidentifications between these two organisms with the Sherlock® system have been reported in previous studies. The growth requirements for *M. haemophilum* would preclude reporting the incorrect identification.

- ***Mycobacterium nonchromogenicum/terrae***

  Three *Mycobacterium terrae* clinical isolates were tested using the Sherlock® system. The isolates were previously characterized by DNA sequencing performed by ARUP Laboratories. Isolates were tested from growth harvested from 7H11 agar. All 3 (100%) of the isolates were correctly identified as *M. nonchromogenicum/terrae* by the Sherlock® system with acceptable SI and sufficient discrimination. However, this identification is also produced by other *Mycobacterium* species which are not in the Sherlock® system library, and this identification cannot be reliably used without further testing.

- ***Mycobacterium xenopi***

  A total of 7 *Mycobacterium xenopi* isolates were tested using the Sherlock® system. This included 5 clinical isolates and 2 CAP isolates (E-02 from 2010 and E-09 from 2012). Two of the clinical isolates were previously characterized by DNA sequencing performed by ARUP Laboratories, and three of the isolates were identified by conventional methods. Isolates were tested from both solid and liquid media. This included 1 isolate grown in MGIT broth, following inoculation with a previously frozen positive specimen concentrate, and 6 isolates grown on 7H11 agar. All 7 (100%) of the isolates were correctly identified by the Sherlock® system with acceptable SI and > 0.200 separation between any secondary choices.

Rapid Growers

- ***Mycobacterium abscessus/chelonae***

  A total of 33 *Mycobacterium abscessus/chelonae* isolates were tested using the Sherlock® system. The clinical isolates were previously characterized by DNA sequencing performed by ARUP Laboratories. Isolates were tested from both solid and liquid media, including 22 isolates seeded into MGIT broth and incubated until they were instrument-positive, 1 isolate received in a MP vial, 12 isolates grown on 7H11 agar, and 1 isolate grown on Lowenstein-Jensen agar. The isolates included CAP isolate E-01 from 2011, and 29 clinical isolates. Twenty-seven (82%) of the isolates were correctly identified by the Sherlock® system with acceptable SI and discrimination. Five (15%) of the isolates produced the correct identification, but with SI below 0.500. One (3%) of the isolates produced no match in the library.

- ***Mycobacterium fortuitum* complex**

  A total of 28 isolates belonging to the *Mycobacterium fortuitum* Complex, were tested using the Sherlock® system. The clinical isolates were previously characterized by DNA sequencing performed by ARUP Laboratories. Isolates were tested from both solid and liquid media, including 12 isolates seeded into MGIT broth and incubated until they were instrument-positive, 19 isolates grown on 7H11 agar, and 1 isolate grown on Lowenstein-Jensen agar. The isolates included *M. fortuitum* ATCC 6841, CAP isolate E-07 from 2011, CAP isolate E-08 from 2012, CAP isolate E-05 from 2013 and 24 clinical isolates. All 28 (100%) of the isolates were correctly identified by the Sherlock® system as *M. fortuitum/peregrinum* with acceptable SI.

- ***Mycobacterium mucogenicum***

  A total of 13 *Mycobacterium mucogenicum* isolates were tested using the Sherlock® system. This included 12 clinical isolates and 1 CAP isolate (E-04 from 2009). The clinical isolates were previously characterized by DNA sequencing performed by ARUP Laboratories. Isolates were tested from both solid and liquid media, including 1 isolate seeded into MGIT broth and incubated until instrument-positive and 12 isolates grown on 7H11 agar. Nine (69%) of the isolates were correctly identified by the Sherlock® system as *M. mucogenicum* with acceptable SI. Two of these nine isolates had profiles with < 85% named peaks, even after repeat testing. The other 4 (31%) specimens produced unacceptable results due to multiple species listed with < 0.200 separation.

NTM not in the Sherlock® Library

Of the 240 NTM tested, a total of 36 (15%) of the isolates are not in the Sherlock® library. Thirteen (36%) of the non-library isolates produced the identification of *M. nonchromogenicum/terrae.* Twelve of these isolates were identified as *M. arupense,* and 1 isolate was unidentifiable by DNA sequencing performed at ARUP Laboratories. Eleven (31%) of the non-library NTM isolates produced unacceptable profiles due to low SI (< 0.500). These isolates included 3 *M. goodii,* 3 *M. nebraskense,* 2 *M. smegmatis,* 1 *M. smegmatis/goodii,* 1 *M. wollinski,* and 1 isolate unidentifiable by DNA sequencing. Seven (23%) of the non-library NTM isolates produced unacceptable profiles due to low discrimination (< 0.200 separation) from secondary choices. This included 1 *M. chubense,* 4 *M. nebraskense,* and 2 isolates unidentifiable by DNA sequencing. Six (19%) of the non-library NTM isolates produced profiles with no match. These included 1 *M. cosmeticum,* 1 *M. elephantis,* 1 *M. florentinum,* 2 *M. shimoidei,* and 1 isolate unidentifiable by DNA sequencing. Three (10%) of the non-library NTM isolates produced incorrect identification with acceptable SI and acceptable discrimination. This included 1 *M. cosmeticum* that was identified as *M. fortuitum/peregrinum,* 1 *M. paraffinicum* identified as MAIS complex, and 1 *M. parascrofulaceum* identified as MAIS complex.

Mycobacterium Validation Summary

Organisms	No. (%) of isolates					
	Isolates Tested	Correct species or Complex	Unacceptable ID Low SI (< 0.500)	Unacceptable ID Low Discrim. (< 0.200 sep.)	Incorrect Identification	No Library Match
M. abscessus/chelonae	33	27 (82)	5 (15)	0	0	1 (3)
M. avium-intracellulare Complex	45	45 (100)	0	0	0	0
*M. flavescens**	1	1 (100)	0	0	0	0
M. gordonae	30	29 (97)	0	0	1 (3)	0
M. fortuitum Complex	28	28 (100)	0	0	0	0
M. haemophilum	3	1 (33)	0	0	2 (67)	0
M. kansasii	12	12 (100)	0	0	0	0
*M. lentiflavum**	2	2 (100)	0	0	0	0
M. marinum	7	4 (57)	2 (29)	3 (43)	0	0
M. mucogenicum	13	9 (69)[1]	0	4 (31)	0	0
M. neoaurum	7	7 (100)	0	0	0	0
*M. scrofulaceum**	1	1 (100)	0	0	0	0
M. simiae	11	8 (73)	1 (9)	2 (18)	0	0
*M. szulgai**	4	3 (75)	0	1 (25)	0	0
M. terrae	3	3 (100)	0	0	0	0
M. tuberculosis Complex	29	29 (100)	0	0	0	0
M. xenopi	7	7 (100)	0	0	0	0
Total number of isolates	240	220 (92)	8 (3)	10 (4)	3 (1)	1 (<1))
NTM not in Library	Isolates Tested	Unacceptable ID Low SI (< 0.500)	Unacceptable ID Low Discrim. (< 0.200 sep.)	Unacceptable ID *M. nonchrom./terrae*	Incorrect Identification	No Library Match
M. arupense	12	0	0	12 (100)	0	0
M. chubense	1	0	1 (100)	0	0	0
M. cosmeticum	2	0	0	0	1 (50)	1 (50)
M. elephantis	1	0	0	0	0	1 (100)
M. florentinum	1	0	0	0	0	1 (100)
M. goodii	3	3 (100)	0	0	0	0
M. nebraskense	4	3 (75)	4 (100)	0	0	0
M. paraffinicum	1	0	0	0	1 (100)	0
M. parascrofulaceum	1	0	0	0	1 (100)	0
M. shimoidei	2	0	0	0	0	2 (100)
M. smegmatis	2	2 (100)	0	0	0	0
M. smegmatis/goodii	1	1 (100)	0	0	0	0
M. wollinski	1	1 (100)	0	0	0	0
Unidentifiable by DNA Seq.	4	1 (25)	2 (50)	1 (25)	0	1
Total non-library NTM isolates	36	11 (31)	7 (19)	13 (36)	3 (11)	6 (17)

* Additional isolates needed for validation.

[1] Two (22%) of the *M. mucogenicum* isolates with an acceptable Similarity Index produced profiles with < 85% named peaks, even after repeat testing.

MP Broth

Isolates growing in BacT/ALERT MP Culture Bottles are frequently received from referring laboratories for identification. Since the initial validation had a limited number of isolates from MP broth, additional isolates were evaluated prospectively. In the initial validation, isolate identifications were confirmed by DNA hybridization with the AccuProbe® or by DNA sequencing. Subsequent isolates were harvested and tested from MP broth and then subcultured to 7H11 agar so that they could be tested again for comparison. A total of 31 isolates were tested from MP broth. This included 19 MAI, 4 *M. gordonae,* 3 *M. tuberculosis* (including 1 *M. bovis* BCG), 2 *M. abscessus/chelonae,* 1 *M. fortuitum,* 1 *M. kansasii,* and 1 *M. mucogenicum.* Seventeen (55%) of these isolates produced correct identifications with acceptable SI and > 0.200 separation from any secondary choices. Five (16%) of the isolates produced unacceptable identifications from MP broth due to poor discrimination from other species. This included 4 MAI and 1 *M. gordonae.* These 5 isolates subsequently produced correct identifications when harvested and tested from 7H11 agar. One (3%) of the isolates from MP broth produced an incorrect identification of *M. tuberculosis* with an unacceptable SI (0.390). Testing from 7H11 agar produced an acceptable identification of MAI. This was confirmed by DNA probe. One (3%) of the isolates from MP broth produced no library match. Subsequent testing from growth on 7H11 agar produced an acceptable identification of *M. mucogenicum.* Two (6%) of the isolates, including 1 MAI and 1 *M. gordonae*, produced unacceptable identifications from both MP broth and 7H11 agar due to insufficient discrimination from other species. Three (10%) of the isolates, including 2 MAI and 1 *M. gordonae,* produced correct identifications from MP broth but had insufficient species discrimination when retested from 7H11 agar. Two (6%) of the isolates from MP broth produced incorrect identifications with acceptable SI and species discrimination. This included 1 MAI and 1 *M. fortuitum* that were incorrectly identified from MP broth as *M. gordonae.*

Since BacT/ALERT MP Culture Bottles are submitted from referring laboratories, it is possible that variables, such as isolate age and transport temperatures, may affect the quality of the profiles produced when isolates from MP broth are tested on the Sherlock® system. In order to ensure accuracy of the identifications produced by the Sherlock® system, all isolates received in MP broth will be subcultured to 7H11 agar prior to testing. The only exception would be for isolates that demonstrate cording morphology when examined microscopically. Since this morphologic characteristic is highly specific for *M. tuberculosis*, this identification from an isolate harvested from MP broth would be acceptable as long as the SI is > 0.500, with species separation > 0.200. By testing only cording isolates from MP broth, a shorter turnaround time can be maintained for *M. tuberculosis.*

13.1.3 Aerobic Actinomycetes

The Sherlock® Mycobacteria Identification System has a separate library for aerobic actinomycetes, including *Gordonia, Nocardia, Rhodococcus,* and *Tsukamurella* species. To validate which organisms the system could accurately identify, a variety of isolates from each genus were tested. These isolates were grown on TSA agar with 5% sheep blood for 24-48 h and extracted using the same protocol as for *Mycobacterium* species.

- ***Gordonia* species**

  A total of 14 clinical *Gordonia* isolates were tested using the Sherlock® system. Twelve of these isolates were previously characterized by ARUP Laboratories, and two of the isolates were characterized by PCR performed by the University of Texas Health Center. All 14 (100%) of the isolates were correctly identified to the genus level. However, 3 (21%) of the isolates produced a SI < 0.500 (0.410, 0.470, and 0.478). No other aerobic actinomycetes produced an identification of *Gordonia,* except for 1 *Nocardia* isolate that produced an unacceptable identification due to a low SI, with both *Gordonia* and *Nocardia* listed as possible choices.

- ***Nocardia* species**

  A total of 35 *Nocardia* isolates were tested using the Sherlock® system. This included 34 clinical isolates and *Nocardia farcinica* ATCC 3308. Eleven of the clinical isolates were

previously characterized as *Nocardia* species with conventional methods (colony morphology, partial acid-fast, growth in lysozyme broth, etc.). Thirteen of the clinical isolates were previously characterized by DNA sequencing performed by ARUP Laboratories, and ten of the isolates were characterized by PCR performed by the University of Texas Health Center. This included 8 *N. farcinica,* 7 *N. nova* complex, 1 *N. transvalensis,* 1 *N. brasiliensis,* 3 *N. cyriacigeorgica* complex, 1 *N. abscessus* complex, 1 *N. beijingensis*, and 1 isolate that could not be speciated by DNA sequencing. A total of 29 (83%) of the isolates were correctly identified to the genus level by the Sherlock® system. However, 11 (31%) of those isolates produced identifications with SI < 0.500. Two (6%) of the isolates produced unacceptable identifications due to < 0.200 separation, with both *Nocardia* and *Gordonia* listed as possible choices. Four (11%) of the isolates produced no library match. No other aerobic actinomycetes were incorrectly identified as *Nocardia*.

In addition to genus-level identification, the data were examined to determine the reliability of the system for identifying *N. farcinica* to the species level. This species is known to be resistant to specific therapeutic drugs. Of the 9 known *N. farcinica* isolates, 5 (56%) produced acceptable identifications on the Sherlock® system. One of the isolates produced no library matches, 2 isolates produced correct identifications with SI < 0.500 (0.461 and 0.432), and 1 isolate produced a profile with multiple possible species with < 0.200 separation (first choice, *N. otitidiscaviarum* and last choice, *N. farcinica*). Five other isolates produced identifications for *N. farcinica* with SI > 0.500 and good separation. However, these isolates were not characterized as *N. farcinica* by DNA sequencing (3 *N. cyriacigeorgica* complex, 1 *N. beijingensis*, and 1 isolate not able to be speciated). These data suggest that the system cannot be reliably used to identify *Nocardia farcinica* to the species level.

- ***Rhodococcus* species**

  A total of 12 clinical *Rhodococcus* isolates were tested using the Sherlock® system. Eleven of these isolates were previously characterized by ARUP Laboratories, and one isolate was characterized by API Coryne (BioMèrieux, France). Five (42%) isolates were correctly identified to the genus level with acceptable SI. Three of those isolates were identified as *Dietzia maris,* which was formerly known as *Rhodococcus maris*. This organism is typically identified as *Rhodococcus equi* by the API Coryne. The other 7 (58%) isolates produced the correct identification of *Rhodococcus*, but the SI was < 0.500. No other aerobic actinomycetes were incorrectly identified as *Rhodococcus*.

- ***Tsukamurella* species**

  A total of 11 clinical *Tsukamurella* isolates were tested using the Sherlock® system. Eight of these isolates were previously characterized by ARUP Laboratories, and 3 isolates were characterized by PCR performed by the University of Texas Health Center. Eight (73%) of the isolates were correctly identified to the genus level. One of these isolates produced an identification with a SI < 0.500 (0.398). Two (18%) of the isolates produced profiles with no library match. No other aerobic actinomycetes were incorrectly identified as *Tsukamurella*.

The table below summarizes the results from the testing of the aerobic actinomycete isolates

Organisms	No. (%) of isolates					
	Isolates Tested	Correct Genus	Low SI (< 0.500)	Low Genus Discrim. (< 0.200 sep.)	Incorrect Identification	No Library Match
Gordonia	14	14 (100)	3 (21)	0	0	0
Nocardia	35	29 (83)	11 (31)	2 (6)	0	4 (11)
Rhodococcus	12	12 (100)[1]	7 (58)	0	0	0
Tsukamurella	11	8 (73)	1 (9)	0	0	2 (18)

[1] Three isolates were identified as *Dietzia maris* (formerly *Rhodococcus maris*)

13.2 Precision

Precision testing consisted of evaluating the ATCC run controls for each assay. The controls have performed successfully for over 20 days. Testing was performed by six different users.

14.0 References

1. Mycobacteria Identification System Operating Manual, Version 1.0. MIDI, Inc. February 2003.
2. Standardized Method for HPLC Identification of Mycobacteria; U.S. Dept. of Health and Human Services, CDC, 1996.
3. Versalovic, J, K. C. Carroll, G. Funke, J. H. Jorgensen, M. L. Landry, D. W. Warnock. 2011. Manual of Clinical Microbiology, 10th ed., Vol. 1, ASM Press, Washington, D.C.
4. Kellog, J.A., Bankert, D.A., Withers, G.S., Sweimler, W., Kiehn, T.E., Pfyffer, G.E. (2001). Application of the Sherlock Mycobacteria Identification System Using High-Performance Liquid Chromatography in a Clinical Laboratory. Journal of Clinical Microbiology, 39(3), 964-970.

15.0 Document Control

Reviewed and approved by Microbiology director: (AR) 12/03/2013

Supervisor reviews: (JC) 12/05/2013, Jason Ammons 12/10/2015

Document Control History

3/20/14 Changed reporting for MAIS id from MAI to MAI/M. scrofulaceum per AR in response to recent CAP survey results.

9/3/2015 Added instructions for differentiating MAIS with overnight urea broth test.

12/10/2015 Updated verification for *M. neoaurum* with data from additional isolates.